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Modulation of I_{HERG}/I_{Kr} by Cellular Metabolites: Implication in the Arrhythmogenesis During Myocardial Ischemia

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Modulation of I_{HERG}/I_{Kr} by Cellular Metabolites: Implication in the Arrhythmogenesis During Myocardial Ischemia

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SOMMAIRE

L'ischémie du myocarde induit des altérations de l'électrophysiologie cardiaque selon un mode biphasique. Les anomalies les plus profondes sont associées a la phase précoce, l'ischémie aigue du myocarde (AMI). Elles sont caractérisées par une accumulation extracellulaire d'ions K^+ ($[K^+]_o$–↑), un raccourcissement de la durée du potentiel d'action (PA) et de l'intervalle QT (APD–↓ ou QT–↓), ainsi qu'une accumulation de métabolites de phospholipides, les lysophosphatidylcholines (LPCs). Lors de la deuxième phase, on observe un rallongement de l'APD et de l'intervalle QT ainsi qu'une synthèse "de novo", une accumulation de métabolites de sphingolipides, les céramides, et de TNF-α. Ces modifications sont associées à l'ischémie chronique du myocarde (CMI).

Ces changements opposés de l'APD/QT sont associés à différents types d'arythmies. Il a été montré que la relation entre les perturbations électriques et métaboliques joue un rôle clef dans la genèse des arythmies ischémiques et la mort subite. Cependant les mécanismes ioniques et métaboliques impliqués dans ces modifications séquentielles de l'APD/QT restent mal compris.

Notre hypothèse est que les désordres électriques ischémiques sont la conséquence de la conjugaison de stress métabolique et de déséquilibres électrolytiques. Plus spécifiquement, le courant I_{Kr}/I_{HERG} est augmenté durant la phase précoce, l'AMI. Cette augmentation est principalement causée par une surproduction et une accumulation de LPCs. La potentialisation de I_{Kr}/I_{HERG} par les LPCs contribue de façon critique à la perte de K^+ intracellulaire, a la diminution de l'APD, et par la même, aux arythmies associées a l'ischémie. En revanche, lors de la CMI et l'insuffisance cardiaque, la diminution de l'expression de HERG induite par les céramides et le TNF-α contribue a l'augmentation tardive de l'APD/QT et par conséquent entraîne l'apparition de troubles du rythme.

Afin de vérifier cette hypothèse, nous avons réalisé des études à différents niveaux. En passant par l'organe, la cellule, pour terminer à l'échelon moléculaire. Ces expériences ont été réalisées en utilisant différentes approches combinées, l'électrophysiologie, la pharmacologie, la biochimie, et la biologie moléculaire.

Tout d'abord, nous avons montré que les LPCs augmentent le courant I_{HERG} exprimé dans des cellules HEK-293, et que cette augmentation du courant I_{HERG} entraînait un raccourcissement de l'APD ainsi qu'un potentiel de membrane plus négative; LPC-16 augmentait I_{HERG} de façon plus prononcée pour des potentiels plus négatifs, ceci associé à un raccourcissement plus important de l'APD. De plus, seulement la forme amphiphile avec 16 hydrocarbonés comme le 1-Palmytoyl-lysophosphatidylcholine (LPC-16) et le 1-palmitoyl-lysophosphatidylglycerol (LPG-16) entraînait une augmentation significative du courant I_{HERG}, ainsi qu'un déplacement de son activation vers des potentiels plus négatifs.

En utilisant le modèle d'ischémie sévère du myocarde chez le lapin, nous avons montré que le LPC-16 exogène mimait l'effet de l'ischémie de faible perfusion correspondant à une augmentation de $[K]^+_o$ et de l'intervalle QT ces effets étaient prévenus par des bloqueurs de I_{Kr} tel que le doflétilide, les autres bloqueurs des canaux K^+ n'ayant pas d'effet. De façon consistante, le doflétilide supprime efficacement, les tachyarythmies induites par l'ischémie ou le LPC-16. De plus, le LPC-16 raccourcis de façon remarquable l'APD dans les myocytes ventriculaires gauche de lapin, augmente, de façon réversible et dépendante au potentiel, l'amplitude du courant I_{Kr} dans les myocytes ventriculaires de cochon d'Inde. Le doflétilide supprime cet effet sur le courant I_{Kr}/I_{HERG}, et limite ainsi le raccourcissement de l'APD.

Nous avons montré que lors de la mise en place de la CMI, le courant I_{Kr} transporté par les canaux HERG était une cible majeure pour l'action des céramides et du TNF-α. L'incubation continue de cellules exprimant les canaux HERG, ou de myocytes, avec des céramides ou du TNF-α diminue de façon significative le courant I_{Kr} ou la fonction des canaux HERG. Ces effets sont médiés par la stimulation de ROS (réactive oxygène species) intracellulaires. Puisque les antioxydants tels que la vitamine E ou le MnTBAP suppriment l'effet dépressif des céramides ou du TNF-α sur I_{Kr}/I_{HERG}. De plus, le céramide comme le TNF-α augmentent de façon importante le niveau intracellulaire de ROS, cet effet est prévenu par la vitamine E ou le MnTBAP.

En conclusion, nos résultats suggèrent que l'accumulation de LPC-16 conjuguée a l'augmentation du courant I_{HERG} démasquent un réel couplage entre un catalyseur métabolique et une voie de signalisation ionique, qui conduit à une augmentation du

courant potassium sortant et une réduction de l'intervalle QT lors d'un épisode ischémique. L'ensemble de ce travail constitue la première évidence de l'implication du courant I_{Kr}/I_{HERG} dans l'augmentation post-ischémique du courant potassium sortant et par ce biais dans la diminution de l'intervalle QT. L'inhibition de la production de LPC-16 ainsi que la réduction de son accumulation pourrait constituer une stratégie thérapeutique prometteuse en vue d'atténuer les arythmies létales associées aux maladies ischémiques du coeur. D'un autre côté, la réduction du courant I_{HERG} par les céramides ou le TNF-α principalement en augmentant la production de ROS semble contribuer a la prolongation de l'intervalle QT lors de l'ischémie prolongée du myocarde, ainsi que dans l'insuffisance cardiaque.

Mots clefs: ischémie du myocarde, arythmies, canaux potassium, lysophosphatidylcholine, céramides, TNF-α, ROS.

ABSTRACT

Ischemic myocardium demonstrates characteristic bi-phasic alterations in cardiac electrophysiology. The most profound abnormalities associated with the early phase of acute myocardial ischemia (AMI) are extracellular K^+ accumulation ($[K^+]_o$–↑) and shortening of action potential duration or QT interval (APD–↓ or QT–↓) as well as accumulation of phospholipid metabolites lysophosphatidylcholines (LPCs). These are followed by the subsequent lengthening of APD or QT interval (APD–↑/QT–↑) and *de nova* synthesis and accumulation of sphingolipid metabolite ceramide and tumor necrosis factor–α, as the most prominent electrical and metabolic disturbances in chronic myocardial ischemia (CMI). The opposite changes of APD/QT are often accompanied by different types of arrhythmias. It has been shown that the interplay between the electrical and metabolic perturbations is pivotal in the genesis of ischemic arrhythmias and sudden cardiac death. However, the ionic and metabolic mechanisms underlying the sequential changes of APD/QT remained poorly understood.

We proposed that ischemic electrical disorders are a consequence of interrelated metabolic stress and electrolyte disturbance. More specifically, I_{Kr}/I_{HERG} is enhanced during the early stage of AMI and the enhancement is mainly caused by overproduction and accumulation of LPCs. The enhancement of I_{Kr}/I_{HERG} by LPCs is a critical contributor to intracellular K^+ loss/$[K^+]_o$–↑ and APD–↓ thereby the associated ischemic arrhythmias. Whereas in CMI and failing heart, where HERG impairment by ceramide and TNF-α contributes to the late APD–↑/QT–↑, leading to cardiac arrhythmias. To examine our hypothesis the studies at different levels including organ, cellular, and molecular levels were carried out with combined approaches of electrophysiology, pharmacology, biochemistry and molecular biology.

We first revealed that LPCs enhance I_{HERG} expressed in HEK293 cell and the enhancement of I_{HERG} manifested with shorter APD and at more negative potential; LPCs increased I_{HERG} to a greater extent at more negative potentials and with shorter APD. Furthermore, only the amphiphile with 16 hydrocarbons such as 1-palmitoyl-lysophosphatidylcholine (LPC-16) and 1-palmitoyl-lysophosphatidylglycerol (LPG-16)

were found to produce significant enhancement of I_{HERG} and negative shifts of HERG activation.

By using a rabbit model of acute global myocardial ischemia, we found that exogenous LPC-16 mimicked the low-perfusion ischemia to produce significant $[K^+]_o$–↑ and QT–↓, which were prevented by I_{Kr} blocker dofetilide but not by blockers for other K^+ channels. Consistently, dofetilide efficiently abolished the ventricular tachycardia-arrhythmias induced by LPC-16 or ischemia. Moreover, LPC-16 remarkably shortened APD in rabbit left ventricular myocytes, reversibly and voltage-dependently increased the amplitude of I_{Kr} in guinea pig ventricular myocytes. Dofetilide abolished the I_{Kr}/I_{HERG} enhancing and APD shortening caused by LPC-16.

In the setting of CMI, we identified I_{Kr}/HERG K^+ channel as a new target for the action of ceramide and TNF-α. Chronic exposure of HERG expressing cells or cardiac myocytes to ceramide or TNF-α significantly impaired HERG K^+ channel or I_{Kr} function. The impairment of channel function is mediated by stimulating intracellular reactive oxygen species (ROS) because antioxidants vitamin E or MnTBAP abolished the depressing effects of ceramide or TNF-α on I_{Kr}/HERG. Moreover, either ceramide or TNF-α remarkably elevated the intracellular ROS levels, which was prevented by vitamin E or MnTBAP.

In conclusion, our results suggest that LPC-16 accumulation and HERG enhancement form the coupling between metabolic trigger and ionic pathway that may account for ischemic $[K^+]_o$–↑ and QT–↓. This represents the first documentation of I_{Kr}/HERG as the ionic mechanism for ischemic $[K^+]_o$–↑ and QT–↓. Inhibition of LPC-16 production and accumulation and/or of I_{Kr}/HERG may be a promising therapeutic strategy to attenuate the incidence of lethal arrhythmias associated with ischemic heart disease. On the other hand, HERG/I_{Kr} impairment by ceramide or TNF-α mainly via ROS overproduction may contribute to QT prolongation in prolonged ischemia of myocardium and heart failure. The inhibition of ceramide and TNF-α/TNFR1 signaling may be an effective strategy to prevent not only the arrhythmogenesis but also the cardiac sudden death in chronic myocardial ischemia and CHF.

Key words: Myocardial ischemia, Arrhythmias, Potassium channels, Lysophosphatidylcholine, Ceramide, TNF-alpha, Reactive oxygen species.

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STATEMENT OF AUTHORSHIP

The following is a statement regarding the contributions of co-authors and myself to the **five papers** already published or submitted for publication, included in this thesis.

1. Wang J., Wang H., Han H., Zhang Y., Yang B., Nattel S., and Wang Z. Phospholipid Metabolite 1-Palmitoyl-lysophosphatidylcholine Enhances Human Ether-a-go-go-related Gene (HERG) K^+ Channel Function. ***Circulation***. 2001; 104(22): 2645-8.

In this paper, my supervisors Drs Wang and Nattel offered me close instruction in whole process, generating the initial idea, clarifying the notion, and working out the final version of the paper. I designed and performed the experiments, analyzed the data, and wrote the manuscript.

2. Wang J., Zhang Y., Wang H., Han H., Nattel S., Yang B., and Wang Z. Potential Mechanisms for the Enhancement of HERG K^+ Channel Function by Phospholipid Metabolites. ***Br J Pharmacol***. 2004; 141(4): 586-99.

The initial idea was derived from paper 1. I designed and performed the experiments, analyzed the data, and wrote the manuscript. Yiqiang Zhang participated in analyzing and discussing the data. Dr Wang served in overall supervision, clarified the idea, and producing the final version of the paper.

3. Wang J., Gillis M., Zhang Y., Lin H., Xu C., Yang B., Wang Z. Enhancement of HERG Function by Lysophosphatidylcholine Contributes to Extracellular K^+ Accumulation and "Short QT Syndrome" in the Heart with Acute Global Ischemia. ***Circulation***. (Submitted in July 2005).

The initial idea was prompted by literature review and our previous work. I had been responsible for planning and executing experiments, analyzing data, and writing the manuscript. Marc-Antoine Gillis helped me performing Langendorff perfusion and ECG recording. Yiqiang Zhang took part in discussing the data. Dr Wang provided overall instruction in all aspects, clarified the thoughts, and produced the final version of manuscript.

4. Wang J., Zhang Y., Wang H., Lin H., Yang B., Wang Z. Sphingolipid Metabolite Ceramide Causes Metabolic Perturbation Leading to HERG K^+ Channel Dysfunction and Abnormal Slowing of Cardiac Repolarization. ***J Biol Chem,*** (submitted in July 2005).

I designed the experiments and completed majority of the experimental work, analyzed the data, and wrote the manuscript. Yiqiang Zhang participated in data analysis and Huizhen Wang joined in part of experiments. Dr Wang gave his supervision in whole process, helped me in making my thoughts clear, reorganizing the data, and editing the final version of the manuscript.

5. Wang J.*, Wang H.*, Zhang Y., Gao H., Nattel S., Wang Z. Impairment of HERG K^+ Channel Function by Tumor Necrosis Factor-α: Role of Reactive Oxygen Species as a Mediator. ***J Biol Chem,*** 2004; 279(14): 13289 – 13292. (* indicating both authors contributed equally to this study).

I was responsible for planning and conducting most of the experiments, analyzing the data, and wrote the manuscript. Huizheng Wang participated in doing Western blot experiments. Dr Nattel served as co-supervisor for scientific counsel and help in manuscript preparation. Dr Wang paid overall close supervision, including generating the initial ideas, reorganizing the data, and creating the final version of the article.

ADDITIONAL PUBLICATIONS

(During my PhD Training Period: 05/2000–07/2005)

Peer-reviewed Articles:

1*. **Wang J**, Han H, Zhang Y, Long H, Wang H, Xu D, and Wang Z. HERG K^+ channel conductance promotes H_2O_2–induced apoptosis in HEK293 cells: Cellular mechanisms. ***Cell Physiol Biochem.*** 2004; 14:121 – 134.

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7*. **Wang J**, Zhang Y, Wang H, Han H, Nattel S and Wang Z. Mechanisms for the enhancement of HERG K^+ channel function by phospholipid metabolites. 2003 ***Circulation*** May 20, Page 35

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Note:

(1) For publications in which I am the first author, I generated the idea, designed and performed the experiments, analyzed the data and wrote the manuscripts;

(2) For publications in which I am a 2^{nd} ~3^{rd} co-author, I participated in about 30~40% work of the projects, including idea generation, experiments design and carry-out, data analysis, manuscript correction and discussion, etc.;

(3) * indicating I was involved in major part of the project.

This thesis is dedicated to:

My parents, Zhirong Zhu & Zhonghe Wang
My daughter, Renfei
My wife, Jing
My sisters, Jingming and Jingyue
My brothers, Jingjie and Jingdong

For their love, understanding, encouragement, and ...

TABLE OF CONTENTS

LIST OF SIGNS AND ABBREVIATIONS

$^1O_2$: Singlet oxygen

$^{\bullet}OH$: Hydroxyl radical

$[K^+]_o$-↑: Extracellular potassium accumulation

$[Ca^{2+}]_i$: Intracellular calcium concentration

$[Na^+]_i$: Intracellular sodium concentration

$[Mg^{2+}]_i$: Intracellular magnesium concentration

γ: Single-channel conductance

AA: Amino acid or arachidonic acid

Ach: Acetylcholine

ACS: Acute coronary syndromes

AMI: Acute myocardial ischemia

AT-II: Angiotensin II

ATP: Adenosine triphosphate

AP(s): Action potential(s)

APD: Action potential duration

AVN: Atrioventricular node

Bis: Bisindolylmaleimide

CAD: Coronary artery disease

cAMP: Cyclic adenosine 3', 5'-monophosphate

CHF: congestive heart failure

CHO: Chinese hamster ovary

CICR: Ca^{2+} induced Ca^{2+}-release

CM-H2DFDA: 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate

CMI: Chronic myocardial ischemia

DAD: Delayed early afterdepolarizations

E-C: Excitation-contraction

ECG: Electrocardiogram

EAD: Early afterdepolarizations

E_m: Membrane potential

E_{rev}: Reversal potential

ERP: Effective refractory period

GABA: Gamma-amino butyric acid

H_2O_2: Hydrogen peroxide

HEK: Human embryonic kidney

HERG: Human ether-a-go-go-related gene

I/R: Ischemia/reperfusion

ICU: Intensive care unit

I_f: Pacemaker funny current

IHD: Ischemic heart disease

I_{HERG}: HERG K^+ currents

I_{inj}: Injury current

$I_{L\text{-}Ca}$: L-type Ca^{2+} current

IPC: Ischemic preconditioning

$I_{T\text{-}Ca}$: T-type Ca^{2+} current

I_K: Delayed rectifier K^+ current

I_{K1}: inward rectifier K^+ current

I_{KAA}: Fatty acid and amphiphile activated K^+ current

I_{KACh}: Acetylcholine-sensitive K^+ current

I_{KATP}: ATP-sensitive K^+ current

I_{KNa}: Na^+-activated K^+ current

I_{Kr}: Rapidly-activated delayed rectifier K^+ current

I_{Ks}: Slowly-activated delayed rectifier K^+ current

I_{to}: Transient outward K^+ current

I_{Kur}: Ultrarapidly-activated delayed rectifier K^+ current

IVF: Idiopathic ventricular fibrillation

I-V: Current-Voltage

JNK: Jun N-terminal kinase

KChIP: Potassium channel interacting proteins

KCOs: K^+ channel openers

K_d: Diffusion constant

K_V: Voltage-gated potassium

LPC: Lysophosphatidylcholine

LPG-16: 1-palmitoyl-lysophosphatidylglycerol

M cell: Midmyocardial cells

MI: Myocardial ischemia

minK: Minimal potassium channel

MiRP1: minK related peptide 1

mitoK_{ATP}: Mitochondrial K_{ATP}

MnTBAP: Mn (III) tetrakis(4-benzoic acid) porphyrin chloride;

NBF: Nucleotide binding fold

NCX: Sodium/calcium exchanger

NO: Nitric oxide

NSTEMI: Non–ST-segment elevation myocardial infarction

$O_2^{\bullet-}$: Superoxide radical

PBS: Phosphate-buffered saline

PCr: Phosphocreatine

pH_i: Intracellular pH

pH_o: Extracellular pH

PLA2: Phospholipase A2

P_o: open probability

PTKs: Protein tyrosine kinases

PTX: Pertussis toxin

Q10: Ubiquinone

QT-↓: Shortening of QT interval

RNAi: RNA interference

ROS: Reactive oxygen species

RP: Resting potential

S1P: Sphingosine-1-phosphate

sarcK_{ATP}: Sarcolemmal K_{ATP}

SCD: Sudden cardiac death

SFLL: SFLLRNPNDKYEPF (Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Lys-Tyr-Glu-Pro-Phe), thrombin receptor activator

SM: Sphingomyelin

SMase: Sphingomyelinase

SOD: Superoxide dismutase

SPC: Sphingosylphosphocholine

SQTS : Short QT syndrome

SR: Sarcoplasmic reticulum

TdP: Torsade de pointes

TMD : Transmembrane domain

TNF-α: Tumor necrosis factor-α

TR: Thioredoxin reductase

TTX: Tetrodotoxin

UA: Unstable angina

VF: Ventricular fibrillation

VGCCs: Voltage-gated calcium channels

VT: Ventricular tachycardia

XO: Xanthine oxidase

LIST OF FIGURES AND TABLES

CHAPTER I

CHAPTER II

II-1

II-2

II-3

CHAPTER III

CHAPTER IV

PART I

INTRODUCTION AND THE REVIEW OF THE LITERATURE

CHAPTER I

Electrical and Metabolic Disturbances Underlying Ischemic Arrhythmias in Hearts

The overall purpose of this chapter is to review the updated experimental findings and notions that have shed light on the mechanisms of electrophysiological and metabolic disturbances during myocardial ischemia and ischemic arrhythmias. The specific goals are (1) to set a theoretical foundation for forming my project hypothesis; (2) to raise the questions, which are related to the research project; and (3) to propose the hypothesis and the objectives of the project.

I-1 Perspective: Ischemic Heart Disease – A Global Health Problem

Although a substantial reduction in death rate from cardiovascular causes during the past 50 years, ischemic heart disease (IHD) remains the leading cause of morbidity and mortality in the industrialized world. In the United States, IHD afflicts in excess of 6 million Americans annually (American Heart Association, 2004), it causes about 152,000 deaths per year in UK, and world-wide, one in eight deaths is attributed to IHD (Ghuran & Camm, 2001). Retrospectively, data from clinical electrophysiological studies and randomized trials have revealed that the majority of IHD deaths are caused by ischemic arrhythmias, which appear as the most common pathophysiological cascade involved in lethal arrhythmias degenerating first to ventricular tachycardia (VT), and then to ventricular fibrillation (VF) and later to asystole and sudden cardiac death (SCD) (Mehta *et al.*, 1997; Myerburg & Castellanos, 1997; Zipes & Wellens, 1998).

Furthermore, SCD rates also parallel the rates of IHD as a whole in less-developed countries (Huikuri *et al.*, 2001; Zipes & Wellens, 1998). It has been predicted that between 1990 and 2020, mortality from IHD in developing countries is expected to increase by 120% for women and 137% for men. Predictions for the next 2 decades include a near tripling of IHD mortality in Latin America, the Middle East, and sub-Saharan Africa (Leeder, 2004). Not only is acute myocardial ischemia (AMI) the most common factor triggering VT, thereafter SCD (Davies, 1992), but the risk of SCD in the population who have had chronic myocardial ischemia (CMI) is four-folds higher than the normal population as well (Abildstrom *et al.*, 2002). Therefore, great efforts have been devoting to understand the mechanisms underlying ischemic arrhythmias, and to set the ways for effective treatment, and ultimate prevention of IHD.

Myocardial ischemia (MI) is a condition in which normal myocardial perfusion is arrested, leading to cascades of metabolic and electrophysiological alterations, which are interrelated and are caused by metabolic stress. The results are failure of contraction, deterioration of electrical behavior, and eventual death of the myocytes. At the organism level, the end point may be lethal arrhythmias or mechanical pump failure. Despite a remarkable progress of understanding of the pathophysiological and biochemical aspects of MI, our knowledge of which patients with MI will develop sustained VT remains unclear, and the mechanisms underlying the fatal cardiac arrhythmias triggered by MI are far from elucidated. Apparently, more studies, especially the integrated investigations combining *in vitro* electrophysiological and molecular biological studies and *in vivo* electrophysiological and biochemical investigations are needed to clarify the mechanisms so as to interfere with the pathological process of MI and to open the novel avenue in the treatment and prevention of IHD.

I-2 General Features of Ischemic Arrhythmias

Temporally, ischemic arrhythmias can be categorized into three phases based on the time when they appear: phase I arrhythmias occurring during the first 30 min of ischemia; phase II seen between 5 to 72 hours and phase III arrhythmias of the chronic stage after an infarct. Phase I can be further subdivided in Ia (between 2 and 10 min of ischemia, while the first burst of VT normally occurs) and Ib types (between 15 and 30 min), with the Ib type especially dangerous because they frequently evolve in VF and SCD in human (Carmeliet, 1999; Pogwizd & Corr, 1987).

VT is the most pronounced arrhythmia in MI. It is defined as three or more consecutive beats arising below the atrioventricular node (AVN) with an RR interval of less than 500 ms (>120 beats/min), being frequently diagnosed with the advert of intensive care unit (ICU) monitoring and long-term ambulatory electrocardiogram (ECG) recordings (Akai *et al.*, 2000). VT may be classified by morphological criteria (monomorphic, polymorphic, bundle branch block pattern and axis), by duration (sustained or nonsustained) or by the underlying mechanisms (enhanced automaticity, triggered activity or re-entry). Clinically, MI has been occasionally found to precede the

onset of a monomorphic VT. More frequently, however, AMI triggers the occurrence of polymorphic VT that may predispose to VF rather than a sustained monomorphic VT.

VT followed by VF is the lethal arrhythmia that disposes patients who suffer from MI to sudden death. The earlier and more frequently observed abnormalities in ECG recording are the alterations in QT interval, and the changes of ST segment as well. Very intriguingly, ischemic myocardium demonstrates characteristic sequential alterations in electrophysiology with early QT interval shortening (QT-↓) in acute ischemia and subsequent QT interval lengthening (QT-↑) after a prolonged ischemic period, which are associated with different types of arrhythmias (Boyden & Jeck, 1995; Carmeliet, 1999; Singer *et al.*, 1981). For instance, in phase I, especially phase Ia of AMI, QT-↓ has been frequently observed in a wide variety of animal modals and in human (Friedman *et al.*, 1973; Lazzara *et al.*, 1974; Lazzara *et al.*, 1978). In coincidence with the QT-↓, the most profound electrophysiological disorder in this phase of MI may be extracellular potassium accumulation ($[K^+]_o$-↑). In contrast to the QT-↓ in early phase of AMI, QT-↑ has been found to be a universal change in the CMI (Eick *et al.*, 1976; Lazzara *et al.*, 1975; Wit & Friedman, 1975). Either AMI or CMI, at the cellular and multicellular levels, the electrophysiological disturbances are characterized as depolarization, prolongation of the effective refractory period (ERP), decrease in conduction velocity, and change in excitability. The basis of the electrophysiological disturbance is a group of ions and exchangers, which interplay to set the mechanisms of the electrophysiological disturbances in ischemic myocardium.

I-3 Our Current Understanding of the Potential Ionic Mechanisms for Ischemic Arrhythmias

Cardiac myocytes make electrical continuity as a tissue through low-resistance intercellular gap junction. Each individual cardiocyte is like a small battery with the voltage of -50 to -90 mV across its sarcolemmal membrane (measured from the intracellular space with respect to the bathing solution ground). **Depolarization** is defined as a change in membrane potential (E_m) to more positive voltage, which is mediated by channels or transporters that enable positively charged ions such as Na^+ or Ca^{2+} to enter the cell (***inward currents***) (van Ginneken & Giles, 1991); whereas **repolarization** refers

to any change to more negative voltage, which is mainly mediated via a variety of K^+ channels allowing K^+ movement from the intracellular to extracellular side (***outward currents***) (Marban, 2002; Nattel, 2002). The heart beat is initiated by slow diastolic depolarization of the sinoatrial node (SAN), which drives the membrane potential after action potential (AP) toward the threshold for firing the next AP. Multiple ionic currents with complex interactions are involved in spontaneous diastolic depolarization (Schram *et al.*, 2002; Boyett *et al.*, 2000).

Rapid voltage changes of the mass of the heart are detected as millivolt-sized changes in the surface ECG. Figure 1 depicts the temporal relationships between the electrical activity of a typical ventricular myocytes, as measured using cellular recordings of the transmembrane AP (panel A), and the corresponding ECG (panel B).

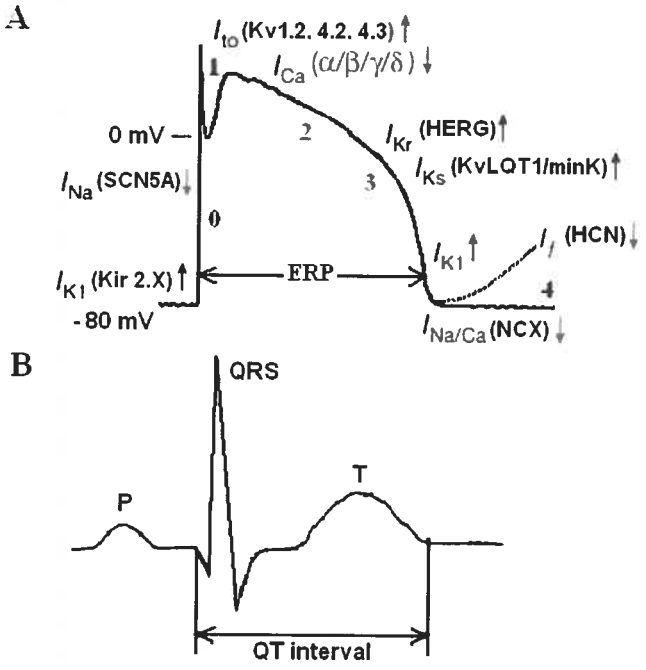

Figure 1. The temporal relationships between the ventricular AP and the corresponding surface ECG. A, a typical ventricular AP with inward current (downward arrows) and outward currents (upward arrows). The molecular basis of each current is indicated in parentheses. Numbers indicate the phases of the AP. **B**, a schematic ECG. QRS complex, P wave, T wave and QT interval are indicated.

The upstroke of the AP at the onset of depolarization produces the spiky 'QRS complex'; repolarization is manifested as the gently rolling T wave. As a first approximation, the time between the beginning of the QRS complex and the end of the T wave — the 'QT interval' — can be used to deduce the overall timing and duration of ventricular depolarization and repolarization. The frequency of QRS complexes and their sequence relative to the smaller P waves produced by atrial activity allow the clinical detection of normal rhythm or arrhythmias.

I-3-1 Properties of Ionic Channels Underlying Cardiac Action Potentials

Over the last 50 years patch-clamp techniques combined with molecular biology have revolutionized the study of ion channels underlying cardiac AP. The AP is virtually an electrical signal integrating with a variety of ***inward and outward currents***, which result from the orchestral opening or closing of number of ion channels and transporters in the sarcolemma membrane. Transmembrane APs form the cellular basis for pacemaker activity, impulse spread, and control of cardiac excitation-contraction coupling (Splawski *et al.*, 2002). APs that originate in the SAN (Lacinova, 2004) and transmit sequentially throughout the atria, atrioventricular node (AVN), His-Purkinje system, and the ventricles, are a summation of precisely orchestrated openings and closings of distinct populations of ion channels. Perturbations in ion channel function and/or structural malformations in heart underlie various cardiac arrhythmias. Moreover, aberrant ion channel function underlies a wide range of diseases in both man and other species (Ashcroft, 2000). Strategic molecular targeting of these proteins will therefore yield novel forms of antiarrhythmic therapy (Ackerman & Clapham, 1997).

Traditionally, the AP can be divided into ***five phases***. In the resting ventricular myocyte (membrane potential around -90 mV), voltage-gated sodium channels are closed. A propagating impulse causes the membrane potential to become less negative, therefore the sodium channels are opened, and the resulting large rapid inward Na^+ flux (I_{Na}) produces ***phase 0*** depolarization. The initial ***phase 1*** repolarization results from the opening of the transient outward potassium channels (I_{to}). During this phase, calcium channels also open, and the long ***phase 2*** plateau of the AP corresponding to ventricular systole (Sorrentino & Reggiani, 1999) reflects a balance between inward calcium current

(I_{Ca}) and outward potassium current through delayed rectifier channels (I_{Kr}, I_{Ks}, and I_{Kur} in atrial myocytes). Inactivation of calcium channels in ***phase 3***, together with ongoing outward K^+ flux through delayed rectifier and inward rectifier channels (I_{K1}) completes repolarization to resting ***phase 4***. Phase 4 corresponds to ventricular filling during diastole (relaxation) and is maintained by the open I_{K1}. In pacemaker myocytes including SAN, AV node cells, and Purkinje fibers (Knopf *et al.*, 1990), the cells begin a spontaneously slow depolarization forming pacemaker current (I_f) rather than remain at the resting potential. This is also referred as phase 4 (Coraboeuf & Nargeot, 1993; ten Eick *et al.*, 1992).

AP configuration and duration vary in specific regions (e.g., atrium versus ventricle) as well as in specific areas within those regions (Figure 2).

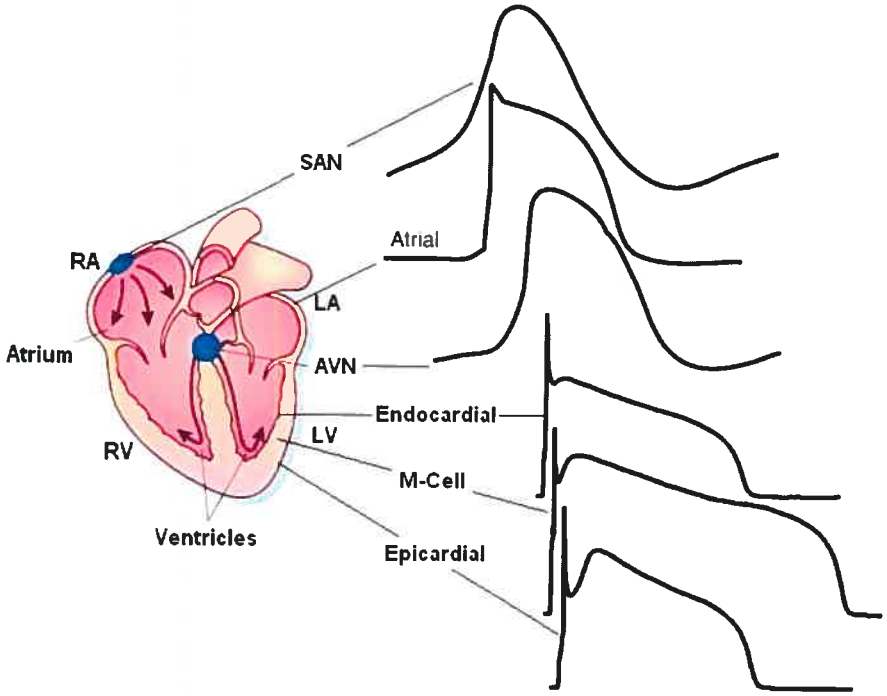

Figure 2. Action potential waveforms are variable in different regions of the heart. Schematic representation of the heart; action potential waveforms recorded in different regions of the heart are illustrated. Action potentials are displaced in time to reflect the temporal sequence of propagation through the heart. [Modified from (Nattel & Li, 2000; Pinto & Boyden, 1999)].

Such physiologic heterogeneities likely reflect variations in expression or function of the repertoire of ion channels and other proteins that constitute cardiac ion currents (Schram *et al.*, 2002). Exaggeration of these heterogeneities, by changes in rate, ion channel mutations, and/or drug exposures, promotes reentrant excitation, a common mechanism for many cardiac arrhythmias. The acute electrophysiological response of a myocyte to exogenous stressors such as myocardial ischemia, autonomic activation, or drugs likely reflects changes in function and gating kinetics of individual ion channels, including channels activated by specific stimuli such as adenosine triphosphate (ATP) depletion, muscarinic stimulation, or stretch.

I-3-1-1 Sodium (Na^+) Channels

In the heart, voltage-gated Na^+ channels determine the amplitude and slope of the AP upstroke, which are especially important in the control of impulse conduction velocity, and in the maintenance of appropriate waves of excitation through the working myocardium. Dysfunction of these channels can lead to at least 6 types of cardiac rhythm disorders: long QT syndrome (LQTS) (Wang *et al.*, 1995b; Wang *et al.*, 1995a; Wang *et al.*, 1996; Wang *et al.*, 1998a), Brugada syndrome (Brugada & Brugada, 1992; Antzelevitch *et al.*, 2002), idiopathic ventricular fibrillation (Bezzina *et al.*, 2001; Akai *et al.*, 2000), nonfamilial arrhythmia susceptibility (Splawski *et al.*, 2002), conduction system disease (Schott *et al.*, 1999), and sick sinus syndrome (Benson et al., 2003). These conditions are characterized by ventricular tachyarrhythmia, heart block, or atrial bradyarrhythmia. Very recently, Na^+ channels have been identified to be associated with atrial fibrillation and heart failure (Olson *et al.*, 2005; Chen *et al.*, 2003b). All those disorders can be traced to the abnormal molecular structure and expression of Na^+ channels.

Na^+ channels are believed to consist of a principal pore-forming α-subunit (260 kDa) and two auxiliary β-subunits (36 and 33 kDa each) (Fozzard & Hanck, 1996). In mammals, the genes encoding $Na_v1.1$, 1.2, 1.3, and 1.7 (human genes SCN1A, SCN2A, SCN3A and SCN9A) are clustered on human chromosome 2q and appear to have arisen as duplications of a founder gene (Lopreato et al., 2001). There is a second cluster of three genes on human chromosome 3p, all of which encode tetrodotoxin (Satin *et al.*,

1992)-resistant channels. One of these, SCN5A (also designated as hH1) encoding $Na_V1.5$, is expressed in heart and brain (Head & Gardiner, 2003).

The α-subunit has four structurally similar domains (DI-DIV); each domain has six helical transmembrane segments (S1-S6). The S4 segments of each domain contain a large number of positive charged residues serving as voltage sensor for channel activation and coupled-inactivation (Kontis et al., 1997). The short intracellular segment between DIII and DIV has been identified as the fast inactivation gate. The channel pore is formed by four P-loop regions (P region) linking S5 and S6 segment in each domain. The P region is deduced to be composed of twenty-residues forming the lining of the outer vestibule responsible for ion selectivity and toxin affinity. The more detailed investigations reveal that the primary structure in each P-loop of Na^+ channels is unique, which differs from Ca^{2+} channels in which each P-loop is the same, therefore rendering each of them to different function. For instance, a lysine in the P region of DII critically selects for Na^+ over Ca^{2+} (Heinemann et al., 1992); a cysteine in the P region of DI renders the cardiac channel insensitive to blockade by tetrodotoxin (TTX) or saxitoxin (STX), but sensitive to Cd^{2+} or Zn^{2+} (Satin *et al.*, 1992).

The β-subunits consist of a single transmembrane domain, a small intracellular C-terminal region and a large extracellular N-terminal domain with an immunoglobulin-like fold (Stevens et al., 2001). Three different isoforms of auxiliary β-subunits are expressed in cardiac tissue from a range of species including rodents, sheep and human (Dhar *et al.*, 2001; Fahmi *et al.*, 2001; Qu *et al.*, 1995) and are known to modulate the kinetics of the cardiac sodium channel α-subunit (Kupershmidt et al., 1998), as well as to regulate the expression of sodium channel α-subunits in the heart (Dhar *et al.*, 2001), and therefore attribute to the electrical properties of whole channels.

I-3-1-2 Calcium (Ca^{2+}) Channels

Voltage-dependent calcium channels (VGCCs) in cardiac myocytes are essential for regulating the influx of Ca^{2+} ions across the sarcolemmal membranes, in response for the upstroke of the AP in the SAN and AVN and play an important role in determining the plateau and eventual spike-dome appearance of the AP in other cardiac cells. Moreover, the Ca^{2+} influx behalves as the electrical signaling player involving in

initiating intracellular events such as Ca^{2+} induced Ca^{2+}-release (CICR) from sarcoplasmic reticulum (SR) (Singer *et al.*, 1981), excitation-contraction (E-C) coupling, and modulation of gene expression. Intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) also modulates the conductance of Ca^{2+}-activated K^+ and Cl^- currents (Wehrens & Marks, 2004). The blockade of Ca^{2+} entry through the cardiac VGCC represents one of the principal approaches for the treatment of IHD.

On the basis of electrophysiological and pharmacological characteristics, VGCCs have been classified into T-, L-, N-, P-, Q-, and R-types (Yunker, 2003). In the sarcolemmal membrane of heart cells two types of Ca^{2+}-permeable channels have been described and differentiated: the L-type ("long lasting" and "large") and T-type ("transient" and "tiny") channels and a background channel (McDonald *et al.*, 1994). The heterogeneity of the T- and L-type channel density in different region of the heart has been long recognized and described (Bourinet *et al.*, 2004; Yunker & McEnery, 2003).

VGCCs are heteromultimeric protein complexes. The three-dimensional structure of the bovine cardiac L-type calcium channel has recently been resolved (van der Heyden *et al.*, 2005;Wang *et al.*, 2004c). The largest subunit (~190–240 kDa) is the pore-forming α_1 subunit, which is associated with an intracellularly located β subunit (~55 kDa) and a mostly extracellularly located disulfide-linked $\alpha_2\delta$ subunit (~170 kDa). Several α_1 subunits have been identified to date (α1A-α1I, α1S, now termed $Ca_V1.1$- $Ca_V3.3$), and the α_{1C} isoform is the one that is expressed at high levels in cardiac muscle, but also in smooth muscle and in the brain (Catterall, 2000; Keef *et al.*, 2001; Jiang *et al.*, 2000a; Striessnig, 1999).

The α_{1C} subunit interacting with accessory subunits and especially the β subunit is required to form fully functional Ca^{2+} channels and/or to alter certain channel properties. Accessory subunits determine the activation and inactivation kinetics of the channels. The β subunit also controls targeting of the α_{1C} subunit to the membrane. A highly conserved 18-amino acid sequence in the cytoplasmic loop connecting DI and DII has been identified as the interaction domain of the α_{1C} subunit for the β subunit. The $\alpha_2\delta$ complex, which is less tightly associated with the α_{1C} subunit, consists of an extracellularly located α_2 subunit linked to a hydrophobic membrane-spanning δ subunit. The α_2 subunit and δ subunit are encoded by a single gene. The mature forms of these subunits are derived by

post-translational proteolytic processing, but they remain associated through a disulfide bond. The extracellular α_2 subunit interacts with the S5–S6 linker in DIII of the α_{1C} subunit (Norman & Leach, 1994; Striessnig, 1999).

I-3-1-3 Pacemaker Channels (f-Channels)

In 1979, a current named as funny current (I_f, f for "funny" because of its peculiar features) was recorded from small voltage-clamped SAN preparations (Brown *et al.*, 1979). I_f was inwardly activated on hyperpolarization in the appropriate (diastolic) range of potentials, and increased by adrenaline. Following the original report, the I_f current was described in several cardiac and noncardiac cells, but the most detailed analysis was conducted in SAN cells. I_f plays an important roles both in initiating normal rhythmic activity and in mediating the responses to adrenergic and muscarinic neurotransmitters (Baruscotti & DiFrancesco, 2004; DiFrancesco, 1995; DiFrancesco & Tromba, 1988).

Unlike the majority of other voltage-gated channels in which the permeability is normally selective for a single ion species, f-channels have a mixed permeability to both Na^+ and K^+ cations (DiFrancesco, 1981). The voltage range of current activation is highly variable and depends on structural and modulatory factors. In SAN myocytes, the I_f is time- and voltage dependent inward current upon hyperpolarization, activation threshold can be as positive as -40/-45 mV and being fully activated at -100 mV, and the $V_{1/2}$ for activation is ~-80mV, which ensures the current contribution within the whole of the pacemaker phase (Cerbai *et al.*, 1999; DiFrancesco & Mangoni, 1994; van Ginneken & Giles, 1991).

Despite the importance of f-channels to functional properties of cardiac myocytes, its cloning was only achieved nearly three decades after its original description and was accomplished by chance (Santoro *et al.*, 1997). The newly identified sequence bore the hallmarks of a voltage-dependent K^+-permeable ion channel: six putative transmembrane domains and an S4-charged α-helix, plus the expected cyclic nucleotide binding domains at the intracellular C-terminus. Following this first sequence, other isoforms were identified (Gauss *et al.*, 1998; Ishii *et al.*, 1999; Ludwig *et al.*, 1998; Seifert *et al.*, 1999), this group of channels, known as the hyperpolarization-activated cyclic nucleotide-gated family (Pachucki *et al.*, 1999), comprises four members (HCN1 to HCN4) and belongs to

the superfamily of voltage-gated potassium (K_V) channels, with which they share many similarities. The four isoforms share a homology of ~60% in amino acid (Wang *et al.*, 1996) sequence identity and express in heart except for HCN3 that only in brain (Kaupp & Seifert, 2001). The functional expression of these channels resulted in currents with the hallmarks of the cardiac I_f or of its neuronal equivalent (termed I_h). Although the properties of different HCN isoforms differ quantitatively, all isoforms yield currents that are activated by hyperpolarization, carry K^+ and Na^+, are blocked by Cs^+ in a voltage-dependent manner, and are modulated by a direct action of cAMP on the cytoplasmic side of the channel (Santoro *et al.*, 1997).

There is surprisingly little direct evidence implicating I_f in either acquired or inherited disease. Perhaps the best-studied case is in cardiac ventricular muscle, where increases in I_f magnitude or shifts in voltage dependence have been associated with certain cardiovascular diseases such as spontaneous hypertension in rats (Cerbai *et al.*, 1994), human failing hearts (Cerbai *et al.*, 1997; Cerbai *et al.*, 2001). It was also reported that HCN2 message level varies with thyroid hormone and/or thyroid hormone receptor level, perhaps contributing to the rapid heart rate associated with hyperthyroidism (Gloss *et al.*, 2001; Pachucki *et al.*, 1999). In addition to these acquired diseases, a recent report describes the first known human mutation in an HCN4 gene. The mutation, identified by genotyping a patient with sinus bradycardia and atrial ventricular fibrillation, results in truncation of the terminal portion of the HCN4 C-terminal, including the CNBD, which leads to a pronounced reduction in the magnitude of I_f (Schulze-Bahr *et al.*, 2003).

I-3-1-4 Potassium (K^+) Channels

Cardiac K^+ currents (I_K) are a large family of a variety of K^+ channels with different characteristics. I_K can be distinguished on the basis of differences in their functional and pharmacological properties. In mammalian cardiac cells, K^+ channels can be categorized as voltage-gated (K_V) and ligand-gated channels. The first category includes the rapidly activating and inactivating transient outward current (I_{to}) (Wang *et al.*, 1999), the ultrarapid-activated delayed rectifier (I_{Kur}) (Wang *et al.*, 1993), rapid-activated delayed rectifier (I_{Kr}) and slow-activated delayed rectifier (I_{Ks}) (Volders *et al.*, 1999; Wang *et al.*, 1994) and the inward rectifier (I_{K1}) (Wang *et al.*, 1998b), whereas the ligand-

gated channels include those activated by a decrease in the intracellular concentration of ATP (K_{ATP}) (Wilde *et al.*, 1989), activated by acetylcholine (K_{Ach}) (Koumi & Wasserstrom, 1994; Yang *et al.*, 1996), Na^+-activated K^+ currents (I_{KNa}) (Kameyama *et al.*, 1984; Luk & Carmeliet, 1990; Noda *et al.*, 1984), arachidonic acid-activated K^+ currents (I_{KAA}) (Kim & Duff, 1990), and Ca^{2+}- Activated K^+ Channels (K_{Ca}) (Cao *et al.*, 2005; Xu *et al.*, 2002).

Cardiac K^+ channels play a pivotal role in defining resting potential, cell excitability and membrane repolarization and thereby the likelihood of arrhythmias. The configuration and duration of the cardiac APs vary considerably among species and different cardiac regions (atria vs. ventricle) and specific areas within those regions (epicardium vs. endocardium) (Figure 2). This heterogeneity mainly reflects differences in the type and/or expression patterns of the K^+ channels that participate in the genesis of the cardiac AP. Changes in the expression of K^+ channels explain the regional variations in the morphology and duration of the cardiac AP among different cardiac regions and are influenced by heart rate, intracellular signaling pathways, drugs and cardiovascular disorders (Coetzee *et al.*, 1999; Nerbonne, 2000; Snyders, 1999). Moreover, the expression and properties of K^+ channels are not static but are influenced by heart rate, neurohumoral state, pharmacological agents, cardiovascular diseases (cardiac hypertrophy and failure, myocardial ischemia and infarction) and arrhythmias.

I-3-1-4-1 Transient Outward K^+ Current (I_{to})

Cardiac I_{to} is responsible for early rapid repolarization (Smith *et al.*, 1995) and determines the height of the early plateau, thus influencing activation of other currents that control repolarization, mainly $I_{L\text{-}Ca}$ and the delayed rectifier K^+ current (I_K). Furthermore, variations in cardiac repolarization associated with I_{to} differences strongly influence intracellular Ca^{2+} transient by modulating Ca^{2+} entry via $I_{L\text{-}Ca}$ and sodium/calcium exchange (NCX), potentially exacerbating impaired Ca^{2+} cycling in heart disease (Sah *et al.*, 2003).

I_{to} density is 4- to 6-fold higher in atrial tissue, Purkinje fibers, epicardial and midmyocardial (M) cells than in the endocardial cells (Nabauer *et al.*, 1996; Yan & Antzelevitch, 1996). The prominent epicardial I_{to} contributes to the selective electrical

depression of the epicardium during ischemia and to the development of a marked dispersion of repolarization between normal and ischemic epicardium and between epicardium and endocardium, thereby providing the substrate for reentrant arrhythmias (Lukas & Antzelevitch, 1993; Yan *et al.*, 2004). CMI and infarction are associated with prolongation of the APD, an effect that has been attributed, in part, to a downregulation of I_{to} (Oudit *et al.*, 2001; Pinto & Boyden, 1999; Tomaselli & Marban, 1999). However, there is also an evidence of the up-regulation of I_{to} in cardiac myocytes after induced myocardial infarction (Pinto & Boyden, 1999; Yao *et al.*, 1999). Paradoxically, using the dynamic clamp technique which allows quantitative 'insertion' of simulated conductance in real, biological cells, bridging pure computer modeling and experimental electrophysiology, it has been verified that I_{to} does not significantly affect the APD of canine ventricular myocytes, and that the I_{to} gradient is not a significant contributor to the transmural APD dispersion in the canine ventricle (Sun & Wang, 2005).

$K_V4.3$ channels are the leading candidate for encoding I_{to} in human and dog (Kaab *et al.*, 1998; Wang *et al.*, 1999), although a number of K_V α-subunits, such as $K_V1.4$, $K_V3.4$, $K_V4.2$, and $K_V4.3$ known for generating I_{to}-like current when heterologously expressed, have become primary candidates for cardiac I_{to}. Potassium channel interacting proteins (KChIP) are Ca^{2+}-binding proteins that act as β-subunits interacting with the cytoplasmic amino termini of K_V4 α-subunits and modify I_{to} features (Sanguinetti, 2002). For instance, KChIP2, when coexpressed with $hK_V4.3$, increases surface channel density and current amplitude, slows the inactivation, accelerates the recovery from inactivation and shifts the half-maximal inactivation to more positive potentials (An *et al.*, 2000; Pourrier *et al.*, 2003a). Thus, features of $K_V4.3$/KChIP2 currents closely resemble those of I_{to}. In human ventricle KChIP2 mRNA is 25-fold more abundant in the epicardium than in the endocardium, and this gradient parallels the gradient in I_{to} expression, while $K_V4.3$ mRNA is expressed at equal levels across the ventricular wall. Thus, transcriptional regulation of the KChIP2 gene is the primary determinant of I_{to} expression across the ventricular wall (Rosati *et al.*, 2001).

I-3-1-4-2 Ultra-rapid Delayed Rectifier K^+ Current (I_{Kur})

I_{Kur} is present in human and dog atrium and throughout rat and mouse heart. Its density is similar in cells isolated from different regions of the canine right atrium (Brouillette *et al.*, 2004; Feng *et al.*, 1998; Nattel *et al.*, 1999; Roden *et al.*, 2002; Wang *et al.*, 1993). I_{Kur} is the predominant delayed rectifier current responsible for human atrial repolarization. $K_V1.5$ (613 amino acids) encodes the α-subunit of the I_{Kur} channel (10–14 pS). Heterologous expression of $K_V1.5$ results in a delayed rectifier current with the biophysical and pharmacological characteristics of I_{Kur} and exposure to $K_V1.5$ antisense oligodeoxynucleotides-AsODN specifically inhibits I_{Kur} density in human atrial myocytes (Nattel *et al.*, 1999; Wang *et al.*, 1993). Cyclic adenosine 3', 5'-monophosphate (cAMP), mechanical stretch and hyperthyroidism increase, whereas extracellular acidosis, phenylephrine, and hypothyroidism decrease $K_V1.5$ expression (Guo *et al.*, 1998; Le Bouter *et al.*, 2003; Nattel *et al.*, 1999). $K_V1.5$ mRNA levels decrease in hypertensive hypertrophied rat ventricle (Matsubara *et al.*, 1993) and in the epicardial border zone of the infracted canine ventricle (Pinto & Boyden, 1999).

I_{Kur} is a substantial current in human atrial myocytes (Wang *et al.*, 1993). Kinetic analysis in human atrial cells indicates that I_{Kur} has amplitude that is at least as large, and often larger, than that of I_{to} (Schaffer *et al.*, 1998). Selective inhibition of I_{Kur} with pharmacologic probes suggests a significant role for the current in repolarizing human (Wang *et al.*, 1993), canine (Yue *et al.*, 1996), and mouse (Fiset *et al.*, 1997) cardiomyocytes. Mathematical models of the human atrial AP based on formulations of directly measured ionic currents also indicate a role for I_{Kur} in repolarization (Courtemanche *et al.*, 1998; Courtemanche *et al.*, 1999; Nygren *et al.*, 1998). In atrial fibrillation (AF) I_{Kur} appears to be downregulated (Van Wagoner *et al.*, 1997), the morphologic changes associated with tachycardia-induced cellular remodeling (Yue *et al.*, 1997) result in a clearer AP prolongation from I_{Kur} inhibition in AF-type myocytes than in normal ones (Courtemanche *et al.*, 1999). Thus, inhibition of I_{Kur} would be expected to prolong human atrial refractory period and protect against atrial reentrant arrhythmias like AF. At the same time, I_{Kur} is absent in human ventricular myocytes (Li *et al.*, 1996). Therefore, the development of I_{Kur}-selective blockers could result in atrial-specific antiarrhythmic drugs. If this proves to be feasible, it would be a major therapeutic advance, because the most important limitation of AP-prolonging antiarrhythmic therapy

2003; Roden *et al.*, 2002; Splawski *et al.*, 2000). Whereas gain of function in I_{Kr} has been associated with "short QT-syndrome" (Brugada *et al.*, 2004), a new clinical entity originally described as an inherited syndrome (Gussak *et al.*, 2000), also leading to the development of lethal arrhythmia and SCD, although the different phenotypes are attributed to the alterations in I_{Kr} function.

Molecular Basis and Structure

The human *ether a-go-go*-related gene (HERG) product constitutes the α-subunit (1159 amino acids) underlying I_{Kr} (Keating & Sanguinetti, 2001; Sanguinetti *et al.*, 1995; Trudeau *et al.*, 1995) and the KCNE2 gene product minK-related peptide 1 (MiRP1) (123 amino acids) may contribute to I_{Kr} as a β-subunit (Abbott *et al.*, 1999; Tristani-Firouzi & Sanguinetti, 2003).

HERG was initially isolated by screening a human hippocampal cDNA library with a mouse homolog of *ether-a-go-go,* a *Drosophila* K^+ channel gene (Warmke & Ganetzky, 1994). The function of HERG was unknown when isolated, but it was strongly expressed in the heart and was postulated to play an important role in repolarization of cardiac APs (Curran *et al.*, 1995). This notion was verified and moreover, following the discovery HERG has become one of the hottest targets in electrophysiological and pharmacologic studies.

HERG channels represent a typical potassium channel protein composed of six α-helical transmembrane segments, S4 functions as a voltage sensor, and a highly selective ion conduction pathway located in the linker between transmembrane segments S5 and S6 (Tseng, 2001). Predicted topologies of potassium channels suggest only very small portions of the tetrameric HERG protein are exposed to the ER lumen, with large N- and C-terminal domains including the Per, Arnt, and Sim (PAS) and cyclic-nucleotide binding domains (cNBD) projecting into the cytoplasm (Morais Cabral *et al.*, 1998). Since native I_{Kr} and HERG channels expressed in heterologous systems differ in terms of gating, regulation by external K^+ and single channel conductance (Abbott *et al.*, 1999; Tristani-Firouzi & Sanguinetti, 2003; Tseng, 2001; Sanguinetti *et al.*, 1995; Zhou *et al.*, 1998b), it suggests the presence of a modulating β-subunit that co-assembles with HERG to reconstitute native I_{Kr}. A potential candidate is found to be MiRP1 which when coexpressed with HERG shifts the HERG activation curve in the positive direction,

accelerates the rate of deactivation, decreases single channel conductance (from 13 to 8 pS) and mediates the direct stimulatory effect of cAMP on HERG/MiRP1 channels (Abbott *et al.*, 1999; Cui *et al.*, 2000). MiRP1 reduces channel sensitivity to $[K^+]_o$ and enhances sensitivity to clarithromycin (Abbott *et al.*, 1999) but not to dofetilide, E-4031 or quinidine (Weerapura *et al.*, 2002). MiRP1 can also modulate KCNQ1, $K_V4.2$ and even the distinctly related hyperpolarization-activated cation (HCN) pacemaker channels (Tristani-Firouzi & Sanguinetti, 2003). However, the specific contribution of MiRP1 to native I_{Kr} is still controversial. For instance, currents resulting from coexpression of HERG and MiRP1 did not resemble native I_{Kr} properties closer when compared with HERG in the absence of MiRP1 (Ehrlich *et al.*, 2004; Weerapura *et al.*, 2002).

HERG and MiRP1 have been identified as the loci of mutations associated with LQT2 and LQT6 of the Romano –Ward variant of LQTS, respectively (Kass & Moss, 2003; Roden *et al.*, 2002; Splawski *et al.*, 2000). More than 100 mutations in the HERG and KCNH2 gene have been described, including frameshifts, insertions, deletions and missense and nonsense mutations (Kass & Moss, 2003; Splawski *et al.*, 2000; Zhou *et al.*, 1998a; Rajamani *et al.*, 2002). Mutant channels cause a net reduction in outward K^+ current during repolarization that can result from different mechanisms, including generation of nonfunctional channels, altered channel gating and abnormal protein trafficking (Ficker *et al.*, 2003; Rajamani *et al.*, 2002; Zhou *et al.*, 1998a).

Expression

There is the enormous variation as to the tissue expression of I_{Kr}, or HERG and MiRP1, depending on the different species, tissues, and (patho) physiological conditions. I_{Kr} has been identified in human atrial and ventricular myocytes, rabbit SA and AVN cells and Purkinje fibers (Nerbonne, 2000; Schram *et al.*, 2002). In rabbit SA myocytes HERG channels play an important role in the pacemaker activity and I_{Kr} blockers decrease the maximum rate of diastolic depolarization (Verheijck *et al.*, 1995). In rats I_{Kr} density is higher in atria than in ventricles, while in humans HERG expression is higher in the ventricles (Pond *et al.*, 2000). MiRP1 expression is rather sparse in ventricular tissue (Pourrier *et al.*, 2003b). In canine atria I_{Kr} density is larger in the AV ring region and the left atrial wall than in crista terminalis, pectinate muscles, appendage cells and right atrial wall, which may account for the shorter APD of the left atria (Feng *et al.*, 1998; Schram

et al., 2002). However, in the canine ventricle I_{Kr} is distributed homogeneously (Liu & Antzelevitch, 1995), while in guinea pig left ventricle, I_{Kr} is smaller in subendocardial than in midmyocardial or epicardial myocytes (Bryant *et al.*, 1998b). In rabbit left ventricle, I_{Kr} is greater in the apex than in the basal regions and I_{Kr} blockers cause more significant APD prolongation in the apex than in the base, increasing the regional dispersion of APD (Cheng *et al.*, 1999). I_{Kr} density is reduced in myocytes from infarcted canine ventricle (Jiang *et al.*, 2000b). It, however, increases in subendocardial Purkinje fibers from the 48 h infarcted heart (Pinto & Boyden, 1999), an effect that may increase the proarrhythmic effects of I_{Kr} blockers in patients with myocardial infarction.

Modulation

HERG channels can be modulated via the cAMP-PKA (Cui *et al.*, 2000; Kiehn, 2000), PKC, PKB (Zhang *et al.*, 2003b), phosphatidyl 4,5-biphosphate (PIP_2) pathways (Bian *et al.*, 2001; Bian *et al.*, 2004). Very intriguingly, the channels have been found to be regulated by a great number of drugs (Tamargo *et al.*, 2004) as well as a variety of physiologically or pathophysiologically produced metabolites (Wang *et al.*, 2001; Wang *et al.*, 2004b).

Activation of β-adrenergic receptors and elevation of intracellular cAMP levels regulate HERG channels both through PKA-mediated effects and by direct interaction with the protein (Cui *et al.*, 2000; Thomas *et al.*, 2004). PKA activation reduces HERG current amplitude and induces a depolarizing shift in the voltage-dependent activation curve(Cui *et al.*, 2000; Kiehn, 2000). This shift is inhibited by specific PKA inhibitors (H89, KT5720) and in channels missing all PKA phosphorylation sites (S283A, S890A, T895A, S1137A), while coexpression of HERG with KCNE1 or KCNE2 accentuates the cAMP-induced voltage shift (Cui *et al.*, 2000). However, isoproterenol increases I_{Kr} in guinea pig ventricular myocytes, an effect that was inhibited by bisindolylmaleimide (Bis) (Kiehn, 2000). Isoproterenol also increases I_{Kr} and this effect is inhibited by H89, but not by Bis in rabbit SA cells (Lei *et al.*, 2000). These results suggest that modulation of I_{Kr} may be species-specific and tissue-specific and may depend strongly on the experimental conditions as well.

HERG channels are modulated by PKC independently of direct phosphorylation of the channel (Thomas *et al.*, 2003). PMA causes a positive shift of activation and reduces

I_{Kr} and its effects can still be observed when the PKC-dependent phosphorylation sites are deleted by mutagenesis. Changes in PIP_2 levels result from activation of several adrenergic and muscarinic receptors. PIP_2 increases HERG current and shifts the voltage-dependence of activation in a hyperpolarizing direction (Bian *et al.*, 2001;Bian *et al.*, 2004). Moreover, in cells coexpressing the α_{1A}-receptor and HERG, phenylephrine reduces HERG currents and this effect is prevented by PIP_2 but not by PKC inhibition, suggesting that the mechanism is due to G-protein-coupled receptor stimulation of PLC resulting in the consumption of endogenous PIP_2.

I_{Kr}/HERG channels are the primary targets of class III antiarrhythmic drugs of the methanesulfonanilide group (almokalant, dofetilide, D-sotalol, E-4031, ibutilide, and MK-499). These drugs produce a voltage- and use-dependent block, shorten open times in a manner consistent with open-channel block and exhibit low affinity for closed and inactivated states (Busch *et al.*, 1998; Mitcheson *et al.*, 2000; Tristani-Firouzi & Sanguinetti, 2003; Tseng, 2001). I_{Kr} blockers prolong atrial and ventricular APD (QT prolongation) and refractoriness in the absence of significant changes in conduction velocity (AH, HV and PR intervals) (Sanguinetti & Salata, 1996). In animal models, I_{Kr} blockers suppress ventricular tachycardia induced by programmed electrical stimulation or a new ischemic insult in dogs with prior infarct (Nattel & Singh, 1999; Sanguinetti & Salata, 1996). However, these drugs are probably not effective against triggered activity or increased automaticity (Sanguinetti & Salata, 1996; Tamargo *et al.*, 2004). An increasing number of drugs with diverse chemical structures block I_{Kr}, delay ventricular repolarization, prolong the QT interval (acquired LQTS) and induce TdP (Clancy *et al.*, 2003; Redfern *et al.*, 2003). There have been extensive efforts to develop I_{Kr} channel blockers as a new antiarrhythmic agent for atrial or ventricular fibrillation, since it was demonstrated that selective blockade of I_{Kr} in the heart is not deleterious for the total mortality in fatal ventricular arrhythmia patients (Lee *et al.*, 2003).

I-3-1-4-4 Slow Delayed Rectifier K^+ Current (I_{Ks})

Biophysical Features

I_{Ks} is slowly activated at potentials positive to −30 mV with a linear I–V relationship, reaching half-maximum activation at +20 mV (Kurokawa *et al.*, 2001;

Sanguinetti & Jurkiewicz, 1990; Tristani-Firouzi & Sanguinetti, 2003). The current only shows activation and no inactivation. Deactivation is slow in guinea pig but relatively fast in dog and rabbit (Carmeliet, 1992;Gintant, 1996; Liu & Antzelevitch, 1995). Thus, in guinea pigs, the slow deactivation of I_{Ks} results in a reduction of outward current that contributes to the slow diastolic depolarization of SA node cells (Kurokawa *et al.*, 2001).

Function and Tissue Expression

Like I_{Kr}, I_{Ks} contributes to human atrial and ventricular repolarization. It, however, particularly plays the role during APs of long duration and is a dominant determinant of the physiological heart rate-dependent shortening of APD. As heart rate increases, I_{Ks} channels have less time to deactivate, leading to an accumulation of open channels and a faster rate of repolarization (Delpon *et al.*, 1995; Jurkiewicz & Sanguinetti, 1993).

I_{Ks} density is greater in atrial than in ventricular myocytes and in subepicardial and M cells than in subendocardial cells (Bryant *et al.*, 1998b), but is smaller in apical than in basal myocytes of the rabbit left ventricle in guinea pigs (Cheng *et al.*, 1999). There are no differences in I_{Ks} density among cells from different regions of the canine right atria (Feng *et al.*, 1998), while in the canine ventricle, I_{Ks} density is higher in epicardial and endocardial cells than in the M cells (Liu & Antzelevitch, 1995) and in right than in left ventricular M cells (Volders *et al.*, 1999). The smaller I_{Ks} density in M cells may explain their steeper APD-rate relations and their greater tendency to display pronounced action potential prolongation and to develop EAD at slow heart rates or in response to QT prolonging drugs (Liu & Antzelevitch, 1995). I_{Ks} density is downregulated in all layers of the left ventricle of failing canine hearts (Li *et al.*, 2002a) and a decrease in I_{Ks} and KCNQ1/KCNE1 mRNA levels is found in myocytes from infarcted canine ventricle (Jiang *et al.*, 2000b).

Molecular Basis

I_{Ks} is composed of four pore-forming K_VLQT1 (KCNQ1, 676 amino acids) and two accessory minimal potassium channel (minK) (KCNE1, 129 amino acids) β-subunits (Barhanin *et al.*, 1996; Chen *et al.*, 2003a; Sanguinetti *et al.*, 1996; Suessbrich & Busch, 1999). minK exhibits a single transmembrane spanning domain; the N-terminus is extracellular and the C-terminus intracellular. Heterologous expression of K_VLQT1 produces rapidly activating, slowly deactivating currents, while minK, by itself, does not

form functional channels. Coexpression of K_VLQT1/minK slows activation and deactivation kinetics, shifts the voltage dependence of channel activation to more positive potentials and increases the macroscopic current amplitude, thus reproducing the biophysical properties of the native cardiac I_{Ks} (Sanguinetti *et al.*, 1996).

minK is abundant in the SA node, but less abundant although homogeneously distributed throughout the ventricular wall and in the mouse heart expression is largely restricted to the conducting system. An alternatively spliced variant of K_VLQT1 with a N-terminal deletion that produces a negative suppression of K_VLQT1 is preferentially expressed in the M cells, which is consistent with the lower I_{Ks} density in this region (Pereon *et al.*, 2000). K_VLQT1 and minK expression and I_{Ks} density increases in ventricular myocytes from hypothyroid rats, but decreases in hyperthyroidism (Le Bouter *et al.*, 2003) and in myocytes from infarcted canine ventricle (Jiang *et al.*, 2000b). Mutations in K_VLQT1 and minK are associated with types 1 (LQT1) and 5 (LQT5) of the Romano–Ward variant of the LQTS, respectively, and the Jervell–Lange–Nielsen syndrome associated with deafness arises in children who inherited abnormal K_VLQT1 or minK alleles from both parents (Schulze-Bahr *et al.*, 1997; Splawski *et al.*, 2000).

Modulation

I_{Ks} is upregulated via the PKA pathway (Kaczmarek & Blumenthal, 1997; Blumenthal & Kaczmarek, 1994). Stimulation of PKA by cAMP, phosphodiesterase inhibitors and β-adrenergic agonists increases I_{Ks} density and produces a rate-dependent shortening of the APD (Lo & Numann, 1998; Marx *et al.*, 2002). Phosphorylation of the K_VLQT1 subunit by PKA blunts quinidine- and 293B-induced block, probably by inducing a change in the K_VLQT1 conformation that modifies the drug access to the blocking site (Yang *et al.*, 2003). Thus, β-adrenergic stimulation, itself a potent proarrhythmic stimulus, may also decrease the antiarrhythmic effects of I_{Ks} blockers. PKC modulation of I_{Ks} current is complex, suggesting that there are two functionally distinct PKC sites on K_VLQT1–minK channels (Lo & Numann, 1998). Moreover, premodulation by PKC prevents I_{Ks} modulation by PKA, and PKC has no effect on I_{Ks} current after potentiation by PKA. These data indicate that I_{Ks} is modulated by PKC and PKA in a mutually exclusive manner and suggest that multiple interacting phosphorylation sites are involved. Endothelin-1 inhibits the I_{Ks} enhanced by

isoproterenol and forskolin and prolongs APD duration via the endothelin receptor A pertussis toxin (PTX)-sensitive G protein/PKA pathway (Washizuka *et al.*, 1997).

In guinea pig cardiac myocytes, the stimulation of G protein-coupled ATP receptor by extracellular ATP markedly enhances I_{Ks} through a mechanism that appears to be independent of either the activation of PKC or the elevation of intracellular Ca^{2+} (Matsuura & Ehara, 1997; Matsuura *et al.*, 1996). Moreover, it was recently demonstrated in guinea pig ventricular myocytes that the endogenous membrane PIP_2 has a potent inhibitory action on I_{Ks} channels (Ding *et al.*, 2004).

Some fatty acids such as docosahexaenoic acid and lauric acid significantly enhanced I_{Ks}. Eicosapentaenoic acid, however, was without significant effect on current magnitude, although it reduced the rate of activation. These results suggest that not all fatty acids target the channel and produce the same effect. If K_VLQT1 was expressed in *Xenopus* oocyte alone, docosahexaenoic acid, lauric acid, and oleic acid did not augment current, suggesting that minK confers fatty acid sensitivity to I_{Ks} (Doolan *et al.*, 2002). Lowering $[K^+]_o$ and $[Ca^{2+}]_o$ also increase I_{Ks} (Kurokawa *et al.*, 2001; Tristani-Firouzi & Sanguinetti, 2003).

I-3-1-4-5 Inward Rectifier K^+ Current (I_{K1})

I_{K1} is the current responsible for maintaining the negative resting potential in cardiac cells. The current is strong rectifier that passes K^+ ions over a limited range of membrane potentials (Lopatin & Nichols, 2001). At negative membrane potentials I_{K1} conductance is much larger than that of any other current, and so it clamps the resting membrane potential close to the K^+ equilibrium potential (~-90mV). Upon depolarization, I_{K1} channels close almost immediately, remain closed throughout the plateau and open again at potentials negative to −20 mV. Thus, I_{K1} contributes to terminal phase 3 of AP. I_{K1} density is higher in ventricular than in atrial myocytes, but is similar in epicardial, M and endocardial cells in canine and guinea pig hearts (Schram *et al.*, 2002). I_{K1} density is very low in SA and AV pacemaker cells (Irisawa *et al.*, 1993) and, therefore, the maximum diastolic potential is more depolarized than in atrial and ventricular myocytes.

Kir2.1 subunits (427 amino acids) encoded by the KCNJ2 gene, coassemble to form tetrameric channels (Lopatin & Nichols, 2001). Several I_{K1} channels with

conductance of 9, 21, 35 and 41 pS are recorded in human atrial myocytes (Wible *et al.*, 1995). Likewise, different gene families (Kir2.1–2.3) have been found in human heart encoding I_{K1} (Lopatin & Nichols, 2001). Kir2.1 overexpression increases I_{K1} density, shortens the APD and hyperpolarizes the resting membrane potential in guinea pig myocytes, while genetic suppression of I_{K1} results in opposite changes and in a pacemaker phenotype accelerated by isoproterenol (Miake *et al.*, 2003). Reduced I_{K1} density is observed in canine Purkinje cells after myocardial infarction (Pinto & Boyden, 1999) and in a 3-day-old infarcted rat heart, but this reduction is greater in epicardial than in endocardial myocytes (Yao *et al.*, 1999). The ventricular myocytes from patients with idiopathic dilated cardiomyopathy exhibit decreased channel activity, longer APD and lower resting membrane potentials than those from patients with ischemic cardiomyopathy (Koumi *et al.*, 1995). The downregulation of I_{K1} produces membrane depolarization, prolongation of the APD and both early and delayed afterdepolarizations (Nabauer & Kaab, 1998; Tomaselli & Marban, 1999).

I-3-1-4-6 Acetylcholine-induced K^+ Current (I_{KACh})

The heart rate decrease mediated by the parasympathetic branch of the autonomic nervous system involves the release of acetylcholine (ACh) from post-ganglionic cholinergic neurons onto SAN and AVN cells as well as atrial myocytes (Wickman & Clapham, 1995). Ach binds M_2 muscarinic receptors on these cells, triggering the activation of PTX-sensitive G proteins. The activated G_α and $G_{\beta\gamma}$ subunits in turn activate a distinct class of inwardly rectifying potassium channel (I_{KACh}) (Mark & Herlitze, 2000; Wickman & Clapham, 1995). Activation of I_{KACh} hyperpolarizes the membrane potential, slows the spontaneous firing rate of the pacemaker cells of the SAN and AVN and delays AV conduction (Carmeliet, 1999; Snyders, 1999).

I_{KACh} density is about 6 times greater in the atrium than in the ventricle (Schram *et al.*, 2002) and results from a heterotetrameric complex formed by the homologous Kir3.1 (501 amino acids)/GIRK1 and Kir3.4 (419 amino acids)/GIRK4 potassium channel subunits (Corey & Clapham, 1998; Dascal *et al.*, 1993; Krapivinsky *et al.*, 1995; Kubo *et al.*, 1993; Silverman *et al.*, 1996). Kir3.1 was proposed initially to constitute an integral subunit of both neuronal and cardiac G protein-gated potassium channels (Kofuji *et al.*,

1995; Krapivinsky *et al.*, 1995; Velimirovic *et al.*, 1996). Recent studies, however, have presented evidence for the existence of native G protein-gated potassium channels that do not contain Kir3.1 (Corey & Clapham, 1998; Inanobe *et al.*, 1999; Jelacic *et al.*, 2000). Indeed, Kir3.4 homotetrameric complexes have been identified in heart atrial tissue and were proposed to contribute significantly to macroscopic I_{KACh} current (Bender *et al.*, 2001; Corey & Clapham, 1998).

I_{KACh} activity can be stimulated by intracellular ATP, PIP_2 and ET_A endothelin, μ opioid, α_2-adrenergic and A1 adenosine receptor agonists (Carmeliet, 1999; Cho *et al.*, 2002; Shieh *et al.*, 2000) and inhibited by intracellular acidification and several antiarrhythmic drugs (Brandts *et al.*, 2000; Guillemare *et al.*, 2000; Inomata *et al.*, 1993). Disopyramide, procainamide and pilsicainide mainly block the muscarinic receptors, while flecainide and propafenone act as open channel blockers (Guillemare *et al.*, 2000). Atrial K_{ACh} channels are inhibited by membrane stretch, possibly serving as a mechanoelectrical feedback pathway, a property conferred by the Kir3.4 subunit (Ji *et al.*, 1998). Vagal stimulation produces a nonuniform shortening of the atrial APD and refractoriness mediated by activation of I_{KACh}, an effect that may contribute to the perpetuation of AF (Liu & Nattel, 1997). Chronic AF reduces I_{KACh} density possibly to counteract the AF-induced nonuniform shortening of the atrial refractoriness (Dobrev *et al.*, 2001).

I-3-1-4-7 ATP-sensitive K^+ Current (I_{KATP})

I_{KATP} is an inwardly rectifying current identified first from isolated guinea pig ventricular myocyte (Noma, 1983). After this landmark finding, ATP-sensitive K^+ channels (K_{ATP}) underlying I_{KATP} have also been shown to exist in other tissues and appear to consist of various subtypes depending on the tissue or organelle studied (Gross & Peart, 2003; Isomoto & Kurachi, 1997).

Cardiac K_{ATP} are inhibited by physiological intracellular ATP levels (ATP_i) and activated by ADP_i, thus coupling cell metabolism (reflected by ADP/ATP ratio) to membrane potential (Seino & Miki, 2003; Yellen, 2002; Yokoshiki *et al.*, 1998). It was initially demonstrated that opening of the K_{ATP}, induced by hypoxia, ischemia, or pharmacological K_{ATP} openers increased K^+ efflux (Carmeliet, 1999; Gasser & Vaughan-

Jones, 1990) and shortened the cardiac APD by accelerating phase 3 repolarization (Faivre & Findlay, 1990; Isenberg *et al.*, 1983), and therefore plays a role in arrhythmogenesis during the early phase of myocardium ischemia (Cole *et al.*, 1991; Noma, 1983; Sanguinetti & Salata, 1996). However, the existing data regarding the role of K_{ATP} in ischemic $[K^+]_o$-↑ have been conflicting and challenged (see later in details). Actually, accumulating evidence has revealed that the opening of the myocardial K_{ATP}, especially mitochondrial K_{ATP} (mitoK_{ATP}) rather than the sarcolemmal K_{ATP} (sarcK_{ATP}) channel are mainly responsible for cardioprotective function against various stresses, including ischemia and hypoxia (Gogelein, 2001; Gross & Peart, 2003; Grover & Garlid, 2000).

In the view of molecular basis, cardiac K_{ATP} channels result from the coassembly of four inwardly rectifying channel α-subunits (Kir6.2) and four regulatory SUR2A subunits (Seino & Miki, 2003; Yokoshiki *et al.*, 1998). Kir6.2 subunits confer inhibition by ATP (Tucker *et al.*, 1997). The SUR2A subunit has three transmembrane domains (TMD0, TMD1, and TMD2), each of which consists of five, five, and six membrane spanning regions and two nucleotide binding folds (NBF-1 and NBF-2) located in the loop between TMD1 and TMD2 and in the C-terminus, respectively (Conti *et al.*, 2001; Seino & Miki, 2003). The SUR2A subunit confers sensitivity to MgADP, sulfonylureas and K^+ channel openers and ATP hydrolysis at each NBF gates the K^+ permeation through the Kir6.2 (Seino & Miki, 2003; Yokoshiki *et al.*, 1998). There is an endoplasmic reticulum retention motif in the C-terminal region in Kir6.2 and in an intracellular loop between TMD1 and NBF-1 in SUR2A that prevents their surface expression in the absence of the other subunit (Zerangue *et al.*, 1999). Kir6.2−/− mice lack K_{ATP} and present an aberrant regulation of cardiac excitability, inadequate Ca^{2+} handling, ventricular arrhythmias and sudden death following sympathetic stimulation (Zingman *et al.*, 2002). These results suggest that Kir6.2 is required for adaptation to stress.

K_{ATP} are blocked by sulfonylureas (i.e. glibenclamide, glicazide, glipizide, glimepiride, tolbutamide), glinides (repaglinide, nateglinide) and various antiarrhythmic drugs (Tamargo *et al.*, 2004). Several factors desensitize K_{ATP} to inhibition by ATP_i, including nucleotide diphosphates, lactate, oxygen-derived free radicals and adenosine A1 receptor stimulation (Seino & Miki, 2003; Yokoshiki *et al.*, 1998). Cardiac I_{KATP}

blockers prevent the shortening of the APD and the incidence of ventricular fibrillation during the myocardial ischemia, even when in models of ischemia–reperfusion they are mainly arrhythmogenic (Gogelein, 2001; Grover & Garlid, 2000; Sanguinetti & Salata, 1996). The clinical effects of glibenclamide are also contradictory in type 2 diabetic patients with coronary artery disease (Brady & Terzic, 1998). Moreover, since K_{ATP} are present in pancreatic β-cells and smooth muscle (Seino & Miki, 2003), I_{KATP} blockers can produce hypoglycemia and coronary vasoconstriction, effects that may preclude their interest as antiarrhythmic agents. Cardioselective I_{KATP} blockers (clamikalant, HMR 1098) inhibit hypoxia-induced APD shortening and prevent ventricular fibrillation induced by coronary artery occlusion in post-infarcted conscious dogs at doses that have no effect on insulin release, blood pressure or coronary blood flow (Gogelein, 2001; Grover & Garlid, 2000). Thus, they may represent a new therapeutic approach to the treatment of ventricular arrhythmias in patients with coronary heart disease.

K_{ATP} can be activated indirectly by PKA-coupled and other protein kinase-coupled receptors via phosphorylation of channel proteins or by depleting cellular ATP levels (Babenko *et al.*, 1999). Both PKC and adenosine A1 receptor activation enhance K_{ATP} and these effects are suppressed by chelerythrine or bisindolylmaleimide (Light *et al.*, 1996). K_{ATP} activated by glucose-free anoxia close immediately upon reoxygenation, but activation of both novel and conventional PKC isoforms contributes to the persistent opening of the channels under these circumstances (Ito *et al.*, 2001). Protein tyrosine kinases (PTKs) are also mediators of ischemic preconditioning (IPC). In guinea pig ventricular myocytes the PTK inhibitor genistein elicited I_{KATP}. Stimulation of receptor PTKs with epidermal growth factor, nerve growth factor or insulin attenuates, while the protein tyrosine phosphatase inhibitor orthovanadate prevents the effects of genistein on I_{KATP} (Stadnicka *et al.*, 2002). These results suggest that the PTK-protein tyrosine phosphatase signaling pathway may be one of the regulators of cardiac sarcK_{ATP}.

PIP_2 directly interacts with positively charged residues (R176, R177) at the C-terminus of Kir6.2 subunit stabilizing the open state of the channel and antagonizes ATP inhibition of K_{ATP} (Fan & Makielski, 1997; Lopatin & Nichols, 2001), whereas breakdown of PIP_2 by PLC enhances the ATP-sensitivity of K_{ATP} channels (Xie *et al.*, 1999). Moreover, PIP_2 mimics ATP in preventing current rundown and in rescuing

activity after rundown (Fan & Makielski, 1997). It has been also reported that the release of nitric oxide (NO) and bradykinin during ischemia can play a role in IPC. K_{ATP} are activated via the NO-cGMP-PKG signaling pathway, which phosphorylates some serine–threonine residues and this activation is reversed by protein phosphatase 2A (Han *et al.*, 2002). PKC-ε and PKC-η are activated by NO donors in rabbit hearts (Ping *et al.*, 1999) and their infarct-size limiting effects are abolished by chelerythrine. Interferon-α inhibits I_{KATP} in rabbit ventricular cells and this effect is blocked by genistein, but is not affected by H-7, an inhibitor of PKC and PKA (Nishio *et al.*, 1999). These findings suggest that tyrosine kinase-mediated inhibition of I_{KATP} by cytokines may aggravate cell damage during myocardial ischemia.

I-3-2 Synopsis of Ionic Channels Underlying Cardiac Action Potentials

The normal electrophysiological behavior of the heart is determined by the ordered propagation of excitatory stimuli resulting in rapid depolarization and slow repolarization, generating APs in individual myocytes. In a prototypical fast response cardiac cell (i.e., from atrium, ventricle, or the Purkinje system), the membrane is highly permeable to K^+, as demonstrated by the fact that the reversal potential for K^+ is very close to the resting membrane potential. This permeability reflects the fact that inward rectifier K^+ channels in the membrane are open at rest. A change in the potential across the cell (due to a propagating impulse or an experimentalist's stimulus) is sensed by the Na^+ channel protein, which alters its conformation to open, allowing a large, rapid Na^+ influx, producing the typical rapid phase 0 depolarization (see Figure 2). In some cells, a rapid phase 1 repolarization then ensues, because of outward movement of K^+ via transient outward channels. During phase 0 and phase 1, Ca^{2+} channels open. Phase 2, the characteristically long (hundreds of milliseconds) plateau phase of the AP, reflects a balance between inward current, largely through L-type Ca^{2+} channels, and outward current, largely through delayed rectifier K^+ channels. The net outward current during phase 3 repolarization is provided by delayed rectifier K^+ channels, along with inactivation of Ca^{2+} channels. Final repolarization is accomplished by outward movement of K^+ through inward rectifier channels. Slow response cells, those in the SAN and in the AVN, demonstrate slow depolarization during phase 4, a manifestation of pacemaker

channel activity. Furthermore, a rapid phase 1 upstroke is absent, and initial depolarization is accomplished by opening of L-type (and perhaps T-type) Ca^{2+} channels. Other electrogenic behaviors (due to exchangers and pumps) are readily demonstrated in cardiac tissue and are crucial in maintaining intracellular ionic homeostasis in the face of large ion fluxes accompanying each AP. At the most generic level, abnormalities of impulse generation, propagation, or the duration and configuration of individual cardiac APs form the basis of disordered cardiac rhythm.

Ischemic arrhythmias electrically behavior as APD/QT interval shortening at the very early stage (< 15 min) of blood flow occlusion and then manifest as APD/QT interval prolongation in the late phase and chronic myocardial ischemia. The common consequence is ventricular tachyarrhythmia and VT, and eventually SCD. During myocardial ischemia the function of channels involving in the AP is altered in response to the metabolic stress characterized by a deficient energenic input as well as deficient waste removal, leading to a cascade of metabolic and electrophysiological events. The cascade is identified by the reduced availability of oxygen and substrate, by the disturbances of a variety of ions, and by the intracellular and extracellular accumulation of metabolic by-products, such as the lipid metabolites (including amphiphile like lysophosphatidylcholine, free fatty acids, and sphingolipid ceramide), lactate, taurine, etc, as well as by the overproduction of stress signaling molecules, such as radicals and cytokines (Beresewicz *et al.*, 2002; Carmeliet, 1999; Cui *et al.*, 2004; Fahim & Halim, 2004; Murase *et al.*, 2000). These alterations, with concomitant stimulation of adrenergic, purinergic, and muscarinic receptors, as well as the genesis of stretch produce the profound effects on ion channels and exchangers involving in the APs, thereby the development of lethal ventricular arrhythmias.

I-4 Disturbances of Ion Concentrations during Myocardial Ischemia

Like other types of mammalian cells, the cardiomyocyte is enclosed by an approximately 80-Å-thick bimolecular lipid membrane. This lipid bilayer serves as an excellent electrical insulator, permitting passage of very few ions, allowing the myocyte to maintain ion concentrations in its cytosol that are different from those in the extracellular fluid (see Table 1). By generating ionic concentration differences across the

lipid bilayer, the sarcolemmal membrane of myocyte is capable of storing potential energy in the form of electrochemical gradients, which are used to drive various transport processes, to convey electrical signals like the AP. In myocardial ischemia the typical alterations in ion concentrations, which are attributable to the arrhythmogenesis are accumulation of extracellular potassium concentration (Weiss & Shine, 1982b), increase in intracellular calcium concentration ($[Ca^{2+}]_i$-↑), intra- and extracellular acidosis, as well as intracellular sodium concentration ($[Na^+]_i$-↑) and depletion of intracellular magnesium ($[Mg^{2+}]_i$-↓). In this introduction, only the first two alterations are addressed hereafter.

Table 1. Intracellular and Extracellular Ion Concentrations in Cardiocytes

Ion	Extracellular Concentration (mM)	Intracellular Concentration (mM)	Equilibrium Potential (mV)**
Na^+	145	10	+71
K^+	4	135	-94
Ca^{2+}	2	10^{-7} M	+132
Mg^{2+}	1	1	0
H^+	$10^{-7.4}$ M or pH 7.4	$10^{-7.2}$ M or pH 7.2	
Cl^-*	120	20	-48

Total $[Ca^{2+}]_i$ is about 2 mM, and total $[Na^+]_i$ is about 30 mM. In both cases, most of the ion is sequestered in intracellular organelles or is bound to proteins. Values given are estimates of the free concentration in the cytoplasm.

* Like other cells in mammal, the cardiac myocytes contain equal quantity of positive (+) and negative (-) charges (that is, be electrically neutral). Thus, in addition of Cl^-, the cells contain many other anions, such as HCO_3^-, PO_4^{3-}, proteins, nucleic acids, metabolites carrying phosphate and carboxyl groups, etc. (not listed in this table).

** Equilibrium Potentials were calculated from the Nernst equation at 37°C assuming equal intra- and extracellular activity coefficients.

I-4-1 Extracellular K^+ Accumulation ($[K^+]_o$-↑)

Under physiological conditions, $[K^+]_o$ is low and $[K^+]_i$ is high (see Table 1). Passive K^+ efflux is compensated by active K^+ influx via the Na^+-K^+ pump. During myocardial ischemia, this dynamic equilibrium is broken, and external K^+ accumulates. Increase in $[K^+]_o$ or net cellular K loss is one of the earliest consequences of myocardial ischemia. The external K^+ accumulates has profound electrophysiological effects (Janse & Wit, 1989). The most deteriorative effect is membrane depolarization, which leads to slowing of conduction and altered refractoriness, in concert with other factors, triggers reentrant ventricular arrhythmias causing SCD.

I-4-1-1 The Temporal Course of $[K^+]_o$-↑ during Myocardial Ischemia

$[K^+]_o$-↑ in the myocardium following net cellular K^+ loss ($[K^+]_i$-↓) is one of the earliest consequences of myocardial hypoxia and ischemia, beginning within 15-30s (Carmeliet, 1999; Gasser & Vaughan-Jones, 1990; HARRIS *et al.*, 1954; Hirche *et al.*, 1980; Weiss & Shine, 1982b; Weiss & Shine, 1982a; Weiss & Shine, 1986; Zeng *et al.*, 1995). During the first 10–15 min of myocardial ischemia, $[K^+]_o$ rises 4-5 fold from its normoxic value (Hirche *et al.*, 1980) to a plateau level of 6–11 mM above its initial value. During this plateau phase, $[K^+]_o$ remains almost constant for several minutes, and, in some preparations, it may eventually decrease (Knopf *et al.*, 1990; Wilde *et al.*, 1988). Thereafter, a slower second increase of $[K^+]_o$ takes place simultaneously with the arrest of glycolytic activity and cell death (Sakamoto *et al.*, 1998; Sakamoto *et al.*, 1997). This triphasic pattern is qualitatively similar in hearts of different species, but the time course is influenced by heart rate (Weiss & Shine, 1982a; Weiss & Shine, 1986; Wilde *et al.*, 1990; Wilde & Aksnes, 1995) and experimental conditions (Carmeliet, 1999; Wilde & Aksnes, 1995). APD-↓ or QT-↓ is associated with these changes of $[K^+]_o$. It is well known that $[K^+]_o$-↑ is related to profound modifications of the electrical behavior of ischemic cardiomyocytes. In particular, it contributes to depolarization of resting membrane potential, APD-↓, decrease in the maximum upstroke velocity of AP, and decrease in excitability. These electrophysiological alterations are pivotal in the genesis of ischemic arrhythmias and SCD. Noticeably, APD-↓ or QT-↓ favors the reentrant arrhythmias and DADs, a reproducible pattern of dysrhythmic activities during acute

myocardial ischemia (Wilde *et al.*, 1988). This typical pattern of ischemic electrical disorders and arrhythmias are therefore tentatively referred as "short QT syndrome" ("SQTS").

Because of the importance of $[K^+]_o$-↑ along with "SQTS" during acute myocardial ischemia, the mechanisms responsible for those disorders have been a subject of intensive research. It is generally believed that ischemic electrical disorders are a consequence of interrelated electrolyte disturbance and metabolic stress. In other words, ischemic $[K^+]_o$-↑ and "SQTS" are an event involving the interplay between ionic channels and metabolic substrates. To aid in the comprehension of the mechanisms underlying those electrical and metabolic disturbances, it is necessary to depict the ionic mechanisms and metabolic regulation for ischemic $[K^+]_o$-↑ and "SQTS".

The temporal course of $[K^+]_o$-↑ during myocardial ischemia typically occurs in three phases. $[K^+]_o$-↑ or net cellular K^+ loss begins within 15-30 seconds after the occlusion of a coronary artery, then the increase reaches a plateau after 3-10 min and after that is followed by a third slower increase starting between 15 and 30 min (Wilde & Aksnes, 1995) (Figure 3).

As a matter of fact, the temporal course of the actual levels of $[K^+]_o$ vary widely among species, the models used, the rate of beating or pacing, and the activation of the sympathetic nervous system. Plateau level of $[K^+]_o$ is highest (20 mM) in the isolated guinea pig heart (Kleber, 1983), 10-11 mM in the in situ pig (Firek & Weingart, 1995) and the rabbit perfused septum (Weiss & Shine, 1982a), and 8-9 mM in the Langendorff-perfused rat (Wu & Corr, 1992). The perfused rabbit papillary muscle shows a broad range of $[K^+]_o$ levels from 8 to 20 mM that depend on the diameter of the preparation. The difference has been correlated to a much more pronounced acidosis in the center of thick preparations (Cascio *et al.*, 1992). Rate of stimulation is important between 0 and 60-90 min^{-1}, but not above this value (Harper, Jr. *et al.*, 1993; Weiss & Shine, 1986). In the absence of stimulation, $[K^+]_o$ accumulation starts with a delay and its rate of accumulation is clearly less rapid; an outspoken plateau is not present. Stimulation of the sympathetic nervous system on the other hand causes a faster rise in $[K^+]_o$ (Wilde *et al.*, 1988).

During the plateau, $[K^+]_o$ is constant or slowly changing in the positive or negative direction (Firek & Weingart, 1995; Fleet *et al.*, 1985). The preparation remains excitable

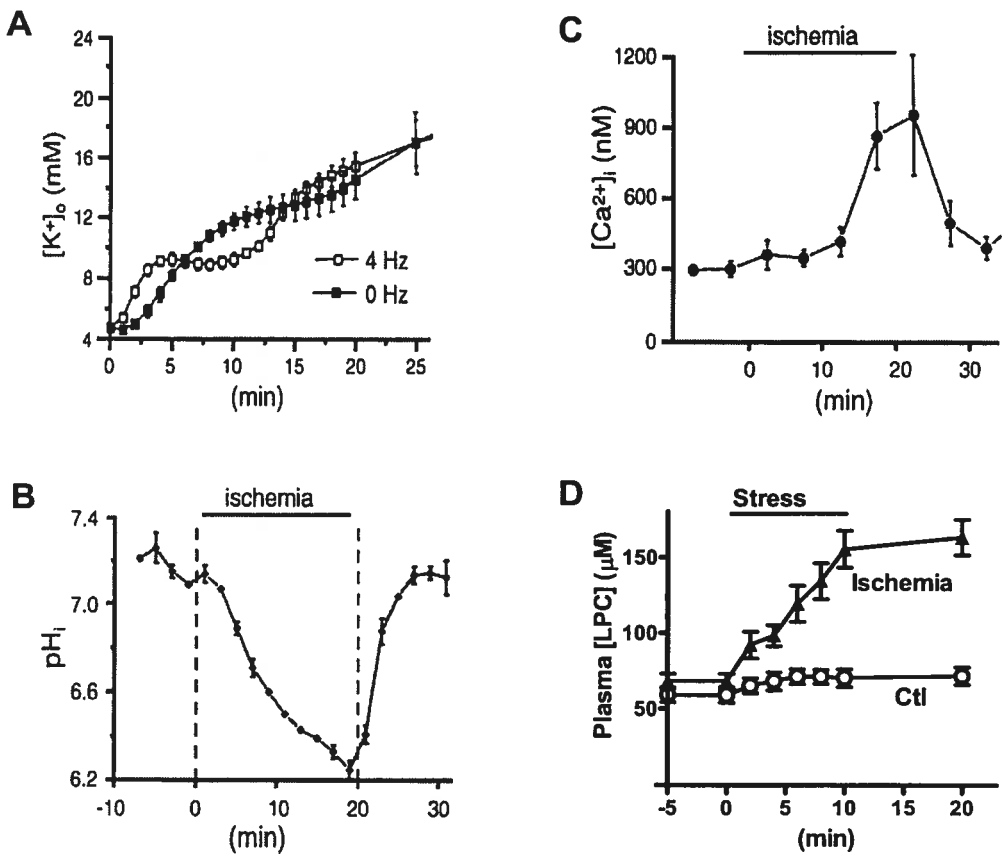

Figure 3. Time-courses of $[K^+]_o$, pH_i, $[Ca^{2+}]_i$, and plasma LPC during ischemia. A: increase of $[K^+]_o$ during ischemia in Langendorff-perfused quiescent and stimulated (4 Hz) rat hearts, measured with K^+-sensitive electrodes inserted in mid left myocardium. Note faster rise but lower plateau during stimulation; effect on plateau has been interpreted as due to activation of Na^+-K^+ pump. [Modification from (Wilde & Aksnes, 1995).] **B:** fall in intracellular pH (^{31}P-NMR) during ischemia and fast recovery upon reperfusion in perfused ferret hearts. [Modification from (Marban *et al.*, 1990).] **C:** changes in free $[Ca^{2+}]_i$ (NMR with 5F-BAPTA) in perfused ferret hearts during 20 min of ischemia followed by reperfusion. Increase in $[Ca^{2+}]_i$ is delayed compared with changes in $[K^+]_o$, or pH. [Modification from (Marban *et al.*, 1990).] **D:** changes in coronary sinus concentration of LPC in patients in response to atrial pacing. Results are shown for patients with and without coronary heart disease as judged by a positive stress test. [Modification from (Sedis *et al.*, 1990).]

or regains its excitability. When stimulation is ceased, $[K^+]_o$ falls, suggesting an active Na^+-K^+ pump (Weiss & Shine, 1986). It is also reported that catecholamines are massively released from nerve endings and cells (Schomig *et al.*, 1987), glycolysis is stimulated, and lactate production is increased during the plateau. The plateau level of $[K^+]_o$ is dependent on glycolytic activity (Wilde *et al.*, 1988). When the heart is depleted of glycogen during successive ischemia periods, lactate production (Gwilt *et al.*, 1993) and acidification (Firek & Weingart, 1995) are less, and $[K^+]_o$ rises faster.

In the late phase of acute ischemia (over 20-30 min), $[K^+]_o$ starts to rise again. This phase is accompanied by inexcitability, an increase of longitudinal resistance, elevation of the resting tension, and development of rigor contracture (Cascio *et al.*, 1990;Weiss & Shine, 1982b). Lactate production stops (Fiolet *et al.*, 1984), and extracellular pH (pH_o) remains at a low level. The metabolic changes suggest that anaerobic glycolysis is blocked.

I-4-1-2 Effects of $[K^+]_o$-↑ on Ion Channels and Carriers

The effect of the $[K^+]_o$-↑ on channels is mainly indirect via the depolarization of sarcolemma membrane it causes. The extent of depolarization during acute myocardial ischemia is responsible for a partial or complete inactivation of the fast Na^+ channel, the T-type Ca^{2+} channel, and I_{to}, and I_{Kur}. Recovery from inactivation of these currents is slowed and is accompanied by a decrease in excitability, prolongation of refractoriness, and conduction slowing of the AP.

For the majority of K^+ channels the conductance is increased when $[K^+]_o$ rises; I_{K1} is the most sensitive one (Carmeliet, 1987), but the voltage-activated currents such as I_{Kr} (Scamps & Carmeliet, 1989) and I_{to} (Firek & Giles, 1995), as well as the ligand-gated K^+ channels such as the fatty acid and amphiphile activated K^+ channels (K_{AA}), the K_{ACh}, the K_{ATP}, and the K_{Na} all increase their conductance and carry more current at elevated $[K^+]_o$. In the case of I_{K1} and I_{Kr}, the mechanism is less pronounced inward rectification, consequent to a smaller block by intracellular cations (I_{K1}) or smaller inactivation (I_{Kr}). These changes again will stabilize the E_m and reduce excitability. Worthwhile to mention is the enhanced conductance of I_f of which the Na^+-carrying capability is increased (Frace *et al.*, 1992; Ho *et al.*, 1993). Because this current is only activated at hyperpolarized

levels, the functional implications of this change remain limited, except transiently during reperfusion.

I-4-1-3 Effects of $[K^+]_o$-↑ on Electrophysiological Properties

At the multicellular level, the changes by $[K^+]_o$-↑ will cause the cell to depolarize and the AP to be reduced in amplitude, rate of rise, and duration. Inactivation of the Na^+ conductance, concomitant to the depolarization, is responsible for the fall in AP amplitude and rate of rise and the decrease in excitability. Transiently, however, excitability may be increased, the reason being that the depolarization, although causing inactivation of the I_{Na}, at the same time moves the E_m closer to threshold with a resultant reduction in the current required to reach threshold (Dominguez & Fozzard, 1970). At depolarized levels, the recovery from inactivation is slower; in the presence of increased $[K^+]_o$, the result is a prolongation of the postrepolarization refractoriness (Janse & Wit, 1989). Conduction velocity is depressed and slowed, especially during the relative refractory period (Kleber, 1987). APD-↓ is mainly due to an increase of the I_{K1} and I_{Kr} conductance and to a lesser extent to a decrease of Na^+ conductance. The changes in excitability, refractoriness, and conduction, together with the shortening of the APD, favor the occurrence of reentrant arrhythmias.

I-4-2 Increase in Intracellular Ca^{2+} Concentration ($[Ca^{2+}]_i$-↑)

I-4-2-1 Ca^{2+} Distribution

Under normal conditions, concentrations of Ca^{2+} differ in the cytosol, the SR, the mitochondria, and the nucleus (Piper *et al.*, 1993). Ca^{2+} plays a different role in each compartment, and modulates the activity of myofilaments in the cytosol and the activity of ionic channels in the plasma membrane. The SR acts as a store for Ca^{2+} and modulates contraction and relaxation. In the mitochondria, the free $[Ca^{2+}]$ regulates the activity of three dehydrogenases (pyruvate dehydrogenase, α-oxoglutarate dehydrogenase, and NAD-dependent isocitrate dehydrogenase) and adapts in that way energy production to energy utilization. In the nucleus, Ca^{2+} modulates gene expression.

During myocardial ischemia, diastolic free $[Ca^{2+}]_i$ rises with some delay. The delay is variable, and quite important differences in time course have been reported, depending on the model. In studies of ischemia (Friedman *et al.*, 1973), the delay attained 10-20 min and the rise in $[Ca^{2+}]_i$ slightly preceded the development of contracture and uncoupling of the gap junctions (Figure 3) (Marban *et al.*, 1990; Wagner *et al.*, 1990). A much shorter delay between 2 and 5 min has been described in other studies (Kihara *et al.*, 1989; Mohabir *et al.*, 1991b). A large variation in time course also exists in experiments on single cells subjected to hypoxia or metabolic inhibition (Russ *et al.*, 1996). The reasons for these divergent results are not known but are probably related to the intensity of the metabolic blockade.

I-4-2-2 Mechanisms underlying $[Ca^{2+}]_i$-↑

The $[Ca^{2+}]_i$-↑ during myocardial ischemia results from a less efficient removal from the myocytes via the Na^+/Ca^{2+} exchanger (NCX) and a reduced Ca^{2+} uptake in the sarcoplasmic reticulum (SR), an increased inward Ca^{2+} leak through the plasma membrane, and displacement of Ca^{2+} from binding sites in the cytoplasm and in mitochondria by H^+ (Carmeliet, 1999).

The most important mechanism for removal of Ca^{2+} from the cell is the NCX, which is responsible for 77% of the Ca^{2+} extrusion (Lamont & Eisner, 1996; Russ *et al.*, 1996). During ischemia the NCX is less efficient because of $[Na^+]_i$-↑ and $[H^+]_i$-↑. An $[Na^+]_i$-↑ promotes the reversed mode of the NCX whereby Ca^{2+} is entering the cell and Na^+ is removed (Haigney *et al.*, 1994; Smith & Allen, 1988; Tani & Neely, 1989). All mechanisms responsible for an $[Na^+]_i$-↑ thus also contribute to the $[Ca^{2+}]_i$-↑ via the NCX. The efficiency of the exchanger is furthermore reduced by acidosis (Doering & Lederer, 1993; Philipson *et al.*, 1982) and the influence of radicals (Coetzee *et al.*, 1994).

Coincident with metabolic inhibition occurring myocardial ischemia background channels carrying Ca^{2+} inward become more important as they are activated by radicals (Jabr & Cole, 1995; Wang *et al.*, 1995c). Inward leak of Ca^{2+} also occurs via non-specific cation channels that are activated by $[ATP]_o$ (Friel & Bean, 1988; Hirano *et al.*, 1991), mechanical stretch (Craelius *et al.*, 1988; Kim, 1993; Sasaki *et al.*, 1992), and a rise in $[Ca^{2+}]_i$ itself (Colquhoun *et al.*, 1981; Ehara *et al.*, 1988; Matsuda, 1983).

I-4-2-3 Effects of $[Ca^{2+}]_i$-↑ on Currents and Carriers

An $[Ca^{2+}]_i$-↑ modulates multiple currents in cardiac myocytes. $I_{Ca\text{-}L}$ *per se* is very sensitive to $[Ca^{2+}]_i$-↑ and shows an increased rate of inactivation, limiting in this way the Ca^{2+} load of the cell (McDonald *et al.*, 1994). In contrast, the current may also undergo facilitation for moderate increases of the Ca^{2+} level (Kass *et al.*, 1978). Some currents rely on $[Ca^{2+}]_i$ for their activation, such as I_{NSC} (Colquhoun *et al.*, 1981; Ehara *et al.*, 1988), the Ca^{2+} release channel (Cannell *et al.*, 1995a; Fabiato, 1985), and the NCX (Miura & Kimura, 1989). An $[Ca^{2+}]_i$-↑ stimulates the NCX and counteracts the Na^+-induced inactivation in this way (Matsuda, 1996). Other currents are modulated; an increase is seen for the $I_{Ca\text{-}T}$ (Tseng & Boyden, 1991), I_{Ks} (Nitta *et al.*, 1994; Tohse *et al.*, 1987), I_{Kr} (Scamps & Carmeliet, 1989), I_f (Hagiwara & Irisawa, 1989), the Na^+-K^+ pump (Gao *et al.*, 1996). Two currents are inhibited: the I_{K1} (Martynyuk *et al.*, 1996; Mazzanti & DeFelice, 1990; Mazzanti & DiFrancesco, 1989), a phenomenon comparable to the block by $[Mg^{2+}]_i$, and the gap junction channel (De Mello, 1975; Noma & Tsuboi, 1987).

I-4-2-4 Electrophysiological Effects of $[Ca^{2+}]_i$-↑

Enhancement of $I_{L\text{-}Ca}$ and $I_{T\text{-}Ca}$ inactivation induced by Ca^{2+}, increase of the transient Ca^{2+}-activated Cl^- current, and of the I_{Kr} and I_{Ks} will lead to APD-↓, which is prominent at the early phase of acute myocardial ischemia. As the $[Ca^{2+}]_i$-↑ is more pronounced in Ca^{2+} overload, EAD as well as DAD are generated, and may result in arrhythmias (Kihara *et al.*, 1989; Thandroyen *et al.*, 1991). One of the mechanisms underlying EAD is reactivation of $I_{L\text{-}Ca}$ (Ming *et al.*, 1994), but also release of Ca^{2+} from the SR (Priori & Corr, 1990), followed by activation of the "transient inward current" can cause EAD. The transient inward current is a composite current and is carried through the Cl^- channel (Papp *et al.*, 1995; Sipido *et al.*, 1995), the NSC channels (Cannell & Lederer, 1986; Han & Ferrier, 1992), and the NCX (Han & Ferrier, 1995; Laflamme & Becker, 1996; Nattel, 2002). Besides cytoplasmic Ca^{2+}, the other factors such as ATP (Xu *et al.*, 1996), the phosphorylation state of the channel (Patel *et al.*, 1995;Valdivia *et al.*, 1995), and luminal $[Ca^{2+}]$ (Sitsapesan & Williams, 1994) are also involved in determining the efficiency of the release mechanism (Cannell *et al.*, 1995b). Under conditions of moderate Ca^{2+} load, the release is linearly related to the luminal $[Ca^{2+}]$ concentration and

is not propagated. With increase of Ca^{2+} load, however, release becomes regenerative and propagated (Bassani *et al.*, 1995; Han *et al.*, 1994; Janczewski *et al.*, 1995; Satoh *et al.*, 1997). Under these conditions, release may be spontaneous and occur in the absence of trigger Ca^{2+}. The result is EAD during the AP or DAD, following repolarization. Important to note is that Ca^{2+} overload also increases the tendency of Purkinje fibers to become spontaneously active; the activation curve of the I_f current is shifted in the positive direction (Hagiwara & Irisawa, 1989; Zaza *et al.*, 1991), which favors activation of the current and increases the rate of diastolic depolarization. The fall in gap junction conductance by $[Ca^{2+}]_i$ further enhances the probability for arrhythmias by reducing conduction (De Mello, 1975; Noma & Tsuboi, 1987).

I-5 Metabolic Disturbances during Myocardial Ischemia

In concert with the ionic disturbances a variety of noxious metabolites accumulate within the ischemic region, impinge on the sarcolemma, and interact with integral membrane proteins including ion channels and exchangers, and thereby contribute to electrophysiological derangements. Over the last decade a great mount of experimental evidence has shed light on understanding of the substances involved, their sites of synthesis and subcellular distribution, and the factors that modulate their accumulation, as well as provided insight into the mechanisms by which they contribute to ischemic arrhythmias. In this introduction, only radicals, LPC, Ceramide, and TNF-α are addressed. This does not exclude the importance of other metabolites but rather supports the notion that multiple factors interact to contribute to the marked heterogeneous electrophysiological disturbances during myocardial ischemia.

I-5-1 Phospholipid Metabolites and Ischemic Arrhythmias

I-5-1-1 Accumulation of Lysophosphatidylcholine during Myocardial Ischemia

The cell surface membrane or sarcolemma is primarily composed of phospholipid, cholesterol, and proteins which together form a complex dynamic structure which retains the intracellular contents, regulates ion homeostasis, governs nutrient transport, and transduces extracellular signals into the cells. The principal classes of sarcolemmal

phospholipids are, according to their relative abundance, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, and phosphatidylserine (Table 2).

Table 2. Phospholipid Composition of Myocardial Sarcolemma

Phospholipid Class	Relative Composition (%)
Phosphatidylcholine (PC)	47
Phosphatidylethanolamine (PE)	28
Sphingomylin	11
Phosphatidylinositol (PI)	6
Phosphotidylserine (PS)	5
Lysophosphatidylethanolamine (LPE)	2
Lysophosphatidylcholine (LPC)	1

[Adapted from (Corr *et al.*, 1995) with modification]

The sarcolemmal phospholipids are composed of a charged polar headgroup region and two non-polar covalently bound long-chain aliphatic hydrocarbon moieties. Under the situation of myocardial ischemia, the phospholipids (e.g. phosphatidylcholine) are metabolized to generate lysophosphatidylcholines (LPCs). LPCs are amphiphile possessing a charged headgroup like phospholipids but only a single aliphatic hydrocarbon chain as a consequence of the hydrolytic cleavage of one of the two aliphatic hydrocarbon groups of phosphatidylcholine (Choy *et al.*, 1997; Hatch *et al.*, 1989). The major species of LPCs produced in ischemic myocardium is 1-palmitoyl-lysophosphatidylcholine (LPC-16), which possesses 16 hydrocarbons in its aliphatic chain. As an intermediate of metabolism of phosphatidylcholine, LPC-16 is present in a variety of mammalian tissues (Prokazova *et al.*, 1998) and accumulates rapidly in the heart during myocardial ischemia and in diabetic cardiomyopathy (Corr *et al.*, 1995; Kinnaird *et al.*, 1988; Makino *et al.*, 1987; Saffitz *et al.*, 1984). The time course of LPCs release from ischemic human myocardium parallels the time course of early ischemic ventricular arrhythmia (Sedlis *et al.*, 1997). Accumulation of LPC-16 is thought to be a major contributor to the development of cardiac arrhythmias during myocardial ischemia

(Corr *et al.*, 1987a; Corr *et al.*, 1987b; Fazekas *et al.*, 1992; Man, 1988; Saffitz *et al.*, 1984).

I-5-1-2 Effects of LPCs on Currents and Carriers and the Mechanisms

In guinea-pig ventricular myocytes, LPC-16 at concentrations over 10 μM irreversibly depressed the amplitude of I_{Na} within 0.5-3 min, retarded the time course of activation and inactivation of I_{Na} (Burnashev *et al.*, 1991; Sato *et al.*, 1992; Shander *et al.*, 1996), and the reduction of I_{Na} was followed by cell contracture or cell death (Sato *et al.*, 1992). It was further revealed that LPC modulates I_{Na} in these cells by a pathway that involves both PKC-dependent and tyrosine kinase dependent phosphorylation (Watson & Gold, 1997). In rat ventricular cells, applying LPC (9-25 μM) to the inner side of the membrane reduced peak I_{Na} and prolonged the time course of I_{Na} inactivation (Burnashev *et al.*, 1991). It has been reported that the $I_{L\text{-}Ca}$ was reduced (Clarkson & ten Eick, 1983; Wu & Corr, 1992; Ziolo *et al.*, 2001), or not changed (Liu *et al.*, 1991), or increased (Sedlis *et al.*, 1983; Ver *et al.*, 1992; Woodley *et al.*, 1991). LPC (10-50 μM) causes a concentration-dependent decrease in outward K^+ conductance, providing one possible explanation for the mechanism responsible for the decrease in resting membrane potential (Clarkson & ten Eick, 1983; Kiyosue & Arita, 1986). It was also reported that exogenous LPC induces a Ca^{2+} permeable I_{NSC} in cardiac myocytes (Magishi *et al.*, 1996), the mechanism involved in a G_i/G_o-coupled receptor and Rho-mediated pathway (Li *et al.*, 2002b). LPC-16 markedly reduces the single-channel conductance of I_{K1} with no change in P_o (Clarkson & ten Eick, 1983; Kiyosue & Arita, 1986; Sato *et al.*, 1993).

In the whole the different mechanisms have been proposed to explain the effects of LPCs on channels and carriers based on the experimental data and the chemical properties of LPCs: (1) Relatively low concentrations (<5μM) of LPCs may interact directly with the channel protein (Lamers *et al.*, 1984). Because of their charge, they can affect the permeation pathway or change the local concentrations of the substrate ion or other important ions or by interfering with the charge of the phospholipids surrounding the channels, thereby affecting the gating characteristics of channels (Inoue & Pappano, 1983). (2) High concentrations (>20μM) may incorporate in the myocardial sarcolemma thereby affect membrane fluidity and/or disrupt the cytoskeleton. Incorporation of LPCs in the membrane has been measured experimentally and results in a significant

perturbation of the bilayer structure (Gruver & Pappano, 1993). Mechanical destabilization and dysfunction of membrane proteins can also be the result of disruption of the cytoskeleton. Such destabilization has been shown to occur with LPCs (Undrovinas *et al.*, 1995). (3) By activating LPCs receptors anchoring in the cell membrane, and then modulating ion channel functions via a variety of intracellular signaling pathways, such as PIP_2, PKA, PKB (Thors *et al.*, 2003), PKC, and tyrosine kinase mediated pathways.

I-5-1-3 Electrophysiological Effects of LPCs

APD-↓ by LPC-16 has been documented in rabbit atrial and ventricular cells (Fazekas *et al.*, 1992), guinea pig ventricular cells (Liu *et al.*, 1991), and canine ventricular cells (Saffitz *et al.*, 1984) and Purkinje fibers (Corr *et al.*, 1995). LPC-16 levels correlate with the occurrence of arrhythmias in ischemic and diabetic hearts (Corr *et al.*, 1987b; Kinnaird *et al.*, 1988; Man, 1988). Intriguingly, the concentrations of LPC-16 required to elicit these effects are decreased three-fold by a reduction in pH to 6.7, analogous to that in ischemic myocardium in vivo (Corr *et al.*, 1981; Snyder *et al.*, 1981). Coincidentally, it was reported that LPC-16 decreases tissue K^+ content by ~15%, an effect associated with gradual APD shortening and increased K^+ efflux (Goldhaber *et al.*, 1998). Pretreatment with cromakalim to selectively activate K_{ATP} channels shortened APD and had no effect on net tissue K^+ content during control aerobic perfusion. However, cromakalim increased net K^+ loss during exposure to LPC-16 (Corr *et al.*, 1981; Goldhaber *et al.*, 1998; Snyder *et al.*, 1981). Thus inhibition of LPC-16 production and accumulation may be a promising therapeutic strategy to attenuate the incidence of lethal arrhythmias associated with ischemic heart disease (Corr *et al.*, 1995; Corr & Yamada, 1995; Giffin *et al.*, 1988; Man, 1988).

It has been suggested that increased K^+ efflux during myocardial hypoxia and ischemia may result from extrusion of intracellularly generated anions such as lactate and inorganic phosphate (Pi) as a mechanism of balancing transsarcolemmal charge movement (Gaspardone *et al.*, 1986). Particularly, net cellular L-lactate efflux associated with accelerated anaerobic glycolysis has been implicated as a potential cause of the marked cellular K^+ loss contributing to lethal cardiac arrhythmias in ischemic heart. However, the data against this notion also exist. In isolated arterially perfused rabbit

interventricular septa, selective inhibition of glycolysis caused a marked increase in K^+ efflux despite a fall in lactate production and maintenance of normal cellular high-energy phosphate content (Weiss *et al.*, 1989). In isolated rat hearts rendered globally ischemic, no correlation between early lactate and K^+ efflux rates was found (Kantor *et al.*, 1990).

I-5-2 Sphingolipid Metabolites and Myocardial Ischemia

Although sphingolipid is an abundant component in myocardial sarcolemma, it is only recently that the first evidence was provided in favor of a biomodulatory role of some sorts of sphingolipid, in particular the sphingomyelin (SM) metabolites (Hannun & Luberto, 2000; Spiegel & Merrill, Jr., 1996). A wealth of reports has now accumulated, on a wide variety of cell types of different animal species, supporting an unquestionable role of sphingolipid in regulating numerous cell functions and possibly mediating the effects of extracellular stimuli (Alewijnse *et al.*, 2004; Levade *et al.*, 2001; Tabas, 2004; Levade *et al.*, 2001; Tabas, 2004; Alewijnse *et al.*, 2004). Some studies have indicated that the sphingolipid metabolites are involved in the reduction of myocardial contractility during ischemia and reperfusion (Oral *et al.*, 1997).

Upon various stimuli, cells metabolize SM from the cellular plasma membrane to form sphingosylphosphorylcholine (SPC) or ceramide. The latter can be further metabolized to sphingosine and then sphingosine-1-phosphate (S1P). Ceramide can be also generated from pamitoyl CoA and serine through *de novo* synthesis. Once generated ceramide can serve as a key second messenger molecule that is capable of mediating multiple physiological effects including regulation of cellular differentiation, proliferation, and apoptosis by activating a variety of signaling cascades, including those triggered by cytokines, growth factors, and stress (Kolesnick, 2002; Mathias *et al.*, 1998; Ohanian & Ohanian, 2001). Ceramide generation can be triggered by diverse stimuli in a wide variety of cell types during apoptosis induced by TNF-α, serum deprivation, ischemia or hypoxia, and other insults (Figure 4) (Kolesnick, 2002; Mathias *et al.*, 1998; Ohanian & Ohanian, 2001).

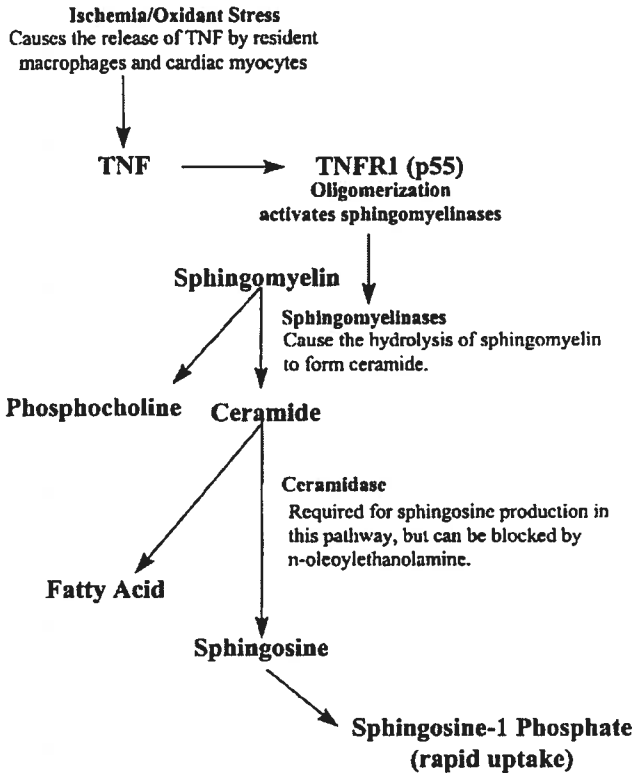

Figure 4. Schematic of agonist induced generation of sphingosine after activation of sphingomyelinases to cause sphingomyelin hydrolysis and ceramide production. [Adapted from (Friedrichs *et al.*, 2002)].

I-5-2-1 Accumulation of Ceramide during Myocardial Ischemia

Unlike phospholipid metabolites such as LPC-16 that accumulates very rapidly during the early phase myocardial ischemia, accumulation of ceramide is pronounced progressively during myocardial ischemia. In anesthetized rats, 30 min of ischemia had no apparent effect on ceramide concentrations in the myocardium, while 30 min of ischemia followed by 3 h of reperfusion resulted in a significant increase in ceramide by 48 % (Zhang *et al.*, 2001). Moreover, different ceramide may play different roles in the process of ischemic injury as demonstrated that only ceramide containing palmitic, stearic, oleic, linoleic, and arachidonic acid accumulate significantly in ischemic rat hearts (Beresewicz *et al.*, 2002). However, it has also been reported that the activation of neutral

sphingomyelinase (NSMase) and accumulation of ceramide is one of the earliest responses of cardiac myocytes to hypoxia and reoxygenation. Ceramide increased abruptly after reoxygenation, peaking at 10 minutes with 225±40% of the control level. NSMase activity was induced with similar kinetics, and both activities remained elevated for several hours. c-Jun N-terminal kinase (JNK) was also activated within the same time frame. Treatment of cardiac myocytes with extracellular ceramide also activated JNK. Pretreating cells with antioxidants quenched sphingomyelinase activation, ceramide accumulation, and JNK activation (Hernandez *et al.*, 2000a).

I-5-2-2 Effects of Sphingolipid Metabolites on Currents and Carriers

Numerous studies have indicated that SM metabolites can alter intracellular ion concentrations in the heart by regulating the activity of various ion channels in the membranes of the sarcoplasmic reticulum and/or in the cell membrane. Sphingosine was consistently found to reduce free $[Ca^{2+}]_i$ in neonatal rat (McDonald *et al.*, 1994), adult rat (Amadou *et al.*, 2002; McDonald *et al.*, 1994), guinea-pig (Sugishita *et al.*, 1999b) and feline cardiomyocytes (Oral *et al.*, 1997). It has been reported that ceramide and S1P can increase free $[Ca^{2+}]_i$ in cardiomyocytes from adult (Liu & Kennedy, 2003;Relling *et al.*, 2003) and neonatal rats (Nakajima *et al.*, 2000).

Sphingosine, SPC and S1P were reported to inhibit rat cardiac Na^+ channels (MacDonell *et al.*, 1998; Yasui & Palade, 1996). Interestingly, the S1P effects on the channel were not PTX-sensitive (MacDonell *et al.*, 1998). Sphingosine-induced decrease in $I_{Ca\text{-}L}$ has been reported in cardiomyocytes from neonatal rats (McDonough *et al.*, 1994), adult rats (McDonough *et al.*, 1994; Yasui & Palade, 1996) and cats (Friedrichs *et al.*, 2002). A reduced L-type Ca^{2+} channel activity was also reported in rat cardiomyocytes for SPC (Yasui & Palade, 1996) and ceramide (Liu & Kennedy, 2003). In rabbit isolated sinoatrial node cells, S1P did not affect the basal activity of L-type Ca^{2+} channels but reversed the effects of the β-adrenergic agonist isoprenaline (Guo *et al.*, 1999). More recently, it was reported that sphingosine exerts direct inhibitory effects on the AP and $I_{Ca\text{-}L}$ in isolated feline myocytes (Friedrichs *et al.*, 2002), consistent with previously reported sphingosine activity on $I_{Ca\text{-}L}$ in isolated rat myocytes (McDonough *et al.*, 1994;

Yasui & Palade, 1996). Thus, it appears that all SM metabolites exert some inhibitory effect upon cardiac L-type Ca^{2+} channels.

In the view of SM metabolites on cardiac K^+ channels, it has been consistently found that S1P activates I_{KACh} in guinea-pig atrial myocytes (Bunemann *et al.*, 1995; Bunemann *et al.*, 1996; Himmel *et al.*, 2000; Liliom *et al.*, 2001; van Koppen *et al.*, 1996), in freshly isolated myocytes from mouse and human atrium (Himmel *et al.*, 2000) and in rabbit isolated sinoatrial node cells (Guo *et al.*, 1999). The S1P effects in the guinea-pig atrium were inhibited by pre-treatment with PTX (Bunemann *et al.*, 1995) or with suramin (Himmel *et al.*, 2000). SPC was found to mimic the S1P effects in guinea-pig atrial myocytes, and the two SMM caused cross-desensitization of each other (Bunemann *et al.*, 1996; Liliom *et al.*, 2001). Since these studies observed no cross-desensitization with acetylcholine acting on muscarinic receptors, it was proposed that S1P and SPC may act on the same receptor. It was further proposed that S1P and SPC account for the plasma-induced activation of I_{KACh} in guinea-pig atrial myocytes (Liliom *et al.*, 2001). In contrast to S1P, SPC did not activate I_{KACh} in freshly isolated myocytes from mouse and human atrium (Himmel *et al.*, 2000). In rabbit isolated sinoatrial node cells, S1P did not alter basal activity of the I_f channel but reversed the isoprenaline effects (Guo *et al.*, 1999). In rat ventricular myocardium, S1P did not significantly affect the inward rectifier K^+ channel (MacDonell *et al.*, 1998).

The role of ceramide in modulating non-cardiac K^+ channel function has also recently been revealed, including inward rectifier K^+ currents, voltage-gated shaker type K^+ channels, and the voltage-gated n-K^+ channels in lymphocyte (Gulbins *et al.*, 1997; Hida *et al.*, 1998; Wu *et al.*, 2001; Zhang *et al.*, 2002), indicating that ceramide may be involved in regulating cell excitability and membrane potentials. Too few data are available to determine ceramide effects on cardiac ion homeostasis.

I-5-2-3 Cardiac Effects of Sphingolipid Metabolites

In cat myocytes, sphingosine markedly decreased APD and lowered the plateau of AP. After administration of TNF-α, sphingomyelin, C2-ceramide, or sphingosine, only C2-ceramide and sphingosine depressed cardiac function in normal rats (Friedrichs *et al.*, 2002). NCX activity was studied in transfected Chinese hamster ovary (CHO) cells expressing the wild-type cardiac NCX1.1 or mutants created by site-directed mutagenesis.

The activity of the wild-type exchanger, but not exchanger mutants deficient in Ca^{2+}-dependent activation, was inhibited by ceramide and sphingosine (Condrescu *et al.*, 2002). C2 ceramide (1 nM-1 μM) caused a concentration-dependent inhibition of $I_{Ca\text{-}L}$ and increased the rate of $I_{Ca\text{-}L}$ inactivation without altering its gating properties in adult rat ventricular myocytes (Schreur & Liu, 1997). An inactive ceramide analog failed to inhibit $I_{Ca\text{-}L}$. At submaximal concentrations, effects of C2 ceramide and IL-1 beta on $I_{Ca\text{-}L}$ were additive and saturable. In the presence of a maximally effective concentration of IL-1 beta, C2 ceramide had no further effect on $I_{Ca\text{-}L}$. These results suggest that ceramide mediates IL-1 beta-induced suppression of cardiac $I_{Ca\text{-}L}$.

Volume-sensitive chloride channels ($I_{Cl,vol}$) are known to play a key role in the regulation of cell volume, thereby apoptosis, by a mechanism called apoptotic volume decrease (AVD). In adult rabbit ventricular cardiomyocytes, exposure of cardiomyocytes to 1 μM doxorubicin, a stimulus for generating ceramide, induced a rapid and significant reduction in cell volume of cardiomyocytes (average of 15%), i.e. AVD as well as increases in the early markers of apoptosis. The crucial role of $I_{Cl,vol}$ during AVD and apoptosis was confirmed using C2-ceramide, demonstrating that activation of $I_{Cl,vol}$ plays a major role in the mechanism leading to cell shrinkage and apoptosis-induced AVD by C2-ceramide (d'Anglemont *et al.*, 2004). Using an adult rat ventricular myocyte culture model, it was found that Ca^{2+} modulates Ceramide-induced cardiac myocyte apoptosis through the coupling of B-type Ca^{2+} channels in mitochondrial membrane (Henaff *et al.*, 2002).

The effects of SM metabolites on cardiac rhythm have been investigated using cultured cardiomyocytes, isolated sinoatrial node cells, isolated hearts and *in vivo* studies. These studies support the notion that SM metabolites lower heart rate under most but not all conditions. It was found that S1P increases SA rate in dogs (Sugiyama *et al.*, 2000b) but reduces the basal rate and reverse the effects of the β-adrenergic agonist isoprenaline in rabbits (Guo *et al.*, 1999). Sphingosine reduced the beating rate of neonatal rat isolated cardiomyocytes (Benediktsdottir *et al.*, 2002). SPC reduced the rate of isolated guinea-pig hearts (Liliom *et al.*, 2001). The systemic administration of S1P reduced heart rate in rats (Benediktsdottir *et al.*, 2002; Sugiyama *et al.*, 2000a), but in some studies this effect was seen only at high doses (Friedrichs *et al.*, 2002) or not at all (Bischoff *et al.*, 2000;

Bischoff *et al.*, 2001). Two recent studies in mice also described S1P-induced lowering of heart rate (Forrest *et al.*, 2004; Sanna *et al.*, 2004). Most interestingly, this effect was abolished in SIP_3 receptor knockout mice (Sanna *et al.*, 2004), and the ability of several S1P receptor agonists to lower heart rate in wild-type mice correlated better with their potency at $S1P_3$ than at $S1P_1$ receptors (Forrest *et al.*, 2004). Taken together, it appears that SM metabolites, particularly S1P, lower heart rate under most conditions, but these effects may not be of sufficient magnitude to be detectable in all cases, particularly *in vivo*.

The above signaling studies would also predict SM metabolites to cause negative inotropic effects. This has been tested at the level of isolated cardiomyocytes, isolated myocardial strips, isolated hearts and *in vivo*. Sphingosine was shown to inhibit contractions of isolated cardiomyocytes from both cats (Oral *et al.*, 1997) and guinea-pigs (Sugishita *et al.*, 1999b). Sphingosine also mimicked the negative inotropic effects of TNF-α in rat right ventricular trabeculae, and this occurred by impairing the economy of chemo-mechanical energy transduction (Hofmann *et al.*, 2003). In line with these *in vitro* observations, sphingosine caused negative inotropy upon systemic administration in rats *in vivo* (Sugiyama *et al.*, 2000b). Moderate negative inotropic effects have also been reported for S1P in isolated dog heart (Sugiyama *et al.*, 2000b). Studies on inotropic effects of ceramide are less consistent. While it was found that ceramide increased contraction of cardiomyocytes and hearts isolated from adult rats (Liu & Kennedy, 2003; Relling *et al.*, 2003), another study detected negative inotropic effects of ceramide upon i.v. administration in rats (Friedrichs *et al.*, 2002). Thus, the majority of studies support the notion that SM metabolites, particularly sphingosine, will reduce cardiac contractility.

The negative chronotropic and inotropic effects of SM metabolites make them a logical candidate to mediate impaired cardiac function under pathophysiological conditions, particularly in the context of hypoxia and ischemia. Hypoxia and ischemia lead to the formation and release of ceramide in neonatal rat cardiomyocytes (Bielawska *et al.*, 1997), and of sphingosine in adult rat isolated cardiomyocytes (Cavalli *et al.*, 2002), in Langendorff-perfused rabbit heart (Cavalli *et al.*, 2002) and in dog heart *in vivo* (Thielmann *et al.*, 2002). This is further supported by the detection of hypoxia-induced activation of NSMase in cardiac myocytes (Hernandez *et al.*, 2000b). Hypoxia/ischemia-

induced ceramide and sphingosine formation appear to primarily have negative effects on cardiac function.

Taken together, these data demonstrate that ceramide and sphingosine are formed during cardiac hypoxia/ischemia, contribute to the myocardial damage under these conditions and mediate at least some of the adverse effects of TNF-α in this regard. In contrast, conversion of ceramide and sphingosine to S1P will counteract these effects and is cardioprotective. Therefore, it has been proposed that manipulation of the relative balance between ceramide and sphingosine on the one hand and S1P on the other hand may be a possible target for the treatment of myocardial ischemia.

I-5-3 Cytokines and Myocardial Ischemia/Heart Failure

Cytokines, including tumor necrosis-α (TNF-α) (Aukrust *et al.*, 1999; Nozaki *et al.*, 1997; Pudil *et al.*, 1999), interleukin 1-β (IL1-β) (Aukrust *et al.*, 1999; Pudil *et al.*, 1999), and interleukin 6 (IL-6) (Aukrust *et al.*, 1999; MacGowan *et al.*, 1997; Pudil *et al.*, 1999; Roig *et al.*, 1998; Tsutamoto *et al.*, 1998), have been shown to be released by the cardiac myocyte immediately after onset of ischemia (Edmunds & Woodward, 1998). The plasma levels of these cytokines are also known to increase in patients with myocardial ischemia and chronic heart failure (Aukrust *et al.*, 1999; Deswal *et al.*, 2001; Edmunds & Woodward, 1998; Kawamura *et al.*, 2001; Torre-Amione *et al.*, 1996; Tsutamoto *et al.*, 1998). Cytokines have been implicated in the syndrome of chronic heart failure for over a decade (Adamopoulos *et al.*, 2001). A growing body of evidence suggests that the inflammatory cascade triggered by the binding of TNF-α to TNF-α receptor I and II (p55TNFR, p75TNFR) are directly responsible for the acute deleterious effects observed in the myocardium (Kapadia *et al.*, 1995; Oral *et al.*, 1997), and are deeply involved in the development of ventricular dysfunction and ventricular remodeling as well (Bryant *et al.*, 1998a; Finkel *et al.*, 1992; Kapadia *et al.*, 1995; Oral *et al.*, 1997; Zhang *et al.*, 2005).

I-5-3-1 Accumulation of TNF-α during Myocardial Ischemia/Heart Failure

In healthy hearts, the TNF-α concentration is very low (Aker *et al.*, 2003; Arras *et al.*, 1996). In response to acute myocardial ischemia/reperfusion, TNF-α is released from

macrophages, monocytes, mast cells, and cardiomyocytes *per se* within minutes (Aker *et al.*, 2003; Arras *et al.*, 1996; Bellisarii *et al.*, 2001; Frangogiannis *et al.*, 1998; Schulz *et al.*, 2004). Subsequently, the myocardial TNF-α concentration increases within the area at risk (Frangogiannis *et al.*, 1998; Gurevitch *et al.*, 1996; Irwin *et al.*, 1999). With prolongation of ischemia and development of cardiomyocyte necrosis, the TNF-α concentration increases also in the surrounding viable portions of the myocardium (Dorge *et al.*, 2002; Ono *et al.*, 1998; Thielmann *et al.*, 2002).

In animals (Aker *et al.*, 2003; Marin-Garcia *et al.*, 2001; Recchia *et al.*, 2000) and patients with severe heart failure (Aukrust *et al.*, 1999; Ceconi *et al.*, 1998; Levine *et al.*, 1990; Munger *et al.*, 1996; Testa *et al.*, 1996; Torre-Amione *et al.*, 1996), the serum TNF-α concentration is increased, and the increase is an independent predictor of mortality in patients with advanced heart failure (Deswal *et al.*, 2001; Ferrari *et al.*, 1995; Rauchhaus *et al.*, 2000). While increased myocardial TNF-α concentration secondary to ischemia or infarction induces contractile dysfunction, this does not necessarily imply that myocardial dysfunction is always associated with an increased myocardial TNF-α concentration. Indeed, in some experimental (Recchia *et al.*, 2000) and clinical (Munger *et al.*, 1996) studies, the TNF-α concentrations did not differ in paired arterial and coronary sinus blood samples, suggesting that TNF-α is of peripheral origin. This conclusion was further supported by results obtained in rabbits with pacing-induced heart failure, in which the myocardial TNF-α concentration remained unchanged, but the increased serum TNF-α concentration closely correlated to the increased hepatic TNF-α concentration (Aker *et al.*, 2003).

I-5-3-2 Effects of TNF-α on Cardiac Channels and Carriers

In the cultured mature ovine oligodendrocytes, brief exposure of TNF-alpha does not elicit membrane depolarization or consistent changes in cytosolic Ca^{2+} levels. However, prolonged exposure leads to inhibition of process extension, membrane depolarization and a decrease in the amplitudes of both inwardly rectifying and outward K^+ currents (Soliven & Szuchet, 1995).

TNF-α inhibited cardiac $I_{Ca\text{-}L}$ and contractile Ca^{2+} transients in adult rat ventricular myocytes. Thus, it is possible that the negative inotropic effects of TNF-α are the result of TNFRI-mediated blockade of cardiac excitation-contraction coupling (Krown *et al.*, 1995). In isolated guinea-pig ventricular myocytes, TNF-α (500 U/ml) decreased both peak systolic $[Ca^{2+}]_i$ and the amplitude of cell shortening without detectable reductions in $I_{Ca\text{-}L}$ within 5 min. TNF-α, however, clearly depressed $I_{Ca\text{-}L}$ and the amplitude of cell shortening in β-adrenergic stimulated cells, significantly increased the content of sphingosine in isolated heart. The effects of low dose sphingosine (5 μM) mimicked those of TNF-α on cardiac myocytes, indicating TNF-α produced an acute negative inotropic effect via a sphingomyelin-dependent pathway (Sugishita *et al.*, 1999a). In H9c2 cardiomyocytes, TNF-α (10-50 ng/mL) depresses the depolarization effect of 80 mM KCl on ^{45}Ca uptake; this effect can be attributed to the negative inotropic effect of TNF-α that reduces cardiac contractility and attenuates Ca^{2+} influx and release by SR. In the same study, TNF-α activates K_{ATP} channels, and this effect is blocked by glybenclamide (El Ani & Zimlichman, 2003).

Overall the data on the modulation of cardiac channels and carriers by TNF-α is sparse, however, it has been speculated that TNF-α does play an important part in the modulation that is involved in a variety of pathophysiological situations in heart, especially heart failure (Ceconi *et al.*, 1998; Ferrari *et al.*, 1995; Munger *et al.*, 1996; Rauchhaus *et al.*, 2000; Testa *et al.*, 1996; Wang *et al.*, 2005).

I-5-3-3 Deleterious Effects of TNF-α on Heart

A growth body of evidence from both animal and human studies have revealed that TNF are elevated in patients with heart failure (Dutka *et al.*, 1993; Levine *et al.*, 1990; McMurray *et al.*, 1991; Torre-Amione *et al.*, 1996). The overexpression of TNF may contribute to disease progression in heart failure, via virtue of the direct toxic effects that this cytokine exerts on the heart and the peripheral circulation (Packer, 1995;Seta *et al.*, 1996). In addition to the well-recognized deleterious effects of TNF, including left ventricular dysfunction (Bozkurt *et al.*, 1998), left ventricular remodeling, cardiac myocyte apoptosis (Bozkurt *et al.*, 1998; Krown *et al.*, 1996), and endothelial cell

dysfunction (Agnoletti *et al.*, 1999), there were also several intriguing lines of experimental evidence that suggested that TNF might contribute to depressed rate heart rate variability in patients with heart failure. For example TNF had been shown to alter ß-adrenergic signaling in isolated cell culture models (Chung *et al.*, 1990; Gulick *et al.*, 1989). Moreover, mice harboring cardiac restricted overexpression of TNF were shown to have blunted responsiveness to isoproterenol (Kubota *et al.*, 1997). Moreover, TNF is an independent predictor of depressed heart rate variability in patients with heart failure. The overexpression of TNF and subsequent loss of beta-adrenergic responsiveness contributes to the decrease in heart rate variability observed in heart failure (Malave *et al.*, 2003).

Using a rat heart ischemia-reperfusion model, it was demonstrated that FR167653, a newly synthesized cytokine inhibitor, significantly reduced ischemia-activated myocardial TNF-α mRNA expression and TNF-α production and as well as inhibited p38 MAPK activation. Its administration improved recovery of cardiac contractile function during reperfusion. This suggested that cytokine inhibition is significant as a method for myocardial protection against ischemia-reperfusion injury (Aleshin *et al.*, 2004; Hoshida *et al.*, 2000).

Very recently it has been reported that isolated rat hearts from age-matched adult males and females were perfused (Langendorff), and myocardial contractile function was continuously recorded. After ischemia/reperfusion (I/R), myocardium was assessed for expression of TNF-α, IL-1β, and IL-6 (RT-PCR, ELISA); IL-1α and IL-10 mRNA (RT-PCR); and activation of p38 MAPK (Western blot). All indexes of postischemic myocardial function [left ventricular developed pressure, left ventricular end-diastolic pressure, and maximal positive (+dP/dt) and negative (–dP/dt) values of the first derivative of pressure] were significantly improved in females compared with males. Compared with males, females had decreased myocardial TNF-α, IL-1β, and IL-6 (mRNA, protein) and decreased activation of p38 MAPK pathway. These data demonstrate that hearts from age-matched adult females are relatively protected against I/R injury, possibly due to a diminished inflammatory response (Wang *et al.*, 2005).

In summary, a number of investigation have shown that TNF-α mimics some aspects of the so-called chronic myocardial ischemia and heart failure phenotypes, including (but not limited to) electronic and anatomic remodeling, progressive left

ventricular dysfunction, pulmonary edema, fetal gene expression, and cardiomyopathy (Diwan *et al.*, 2004; Mann, 2002). The mechanisms underlying the deleterious effects of TNF-α on cardiac functions have also been intensively exploited. To date a couple of hypotheses have been proposed with some support from various experimental results. The most conceivable mechanism is that the cardiac effects of TNF-α executive via the excessive generation of reactive oxygen species (ROS) (Giordano, 2005).

I-5-4 ROS and Myocardial Ischemia/Heart Failure

A great number of in vitro and in vivo studies have demonstrated the excessive production of ROS in the cardiovascular system in response to various stressors and in the failing heart (Cesselli *et al.*, 2001; Sabri *et al.*, 2003; Sawyer *et al.*, 2002; Suematsu *et al.*, 2003). Furthermore, the results from animal studies have also revealed that antioxidants and ROS defense pathways can ameliorate ROS-mediated cardiac abnormalities (Chen *et al.*, 1996; Conrad *et al.*, 2004b; Ho *et al.*, 1998; Yen *et al.*, 1996).

I-5-4-1 Generation and Counterbalancing of ROS in Heart

Atoms or molecules with unpaired electrons are designated free radicals and are highly reactive entities that can readily participate in a variety of chemical/biochemical reactions. In the electron transport chain of oxidative phosphorylation, 4 electrons are required to the full reduction of oxygen to water (H_2O) as a terminal event. The sequential donation of electron to O_2 during the process can generate ROS as intermediates, which including free radicals, peroxides, and singlet oxygen, and "electron leakage" can also contribute to the generation of ROS (Davies, 1995; Genova *et al.*, 2003; Miwa & Brand, 2003). Donation of a single electron to O_2 results in the formation of the superoxide radical ($O_2^{\bullet-}$). Donation of a second electron yields peroxide, which then undergoes protonation to produce hydrogen peroxide (H_2O_2). Donation of a third electron, such as occurs in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$), results in production of the highly reactive hydroxyl radical ($^{\bullet}OH$). Finally, donation of a fourth electron yields H_2O. Singlet oxygen ($^1O_2$), a very short-lived and reactive form of O_2 in which the outer electrons are raised to a higher energy state, can be formed by a variety of mechanisms,

including the Haber-Weiss reaction ($H_2O_2 + O_2^{\bullet-} \rightarrow {}^{\bullet}OH + OH^- + {}^1O_2$) (Toufektsian *et al.*, 2001).

ROS can be formed at a very low concentration in the heart as a byproduct of normal cellular aerobic metabolism (Davies, 1995; Ide *et al.*, 1999). It has been revealed that the several mechanisms responsible for ROS production; they can be produced by xanthine oxidase (XO), NADPH oxidases, cytochrome P450; by autooxidation of catecholamines; and by uncoupling of NO synthase (Griendling *et al.*, 2000; Sawyer *et al.*, 2002; Seshiah *et al.*, 2002; Xia *et al.*, 1998). The ROS generation in the heart can be induced by the action of cytokines and growth factors as well. Angiotensin II (ATII), PDGF, and TNF-α, for example, can induce H_2O_2 and $O_2^{\bullet-}$ formation via activation of the NADPH oxidase (Bendall *et al.*, 2002; Heymes *et al.*, 2003; Sabri *et al.*, 2003; Sauer *et al.*, 2004; Seshiah *et al.*, 2002; Thannickal & Fanburg, 2000).

In normal heart, there are several cellular mechanisms that counterbalance the production of ROS, including enzymatic and nonenzymatic pathways (Nordberg & Arner, 2001). Among the best-characterized enzymatic pathways are catalase and glutathione peroxidase, which coordinate the catalysis of H_2O_2 to H_2O, and the superoxide dismutases (SODs), which facilitate the formation of H_2O_2 from $O_2^{\bullet-}$ (de Haan *et al.*, 2004; Kirkman *et al.*, 1999; Ursini *et al.*, 1995). Thioredoxin and thioredoxin reductase (TR) together form an additional enzymatic antioxidant and redox regulatory system that has been implicated in a wide variety of ROS-related processes (Nordberg & Arner, 2001). Thioredoxin and TR can catalyze the regeneration of many antioxidant molecules, including ubiquinone (Q10), lipoic acid, and vitamin C, and as such constitute an important antioxidant defense against ROS. Deletion of TR results in developmental heart abnormalities and in cardiac death secondary to a severe dilated cardiomyopathy (Conrad *et al.*, 2004a). Nonenzymatic mechanisms include intracellular antioxidants such as the vitamins E, C, and β-carotene, Q10, lipoic acid, urate, and glutathione, which acts as a reducing substrate for the enzymatic activity of glutathione peroxidase (Nordberg & Arner, 2001).

During myocardial ischemia the generation of ROS is amplified (Carmeliet, 1999; Kramer *et al.*, 1994; Kukreja & Hess, 1992). It had been widely appreciated that ROS are produced primarily with the reintroduction of oxygen following ischemia. However, there

is much literature to support the notion that ROS can be abundantly generated during myocardial ischemia *per se* (Becker, 2004; Hess & Manson, 1984; Kevin *et al.*, 2003; Nohl & Jordan, 1986). With ischemia the respiratory cytochromes in mitochondria become redox-reduced allowing them to directly transfer (i.e. "leak") electrons to O_2 (Becker *et al.*, 1999; Nohl & Jordan, 1986). A redox-reduced myocyte in the presence of O_2 appears capable of producing large amounts of ROS, mostly superoxide anions (Hess & Manson, 1984; Kevin *et al.*, 2003; Nohl & Jordan, 1986). Intriguingly, it has been found very recently that there is a positive feedback loop of "ROS-induced ROS release" (Zorov *et al.*, 2000). In isolated adult rat cardiomyocytes, "triggering" or "inducing" ROS can be created via intracellular photoactivation of tetramethylrhodamine compounds. These triggering ROS were associated with mitochondrial depolarization along with mitochondrial permeability transition induction. Observed simultaneously with mitochondrial permeability transition induction was a large burst of ROS from individual mitochondria. These ischemia-generated ROS may contribute to a variety of direct cellular oxidant damage and indirect detrimental roles via different signaling pathways (Carmody & Cotter, 2001).

I-5-4-2 Effects of ROS on Channels and Transporters

The excessive amount of radicals leads to changes in proteins, lipids, and DNA and RNA molecules (Bhatnagar, 1994). Sulfhydryl groups of proteins are oxidized and disulfide bridges are formed, resulting in disturbances of the ion permeation or gating of ionic channels, decrease of transport capacity of carrier molecules, and activation of enzymes (Tan *et al.*, 1995; Ward & Giles, 1997). Membrane lipids undergo peroxidation and change indirectly the behavior of channels. These peroxyradicals oxidize proteins, cholesterol, and other FA especially polyunsaturated FA, thus propagating the reaction in a chain-type way (Kramer *et al.*, 1994). General membrane damage secondary to ROS-mediated lipid peroxidation is one mechanism by which this can occur; however, more specific ROS-mediated effects also contribute. The interplay of ROS with proteins and lipids results in a variety of alterations in cardiac ion channels and transporters. Briefly, I_{Na}, I_{Ca}, I_K except for I_{KATP} as well as NCX and Na^+-K^+ pump are inhibited by ROS.

Information on the effect of free radicals on the Na^+ channel is scarce. In the frog ventricle, application of tert-butylhydroxyperoxide causes a gradual reduction in I_{Na} with a shift of the E_{rev} in the negative direction (Bhatnagar *et al.*, 1990). In rat ventricular cells, the most prominent effect of H_2O_2 is a slowing of inactivation of the TTX-sensitive current (Ward & Giles, 1997).

ROS can target L-type calcium channels on the sarcolemma and suppress the I_{CaL} (Guerra *et al.*, 1996). Inhibition of the I_{CaL} occurs in the guinea pig (Cerbai *et al.*, 1991) and is accompanied by a fall in Ca^{2+} transient (Goldhaber & Liu, 1994). In the ferret, the effect depends on the type of radicals; exposure to oxygen free radicals and S-nitrosothiols increases the current. Under the influence of H_2O_2, a Ca^{2+}-permeable leak channel is induced in cultured rat ventricular myocytes (Wang *et al.*, 1995c). A nonselective cation channel is activated in guinea pig ventricular myocytes after extracellular or intracellular exposure of ROS (Jabr & Cole, 1995; Shattock *et al.*, 1991; Tarr *et al.*, 1994). Oxidative stress activates the SR Ca^{2+} channel probably by disulfide bound formation (Kawakami & Okabe, 1998; Shattock *et al.*, 1990). Binding of ryanodine is decreased (Holmberg *et al.*, 1991). The Ca^{2+}-ATPase of the SR is blocked in a sensitive way by oxidative stress, and the inhibition can be neutralized by dithiothreitol (Eley *et al.*, 1991; Kukreja *et al.*, 1991). ROS depress the activity of the sarcoplasmic reticulum Ca^{2+} ATPase SERCA2, a membrane calcium pump that has been shown to play a crucial role in cardiac calcium handling and as a determinant of myocardial contractility. SERCA2 expression is concomitantly reduced in cardiomyocytes stimulated to hypertrophy via ROS-associated signaling pathways (Kaplan *et al.*, 2003).

Most of I_K with the exception of I_{KATP} are inhibited by exposure to ROS. The I_{to} in rat atrial myocytes is decreased secondary to a shift in the inactivation curve to the left by exposure to 1 mM tert-butylhydroperoxide (Pike *et al.*, 1993). A decrease also occurs by exposure to oxygen radicals for the delayed I_K (Pike *et al.*, 1993; Tarr *et al.*, 1995; Valenzeno *et al.*, 1993) and the I_{K1} (Jabr & Cole, 1993; Nakaya *et al.*, 1992; Shattock *et al.*, 1991). In contrast to the well-documented decrease in delayed I_K by exposure to oxygen radicals, exposure to thimerosal, know to oxidize sulfhydryl groups into disulfide bridges increases I_{Ks} in canine ventricular myocytes (Yao *et al.*, 1997). The I_{KATP} appears after some delay upon exposure to oxidative stress (Jabr & Cole, 1993). Oxidation of

sulfhydryl groups by thimerosal treatment indeed changes the ATP sensitivity of the channel and causes faster activation during ischemia (Coetzee *et al.*, 1995). Similar effects can be obtained by partial proteolysis in the presence of trypsin (Fan & Makielski, 1993).

Direct measurement of the of the Na^+/Ca^{2+} exchange current in patch-clamp experiments with controlled Ca^{2+} and Na^+ concentrations showed a decrease under oxidative stress (Coetzee *et al.*, 1994; Goldhaber, 1996). In contrast, stimulatory effects have been described in flux studies in cardiac vesicles (Reeves *et al.*, 1986), in hearts subjected to hypoxia-reoxygenation (Dixon *et al.*, 1987), and in voltage-clamped guinea pig ventricular myocytes treated with H_2O_2 (Goldhaber, 1996). In those cases, Ca^{2+} and Na^+ concentrations were not "clamped," and stimulation may be due to indirect concentration changes. The Na^+-K^+ pump current is reduced (Shattock & Matsuura, 1993). The inhibition correlates with a decrease in specific ouabain binding and enzyme activity (Kako *et al.*, 1988; Kim & Akera, 1987).

I-5-4-3 Electrophysiological changes caused by ROS

There are two distinct stages in the development of symptoms in cardiac cells exposed to ROS. During a first stage, upstroke velocity and conduction of the AP are reduced; the plateau is prolonged, and EAD may appear on the slow phase of repolarization (Firek & Beresewicz, 1990; Hayashi *et al.*, 1989; Ward & Giles, 1997). Repolarization is followed by DAD (Firek & Beresewicz, 1990; Hayashi *et al.*, 1989; Kukreja *et al.*, 1991). Eventually, the cell may depolarize and show continuous oscillations at the plateau level. In a second stage, extra systoles or spontaneous activity is still present, but the APD is gradually shortened (Beresewicz & Horackova, 1991). When the APD becomes very short (Jabr & Cole, 1993), the diastolic potential shifts in the hyperpolarized direction (Barrington *et al.*, 1988), and the cell becomes inexcitable and goes into irreversible contracture.

Although proarrhythmic effects of oxygen radicals can be expected (Shattock *et al.*, 1991) from the occurrence of EAD and DAD and from the fall in upstroke velocity and gap junction conductance, their role in ischemic arrhythmias has been uncertain. Arguments have been based on the use of antioxidants, but their effect seems to be

species dependent. In the rat, an antioxygen radical treatment mostly provides protection against arrhythmias (Bernier *et al.*, 1989; Hagar *et al.*, 1991; Woodward & Zakaria, 1985), whereas such protection is absent in the dog (Euler, 1995). Antioxygen radical treatment may provide a better protection against delayed rather than early ventricular arrhythmias (Coetzee *et al.*, 1990).

I-6 Electrophysiological Mechanisms Underlying the Ischemic Arrhythmias

It is generally appreciated that the occurrence of lethal arrhythmias in human is the result of the interplay between substrate, trigger, and modulating factors (Coumel, 1987; Nattel, 2002). Substrate factors may be considered to be the functional changes brought about by ischemia, creating the setting for functional reentry circuits within the ischemic myocardium or the anatomical arrangement of surviving myocardial fibers within a healed infarct, which provides an anatomically defined reentrant circuit. The basic electrophysiological mechanisms underlying the genesis of cardiac arrhythmias include automaticity, triggered activity (EAD and DAD), and reentry (Carmeliet, 1999).

It has been well documented that the prominently electrophysiological changes in cellular and multicellular level during myocardial ischemia are sarcolemmic membrane depolarization, APD alterations (shortening accompanied with high frequent DAD during the early phase of acute ischemia and lengthening going with intensified EAD in the following period), ERP-↑, decrease in conduction velocity, change in excitability, and $[K^+]_o$-↑ (Carmeliet, 1999; Coronel *et al.*, 1989; Coronel *et al.*, 1991; Verkerk *et al.*, 1996; Watanabe *et al.*, 1997). Furthermore, the basis of the electrophysiological changes relies upon the interplay between the various ion channels, ion exchangers and the modulators, which derive either from the electric disturbances such as $[K^+]_o$-↑ or from the accumulation of metabolic substrates like LPC and ROS during myocardial ischemia. However, the exact mechanisms underlying ischemic arrhythmias remain obscure. For instance, in human, phase I arrhythmias during acute ischemia often results in VF and SCD. Reentry and DADs have been proposed as the major mechanisms responsible for the phase I arrhythmias in ischemic myocardium (Carmeliet, 1999; Pogwizd *et al.*, 1986; Pogwizd & Corr, 1987). However, the notion has been challenged with the new experimental evidence upon which EAD and wavebreak leading to wavelet formation

might be the hallmark in the mechanism of initiation and maintenance of VF (Cao *et al.*, 1999; Zaitsev *et al.*, 2003). Noticeably, APD-↓ or QT-↓ favors the reentrant arrhythmias and DADs, a reproducible pattern of dysrhythmic activities during acute myocardial ischemia (Wilde *et al.*, 1988). In contrast to acute myocardial ischemia in which APD-↓ or QT-↓ is the fundamental electric disturbance responsible for cardiac arrhythmias, in chronic ischemia the prominent electric disturbance is the lengthening of APD (APD-↑) or QT-↑, though both acute and chronic myocardial ischemia manifest some electrophysiological disturbances in common. In this review I only address two themes, one is about the mechanisms for $[K^+]_o$-↑ and APD-↓ during the acute myocardial ischemia, the other is the mechanisms underlying the chronic ischemic arrhythmias.

I-6-1 Electrophysiological Mechanisms for ischemic $[K^+]_o$-↑ and APD-↓

Because of the importance of $[K^+]_o$-↑ along with APD-↓ during the early phase of acute myocardial ischemia, the mechanisms responsible for it have been a subject of intensive research. It is generally believed that $[K^+]_o$-↑ and APD-↓ are of multifactorial nature with 3 major determinants (Carmeliet, 1999; Wilde *et al.*, 1988): increased passive K^+ efflux, reduced active K^+ influx, and diminished extracellular space. **Increased passive K^+ efflux** is a prerequisite for ischemic $[K^+]_i$-↓ or $[K^+]_o$-↑ and APD-↓. Experimental studies support the hypothesis that K^+ efflux increases rapidly during acute myocardial ischemia; this increase is most likely due to a net increase in the conductance of K^+ channels. **Reduced active K^+ influx** is a warranty for ischemic $[K^+]_o$-↑. This is achieved by reduction of the Na^+-K^+ pump activity. Ischemic conditions make a moderate inhibition of the pump plausible: metabolic stresses have been found to reduce pump activity (Carmeliet, 1999; Kleber, 1983; Shattock & Matsuura, 1993; Shen & Pappano, 1995). Owing to the remarkable capacity of the pump (150 pA or 3×10^{-15} mol/sec), small reduction of the activity can render dramatic changes of $[K^+]_o$. The normal reversal potential (E_{rev}) of the pump is -180 mV. It may however drop to –60 mV which is about the membrane potential of the cells during the plateau of $[K^+]_o$-↑ in ischemic myocardium. The pump, in other words, will stop operating at the potential expected under ischemic conditions (Carmeliet, 1999; Glitsch & Tappe, 1995). **Diminished extracellular space**

produces a concentrating effect on $[K^+]_o$-↑ induced increased passive K^+ efflux and reduced active K^+ influx. Shift of water from extracellular to the intracellular space occurs as a consequence of increase in osmotically active particles in the cell, such as lactate and phosphate. It is estimated that the increase in osmotically active particles can results in a restriction of the extracellular space by 15% after 10 min of ischemia (Carmeliet, 1999; Fiolet *et al.*, 1993; Yan *et al.*, 1996).

Apparently, increased passive K^+ efflux acts as an initiation mechanism for $[K^+]_o$-↑ and APD-↓, decreased active K^+ influx as a maintenance mechanism for $[K^+]_o$-↑, and shrinkage of extracellular space as an amplification mechanism for $[K^+]_o$-↑. However, despite the key importance of increased passive K^+ efflux, the ionic mechanisms are still uncertain and our understanding of pathways along which K^+ leaves the cells have been controversial for nearly two decades.

Cardiac K^+ channels play a pivotal role in defining resting potential, cell excitability and membrane repolarization and thereby the likelihood of arrhythmias. In view of all verified cardiac K^+ currents, some of them have been reported to be downregulated in their function and expression during acute ischemia; such as I_{to} and I_{K1} (Boyden & Jeck, 1995; Carmeliet, 1999; Pinto & Boyden, 1998). These channels as carriers of ischemic K^+ efflux can be excluded. K_{ATP} as a candidate for ischemic $[K^+]_o$-↑ has been extensively studied; yet the results are inconsistent. While either I_{KNa} or I_{KAA} has the potential to be a contributor to ischemic $[K^+]_o$-↑, the existing studies are sparse and do not allow us to reach a definite conclusion. The possible role of I_{Kr} or I_{Ks} has yet to be investigated.

Since K_{ATP} is gated by intracellular ATP and regulated by a variety of cellular metabolites, K_{ATP} channel is considered to be a sensor of the metabolic state of a myocardial cell, and activation of these channels to be an indicator of cellular metabolic compromise (Ashcroft & Ashcroft, 1990; Findlay, 1994). However, K_{ATP} channels do not open unless intracellular ATP level ($[ATP]_i$) drops down to less than 100 μM; the IC_{50} for K_{ATP} inhibition is <100 μM. This implies that K_{ATP} channels normally stay in the closed state because the physiological $[ATP]_i$ is in the order of milliemolar range (5 mM). This is perhaps why the existing data regarding the role of K_{ATP} in ischemic $[K^+]_o$-↑ have been conflicting. Evidence exists that K_{ATP} channels mediate the ischemic K^+ efflux and APD-

↓ and most of the evidence is from studies using K_{ATP} inhibitors or openers; K_{ATP} inhibitors such as glybenclamide have been found to effectively reverse ischemia/hypoxia APD-↓ and the rate of $[K^+]_o$-↑ (Gwilt *et al.*, 1992; Gwilt *et al.*, 1993; Kantor *et al.*, 1990; Wilde *et al.*, 1989; Wilde *et al.*, 1990; Yan *et al.*, 1993). In addition, Glybenclamide also exhibited potent antifibrillatory activity, abolishing irreversible ventricular fibrillation during regional ischemia and during global ischemia. However, there are also experimental findings against K_{ATP} channels as mediators of $[K^+]_o$-↑ and APD-↓ (Allen & Orchard, 1987; Mitani *et al.*, 1991; Shivkumar *et al.*, 1997; Vanheel & de Hemptinne, 1992; Venkatesh *et al.*, 1992; Wilde & Aksnes, 1995). First, the measured $[ATP]_i$ at the time of metabolic blockade of acute ischemia is still in the millimolar range which is much too high to induce massive K_{ATP} (Nichols & Lederer, 1990; Noma, 1983). Second, the time-course of opening of K_{ATP} channels is too slow to explain the start of $[K^+]_o$-↑ within the first minute of ischemia. Third, the results from K_{ATP} openers and inhibitors have been conflicting; Early ischemic $[K^+]_o$-↑ and surface acidification are relatively insensitive to K_{ATP} channel inhibition or activation prior to the ischemic insult (Vanheel & de Hemptinne, 1992) and K_{ATP} opener pinacidil (Kanda *et al.*, 1997). And finally, some blockers of voltage-gated K^+ channels are effective in preventing and reversing ischemic $[K^+]_o$-↑ and APD-↓; these include diltiazem (Sakamoto *et al.*, 1997). The role of K_{ATP} channel in response to myocardial ischemia, therefore, has been questioned. Very recently, using Kir6.2 knockout (KO) hearts provides evidence that the activation of K_{ATP} channels contributes to the shortening of APD, whereas it is not the primary cause of $[K^+]_o$-↑ during early myocardial ischemia (Saito *et al.*, 2005). Thus, it is conceivable that other mechanisms or more specifically other K^+ currents, in addition to K_{ATP}, may also contribute to $[K^+]_o$-↑ and APD-↓ in ischemic myocardium.

I_{KNa} and I_{KAA} are another two candidate channels for ischemic $[K^+]_o$-↑. I_{KNa} is activated by a rise of intracellular Na^+ (Kameyama *et al.*, 1984; Luk & Carmeliet, 1990), a characteristic alteration accompanying $[K^+]_o$-↑ in ischemic myocardium. With respect to the potential role of I_{KNa} in ischemic $[K^+]_o$-↑, the problem is similar to that of K_{ATP}, being whether $[Na^+]_i$ can reach sufficiently high levels needed to activate the channel. Nonetheless, evidence exists that $[Na^+]_i$ close to the membrane can be much higher than in the bulk; gradient and local accumulation are present (Wendt-Gallitelli *et al.*, 1993).

I_{KAA} is an ATP-insensitive K^+ channel with an outwardly rectifying property, with a conductance slightly larger than K_{ATP}, and its open probability is enhanced in acidosis and by stretch, two conditions present during ischemia. Since the level of free fatty acids rises after longer periods of ischemia, it was proposed that I_{KAA} contributes to the late or secondary phase of $[K^+]_o$-↑ (Kim & Duff, 1990). Thus, either of the two currents has the potential to be a contributor to ischemic $[K^+]_o$-↑ and APD-↓. However, it should be noted that the two currents remained insufficiently characterized and their function poorly understood due to the fact that studies on these currents have been sparse.

I-6-2 Electrophysiological Mechanisms for the Arrhythmias during Chronic Myocardial Ischemia

The chronic ventricular arrhythmias (CVA) that occur in human with IHD have been studied extensively by clinical electrophysiologists. Patients who survive after the acute phases of MI may experience a variety of CVA, ranging from single premature depolarization to tachycardia and fibrillation. The CVA significantly increases the mortality in the first year after MI, the greatest risk being within the first 2 month after hospital discharge (Moss *et al.*, 1977). The risk is greater when the premature ventricular depolarization is frequent or complex (multiform QRS morphologies) (Bigger, Jr. *et al.*, 1982; Janse, 2004; Moss *et al.*, 1977; Ruberman *et al.*, 1977). Aside from the premature ventricular depolarization, other factors, including the situations of increased activity of the sympathetic nervous system, electrolyte disturbances, such as low serum potassium levels, and metabolic disorders, such as accumulation of cytokines and ROS, as well as impaired left ventricular function due to molecular, electrical and anatomic remodeling, may all modify both the substrate and the trigger (Marban, 2002; Nattel, 2002).

The electrophysiological remodeling during chronic myocardial ischemia has been revealed to manifest as extremely APD-↑ and ERP-↑ in the cellular level and QT-↑ as reflected in ECG, and which provide the substrates for reentry and EAD-dependent triggered activity, being proposed mechanisms underlying the CVA. It is well established that APD is determined by the balance between plateau inward and outward currents. The APD-↑ can result from either increase in plateau inward currents, mainly $I_{Ca\text{-}L}$, or decrease in repolarizing currents, which orchestrate by I_{K1}, I_{to}, I_{Kr} and I_{Ks}. Moreover, with

the APD-↑ the normal smooth trajectory of repolarization might then be interrupted by EAD (Marban *et al.*, 1986; Marban, 2002; Nattel, 2002). The EAD may induce an AP and produce ectopic firing, and then propagate between different zones of ventricles, which suffer from the chronic ischemia. Along with ERP-↑ and myocardial fibrosis, the abnormal impulse will cause re-entry in the forms of single-circuit or multiple-circuit. The multiple unstable re-entry circuits can coexist simultaneously; render the ischemic ventricle to the situation of tachycardia and fibrillation. A factor predisposing to reentry is the increased dispersion in APD and refractoriness, as has been reported in several studies (Pak *et al.*, 1997; Yan *et al.*, 2001). The majority of ventricular arrhythmias, however, are substrate-related reentry tachycardia, most commonly based on an infarct scar (Brunckhorst & Delacretaz, 2004).

Like in the acute myocardial ischemia while $[Ca^{2+}]_i$ is increased, mainly due to a less efficient removal from the cell via the NCX and a reduced Ca^{2+} uptake in the SR (Carmeliet, 1999; Trafford *et al.*, 1998), whereas in the chronic myocardial ischemia Ca^{2+} efflux is reduced, Ca^{2+} influx is increased because of metabolic inhibition. The Ca^{2+} influx is mediated prominently via background channels and NSC channels that are activated by ROS (Jabr & Cole, 1995; Wang *et al.*, 1995c). This Ca^{2+} influx may contribute APD-↑. However, some experimental data have not favored the notion. It was reported that peak $I_{Ca\text{-}L}$ density of epicardial border zone cells from the 5-day infarcted heart is significantly reduced by 36% compared to control (Aggarwal & Boyden, 1995). Furthermore, this reduction is not due to a decrease in steady-state availability or a prolonged time course of recovery of $I_{Ca\text{-}L}$. Myocytes adjacent to the 8-week infarct in the rabbit heart show a significant decrease in peak I_{CaL} density without a change in current–voltage relations, voltage-dependence or steady-state inactivation kinetics (Litwin & Bridge, 1997). So, the role of I_{CaL} in APD-↑ is still a matter of debate.

In contrast to I_{CaL}, the experimental data have consistently supported the notion that the downregulation of potassium currents, including I_{K1}, I_{to}, I_{Kr} and I_{Ks} is the possible electrophysiological mechanism underlying the APD-↑ in the chronic myocardial ischemia. APs recorded from the epicardial zone cells of infarcted heart usually show no phase 1 or reduced phase 1 of repolarization suggesting a loss in I_{to}. In contrast and as predicted, APs recorded from all cells dispersed from the normal noninfarcted epicardium

show a large and prominent spike and dome morphology. Voltage clamp studies confirm that the I_{to} functions profoundly in the cells (Lue & Boyden, 1992). Similarly, densities of I_{Ks} and I_{Kr} are reduced significantly in cells dispersed from the epicardial border zone of the 5-day canine infarcted heart (Pinto & Boyden, 1998; Pinto & Boyden, 1999).

It is obvious that the chronic myocardial ischemia does result in the electrical remodeling with the characteristics of decreased potassium channel function, particularly reduction of I_{to}, I_{Kr} and I_{Ks}. The possible molecular mechanisms underlying the deduction can be the result of an acquired (versus genetic) change in structure and function of normally expressed channels, a change in the number of functional channels, or a combination of both. From a molecular standpoint, a change in the number of functional channels could be due to changes in the levels of expressed protein or to alterations in channel protein trafficking.

A change in expression of functional channel proteins is most likely due to a change in gene transcription, translation or post translational modification. Transcription, the initial step in protein expression, involves the production of an mRNA copy from a DNA template. It was reported that both the suppressed transcription and function of the native delayed rectifying K currents in myocytes surviving in the infarcted heart. In particular, by 48 h post MI, mRNAs for dKvLQT1, dI_{sK} and dERG are all reduced. By day 5, dKvLQT1 transcripts have recovered, but dI_{sK} and dERG remain reduced. These findings are consistent with loss in function of I_{Ks} and I_{Kr} in the 5-day border zone myocytes (Pinto & Boyden, 1999). More interestingly, in the setting of chronic myocardial ischemia, several autocrine/paracrine factors are activated and may also become important regulators of ion channel function. For instance, the elevated concentrations of TNF-α, ceramide, and ROS are closely associated with chronic ischemic heart. It was revealed very recently that the inward rectifier K^+ currents and voltage-gated shaker type of K^+ current are modulated by ceramide (Kolesnick, 2002; Ohanian & Ohanian, 2001); HERG currents can be downregulated by TNF-α. Furthermore the downregulation is mediated via ROS (Wang *et al.*, 2004a; Zhang *et al.*, 2003a). However, the published data are too preliminary to shed light on the molecular mechanisms underlying the ischemic arrhythmias. Too many questions remain to be

addressed and too many aims should be targeted to fight the IHD, the first killer of humans.

I-7 Questions Raised from Above Review

A tremendous progress has been achieved over the last two decades regarding the electrical and molecular basis of myocardial ischemic arrhythmias. New insight into the basic mechanisms may allow for improved therapeutic approaches in the near future. An improved understanding of the processes leading to the development of the VF substrate may guide to better strategies for prevention of ischemic arrhythmias. Nevertheless, many aspects remain unexplained and the myocardial ischemic arrhythmias remain a significant challenge.

More specifically, in the early phase of acute myocardial ischemia, what are the ionic mechanisms responsible for $[K^+]_i$-↑ and APD-↓? Among the cardiac potassium currents, either voltage-gated ones such as I_{Kr} and I_{Ks} or ligand-gated ones like I_{KATP} or I_{KAA}, which is the dominant contributor governing the repolarization of AP thereby likelihood of cardiac arrhythmias? How is I_{Kr}, a critical component of repolarizing currents, modulated in the media of acute ischemia? Is it reasonable to implicate the HERG enhancement by LPC-16, the evidence in our previous study, in the situation of acute ischemia? Furthermore, what are the molecular mechanisms underlying HERG/I_{Kr} modulation by lysophospholipid metabolites?

With regard to the chronic myocardial ischemia, what are the molecular signals leading from myocardial ischemia and infarction to metabolic, ionic, electrical, and anatomic remodeling? What are the characteristics of the remodeling? Do the sphingolipid metabolite ceramide and TNF-α that are excessively generated after chronic ischemia contribute to the late APD prolongation in ischemic myocardium? If so what is the molecular mechanisms underlying the contribution? Moreover, what are the inter-relationships among metabolic, electrical, and molecular mechanisms?

I-8 Working Hypothesis

1) $[K^+]_o$-↑ and APD-↓ during the early phase of myocardial ischemia are an event consequent to combined metabolic stress and ion channel dysfunction.

Specifically, I_{Kr}/I_{HERG} is enhanced during the stage and the enhancement is mainly caused by LPC-16 overproduction and accumulation. The enhancement of I_{Kr}/I_{HERG} by LPC-16 is a critical contributor to intracellular K^+ loss/$[K^+]_o$-↑ and APD-↓ thereby the associated ischemic arrhythmias;

2) In the setting of the arrhythmias during the prolonged myocardial ischemia the downregulation of I_{Kr}/I_{HERG} caused directly by sphingolipid metabolite ceramide and/or cytokine TNF-α or conjointly via overproduction of ROS contributes to the late APD prolongation in ischemic myocardium. The mechanisms underling this downregulation are the interplays among a variety of signaling pathways, including MAP kinase, PKA, Akt, and PKC. ROS are the endpoint effectors responsible for the downregulation of I_{Kr}/I_{HERG}.

I-9 Specific Objectives of the Project

1) To investigate whether LPC-16 can modulate I_{HERG} and, if so, to characterize the biophysical components of this action and to clarify the potential mechanisms account for LPC modulation of HERG K^+ channel function.
2) To observe $[K^+]_o$-↑ and QT-↓, and the associated arrhythmias in a rabbit model of acute global ischemia, and to evaluate the effects of inhibitors toward I_{Kr}/HERG, I_{Ks}, and K_{ATP} in such a model. The purpose of this study is to obtain a rough picture of the relative contributions of I_{Kr}, I_{Ks} and K_{ATP} to the typical ischemic electrical disturbance;
3) To test whether exogenous LPC-16 can reproduce the same pattern of electrical disorders as seen with the rabbit model of acute ischemia, and to assess the effects of I_{Kr}/HERG blocker, I_{Ks} blocker, and K_{ATP} inhibitor on the electrical disorders caused by exogenous LPC-16. The purpose of this study is to get an idea whether LPC-16 is sufficient to produce the typical ischemic electrical disturbance and if yes, whether this action of LPC-16 is related to any alterations of I_{Kr}, I_{Ks} and K_{ATP};
4) To investigate how LPC-16 modulates the function of I_{Kr} in rabbit ventricular myocytes. The purpose of this study is to decipher the ionic mechanisms by which LPC-16 accumulation during the early stage of acute myocardial ischemia causes $[K^+]_o$-↑ and APD-↓.

I-10 References

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PART II

ORIGINAL CONTRIBUTIONS

CHAPTER II

Modulate of I_{HERG}/I_{Kr} by Phospholipid Metabolites Contributes to the Arrhythmogenesis during the Early Phase of Acute Myocardial Ischemia

There are three articles in this chapter. In the first article we described our finding that apart from being a well-recognized target for drug inhibition, I_{HERG} can also be enhanced by the natural metabolite lysophosphatidylcholine, which profoundly accumulates during the early phase of myocardial ischemia. Furthermore, the mechanism of enhancement involved a substantial positive shift in I_{HERG} inactivation voltage-dependence. This finding, to our knowledge, is the first time that a shift in I_{HERG} inactivation voltage-dependence has been shown to underlie a potentially significant endogenous pathophysiological mechanism. In addition to the potential intrinsic significance of this observation, it may open up new possibilities in exploring the regulation of HERG properties by membrane lipids.

Following the finding we carried out a series of detailed experiments to investigate the potential mechanisms for the enhancement of HERG K^+ channel function by phospholipid metabolites. Our results demonstrate that the enhancement of HERG function by lysophospholipids is specific to the lipids with 16-hydrocarbon chain structure (LPC-16 and LPG-16) and the pattern of voltage dependence is determined by the polar headgroups. The increase in I_{HERG} is best described by direct interactions between lipid molecules and HERG proteins, which are consistent with lack of effects *via* membrane destabilization or modulation by intracellular signaling pathways. These results will be presented in the second article of this chapter.

In the third article we combine *in vivo* and *in vitro* electrophysiological and biochemical approaches to verify that LPC-16 accumulation/HERG enhancement may be the link between metabolic trigger and ionic pathway for ischemic $[K^+]_o$-↑ and QT-↓. This represents the first documentation of I_{Kr}/HERG as the ionic mechanism in ischemic $[K^+]_o$-↑ and QT-↓. Inhibition of LPC-16 production and accumulation and/or of I_{Kr}/HERG may be a promising therapeutic strategy to attenuate the incidence of lethal arrhythmias associated with ischemic heart disease.

II-1 Phospholipid Metabolite 1-Palmitoyl-Lysophosphatidylcholine Enhances Human *Ether-a-Go-Go*-Related Gene (HERG) K^+ Channel Function

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II-1 Phospholipid Metabolite 1-Palmitoyl-Lysophosphatidylcholine Enhances Human *Ether-a-Go-Go*-Related Gene (HERG) K^+ Channel Function

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II-1-1 ABSTRACT

Background Lysophosphatidylcholine (LPC), a naturally occurring phospholipid metabolite, accumulates in the ischemic heart and causes extracellular K^+ accumulation and action potential shortening. LPC has been incriminated as a biochemical trigger of lethal cardiac arrhythmias, but the underlying mechanisms remain poorly understood.

Methods and Results We studied the effect of 1-palmitoyl-LPC (Pal-LPC) on currents resulting from human *ether-a-go-go*-related gene (HERG) expression in human embryonic kidney (HEK) cells using whole-cell patch-clamp techniques. Bath application of Pal-LPC consistently and reversibly increased HERG current (I_{HERG}). The effects of Pal-LPC were apparent as early as 3 minutes after application of the drug, reached maximum within 10 minutes, and were reversible on washout. Pal-LPC increased I_{HERG} at voltages between -20 and +30 mV, with greater effects at stronger depolarization. However, Pal-LPC did not affect the voltage-dependence of I_{HERG} activation. In contrast, Pal-LPC significantly shifted the inactivation curve toward more positive potentials, causing a mean 20.0±2.2 mV shift in half-inactivation voltage relative to control.

Conclusions Our results indicate that apart from being a well-recognized target for drug inhibition, I_{HERG} can also be enhanced by natural substances. An increase in I_{HERG} by Pal-LPC may contribute to K^+ loss, abnormal electrophysiology, and arrhythmia occurrence in the ischemic heart.

Key Words: lysophosphatidylcholines • ion channels • patch-clamp techniques

II-1-2 INTRODUCTION

Lysophosphatidylcholine (LPC) is a naturally occurring intracellular phospholipid metabolite that is present in a variety of mammalian tissues.[1] LPC accumulates rapidly in the heart during cardiac ischemia and in diabetic cardiomyopathy.[2] It has been incriminated as a biochemical trigger of lethal cardiac arrhythmias,[3-8] and it promotes

abnormal rhythmic activity, delayed afterdepolarizations, triggered activity, and intramyocardial reentry. Action potential duration (APD) shortening by LPC has been documented in rabbit atrial and ventricular cells,[4] guinea pig ventricular cells,[5] and canine ventricular cells[6] and Purkinje fibers.[3] LPC levels correlate with the occurrence of arrhythmias in ischemic and diabetic hearts.[7-9]

Inhibitory effects on sodium current[10] and inwardly rectifying K^+ current (I_{K1}) [11] explain the conduction slowing and membrane depolarization produced by LPC, but the mechanisms underlying APD shortening remain incompletely understood. Reduced I_{K1} should, if anything, increase APD. A recent study by Goldhaber et al[12] reported that LPC (20 μmol/L) decreases tissue K^+ content by ~15%, an effect associated with gradual APD shortening and increased K^+ efflux. During acute myocardial ischemia, increased K^+ efflux in the face of maintained Na^+/K^+ pumping results in extracellular K^+ accumulation, a key arrhythmogenic factor. Although increased K^+ efflux can account for some of the important arrhythmogenic effects of LPC, how LPC increases K^+ efflux is essentially unknown. Increased K^+ conductance could account for APD abbreviation and could contribute to increased K^+ efflux; however, LPC is not known to increase cardiac K^+-currents.

The human *ether-a-go-go*-related gene (HERG) encodes the rapid delayed rectifier K^+ current (I_{Kr}),[13] a critically important cardiac repolarizing current in most animals, including humans.[14] Apart from being a molecular target for mutations that generate long-QT syndrome, HERG is also a prime pharmacological target for a wide range of drugs that block the HERG current (I_{HERG}) exclusively; this blockade either confers antiarrhythmic efficacy or produces proarrhythmic cardiotoxicity.[15] The present study was designed to investigate whether LPC can modulate I_{HERG} and, if so, to characterize the biophysical components of this action.

II-1-3 METHODS

II-1-3-1 Cell Culture

HEK293 cells stably expressing HERG (a kind gift from Drs Zhou and January, University of Wisconsin, Madison[16]) were seeded in a 25-cm^2, triangular, cell-culture

flasks and grown in Dulbecco's modified eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 200-μmol/L G418, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells subcultured to ~85% confluency were harvested by trypsinization and stored in Tyrode solution containing 0.5% BSA at 4°C. Electrophysiological recordings were conducted within 10 hours of storage.

II-1-3-2 Whole-Cell Patch-Clamp Recording

Patch-clamp techniques have been described in detail elsewhere.[14] Currents were recorded by whole-cell voltage-clamp with an Axopatch-200B amplifier (Axon Instruments). Borosilicate glass electrodes had tip resistances of 1 to 3 MΩ when filled with (in mmol/L): GTP 0.1, potassium aspartate 110, KCl 20, $MgCl_2$ 1, Mg-ATP 5, HEPES 10, and phosphocreatine 5 (pH 7.2). The extracellular solution contained (in mmol/L): NaCl 136, KCl 5.4, $MgCl_2$ 1, HEPES 5, glucose 10, and $CaCl_2$ 1 (pH 7.4). Experiments were conducted at 36±1°C. Junction potentials were zeroed before formation of the membrane-pipette seal. Series resistance and capacitance were compensated, and leak currents were subtracted. 1-Palmitoyl-LPC (Pal-LPC; Sigma) was dissolved directly into the bath solution at the desired concentrations immediately before each experiment. 1-Palmitoyl-lysophosphatidylglycerol (Avanti Polar Lipid) and membrane-permeable ceramide (Sigma) were used for negative controls.

II-1-3-3 Data Analysis

Group data are expressed as mean±SEM. Comparisons among groups were made by ANOVA (F test), Bonferroni-adjusted *t* tests were used for multiple group comparisons, and paired *t* tests were used for single comparisons. A 2-tailed $P<0.05$ indicated statistically-significant difference. Nonlinear least-square curve-fitting was performed with CLAMPFIT in pCLAMP 8.0 or Graphpad Prism.

II-1-4 RESULTS

Bath application of Pal-LPC reversibly increased I_{HERG} (Figures 1A and 1B) in a concentration-dependent manner (Figure 1D). Pal-LPC increased I_{HERG} at voltages between -20 and +30 mV (Figure 1B), and the F-test indicated the effect was voltage-

dependent (P<0.01, Figure 1C). However, the half-maximum activation voltage, which was determined by the tail currents, was not affected by Pal-LPC (from control to LPC: -26±3 to -24±3 mV, P>0.05), nor was the slope factor k (from control to Pal-LPC: 8±1 to 9±1 mV, P>0.05). The effects of Pal-LPC were apparent (~37% the maximum) as early as 3 minutes after application of the drug, and they reached maximum within 10 minutes (Figure 1E). Under our experimental conditions, ~2.5 minutes are required for the solution to circulate into the bathing chamber, and this implies that Pal-LPC effects took place within 0.5 to 1 minute of exposure. Neither 1-palmitoyl-lysophosphatidylglycerol (5 μmol/L) nor membrane-permeable ceramide (50 μmol/L) produced significant effects on I_{HERG} (Figures 1F and 1G).

Pal-LPC significantly shifted the inactivation curve toward more positive potentials (Figures 2A and 2B), causing a mean 20.0±2.2 mV shift in half-maximum activation voltage for inactivation relative to control. Figure 2C shows Pal-LPC-induced percent change in currents at +20 mV after prepulses to different voltages, and it indicates that Pal-LPC did not alter currents when inactivation was fully removed by prepulses to more negative voltages but it significantly increased current at prepulse voltages allowing for inactivation. The inactivation time constants determined by monoexponential fit to the decaying currents recorded at +20 mV (Figure 2A) were 4.8±0.4 and 6.9±0.9 ms for control and Pal-LPC, respectively (P<0.05). The recovery time constants measured by the inward currents during the hyperpolarizing prepulses were not significantly altered by Pal-LPC (from 1.9±0.1 to 1.7±0.2 ms, P>0.05).

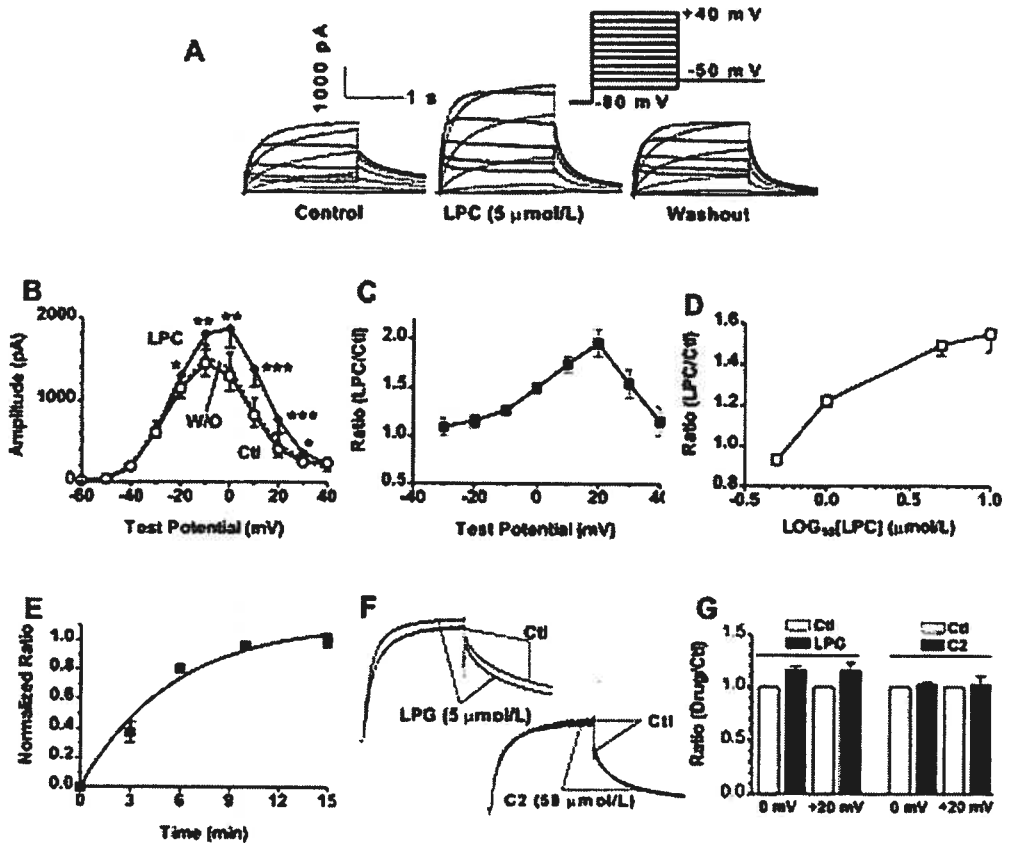

Figure 1. **A**, I_{HERG} recordings in control conditions, 10 minutes after LPC application, and after 10 minutes of LPC washout. **B**, I_{HERG} step current-voltage relations in control conditions (Ctl, ○), 10 minutes after LPC (5 µmol/L; ◊) application, and after 10 minutes of LPC washout (W/O; ▲). **P*<0.05, ***P*<0.01, and ****P*<0.001, LPC versus control (n=10 cells). **C**, Ratio of I_{HERG} in the presence of LPC over control (*P*<0.05 by ANOVA for the voltage-dependence of current increase between -30 and +20 mV). **D**, Concentration-dependence of LPC on I_{HERG} at 0 mV (n=12). **E**, Time-course (time after LPC application) of LPC effects (n=10), expressed as ratios of LPC/control, normalized to 15 minutes. **F** and **G**, Raw and mean data of I_{HERG} before and after application of 1-palmitoyl-lysophosphatidylglycerol (LPG; n=6) or membrane-permeable ceramide (C2; n=6).

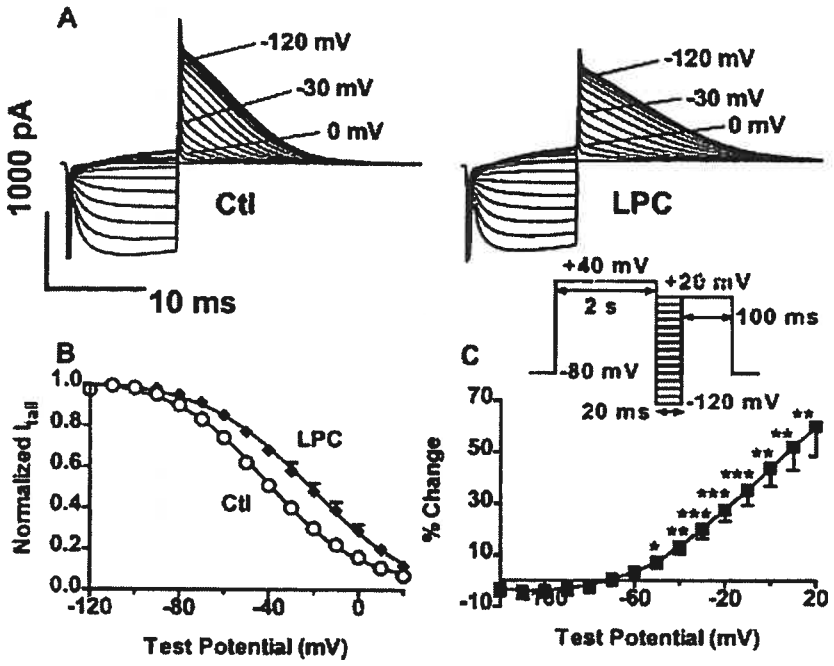

Figure 2. **A**, I_{HERG} recordings (with voltage protocol in inset) before and 10 minutes after LPC application. **B**, I_{HERG} inactivation voltage-dependence. The tail current (I_{tail}) during the test pulse to +20 mV at various prepulse potentials was normalized to the value for a prepulse to -120 mV. Symbols are mean±SEM of 13 experiments, and curves are Boltzmann fits. Half-maximum activation voltage for inactivation was shifted by LPC from -40.9±3.4 to -21.2±1.8 mV (P<0.001). **C**, Ratio of I_{HERG} with LPC over control (P<0.001 for voltage-dependence by ANOVA). *P<0.05, **P<0.01, and ***P<0.001 for LPC versus control.

II-1-5 DISCUSSION

In this study, we demonstrated that Pal-LPC increases I_{HERG} in HEK293 cells. The Pal-LPC-induced increase in I_{HERG} is reversible and occurs within minutes of extracellular application. The current-enhancing effect is in large measure due to a substantial depolarizing shift in I_{HERG} inactivation, such that more current is available at any given voltage within the activation range.

Intracellular K^+ loss and accompanying extracellular K^+ accumulation impairs cardiac electrical activity and can also promote cell death through apoptosis,[17] thereby contributing to the pathophysiology of the ischemic myocardium. ATP-sensitive K^+ current (I_{KATP}) contributes significantly to the rapid increase in cellular K^+ efflux and APD shortening during myocardial ischemia and hypoxia. However, during the first 10 minutes of ischemia in intact hearts, cytosolic ATP concentrations remain ~2 orders of magnitude greater than the ATP concentration, thus causing half-maximal blockade of I_{KATP} in excised membrane patches.[18] It is conceivable that membrane conductance other than I_{KATP} may contribute to APD abbreviation and loss of intracellular K^+.

LPC, a class of lysophospholipids that accumulate in the cell membrane during acute myocardial ischemia, decreases peak but enhances sustained Na^+ current, resulting in a noninactivated component in ventricular myocytes of various species.[10,19,20] LPC also reduces I_{K1}, [11] the major determinant of the membrane resting potential in cardiac cells. These findings can account for the conduction slowing and membrane depolarization caused by LPC, but they do not explain how LPC produces APD shortening [3–6] and K^+ loss.[12] Our observation regarding Pal-LPC effects on I_{HERG} may explain, at least in part, the latter observations. Because Pal-LPC increased I_{HERG} significantly in the range of plateau voltages (-10 to +10 mV), it is quite possible that Pal-LPC promotes the repolarization of cardiac action potentials or APD shortening during acute ischemia and that Pal-LPC-induced I_{HERG} enhancement could also contribute to K^+-efflux.

Many class III drugs produce their antiarrhythmic effects by acting on HERG/I_{Kr} currents. In addition, a wide spectrum of non-antiarrhythmic agents can cause proarrhythmic effects (acquired long-QT syndrome) by inhibiting I_{HERG}. [15] In the present study, we found that I_{HERG} can also be enhanced. Furthermore, the mechanism of enhancement involved a substantial positive shift in I_{HERG} inactivation voltage-dependence. The rapid inactivation mechanism of I_{HERG} is a distinct property and, to our knowledge, this is the first time that a shift in I_{HERG} inactivation voltage-dependence has been shown to underlie a potentially significant endogenous pathophysiological mechanism. In addition to the potential intrinsic significance of this observation, it may open up new possibilities in exploring the regulation of HERG properties by membrane lipids.

Under ischemic conditions, LPC (normally present at 0.5% to 3.5% of total membrane phospholipids), increases to an extent that varies among different species and different tissue compartments. The typical LPC concentration range is 100 to 200 μmol/L. [21] Assuming that ~90% of LPC protein-bound, the free concentration of LPC is 10 to 20 μmol/L. LPC, mainly Pal-LPC,[21–23] accumulation during cardiac ischemia is thought to occur predominantly in the extracellular space.[24] Thus, the concentration of Pal-LPC (5 μmol/L) used in this study is likely relevant to pathophysiological situations.

II-1-6 ACKNOWLEDGEMENTS

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II-2 Potential Mechanisms for the Enhancement of HERG K^+ Channel Function by Phospholipid Metabolites

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II-2 Potential Mechanisms for the Enhancement of HERG K^+ Channel Function by Phospholipid Metabolites

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II-2-1 ABSTRACT

1. Phospholipid metabolite lysophospholipids cause extracellular K^+ accumulation and action potential shortening with increased risk of arrhythmias during myocardial ischemia. Here we studied effects of several lysophospholipids with different lengths of hydrocarbon chains and charged headgroups on HERG K^+ currents (I_{HERG}) expressed in HEK293 cells and the potential mechanisms using whole-cell patch-clamp techniques.

2. Only the lipids with 16 hydrocarbons such as 1-palmitoyl-lysophosphatidylcholine (LPC-16) and 1-palmitoyl-lysophosphatidylglycerol (LPG-16) were found to produce significant enhancement of I_{HERG} and negative shifts of HERG activation, although the voltage dependence of the effects was different between LPC-16 and LPG-16 which have differently charged headgroups. The lipid with 18 hydrocarbons modestly increased I_{HERG}. The lipids with 6 or 24 hydrocarbons had no effect or slightly decreased I_{HERG}.

3. Inhibition or activation of protein kinase C did not alter the effects of LPC-16 and LPG-16. Participation of phosphatidylinositol-4, 5-bisphosphate in I_{HERG} enhancement by LPC-16/LPG-16 was also excluded.

4. Vitamin E augmented the effects of LPC-16/LPG-16 whereas xanthine/xanthine oxidase reduced I_{HERG}: indicating that LPC-16/LPG-16 produced dual effects on I_{HERG}: direct enhancement of I_{HERG} and indirect suppression *via* production of superoxide anion.

5. We conclude that enhancement of HERG function by lysophospholipids is specific to the lipids with 16-hydrocarbon chain structure and the pattern of voltage dependence is determined by the polar headgroups. The increase in I_{HERG} is best described by direct interactions between lipid molecules and HERG proteins, which is consistent with lack of effects *via* membrane destabilization or modulation by intracellular signaling pathways.

Keywords: HERG; lysophospholipids; 1-palmitoyl-lysophosphatidsylcholine; 1-palmitoyl-lysophosphatidylglycerol; protein kinase C; phosphatidylinositol-4,5-bisphosphate; oxidative stress; vitamin E

Abbreviations: Bis, bisindolylmaleimide; I_{HERG}, current carried by HERG K^+ channels; LPC, lysophosphatidylcholine; LPC-6, 1-caproyl- lysophosphatidylcholine; LPC-16, 1-palmitoyl-lysophosphatidylcholine; LPC-18:1, 1-oleoyl-lysophosphatidylcholine; LPC-24,

1-lignoceroyl-lysophosphatidylcholine; LPG-16, 1-palmitoyl-lysophosphatidylglycerol; PDD, phorbol ester 12,13-didecanoate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP_2-Ab, inhibitory antibody against phosphatidylinositol-4,5-bisphosphate; PMA, phorbol 12-myristate 13-acetate; VitE, vitamin E; X/XO, xanthine/xanthine oxidase.

II-2-2 INTRODUCTION

The cell surface membrane or sarcolemma is primarily composed of phospholipid, cholesterol, and proteins, which together form a complex dynamic structure that retains the intracellular contents, regulates ion homeostasis, governs nutrient transports, and tansduces extracellular signals into the cells. The principal classes of sarcolemmal phospholipids are, according to their relative abundance, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine (Corr *et al.*, 1995). The sarcolemmal phospholipids are composed of a charged polar headgroup region and two nonpolar covalently bound long-chain aliphatic hydrocarbon moieties. Under certain situations, for example, in ischemic tissues, the phospholipids (e.g. phosphatidylcholine) can be metabolized to generate lysophosphatidylcholine (LPC), known as a biochemical trigger of ischemic arrhythmias in the heart. LPC is an amphiphile possessing a charged headgroup like phospholipids but only a single aliphatic hydrocarbon chain as a consequence of the hydrolytic cleavage of one of the two aliphatic hydrocarbon groups of phosphatidylcholine (Hatch *et al.*, 1989; Choy *et al.*, 1997). As an intermediate of metabolism of phosphatidylcholine, LPC is present in a variety of mammalian tissues (Prokazova *et al.*, 1998) and accumulates rapidly in the heart during cardiac ischemia and in diabetic cardiomyopathy (Saffitz *et al.*, 1984; Makino *et al.*, 1987; Kinnaird *et al.*, 1988; Corr *et al.*, 1995). Accumulation of LPC is thought to be a major contributor to the development of cardiac arrhythmias during myocardial ischemia (Fazekas *et al.*, 1992; Man 1988; Corr *et al.*, 1987; Saffitz *et al.*, 1984), such as abnormal rhythmic activity, delayed afterdepolarizations (DAD), triggered activity, and intramyocardial re-entry. Action potential duration (APD) shortening by LPC has been documented in rabbit atrial and ventricular cells (Fazekas *et al.*, 1992), guinea pig ventricular cells (Liu *et al.*, 1991), and canine ventricular cells (Saffitz *et al.*, 1984) and Purkinje fibers (Corr *et al.*, 1995). LPC levels correlate with the occurrence of

arrhythmias in ischemic and diabetic hearts (Corr *et al.*, 1987; Kinnaird *et al.*, 1988; Man, 1988). The most profound alterations associated with electrical activities in ischemic myocardium are extracellular K^+ accumulation and shortening of APD. This extracellular K^+ accumulation in the face of maintained Na^+/K^+ pumping and unaltered ATP-sensitive K^+ current is a key arrhythmogenic factor during myocardial ischemia. Coincidentally, a recent study by Goldhaber *et al.* (1998) reported that LPC (20 μM) decreases tissue K^+ content by 15%, an effect associated with gradual APD shortening and increased K^+ efflux.

The ability of LPC to induce arrhythmias is likely accounted at least partly by its ability to affect ion channel functions. Indeed, LPC at concentrations between 5 and 50 μM was found to inhibit I_{K1} in several studies (Clarkson & Ten Eick, 1983; Kiyosue *et al.*, 1984; Sato *et al.*, 1993) and 5 μM LPC caused about 30% decrease in the single-channel conductance of I_{K1} without altering the open probability. LPC inhibits the peak sodium current (I_{Na}) but increases the sustained component of I_{Na} in cardiac cells (Burnashev *et al.*, 1991; Undrovinas *et al.*, 1992). The inhibitory effects of LPC on sodium current and I_{K1} explain LPC-induced conduction slowing and membrane depolarization but do not account for APD shortening. Instead, inhibition of I_{K1} and enhancement of slowly inactivating I_{Na} should, if anything, prolong APD. In our recent study (Wang *et al.*, 2001a), we revealed that LPC significantly enhances the K^+ current expressed by HERG (the human *ether-a-go-go*-related gene), which encodes the rapid delayed rectifier K^+ current (I_{Kr}) (Sanguinetti *et al.*, 1995), one of the key cardiac repolarizing currents in most animals, including man (Wang *et al.*, 1993). LPC increases HERG conductance (I_{HERG}), accelerates I_{HERG} activation and slows I_{HERG} inactivation (Wang *et al.*, 2001a), effects deemed to augment K^+ efflux through HERG channels. These observations provide an explanation for LPC-induced extracellular K^+ accumulation and APD shortening occurring in ischemic myocardium. Yet how LPC modulates I_{HERG}, or how the biochemical substrate (LPC) and the electrical substrate (HERG) for the altered electrical activities in ischemic myocardium interact with each other, remained unclear.

In theory, LPC can act on ion channels through at least three different mechanisms. First, LPC can readily incorporate into the sarcolemma, and incorporation of LPC into the membrane phospholipid bilayer results in significant perturbation of the orderly packed

phospholipid molecules and alteration of normal conformation of integral membrane proteins such as ion channels (Corr *et al.*, 1995). Second, LPC may interact directly with ion channel proteins. LPC has an easy assess to both the intracellular and extracellular sides of membrane and may bind to channel proteins so as to alter the conductance of the channels. And third, HERG modulation by LPC may be mediated through intracellular signaling pathways.

The present study was designed to clarify which of the above three potential mechanisms account for LPC enhancement of HERG K^+ channel function. To this end, we carried out the following experiments: (1) to compare the effects of lysophospholipids with varying lengths of aliphatic hydrocarbon chain on I_{HERG} stably expressed in HEK293 cells; (2) to compare the effects of lysophospholipids with different charged headgroups; (3) to investigate possible participation of some related intracellular signaling pathways in HERG regulation by the lysophospholipids.

II-2-3 METHODS

II-2-3-1 Cell Culture

HEK293 cells stably expressing HERG (a kind gift from Drs Z Zhou and C January) (Zhou *et al.*, 1998) were seeded in a 25 cm^2 triangular cell-culture flask and grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 200 μM G418, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. The cells subcultured to ~85% confluency were harvested by trypsinization and stored in Tyrode solution containing 0.5% BSA at 4°C. Electrophysiological recordings were conducted within 10 h of storage.

II-2-3-2 Whole-Cell Patch-Clamp Recording

Patch-clamp techniques have been described in detail elsewhere (Wang *et al.*, 2001a, 2001b). Currents were recorded by whole-cell voltage clamp with an Axopatch-200B amplifier (Axon Instruments). Borosilicate glass electrodes had tip resistances of 1–3 M when filled with the internal solution containing (mM): 110 potassium aspartate, 20 KCl, 1 $MgCl_2$, 5 Mg-ATP, 10 HEPES (pH 7.2). The extracellular solution contained

(mM): 136 NaCl, 5.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 5 HEPES (pH 7.4). Experiments were conducted at 36±1°C. Junction potentials were zeroed before formation of the membrane-pipette seal. Series resistance and capacitance were compensated and leak currents were subtracted.

All phospholipids were purchased from Avanti Polar Lipid Inc. (Alabaster, AL, U.S.A.) except for phosphatidylinositol-4, 5-bisphosphate (PIP_2), which was purchased from Calbiochem-Novobiochem International (La Jolla, CA, U.S.A.). To simplify the terminology, the following abbreviations are used in this manuscript: LPC-6 for 1-caproyl- lysophosphatidylcholine, LPC-16 for 1-palmitoyl-lysophosphatidylcholine, LPC-18:1 for 1-oleoyl-lysophosphatidylcholine which contains one double bond, LPC-24 for 1-lignoceroyl-lysophosphatidylcholine, and LPG-16 for 1-palmitoyl-lysophosphatidylglycerol. The numbers indicate the number of carbon atoms in the aliphatic chain. LPC-6, PLC-16, and LPG-16 were dissolved directly into the bath solution at the desired concentrations immediately before each experiment. LPC-18 and LPC-24 were dissolved in 100% chloroform as 1000 x stock solutions. Preparation of PIP_2 solution followed the procedures described by Bian *et al.* (2001). Briefly, PIP_2 was dispersed by sonication in water (0.5 mM) for 30 min on ice and then divided into aliquots and keep at -80°C. Before each experiment, an aliquot was thawed and diluted to 10 μM in the pipette solution and sonicated again for 20 min. PIP_2-specific antibody (Assay Designs Inc., Ann Arbor, MI, U.S.A.) was diluted to 60 nM in the pipette solution. To minimize binding of PIP_2 antibody to the pipette wall, 100 μM BSA was included to the pipette solution. The protein kinase C (PKC)-stimulating phorbol ester 12, 13-didecanoate (PDD), phorbol 12-myristate 13-acetate (PMA), bisindolylmaleimide (Bis; PKC inhibitor), xanthine (X), xanthine oxidase (XO), and vitamin E (VitE) were all purchased from Sigma. PDD, PMA, and Bis were prepared as 1000 x final concentration stock solutions in Me_2SO and diluted into patch-clamp recording Tyrode solution at the time of experiments. X was dissolved in 2 N NaOH and diluted in Tyrode solution by 800 times with pH adjusted to 7.4 with HCl. XO was added to the X preparation to form the X/XO superoxide anion (O^{2-}) generating system. VitE was dissolved in ethanol and diluted by 1000 times to reach the final concentration. All the solvents other than the Tyrode solution, which was used to dissolve the drugs used in this study, had an experimental

concentration of 0.1% and for the experiments involving these solvents, the control recordings were made in the presence of 0.1% of such solvents.

II-2-3-3 Data Analysis

Group data are expressed as mean±s.e.m. Comparisons among groups were made by ANOVA (F-test), and Bonferroni-adjusted *t*-tests were used for multiple group comparisons and paired *t*-test was used for single comparison. A two-tailed $P<0.05$ was taken to indicate a statistically significant difference. Nonlinear least-square curve fitting was performed with CLAMPFIT in pCLAMP 8.0 or Graphpad Prism 3.0.

II-2-4 RESULTS

II-2-4-1 Enhancement of I_{HERG} – an Effect Specific to the Lysophospholipids with 16 Aliphatic Hydrocarbon Chains

This part of the studies was designed to test the possibility that lysophospholipids modulate HERG K^+ channel function by altering biophysical properties of membrane owing to their ability to incorporate into the cytoplasmic membrane. This was assessed indirectly from two different aspects. First, effects of lysophospholipids with different lengths of aliphatic hydrocarbon chains (Figure 1) on I_{HERG} were investigated. One would expect that lysophospholipids with longer aliphatic hydrocarbon chains, and thereby greater hydrophobicity and accessibility to the lipid bilayer, should have greater effects on I_{HERG}, if membrane incorporation is indeed required for the actions. Second, since the incorporation of lysophospholipids into sarcolemma can alter sarcolemmal ultrastructure and destabilize the lipid bilayer, the effects induced by LPC would be expected to persist even after the lipids would have been withdrawn from the media. Therefore, by assessing the time course of I_{HERG} recovery to baseline from changes induced by the lipids upon washout of the drugs, one can deduce if a drug acts directly by interacting with the channels or indirectly requiring long-lasting modifications of the lipid bilayer environment in which the channels seat.

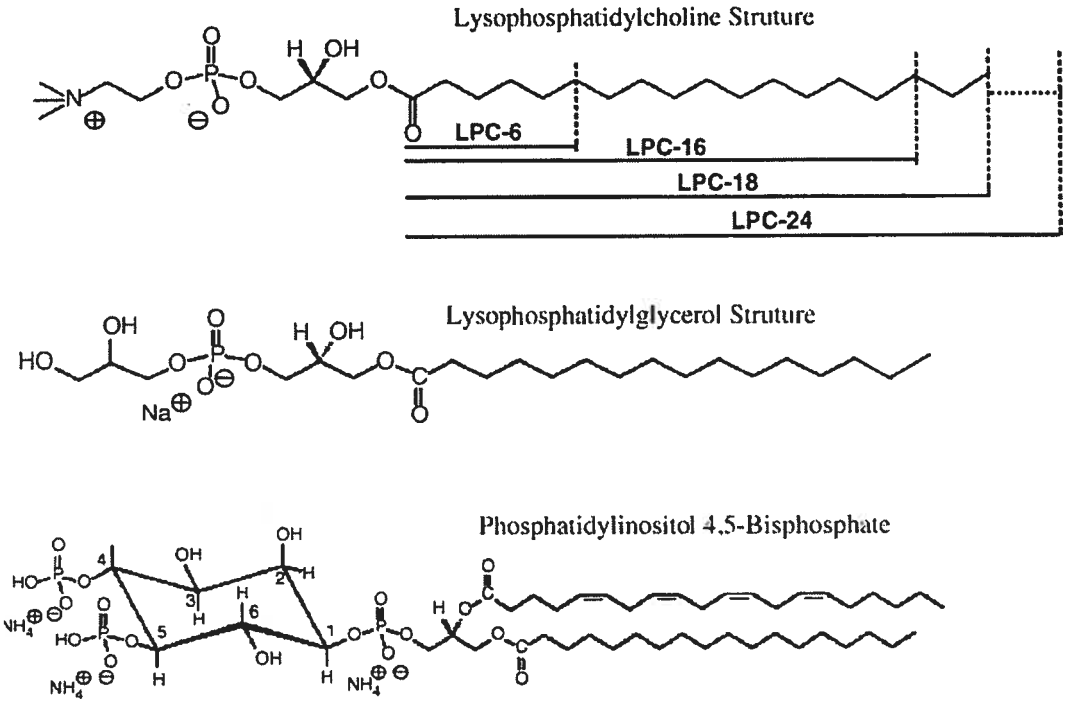


Figure 1. Structures of LPCs, LPG-16 and PIP_2. The length of aliphatic hydrocarbon chain of each lipid used in our study is indicated by the dash lines and the numbers following the short names of the lipids. Note the neutral headgroup of LPC-16 and negatively charged headgroups of LPG-16 and PIP_2.

Depolarizing steps from a holding potential of -80 mV elicited time-dependent activation of I_{HERG} that peaked at -10 mV and decreased in amplitude with stronger depolarization due to the rapid inactivation process of the channels. I_{HERG} was monitored for 10 min after the formation of whole-cell configuration to ensure the stability of the current under the normal Tyrode solution, and the cells with current rundown >10% its initial amplitude were excluded from further experiments. Then, the superfusion was switched to the Tyrode solution containing one of the lysophospholipids and the same current recordings were repeated every 5 min up to 20 min. Recordings made at 15 min

after drug application were analyzed for drug effects. As illustrated in Figure 2 with the averaged data accompanied with the raw data in the insets, LPC-16 (5 μM) and LPG-16 (5 μM) both substantially increased I_{HERG} amplitude. A concentration of 5 μM LPC-16 was chosen because it is within the range of free concentration of LPC (from 5 to 20 μM) in the extracellular space during cardiac ischemia (Liu *et al.*, 1997), and for better comparison the concentration of other lysophospholipids studied was also set to 5 μM unless otherwise specified.

In contrast, LPC-6 (5 μM) and LPC-24 (5 μM) produced some but not statistically significant decreases in I_{HERG}. Since LPC-6 may have weaker accessibility to the cell through the membrane because of its lower hydrophobicity, intracellular application was performed with LPC-6 (5 μM) included in the pipette solution. Yet LPC-6 still failed to increase I_{HERG} but actually slightly reduced the current under such a condition. Moreover, no I_{HERG} enhancement was observed even when extracellular LPC-6 concentration was doubled to 10 μM. LPC-24 at a concentration of 5 μM might form micelles because this concentration is above the critical micelle concentration (1 μM), which might affect its effect on I_{HERG}. Thus, effects of 1 μM LPC-24 were also investigated. However, no changes of I_{HERG} were found (data not shown). By comparison, LPC-18:1 which has similar chain length as LPC-16 produced a slight increase in I_{HERG} amplitude. For example, at 0 mV, I_{HERG} amplitude was 63.7±9.1 pA before and 70.0±11.5 pA (P>0.05, n=8) 10 min after LPC-18 (5 μM) application, an increase by 13.4±3.9%. When the concentration of LPC-18 was elevated to 10 μM, the effects were also correspondingly augmented to 20.3±3.5% increase in I_{HERG} compared with control.

Alterations of I_{HERG} with time before and after LPC-16 or LPG-16 and after washout of the drugs were monitored (Figure 3). I_{HERG} generally demonstrated an initial transient and slight run-up within the first 5 min following whole-cell access and a subsequent also transient and slight rundown within the next 5 min. Addition of LPC-16 or LPG-16 produced rapid increases in I_{HERG} and the increases reached maximum levels within 10 min following drug application. Virtually complete recovery of I_{HERG} was achieved upon washout of the drugs 10 min after switch back to the drug-free solution.

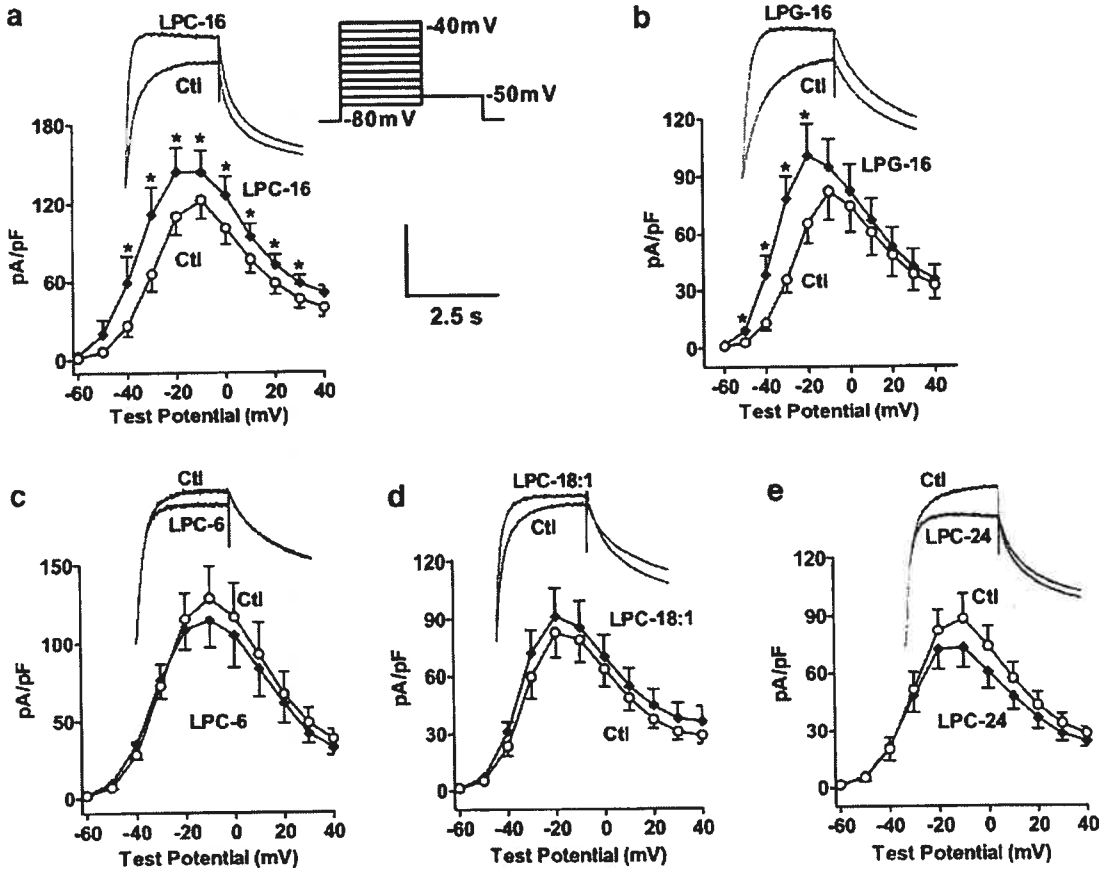


Figure 2. Current density-voltage relationships of I_{HERG} showing the effects of various lysophospholipids on I_{HERG} at various potentials tested. I_{HERG} was elicited by the voltage protocol shown in the inset. Raw traces of I_{HERG} recorded at 0 mV are shown in the insets. Ctl – control data recorded 10 min after the formation of whole-cell configuration; LPC-16, LPC-6, LPC18:1, and LPC-24 – currents recorded 10 min after superfusion with LPCs with 16-, 6-, 18-, and 24- hydrocarbon chains, respectively (LPG-16, lysophosphatidylglycerol with a 16-hydrocarbon chain). Data are means±s.e. from 13 cells for LPC-16, 13 for LPG-16, 10 for LPC-6, 11 for LPC-18:1, and 12 for LPC-24. Note that only LPC-16 and LPG-16 produced marked enhancement of I_{HERG}. * $P<0.05$ vs control (Ctl).

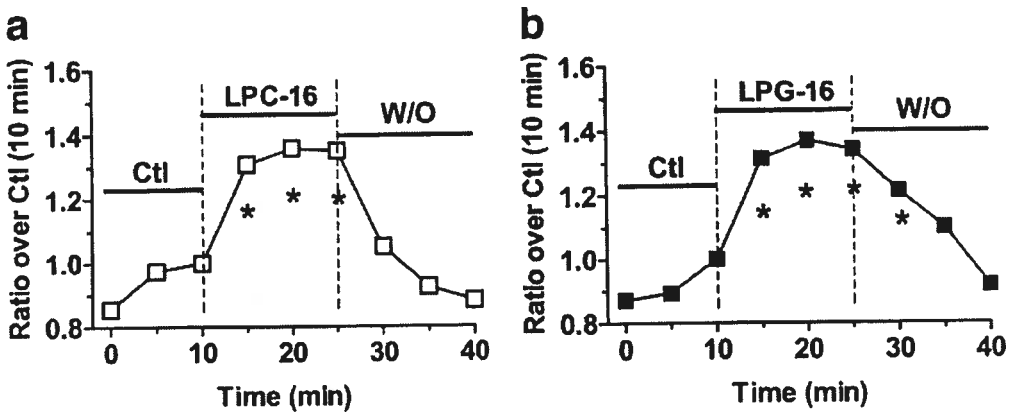


Figure 3. Time course of I_{HERG} before (Ctl) and after application of LPC-16 (a) or LPG-16 (b) and after washout of the drugs. *P<0.05 vs. control (Ctl) 10 min.

II-2-4-2 Lack of Influence of PKC on Lysophospholipid-Induced I_{HERG} Enhancement

LPC has been demonstrated to be able to activate several protein kinases including PKC (Prokazova *et al.*, 1998; Bassa *et al.*, 1999; Motley *et al.*, 2002) and protein kinase A (PKA) (Ahumada *et al.*, 1979). Particularly noteworthy is the ability of LPC to activate PKC because PKC has been importantly implicated in modulation of a variety of ion channels (Fedida *et al.*, 1993; Prokazova *et al.*, 1998; Bassa *et al.*, 1999; Wang *et al.*, 2001b; Motley *et al.*, 2002). To test this notion, we first looked at whether PKC inhibitor Bis could reverse or prevent the increase in I_{HERG} induced by LPC-16. The effects of LPC-16 on I_{HERG} were first established by bathing the cells with the Tyrode solution containing LPC-16 (5 μM) alone for 10 min, followed by addition of Bis (100 nM) to the LPC-16-containing solution for another 15 min. As illustrated in Figure 4c and 4d, Bis did not alter the effects of LPC-16 or of LPG-16, as indicated by roughly the same enhancement of I_{HERG} by LPC-16 (Figure 4a) or LPG-16 (Figure 4b) with and without co-application of Bis. In another experiment, the cells were preincubated with Bis (100 nM) for 30 min, followed by application of Bis and LPC-16. Under such conditions, LPC-16 maintained the same ability to enhance I_{HERG} as without Bis pretreatment (data not shown). Our data thus indicate that inhibition of PKC does not reverse the effects of LPC-

16 on I_{HERG}, neither does it prevent the effects of LPC-16 on I_{HERG}. Likewise, application of neither PDD (1 μM) nor PMA, (1 μM) to activate PKC increased I_{HERG} in the absence of LPC-16 (Figure 4e and 4f). Moreover, PDD or PMA also failed to alter LPC-16- or LPG-16-induced I_{HERG} enhancement.

To ensure that the lack of effects of PKC inhibitors and activators on I_{HERG} modulation by LPC-16 is not due to inappropriate drug concentrations used and/or an absence of PKC signaling pathway in HEK293 cells, we performed two positive control experiments. In the first set of experiment, the effects of PKC inhibitors and activators on transient outward K^+ current (I_{to}) in isolated canine ventricular myocytes were studied since we have previously demonstrated the ability of PKC to modulate I_{to} (Wang *et al.*, 2001b). Application of PDD (1 μM) to the bath suppressed I_{to} by ~35% and the effect was completely reversed by co-application of 50 nM Bis. In the second set of experiments, PKC activities were assayed to verify the ability of PDD to stimulate PKC activation in HEK293 cells. Consistent with our previous study (Wang *et al.*, 2001b), PDD significantly increased PKC activities by ~47%.

II-2-4-3 Lack of Influence of PIP_2 on Lysophospholipid-Induced I_{HERG} Enhancement

PIP_2, a phospholipid to be hydrolyzed to form 1,4,5-inositol trisphosphate (IP_3) and diacyl glycerol (DAG) by phospholipase C, is an intermediate in the G_q protein–IP_3/DAG–PKC signaling pathway. PIP_2 has been shown to increase I_{HERG} amplitude, accelerate I_{HERG} activation, and slow I_{HERG} inactivation (Bian *et al.*, 2001), effects resembling those produced by LPC as described in our previous study (Wang *et al.*, 2001a). This similarity prompted us to hypothesize that PIP_2 may be a mediator for LPC-16-induced I_{HERG} enhancement.

To test the hypothesis, we first tested whether inhibition of PIP_2 could prevent LPC-16 from increasing I_{HERG} by studying the effects of LPC-16 in the presence of the neutralizing monoclonal PIP_2-specific antibody (PIP_2-Ab). PIP_2-Ab (60 nM) was included in the pipette solution and I_{HERG} was recorded every 5 min after formation of whole-cell configuration for 10 min to allow complete dialysis before addition of LPC-16 or LPG-16.

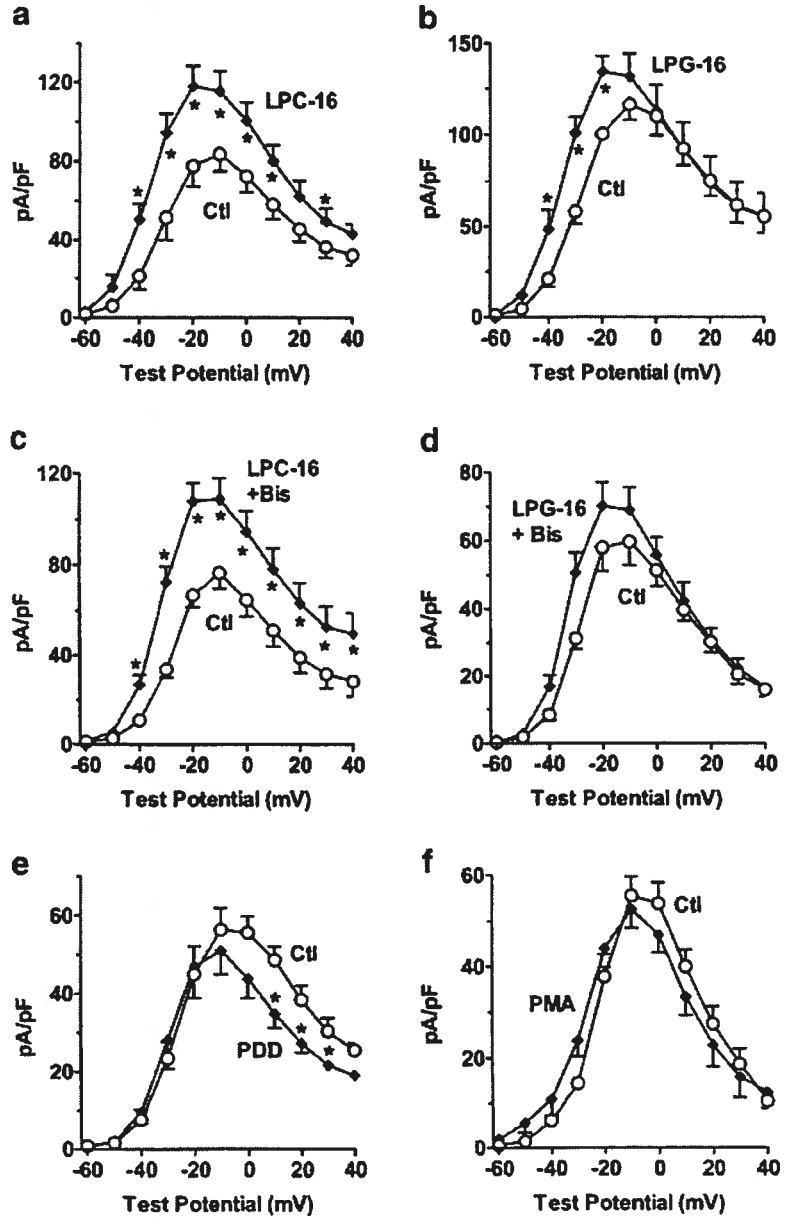


Figure 4. Effects of PKC on LPC-16- or LPG-16-induced I_{HERG} enhancement. (**a,b**) Increases in I_{HERG} by LPC-16 (n=6) (a) and LPG-16 (n=6) (b). (**c**) Inhibition of PKC inhibitor Bis 100 nM) fails to reverse I_{HERG} enhancement induced by LPC-16 (n=8) (c) or LPG-16 (n=5) (**d**). Bis was added to the superfusate containing LPC-16 (5 μM) or LPG-16 (5 μM) 10 min after establishment of I_{HERG} enhancement induced by LPC-16 or LPG-16 alone. (**e, f**) Lack of effects of PKC activators PDD (1 μM, n=5) (e) and PMA (1 μM, n=6) (f) on I_{HERG}. *P<0.05 vs. control (Ctl).

Subsequent addition of LPC-16 (5 μM) or LPG-16 (5 μM) to the bath increased I_{HERG} amplitude to the same extent as without PIP_2-Ab (Figure 5a and b), implying that the presence of PIP_2-Ab did not prevent the increase in I_{HERG} induced by LPC-16 or LPG-16.

To ensure that failure of PIP_2-Ab to reverse the effect of LPC-16 or LPG-16 on I_{HERG} was not due to inappropriate use of the inhibitor, we performed control studies. PIP_2 (10 μM) was included in the pipette solution and I_{HERG} was monitored over a 10-min period right after gaining whole cell access. I_{HERG} increased with time and the activation of I_{HERG} shifted towards hyperpolarizing potentials, consistent with the previous finding reported by Bian *et al.* (2001). I_{HERG} enhancement was abolished when PIP_2-Ab (60 nM) and PIP_2 (10 μM) were co-applied, validating the specificity and effect of PIP_2-Ab. Also noticeable is that the percentage of I_{HERG} increase induced by PIP2 is substantially smaller than that in the presence of LPC-16 or LPG-16, despite that the concentration of PIP_2 used here is two times higher than that of LPC-16 or LPG-16. For instance, at –20 mV, I_{HERG} increase was ~50 and ~45% by LPC-16 and LPG-16, respectively, but was only ~20% by PIP_2.

II-2-4-4 Influence of Antioxidant VitE on Lysophospholipid-Induced I_{HERG} Enhancement

Studies have demonstrated the ability of LPC-16 to enhance reactive oxygen species (ROS) production, specifically superoxide anion (O^{2-}), in cells (Ginsburg *et al.*, 1989; Ohara *et al.*, 1994; Nishioka *et al.*, 1998; Takeshita *et al.*, 2000; Inoue *et al.*, 2001). To investigate the possibility of ROS mediation of LPC-16 action on I_{HERG}, we assessed the influence of VitE (100 μM) on LPC-16- or LPG-16-induced I_{HERG} enhancement. Following a 10-min stabilization period with whole-cell recordings, VitE was co-applied with PLC-16 or LPG-16. Under such a condition, I_{HERG} was consistently increased, even to far greater extents than with LPC-16 or LPG-16 alone (Figure 6a-e). The increases in I_{HERG} at 0 mV were ~45% for LPC-16 and ~20% for LPG-16 in the absence of VitE, and ~70% for LPC-16 and ~110% for LPG-16 in the presence of, VitE. This increased effect of LPC-16 or LPG-16 by VitE cannot be interpreted as a direct enhancing effect of VitE on I_{HERG} because VitE alone failed to affect the current (Figure 6f). The data are explainable if we assume that O^{2-} produced by LPC-16 suppresses I_{HERG}.

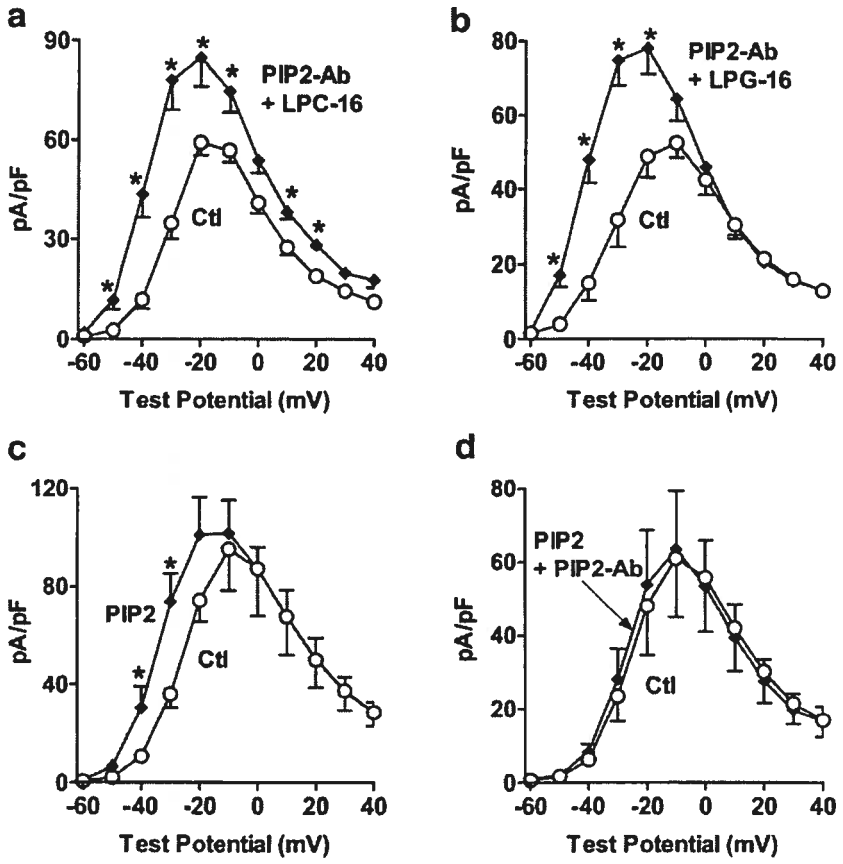


Figure 5. Effects of PIP_2 on LPC-16- or LPG-16-induced I_{HERG} enhancement. (**a**,**b**) Inhibition of PIP_2 by anti-PIP_2 antibody (PIP_2-Ab, 60 nM) fails to prevent I_{HERG} enhancement induced by LPC-16 (a) or LPG-16 (b). PIP_2-Ab was included in the pipette solution. I_{HERG} was monitored for 10 min before the addition of LPC-16 (5 μM) or LPG-16 (5 μM) to ensure complete dialysis of PIP_2-Ab into the cytosol. (**c**) Effects of PIP_2 on I_{HERG}. PIP_2 was applied intracellularly through the pipette solution. I_{HERG} measured at 10 min after membrane rupture was used for analysis (n=6). (**d**) Effects of co-application of PIP_2 and PIP_2-Ab on I_{HERG} (n=6). Note that PIP_2 lost the ability to alter I_{HERG}. $^*P<0.05$ vs. control (Ctl).

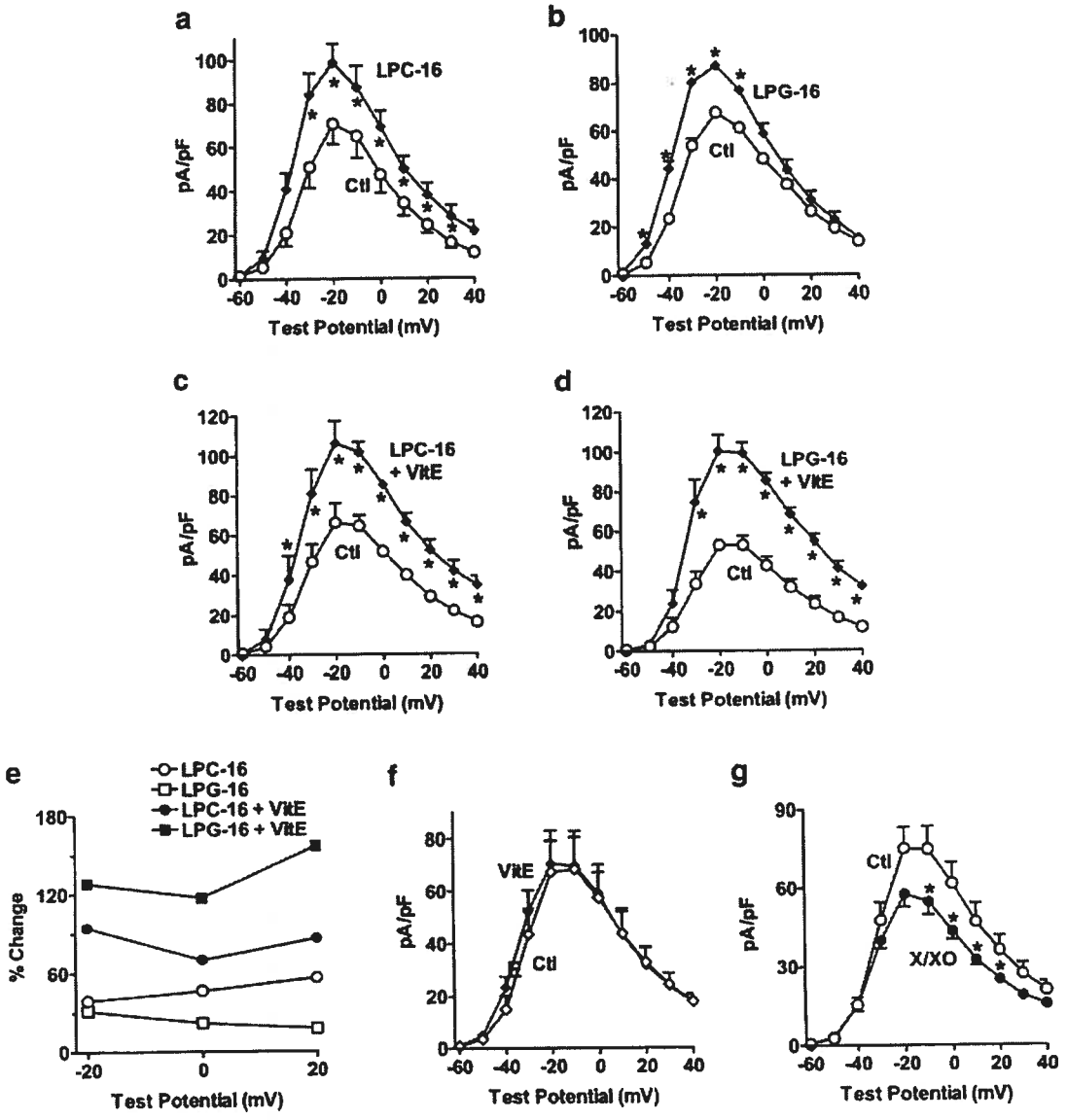


Figure 6. Effects of antioxidant VitE on LPC-16- or LPG-16-induced I_{HERG} enhancement. (**a, b**) Enhancement of I_{HERG} by LPC-16 (a, n=6) or LPG-16 (b, n=6). (**c, d**) Effects of co-application of LPC-16 or LPG-16 and VitE on I_{HERG}. (**e**) Effects of VitE on I_{HERG} (n=4). (**f**) Effects of X/XO on I_{HERG}. Cells were superfused with X/XO (500 μM/5 mU/ml) for >30 min before patch-clamp recordings. (**g**) Comparison of % changes of I_{HERG} with varying drugs over control (Ctl). Note the increased effect of LPC-16 and LPG-16 in the presence of VitE. *P<0.05 vs. Ctl.

The data are explainable if we assume that O^{2-} produced by LPC-16 suppresses I_{HERG}. This has actually been confirmed by our recent studies (Zhang *et al.*, 2003b). Here we further clarify this issue with an additional experiment using X/XO O^{2-}-generating system (Barrington *et al.*, 1988; Aiello *et al.*, 1995). Cells were incubated with or without X/XO (500 μM/5 mU/ml) in the Tyrode solution for ~40 min before I_{HERG} was recorded. I_{HERG} density was smaller in X/XO-treated cells than in X/XO-nontreated cells (Figure 6g).

II-2-4-5 Comparison of Effects of Lysophospholipids with Varying Lengths of Aliphatic Hydrocarbon Chain and Differently Charged Groups on I_{HERG}

Lysophospholipids are amphipathic molecules composed of a polar headgroup and a non-polar aliphatic hydrocarbon chain with varying lengths. To investigate how the differences in the aliphatic hydrocarbon chain and in the polar headgroup determine the effects of lysophospholipids on I_{HERG}, we performed detailed analyses of voltage- and time-dependent properties of I_{HERG} modulation by various lysophospholipids.

Activation properties

From Figure 2, it appears that the effects of LPC-16 and LPG-16 are quite different at different voltages. To clarify this notion in detail, we constructed the normalized I–V relationships with various lysophospholipids. As shown in Figure 7, LPG-16 produced a negative shift of the I–V curve, which resulted in a crossover of I–V curves between control and LPG-16. This phenomenon was not seen with LPC-16. This would imply that while LPC-16 increases I_{HERG} at the full voltage range tested, LPG-16 has an effect only at more negative potentials. This point is better addressed with the percent changes of I_{HERG}, caused by the lysophospholipids, as a function of test potentials shown in Figure 8. LPC-16 produced biphasic voltage-dependent effects on I_{HERG}: between –40 and –10 mV, LPC-16 induced greater I_{HERG} increment at more negative potentials, whereas between –10 and +40 mV it produced great effects at more positive potentials. By comparison, although the effect of LPG-16 at potentials negative to 0 mV was similar to that of LPC-16, it was virtually absent at potentials positive to 0 mV. LPC-18:1 produced similar patterns of voltage dependence as did LPC-16, but the I_{HERG} increase was to a less extent.

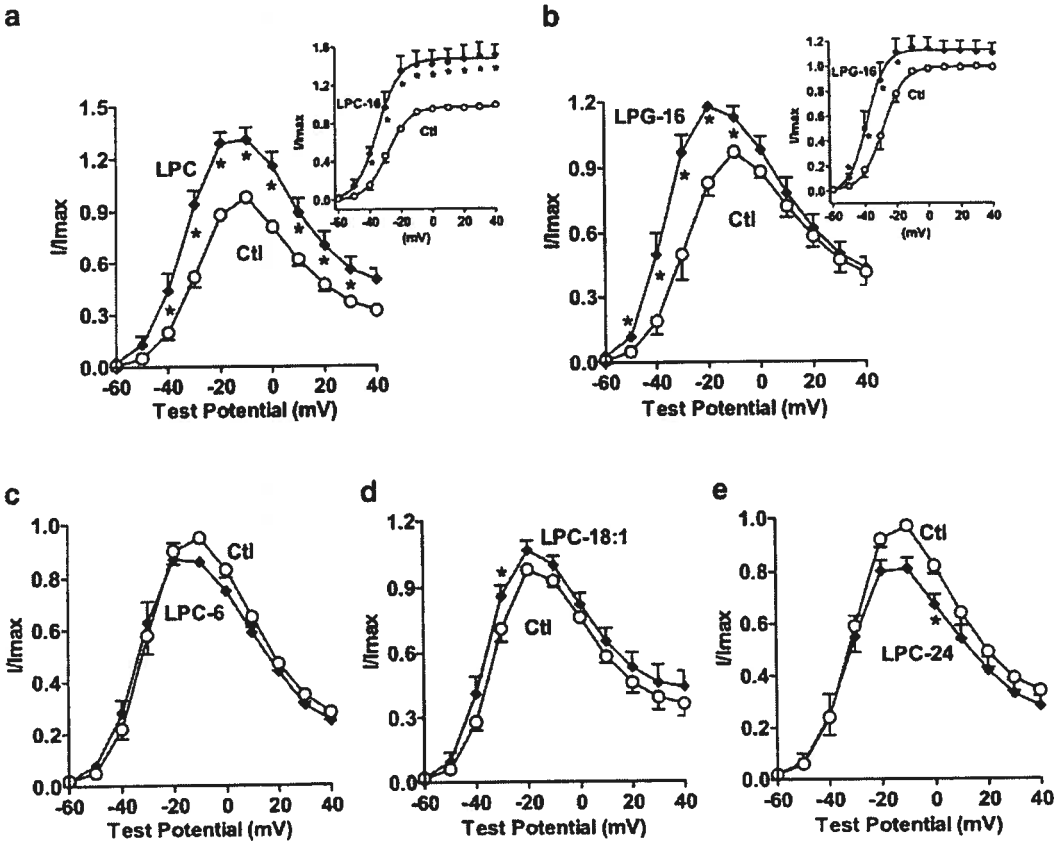


Figure 7. Normalized I–V relationships showing the negative shift of I–V curve by LPG-16 but not by other lysophospholipids. Currents were normalized by dividing step I_{HERG} at various potentials to the corresponding maximum value. Data are means±s.e. from 13 cells for LPC-16, 13 for LPG-16, 10 for LPC-6, 11 for LPC-18:1, and 12 for LPC-24.

To investigate the effects of lysophospholipids on the steady-state voltage-dependent activation property of I_{HERG}, we constructed the activation curves as illustrated in Figure 9. Both LPC-16 (5 μM) and LPG-16 (5 μM) cause significant shifts of activation curves toward hyperpolarizing potentials. The half-maximum activation voltages ($V_{1/2}$) were -29.0±3.6 mV with a slope factor (k) of 7.2 mV for control and -34.6±4.6 mV with slope factor (k) of 7.5 mV for LPC-16 (P<0.05 vs. Ctl), and were -

28.9±3.4 mV (k=6.3 mV) for control and -37.9±4.9 mV (k=5.9 mV) for LPG-16 (P<0.05 vs. Ctl), indicating a 5 mV negative shift for LPC-16 and a 10 mV negative shift for LPG-16. The difference of $V_{1/2}$ shifts between LPC-16 and LPG-16 was statistically significant (P<0.05, unpaired t-test), indicating a greater effect of LPG-16 on the steady-state voltage dependence of I_{HERG}. Other lysophospholipids did not significantly affect I_{HERG} activation curves.

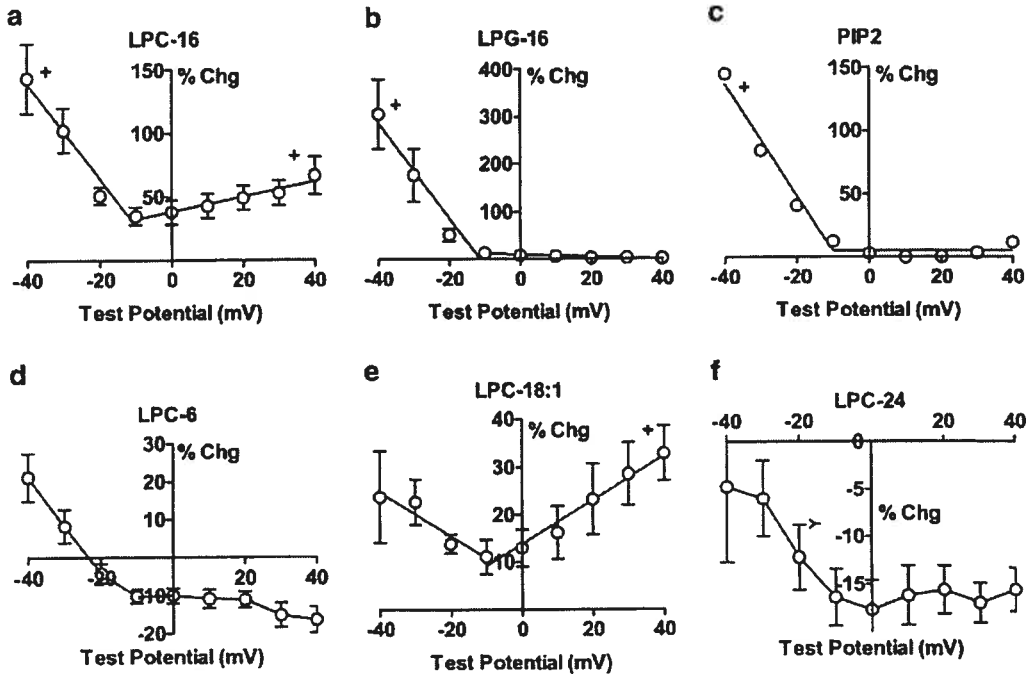


Figure 8. Percent changes of I_{HERG} produced by various lysophospholipids over control as a function of depolarizing voltages. Note the biphasic voltage dependence of I_{HERG} enhancement induced by LPC-16 and LPC-18:1 but not other lysophospholipids. Data are means±s.e. from 13 cells for LPC-16, 13 for LPG-16, eight cells for PIP_2, 10 for LPC-6, 11 for LPC-18:1, and 12 for LPC-24. -P<0.05 F-test indicating voltage dependence.

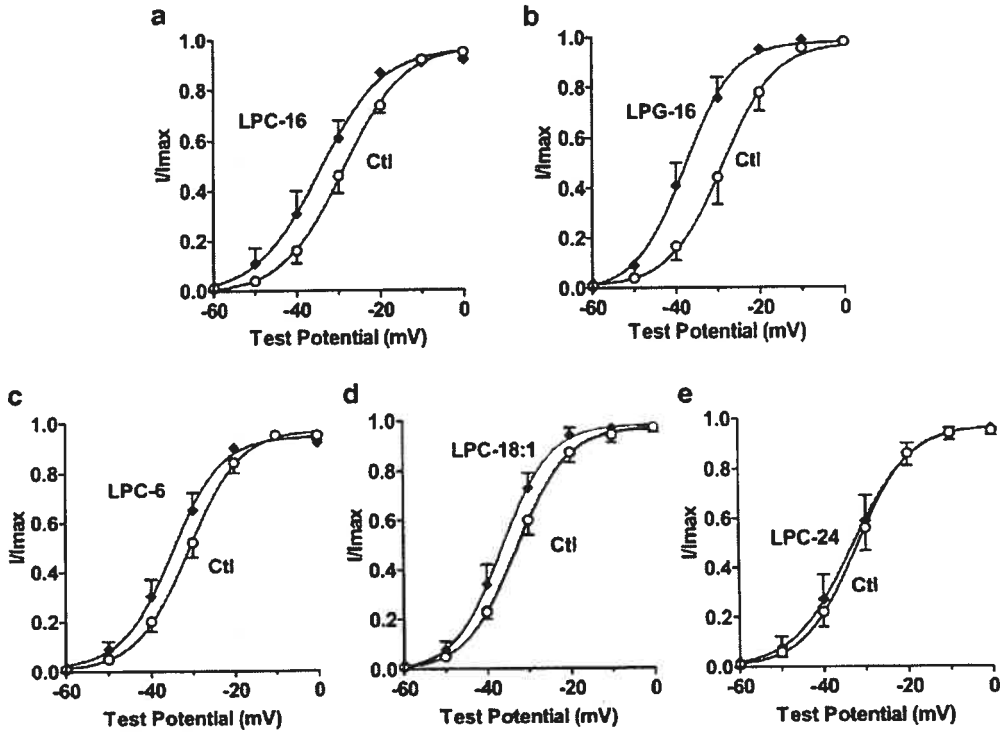


Figure 9. Effects of various lysophospholipids on the steady-state voltage-dependent activation curves. The activation curves were constructed by plotting the conductance *G* as a function of depolarizing potentials. *G* was calculated by normalizing the tail currents at -50 mV by dividing the amplitude of the tail currents measured at various antecedent depolarizing potentials by that of the tail current at +40 mV. Symbols are mean of experimental data and lines represent the Boltzmann fit: $G/G_{max}=1/\{1+\exp[(V_{1/2}-V)/k]\}$, where G_{max} represents the maximal conductance at +40 mV, $V_{1/2}$ is the half-maximal activation voltage, and k is the slope factor. Data are means±s.e. from 13 cells for LPC-16, 13 for LPG-16, 10 for LPC-6, 11 for LPC-18:1, and 12 for LPC-24.

Inactivation properties

The steady-state voltage-dependent inactivation was assessed by the voltage protocol shown in the inset of Figure 10. I_{HERG} was first inactivated by a 2 s depolarizing step to +40 mV and then reactivated to various extents during the hyperpolarizing pulses to various potentials of a 10 ms duration that allowed for full reactivation with minimal deactivation, and the decaying outward currents induced by the subsequent 50 ms depolarizing pulse to +20 mV represent I_{HERG} inactivation. As displayed in Figure 10, LPC-16 caused significant shift (10 mV) of the inactivation curve to depolarizing voltages with inactivation $V1/2$ changed from -54.7±5.6 mV (k=-18.6 mV) to –44.1±4.8 mV (P<0.05 vs. Ctl) (k=-14.8 mV). LPC-18:1 produced a slight non-significant positive shift of I_{HERG} inactivation curve. LPG-16 did not alter I_{HERG} inactivation properties (Figure 10b), nor did LPC-6 and LPC-24. This issue was further addressed by the data presented in the insets of Figure 10 where percent changes of I_{HERG} caused by the lysophospholipids over control are plotted as a function of hyperpolarizing potentials. Obviously, the degrees of I_{HERG} increases induced by LPC-16 and LPC-18:1 significantly depended on the antecedent hyperpolarizing voltages (P<0.05, F-test), with greater effects at more positive potentials (Figure 10a and d). By comparison, the effect of LPG-16 was only weakly voltage-dependent (Figure 10b, P>0.05, F-test).

Kinetics

Effects of various lysophospholipids on the time-dependent properties of I_{HERG} were analyzed. The apparent activation and deactivation time courses were determined by the single exponential fit to the step I_{HERG} during depolarizing pulse to 0 mV and to the tail I_{HERG} upon repolarization to –50 mV, respectively. As shown in Figure 11a and c, the apparent activation time constant was significantly decreased by LPC-16, LPG-16, and LPC18:1, and the apparent deactivation was significantly accelerated only by LPC-16 but not the other lipids studied. Since the apparent activation contains components involving both activation and inactivation processes, it was not sure whether the observed effects of the lipids are truly specific to the activation kinetics. To clarify this point, we performed experiments using the voltage protocols shown in Figure 11b, which allowed data analysis reflecting true activation with minimal inactivation. The results confirmed the ability of LPC-16 and LPG-16 to accelerate the activation time course.

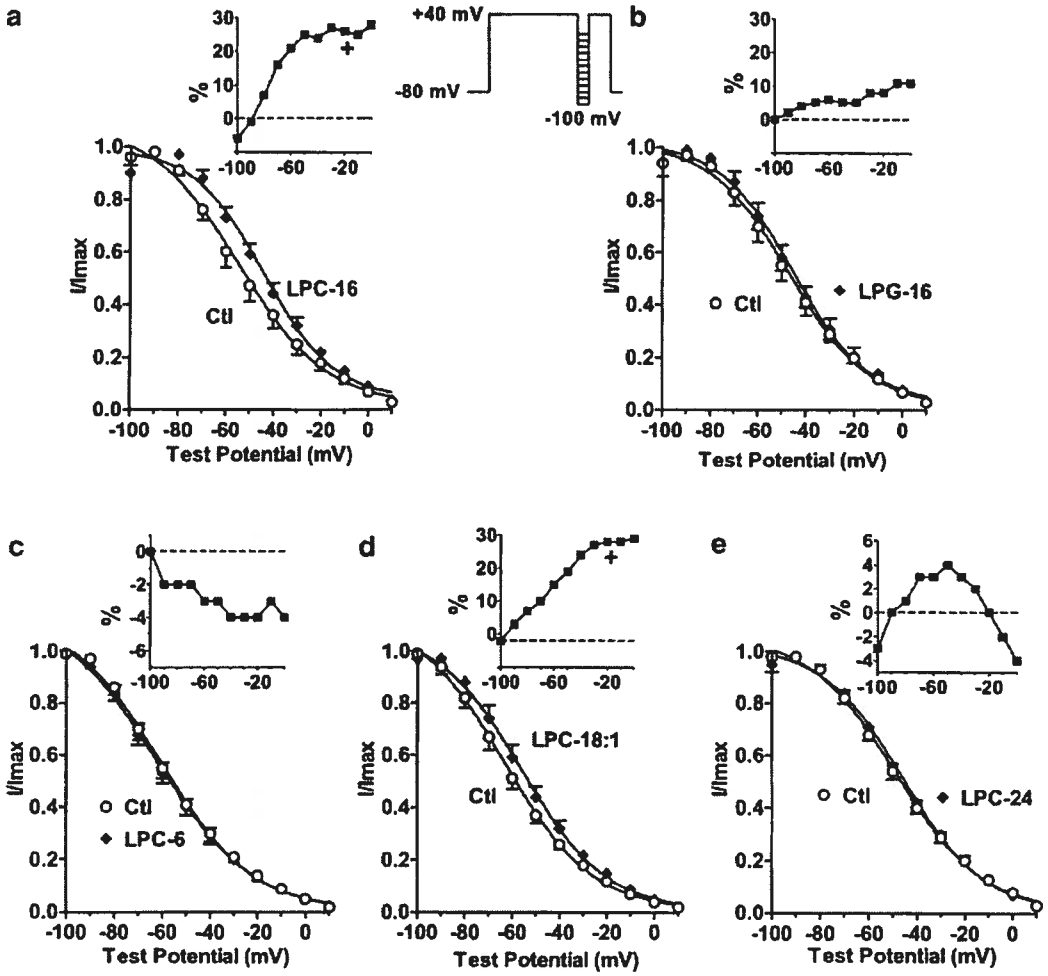
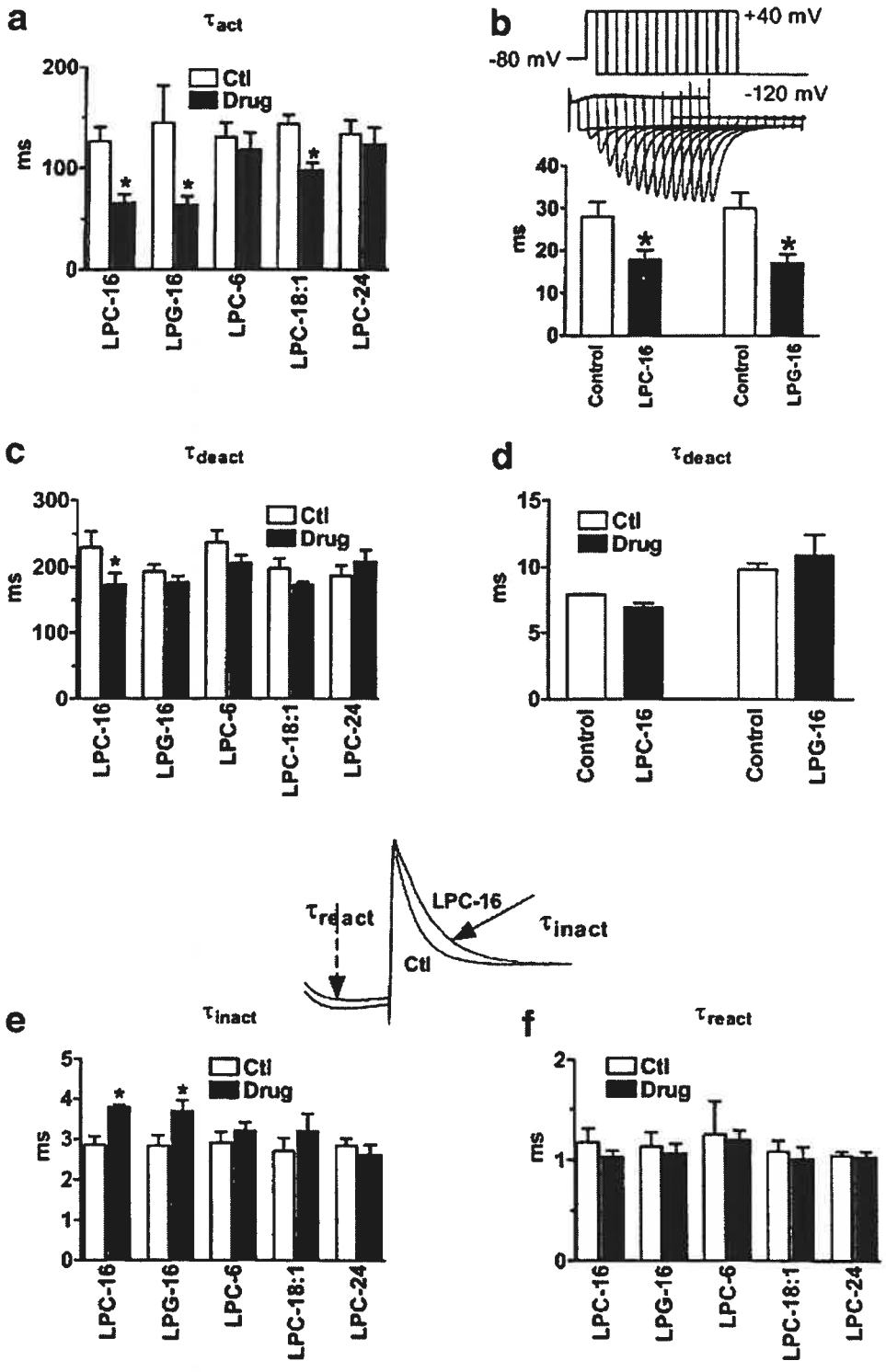


Figure 10. Effects of various lysophospholipids on the steady-state voltage-dependent inactivation curves. The inactivation curves were constructed by plotting the channel availability or conductance *G* as a function of hyperpolarizing potentials. *G* was calculated by normalizing the tail currents elicited at +20 mV by dividing the amplitude of the tail currents measured at various antecedent hyperpolarizing potentials by that of the tail current at -100 mV. Symbols are the mean of experimental data and lines represent the Boltzmann fit: $G/G_{max}=1/\{1+\exp[(V_{1/2}-V)/k]\}$, where G_{max} represents the maximal channel availability at -100 mV, $V_{1/2}$ is the half-maximal activation voltage, and k is the slope factor. Data are means±s.e. from 10 cells for LPC-16, nine for LPG-16, eight for LPC-6, eight for LPC-18:1, and nine for LPC-24. Percent changes of I_{HERG} produced by various lysophospholipids over control as a function of hyperpolarizing potentials are shown in the insets. Data are means±s.e. from 10 cells for LPC-16, 9 for LPG-16, 8 for LPC-6, 8 for LPC-18:1, and nine for LPC-24. +P<0.05 F-test indicating voltage dependence.


a
τact
200
100
0
ms
Ctl
Drug
*
LPC-16
LPG-16
LPC-6
LPC-18:1
LPC-24
b
+40 mV
-80 mV
-120 mV
40
30
20
10
0
ms
Control
LPC-16
Control
LPG-16
c
τdeact
300
200
100
0
ms
Ctl
Drug
LPC-16
LPG-16
LPC-6
LPC-18:1
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LPG-16
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LPC-16
LPG-16
LPC-6
LPC-18:1
LPC-24

Figure 11. Effects of various lysophospholipids on the kinetics of I_{HERG}. (**a, b**) Effects on activation kinetics. The activation time constants (τ_{act}) in (a) were obtained with the single exponential fit to the step I_{HERG} evoked at 0 mV, and τ_{act} in (b) was determined by the single exponential fit to the peak inward currents elicited at –100 mV with antecedent depolarizing steps of varying durations from 5 to 80 ms in 5-ms increments, as shown in the inset. (**c, d**) Effects on deactivation kinetics. The deactivation time constants (τ_{deact}) in (c) were determined by the single exponential fit to the outward tail I_{HERG} elicited at –50 mV preceded by a depolarizing step to 0 mV, and τ_{deact} in (d) was determined by the single exponential fit to the inward tail I_{HERG} elicited at –100 mV. (**e**) Effects on inactivation kinetics. The inactivation time constants (τ_{inact}) were obtained with the single exponential fit to the outward decaying currents elicited at +20 mV (as indicated by the solid arrow in the inset) with the voltage protocol shown. (**f**) Effects on reactivation kinetics. The reactivation time constants (τ_{react}) were determined by the single exponential fit to the inward currents elicited at –100 mV preceded by a 2 s depolarizing step, as indicated by the dash arrow in the inset. $^{*}P<0.05$ vs. Ctl.

However, the deactivation process analyzed with the decaying phase of the inward currents elicited during the brief hyperpolarizing pulse to –100 mV failed to show any significant alterations in the presence of LPC-16 or LPG-16.

The inactivation and reactivation kinetics were analyzed with the voltage protocols shown in the inset of Figure 10 and current traces in Figure 11e. The results in Figure 11e and f indicate that both LPC-16 and LPG-16 significantly slowed the inactivation kinetics and none of the lipids tested appreciably altered the reactivation time course. The slowing of inactivation by LPC-16 and LPG-16 is to a similar extent; the inactivation time constants were 2.9±0.2 ms for control and 3.8±0.1 ms for LPC-16 ($P<0.05$, $n=7$), and 2.8±0.3 ms for control and 3.7±0.3 ms for LPG-16 ($P<0.05$, $n=6$).

II-2-5 DISCUSSION

We compared in this study the effects of several lysophospholipids with different aliphatic hydrocarbon tails and polar headgroups on HERG function. It appears that the enhancement of HERG function is specific to the lysophospholipids with 16 hydrocarbons,

whereas the voltage dependence of the effects is determined by the charged headgroups. Enhancement of I_{HERG} cannot be well explained by altered membrane properties as a result of incorporation of the lipids into sarcolemma. Potential involvement of several intracellular signaling pathways in the effects of LPC-16/LPG-16 on I_{HERG} seems unlikely either. Our data point to direct interaction between LPC-16/LPG-16 and HERG K^+ channel proteins or some other unidentified signaling pathways related to LPC-16/LPG-16 as the mechanisms by which these lipids enhance HERG function. Increase in I_{HERG} by the lysophospholipids may contribute to K^+ loss and action potential shortening in the ischemic heart.

HERG is highly susceptible to regulation by various extracellular and intracellular factors. Of drugs belonging to various categories that have been shown to modulate HERG, all have been reported to inhibit the channel (Taglialatela *et al.*, 1998). This property of HERG channels is responsible for the long QT syndrome induced by drugs or under pathological situations associated with aberrant neuronal functions in many of the clinical cases. However, recent studies from our laboratory (Wang *et al.*, 2001a) and another group (Bian *et al.*, 2001), as well as the present study, revealed the other side of HERG channels: HERG function can also be enhanced. We were the first to report that HERG function is increased by a phospholipid metabolite 1-LPC-16 (Wang *et al.*, 2001a). Subsequently, Bian *et al.* (2001) showed that PIP_2 produced similar enhancing effects on I_{HERG}. More recently, we found that HERG function is largely dependent on the basal activity of protein kinase B (PKB) in the human embryonic kidney cell line and activation of PKB enhances HERG function (Zhang *et al.*, 2003a). The present study further revealed that besides LPC-16, other phospholipid metabolites also have the potential to enhance HERG function; specifically, the lysophospholipids with 16 hydrocarbons such as LPG-16 produces similar effects, as does LPC-16. By comparison, the potency of LPC-16 and LPG-16 is greater than that of PIP_2, in terms of their effects on I_{HERG}; extracellular application of 5 μM of LPC-16 or LPG-16 increased I_{HERG} to a greater extent than intracellular application of 10 μM PIP_2 (see Figure 5) (Bian *et al.*, 2001; Wang *et al.*, 2001a). Lysophospholipids are metabolic intermediates that are massively produced under various metabolic stresses (Hatch *et al.*, 1989; Choy *et al.*, 1997). In ischemic myocardium, unlike PIP_2 which is decreased (Mouton *et al.*, 1991; Liu *et al.*, 1997),

lysophospholipids particularly LPC-16 are rapidly produced and accumulated both extracellularly and intracellularly (Corr *et al.*, 1987; 1995; Man, 1988; Kinnaird *et al.*, 1988; Fazekas *et al.*, 1992). LPC-16 is known to be a causative factor for the occurrence of arrhythmias in the early phase of acute myocardial ischemia. One of the potential mechanisms for LPC-16's arrhythmogenic action is its ability to induce cellular K^+ loss or extracellular K^+ accumulation and shortening of APD, characteristic disorders of cardiac electrophysiology in ischemic hearts (Goldhaber *et al.*, 1998). LPC-16 has been shown to decrease cardiac inward rectifier K^+ current (Clarkson & Ten Eick, 1983; Kiyosue *et al.*, 1984; Sato *et al.*, 1993) and to induce a noninactivating component of inward Na^+ current (Burnashev *et al.*, 1991; Undrovinas *et al.*, 1992). While these properties of LPC may contribute to ischemic arrhythmias, they hardly explain LPC-induced K^+ loss and APD shortening; instead one would expect to see prevention of K^+ loss and lengthening of APD with inhibition of inward rectifier K^+ current and induction of noninactivating Na^+ current. Obviously, some other ion currents may be responsible for LPC's effects on cardiac electrophysiology. The present study provides an alternative mechanism by which LPC could cause cardiac electrical disturbances; LPC increases I_{HERG}, and thereby K^+ efflux, leading to cellular K^+ loss and APD shortening. Moreover, our data show that besides LPC-16, other lysophospholipids such as LPG-16 and LPC-18:1 also have the ability to increase I_{HERG} to varying extents, which could also contribute to the increased risk of ischemic arrhythmias.

A change in the mechanical properties of a bilayer could modify the function of embedded proteins by changing the free energy difference between different conformational states of the protein. At concentrations below their critical micelle concentrations (CMCs), lipids can cause monomer–membrane interaction in which adsorption of monomer into membrane occurs after disaggregation of micellar lipids. Above CMC, there exist micellar–membrane interactions in which desorption of intrinsic membrane phospholipid occurs with formation of mixed micelles in the incubation medium and associated loss of membrane phospholipid, altered membrane permeability and electrical distability or micellar–membrane interactions in which the primary process is adsorption of micellar lipids with localized perturbation of the biophysical characteristics of the membrane bilayer. The effects of amphipathic lipid metabolites on

membrane protein (such as ion channels) function have traditionally been ascribed to their effects on membrane fluidity and membrane deformation energy (DaTorre *et al.*, 1991; Lundbæk & Andersen, 1994). This interpretation is grounded on the following facts: (1) effects of the lipid metabolites are nonspecific, or in other words, the different lipid metabolites produce the same effects on membrane proteins in spite of their different polar headgroups and different lengths of aliphatic chain; and (2) these lipid metabolites are readily incorporated into the sarcolemma. Gross *et al.* (1982) demonstrated that electrophysiological abnormalities occurred in Purkinje fibers and ventricular muscles of canine hearts when only as little as 1% of cellular phospholipid was supplanted by exogenous LPCs, which were incorporated into the sarcolemma. Lundbæk & Andersen (1994) reported that several lysophospholipids affected gramicidin channel function on planar bilayer by altering membrane deformation energy as a result of their incorporation into the bilayer.

It is expected that the lipids examined in this study could all readily incorporate into the sarcolemma. However, our data argue against membrane incorporation as the major mechanism by which lysophospholipids modulate HERG function. Our argument is supported by two lines of evidence. First, the effects of the lipids on I_{HERG} developed rapidly with significant increases occurring within 3 min after application and are readily reversible with complete recovery of I_{HERG} within 5 min after washout of the drugs (see Figure 3). This rapid recovery is unexpected should the lipids take their effects by incorporating into sarcolemma, because once the lipids incorporate into the membrane they will become a part of the membrane composition and can cause permanent destruction of the membrane. Second, the effects of lipids on membrane protein function will vary as a function of their concentrations in the membrane, which can be estimated by membrane adsorption coefficients of various lipids: the higher the concentration of a given lipid in the membrane, the greater the effect. At low aqueous lipid concentrations $[lipid]_0$, the mole fraction of the lysophospholipids in the membrane should approximately be equal to $[lipid]_0$/CMC (Nichols & Pagano, 1981; Lundbæk & Andersen, 1994). The CMCs for the lysophospholipids used in this study are ~7 μM for LPC-16, 600 μM for LPG-16, >7 μM for LPC-6, ~0.4 μM for LPC-18:1, and ~1 μM for LPC-24 and the concentration is 5 μM for all of the lysophospholipids examined. Besides, 1 μM LPC-24

or 10 μM LPC-18:1 was also tested. Accordingly, LPC-18:1 and LPC-24 would be expected to have much higher ratio of membrane incorporation or higher concentrations in the membrane than LPC-16, LPG-16, and LPC-6. Furthermore, LPC-18:1 and LPC-24 have bulkier structures compared with LPC-16, LPG-16, and LPC-6, and should produce more severe derangement of sarcolemma. However, the effects of LPC-18:1 and LPC-24 on I_{HERG} are much smaller or absent relative to LPC-16- or LPG-16. In addition, 5 μM LPC-16 is quite close to its CMC while 5 μM LPG-16 is far below its CMC; yet the effects of these two lipids on I_{HERG} are quite comparable, indicating that membrane concentration or incorporation does not play a major role in defining the effects of these lipids on I_{HERG}.

Lysophospholipids are not simply lipid metabolites producing toxic effects; instead they have been implicated in many signal transduction processes and some of them are actually signaling molecules. By involving in signaling pathways, these lysophospholipids can regulate the function of a variety of proteins, presumably including ion channels. One of the most recognized effects of LPC is its ability to stimulate activation of several protein kinases, such as PKC (Prokazova *et al.*, 1998; Bassa *et al.*, 1999; Motley *et al.*, 2002) and PKA (Ahumada *et al.*, 1979). The potential involvement of PKC in HERG modulation by LPC-16 or LPG-16 was excluded on the ground of failure of the inhibitor to affect the enhancement of I_{HERG} by LPC-16 or LPG-16 (Figure 4) and of failure of PKC activators to mimic the effects of LPC-16/LPG-16. Participation of PIP_2, a component of PKC signaling pathway, in I_{HERG} modulation by LPC-16 and LPG-16 was also ruled out because the effect was not significantly altered by the inhibitory antibody against PIP_2 and also because direct application of PIP_2 produced much smaller enhancement of I_{HERG} than LPC-16 or LPG-16 although the concentration of PIP_2 (10 μM) used was twice as much as LPC-16 or LPG-16 (Figure 6). In agreement with the present work, most of the previous studies have also failed to observe modulatory effects of PKC on I_{HERG} (Kiehn *et al.*, 1998; Bian *et al.*, 2001). Participation of PKA in LPC-16- or LPG-16-induced I_{HERG} enhancement was not examined because previous work has consistently found that PKA simulation actually suppresses I_{HERG} (Kiehn *et al.*, 1998; Cui *et al.*, 2000).

Effects of LPC on cellular functions have also been linked to its ability to enhance production of superoxide anions (O^{2-}) (Ginsburg *et al.*, 1989; Ohara *et al.*, 1994; Nishioka

et al., 1998; Takeshita *et al.*, 2000). There is thus a possibility that I_{HERG} enhancement by LPC is mediated by O^{2-}. However, our data do not support this notion. We have recently found that O^{2-} impairs HERG function (Zhang *et al.*, 2003b). In the present study, we also demonstrated that O^{2-} generating system X/XO (Barrington *et al.*, 1988; Aiello *et al.*, 1995) depresses I_{HERG}. Moreover, the enhancement of I_{HERG} induced by LPC-16 or LPG-16 was augmented in the presence of VitE to scavenge O^{2-} (see Figure 7), indicating that O^{2-} produced by LPC-16/LPG-16 indeed participated in I_{HERG} modulation, but instead of mediating the enhancing effects it exerts an opposite effect counteracting I_{HERG} enhancement. In other words, LPC-16 and LPG-16 actually produce dual effects on HERG function: they depress I_{HERG} *via* production of O^{2-} and increase I_{HERG} by some other mechanisms with a net increase in I_{HERG}. ROS has been implicated in I_{HERG} modulation (Taglialatela *et al.*, 1997; Berube *et al.*, 2001); intriguingly, I_{HERG} was found increased by hydrogen peroxide (H_2O_2) which is supposed to be converted to hydroxyl group (OH^-) to become reactive. Together, these data suggest that different species of ROS may have different effects on I_{HERG}.

Comparison of various LPCs and LPG-16 reveals that only the lipids carrying 16 hydrocarbons in their aliphatic tails are able to affect HERG function to significant extents and the lipids with shorter (such as LPC-6) or longer (LPC-18:1 and LPC-24) tails merely affect or weakly affect I_{HERG}. As a matter of fact, LPC-6 and LPC-24, which are drastically different from LPC16/LPG-16 in terms of their aliphatic chains, virtually suppress I_{HERG} to as much as 20% (Figure 8d and f). Moreover, LPC-16 and LPG-16 caused significant shifts of HERG activation along the voltage axis towards more negative potentials, so did LPC-18:0 although to a less extent, whereas LPC-6 and LPC-24 produced little effect. Furthermore, the activation time course was significantly accelerated by LPC-16, LPG-16, and LPC-18:0 as well, but not by LPC-6 and LPC24. These results could hardly be explained by incorporations of the lipids into the sarcolemma. As estimated based on their CMCs, the membrane incorporation of LPC-16 and LPC24 are quite comparable and that of LPC-18:0 is around 10–20 times higher than LPC-16 and LPC-24; by comparison, the membrane incorporation of LPC-6 and LPG-16 would be at least 100-fold less than LPC-16. The observed effects are quite comparable between LPC-16 and LPG-16 and between LPC6- and LPC-24, but do not follow the

expected order of membrane incorporation. If the effects are viewed as consequences of direct interactions between lipid molecules and HERG proteins and there is a specific 'binding' site for the lipids in the HERG sequence, then our data can be well explained. For example, similar degrees of I_{HERG} enhancement by LPC-16 and LPG-16 are presumably attributable to their identical aliphatic hydrocarbon chain. This implies that 16-hydrocarbon chain structure can best fit the 'binding' site in HERG sequence and that the hydrocarbon chains shorter than 16 may not satisfy the 'binding' requirements while the hydrocarbon chains longer than16 may not have an access to the site. The lipids with similar hydrocarbon chain like LPC-18:0 can also "bind" to the HERG site, but may not in optimal position. This explains why the LPC with 18-hydrocarbon chain is able to enhance I_{HERG}, although the effect is weaker than those of LPC-16/LPG-16. Our data are therefore best described by direct interactions between lipid molecules and HERG channel proteins.

On the other hand, although at a concentration of 5 μM, LPC-16 and LPG-16 produced similar enhancement of I_{HERG}, detailed comparisons reveal that the voltage dependencies of actions are different. For example, LPC-16 showed biphasic voltage dependence on the test potentials that set the open probability of the channels (Figure 8); at potentials negative to –10 mV, the increase was grater at more hyperpolarized voltages, whereas at potentials positive to –10 mV, the increase was greater at more depolarized potentials (Figure 8a). By comparison, the effect of LPG-16 was monophasic; the increase in I_{HERG} was weaker at more positive potentials and was nearly absent at potentials positive to –10 mV (Figure 8b). Similarly, the enhancement of I_{HERG} by LPC-16 was also significantly dependent on the prepulse potentials that set the availability of channels for opening with greater effects at more positive potentials that render smaller channel availabilities (Figure 10a). The effects of LPG-16 did not significantly depend on prepulse potentials (Figure 10b). This is also reflected by the positive shift of the HERG inactivation curve, induced by LPC-16 but not LPG-16 (Figure 10). Noticeably, the voltage dependence of LPC-18:0 mimicked that of LPC-16. The reason for the differences between LPC-16 and LPG-16 and the similarity of LPG-18:0 to LPC-16 is quite obvious: the headgroups in LPC-16 and LPC-18:0 are identical and are neutral with one negatively charged PO_4^- and one positively charged NH_3^+, while the headgroup in LPG-16 is

negatively charged with one PO_4^- (Figure 1). The distinction in the headgroups between LPC-16 and LPG-16 confers their different voltage dependence of effects on I_{HERG} while LPC-16 and LPC-18:0 have the same pattern of voltage dependence because they share the same headgroup. Intriguingly, PIP_2 has a negatively charged headgroup and its effects on I_{HERG} demonstrate characteristic voltage dependence similar to LPG-16 (Figure 8c) (Bian *et al.*, 2001). It has been proposed that PIP_2 may interact with HERG channels by binding to positively charged amino-acid residues in the HERG sequence. LPG-16 might act in the same way as PIP_2. For LPC-16 and LPC-18:0, since they have both positive and negative charges, they might be able to bind both negatively and positively charged amino acids of HERG.

Considering all these arguments, it appears that enhancement of HERG function by LPC-16 and LPG-16 is not mainly due to membrane incorporation of these lipids or to intracellular signaling pathways that are known to be related to the functions of these lipids. We tend to believe that effects of LPC-16 and LPG-16 on I_{HERG} are the consequence of direct interactions between the lysophospholipids molecules and HERG K^+ channel proteins or of some other signaling pathways related to LPC-16/LPG-16 actions. We should admit that our experiments do not provide any direct evidence for the hypothesis of direct lipid–channel interactions. Such evidence requires identification of the binding site(s) for the lipids in HERG sequence by mutagenesis. The possibility that LPC-16/LPG-16 modulate HERG function *via* some unidentified pathways is therefore not excluded.

II-2-6 ACKNOWLEDGEMENTS

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II-3 Enhancement of HERG Function by Lysophosphatidylcholine Contributes to Extracellular K^+ Accumulation and "Short QT Syndrome" in the Heart with Acute Global Ischemia

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II-3 Enhancement of HERG Function by Lysophosphatidylcholine Contributes to Extracellular K^+ Accumulation and "Short QT Syndrome" in the Hearts with Acute Global Ischemia

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II-3-1 ABSTRACT

Background The most profound abnormalities during acute myocardial ischemia are extracellular K^+ accumulation ($[K^+]_o$-↑) and shortening of action potential duration or QT interval (APD-↓ or QT-↓), which are pivotal in the genesis of ischemic arrhythmias and sudden cardiac death. The ionic mechanisms remain obscure.

Methods and Results We performed studies in a rabbit model of acute global myocardial ischemia in order to explore the potential role of delayed rectifier K^+ current (I_{Kr}) as an iconic mechanism for ischemic $[K^+]_o$-↑ and QT-↓. Exogenous LPC-16 mimicked the low-perfusion ischemia to produce significant $[K^+]_o$-↑ and QT-↓. The $[K^+]_o$-↑ and Q-↓ induced by either LPC-16 or ischemia were prevented by I_{Kr} blocker Dofetilide, but not by blockers for other K^+ channels. Consistently, dofetilide efficiently abolished the ventricular tachy-arrhythmias. At the cellular level, LPC-16 remarkably shortened APD and enhanced the function of I_{Kr} and HERG, the pore-forming subunit of I_{Kr}. The effects of LPC-16 were manifested with shorter APD (faster repolarization rate) and at more negative potential (membrane repolarization). Dofetilide abolished the I_{Kr}/HERG enhancing and APD shortening effects of LPC-16.

Conclusions Our results suggest that LPC-16 accumulation/HERG enhancement may be the link between metabolic trigger and ionic pathway for ischemic $[K^+]_o$-↑ and QT-↓. This represents the first documentation of I_{Kr}/HERG as the ionic mechanism in ischemic $[K^+]_o$-↑ and QT-↓. Inhibition of LPC-16 production and accumulation and/or of I_{Kr}/HERG may be a promising therapeutic strategy to attenuate the incidence of lethal arrhythmias associated with ischemic heart disease.

Key Words: ischemia • arrhythmias • ion channels • potassium • myocytes

II-3-2 INTRODUCTION

Myocardial ischemia leads to a cascade of metabolic and electrophysiological events which are interrelated and are caused by metabolic stress, such as intracellular and extracellular accumulation of metabolic by-products like lysophosphatidylcholine (LPC-16), and by extracellular K^+ accumulation ($[K^+]_o$-↑).[1] The results are failure of contraction, deterioration of electrical behavior, and eventual death of the cell. At the organism level, the end point may be lethal arrhythmias or mechanical pump failure. The most profound abnormalities associated with acute myocardial ischemia are $[K^+]_o$-↑ and shortening of action potential duration or QT interval (APD-↓ or QT-↓).

$[K^+]_o$-↑ in the myocardium following net cellular K^+ loss ($[K^+]_i$-↓) is one of the earliest consequences of myocardial hypoxia and ischemia, beginning within 15-30 s.[1-7] During the first 10–15 min of myocardial ischemia, $[K^+]_o$ (or potassium activity) rises 2-3 fold from its normoxic value (5 mmol/L) to a plateau level of 6–11 mmol/L above its initial value. $[K^+]_o$-↑ is related to profound modifications of the electrical behavior of ischemic cardiomyocytes. In particular, it contributes to depolarization of resting membrane potential, decrease in the maximum upstroke velocity of action potential (AP), and decrease in excitability. APD-↓/QT-↓ is also tightly associated with these changes of $[K^+]_o$; increase in K^+ efflux can result in APD-↓ and $[K^+]_i$-↑ as well. These electrophysiological alterations are pivotal in the genesis of ischemic arrhythmias and sudden cardiac death.

Ischemic arrhythmias are generally categorized into three phases based on the time when they appear: phase I arrhythmias occurring during the first 30 min of ischemia, phase II arrhythmias between 5 to 72 hours and phase III arrhythmias of the chronic stage after an infarct. The first burst of ventricular tachycardia occurs between 2 and 10 min of ischemia. In human, phase I arrhythmias often results in ventricular fibrillation and sudden cardiac death. Reentry[1,8] and delayed afterdepolarizations (DADs)[1,9] are the major mechanisms for phase I arrhythmias in ischemic myocardium. Noticeably, APD-↓/QT-↓ favors the reentrant arrhythmias and DADs, a reproducible pattern of dysrhythmic activities during acute myocardial ischemia.[7] Here we refer this typical pattern of ischemic electrical disorders and arrhythmias to as "short QT syndrome". Indeed, pharmacological modulation of $[K^+]_o$-↑ and APD-↓ during acute myocardial ischemia has

been considered as a promising new antiarrhythmic and cardioprotective strategy during myocardial ischemia and reperfusion. Yet our insufficient knowledge of the mechanisms for $[K^+]_o$-↑ and APD-↓ hinders us from benefiting from the promise.

The cell surface membrane or sarcolemma is primarily composed of phospholipid, cholesterol, and proteins which together form a complex dynamic structure which retains the intracellular contents, regulates ion homeostasis, governs nutrient transports, and tansduces extracellular signals into the cells. The principal classes of sarcolemmal phospholipids are, according to their relative abundance, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine.[10] Under certain situations, for example, in ischemic tissues, the phospholipids (e.g. phosphatidylcholine) can be metabolized to generate lysophosphatidylcholines (LPCs). LPCs are amphiphiles possessing a charged headgroup like phospholipids but only a single aliphatic hydrocarbon chain as a consequence of the hydrolytic cleavage of one of the two aliphatic hydrocarbon groups of phosphatidylcholines. The major species of LPCs produced in ischemic myocardium is 1-palmitoyl-lysophosphatidylcholine (LPC-16) which possesses 16 hydrocarbons in its aliphatic chain. As an intermediate of metabolism of phosphatidylcholine, LPC-16 is present in a variety of mammalian tissues and accumulates rapidly in the heart during cardiac ischemia.[10,11-12] Accumulation of LPC-16 is thought to be a major contributor to the development of cardiac arrhythmias during myocardial ischemia,[12-15] such as abnormal rhythmic activity, delayed afterdepolarizations (DAD), triggered activity and intramyocardial reentry, and is regarded as a biochemical trigger of ischemic arrhythmias. APD shortening by LPC-16 has been documented in rabbit atrial and ventricular cells,[13] guinea pig ventricular cells,[16] and canine ventricular cells[1] and Purkinje fibers.[10] LPC-16 levels correlate with the occurrence of arrhythmias in ischemic hearts.[11,14,15] Coincidentally, a recent study by Goldhaber *et al*[17] reported that LPC-16 decreases tissue K^+ content by ~15%, an effect associated with gradual APD-↓ and $[K^+]_o$-↑.

The delayed rectifier K^+ current (I_{Kr}), encoded mainly by human *ether-a-go-go* related gene (HERG), carries outward K^+ flow which is crucial for repolarizing cardiac membrane. I_{Kr} has the potential of being a contributor to ischemic $[K^+]_o$-↑ and APD-↓ and the associated arrhythmias, considering the following notes. First, the only two

existing studies concerning I_{Kr} and ischemia suggest that I_{Kr} is increased during ischemia and infarction. Shinmura *et al*[18] studied the effect of E-4031, a class III antiarrhythmic drug which specifically inhibits I_{Kr}, on ischemia- and reperfusion-induced arrhythmias. They found that E-4031 (100 nM) attenuated loss of K^+ from the ischemic myocardium and concomitant reduction of ischemia-induced arrhythmias. Another study reported by Pinto and Boyden[19] described an E-4031-sensitive K^+ current which was increased in arrhythmogenic subendocardial Purkinje myocytes from the infarcted hearts of dogs. Although the nature of this E-4031 sensitive K^+ current remains to be clarified, it possesses some characteristics of I_{Kr}. Second, HERG is highly susceptible to modulation by various extracellular and intracellular factors. We have recently found that LPC-16 significantly increased I_{HERG} amplitude by shifting the inactivation of the channels to more positive potentials and by slowing the inactivation kinetics.[20,21] The effects took place within 1 min after LPC-16 reached the cells and were readily reversible upon removal of the drug. These data are in line with the fact that LPC-16 causes $[K^+]_o$-↑ and APD-↓ and thus suggest that I_{HERG}/I_{Kr} contribute to the early event of ischemia-induced alterations of cardiac electrical activities. Finally, HERG conductance is known to increase with increasing extracellular K^+, a typical condition in ischemia. It is thus conceivable that I_{Kr} increases both as a cause and as a consequence of $[K^+]_o$-↑.

We proposed that ischemic electrical disorders are a consequence of interrelated metabolic stress and electrolyte disturbance. In other words, ischemic $[K^+]_o$-↑ and APD-↓ are an event involving the interplay between ionic channels and metabolic substrates. The present study was designed to examine this hypothesis. Our results provide supporting evidence for the role of LPC-16 and I_{Kr}/HERG in ischemic $[K^+]_o$-↑ and APD-↓, and suggest that both LPC-16 and I_{Kr}/HERG be the pharmacological targets for the treatment of ischemic cardiac electrical disturbance or arrhythmias.

II-3-3 METHODS

II-3-3-1 Global Myocardial Ischemic Model

New Zealand white rabbits weighing 2.2-2.8 kg of either sex were sedated with a subcutaneous injection of ketamine (50 mg/kg) and xylazine (4mg/kg) and anesthetized to

a surgical plane with intravenous administration of sodium pentobarbital (30 mg/kg). Heparin (1,000units/kg) was delivered intravenously. Heart was then rapidly excised after thoracotomy and placed in cold heparinized Tyrode's solution (4°C).

The aorta was immediately cannulated in a heated chamber filled with humidified room air and the hearts was perfused retrograde in the Langendorff perfusion apparatus at 75 mmHg constant perfusion pressure with modified Tyrode's solution of the following composition (in mmol/L): NaCl 130, KCl 4, $CaCl_2$ 1, $MgCl_2$ 1, $NaHCO_3$ 24, NaH_2PO_4 1.2, and glucose 10. The perfusate was maintained at 35-37°C by a temperature transducer (Physitemp TH8) and oxygenated with 95% O_2 and 5% CO_2. The cava and pulmonary veins was sutured closed and the pulmonary artery was cannulated in order to obtain samples of venous effluent under anaerobic conditions for potassium concentration measurement. The perfusion rate was continuously monitored with a flow meter (transonic T206) and maintained at 30 ml/min for all baseline control periods. The pressure in the aorta was measured with a pressure transducer (WPI BLRP) connected to a strain gage amplifier (EMKA technologies).

II-3-3-2 Experimental Protocols

To mimic global acute ischemia, the perfusion flow was reduced from 30 ml/min to 5 ml/min (1/6 or 17% of baseline control) for 15 min under normothermic condition. The hearts were then subjected to the following experimental procedures.

(1) Baseline recording (30 min, 30 ml/min) — Ischemia (15min, 5 ml/min) — Reperfusion (30 ml/min);

(2) Baseline recording (30 min, 30 ml/min) — K^+ channel blockers (30 ml/min) — Blockers + Ischemia (15 min, 5 ml/min) — Reperfusion (30 ml/min);

(3) Baseline recording (30 min, 30 ml/min) — LPC-16 (30 ml/min) — Reperfusion (30 ml/min);

(4) Baseline recording (30 min, 30 ml/min) — K^+ channel blockers (30 ml/min) — Blockers + LPC (3 μmol/L, 15 min, 30 ml/min) — Reperfusion (30 ml/min).

For experiments involving K^+ channel blockers, drugs were administrated by a Harvard apparatus pump at a rate of 3 ml/min (1/10 dilution). Lysophosphatidylcholine (LPC-16) was purchased from Avanti Polar Lipid Inc (Alabaster, Alabama) and

glibenclamide (GA) were all purchased from Sigma. Dofetilide (Dof, I_{Kr}/HERG blocker) is a gift from Pfizer, and chromanol 293B (293B, I_{Ks} blocker) and HMR (3R, 4S)-(+)-N-[-3-hydroxy-2,2-dimethyl-6-(4,4,4-trifluorobutoxy) chroman-4-yl]- N-methyl-ethanesulfonamide, I_{Ks} blocker) were generously supplied by Aventis. The concentrations of the drugs were set based on our previous and preliminary experiments, as well as on the studies from other laboratories, being around their respective IC_{50} values. PLC-16 was dissolved directly into the bath solution at the desired concentrations immediately before each experiment.[20,21]

II-3-3-3 Measurement of QT Interval

ECG was monitored, on a data acquisition system (EMKA), continuously throughout the experiment and compared to baseline measurements before global ischemia or LPC-16 perfusion. QT intervals were measured on lead II (the negative lead positioned on the base of the right ventricle and the positive lead was inserted at the apex of the heart) from QRS onset to the end of the T wave; biphasic T waves were measured to the time of final baseline return. These QT measurements and simultaneously recorded RR intervals were used to derive heart rate corrected QT intervals (QTc) using Bazett's formula (QTc=QT/(RR)^0.5).[22] ECG was recorded at a sampling rate of 1 KHz and a low pass filter of 0.5 KHz and a notch filter of 60 Hz. The software ECGauto 1.5.7 was used to analyze ECG. ECG parameters were averaged from measures of three consecutive complexes.

II-3-3-4 Measurement of $[K^+]_o$

Effluent (8 ml) was collected every 2 min throughout the whole experimental period. K^+ content was measured by a potassium sensitive electrode (FastFil Combination Model 44-0001, Lazar Research Laboratory Inc. CA). A standard curve was constructed with a series of concentrations of standard KCl solution for each experiment. The readings (in mV) were obtained with a pH meter (Accumet Basic AB15, Fisher Scientific) and the corresponding values were converted to mmol/L concentration in a semilogarithmic paper as the electrode manual instructed.

II-3-3-5 Myocyte Isolation

Cells were isolated from left ventricular endocardium, midmyocardium and right atrium of dogs for collecting action potential (AP) waveforms editing AP-clamp recordings. Myocytes from left ventricular endocardium of rabbits were isolated for investigating the effects of LPC-16 on AP parameters. Myocytes from left ventricle of guinea pigs were isolated for studying the effects of LPC-16 on I_{Kr}.

The enzymatic isolation procedures were the same as previously described.[23-25] The hearts were quickly removed and mounted to a Langendorff perfusion system. The preparation was perfused with Ca^{2+}-containing Tyrode solution at 37°C until the effluent being clear of blood, and then switched to Ca^{2+}-free Tyrode solution (mmol/L: 136 NaCl, 4.7 KCl, 1 $MgCl_2$, 0.33 NaH_2PO_4, 5 HEPES, 10 glucose and 1 $CaCl_2$; pH 7.4) for 20 min at a constant rate of 12 ml/min, followed by perfusion with the same solution containing collagenase (110 U/ml CLS II collagenase; Worthington Biochemical) and 0.1% bovine serum albumin for ~30 minutes. The dispersed cells were stored at 4°C in the medium containing (mmol/L): 20 KCl, 10 KH_2PO_4, 25 glucose, 70 potassium glutamate, 10 β-hydroxybutyric acid, 20 taurine, 10 EGTA, 0.1% albumin and 40 mannitol (pH 7.4).

II-3-3-6 Cell Culture

HEK293 cells stably expressing HERG (a kind gift from Drs. Z Zhou and C January)[26] were seeded in a 25 cm^2 triangular cell-culture flask and grown in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated FBS, 200-μmol/L G418, 100-U/ml penicillin and 100-μg/ml streptomycin. The cells subcultured to ~85% confluency were harvested by trypsinization and stored in Tyrode solution containing 0.5% BSA at 4°C. Electrophysiological recordings were conducted within 10 hrs of storage.

II-3-3-7 Patch-clamp Techniques

The procedure was the same as described in detail elsewhere.[20,21,27] Briefly, a small aliquot of the solution containing the isolated cells were placed in a 1-ml chamber mounted on the stage of an inverted microscope. The cells were perfused at 5 ml/min with the standard extracellular solution (36°C). Ionic currents and action potentials (APs) were

recorded with whole-cell patch-clamp methods in the voltage-clamp mode and the current-clamp mode, respectively, using an Axopatch-200B amplifier (Axon Instruments). Borosilicate glass electrodes (1 mm o.d.) had tip resistances of 1-3 MΩ when filled with pipette solution of the following composition (mmol/L): 110 potassium aspartate, 20 KCl, 1 $MgCl_2$, 5 Mg-ATP, 0.1 GTP, 10 EGTA, 5 phosphocreatine, 10 HEPES, pH adjusted to 7.2 with KOH. Command pulses were generated by a 12-bit digital-to-analog converter controlled by pCLAMP6 software (Axon Instruments). Junction potentials were zeroed before formations of the membrane-pipette seal in Tyrode solution containing (in mmol/L): 136 NaCl, 4.7 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES (pH 7.4). The capacitance and series resistance were both electrically compensated to minimize the duration of the capacitive surge on the current recording and the voltage drop across the clamped cell membrane.

For myocyte experiments, recordings were made under conditions, which minimized the potentially contaminating currents. Contamination by sodium current was prevented by holding the cell at -50 mV. Cadmium chloride (200 μmol/L) was used to inhibit Ca^{2+} current as well as Ca^{2+}-activated chloride current. K_{ATP}, if any, was suppressed by glibenclamide (10 μmol/L) in the perfusate and by 5 mmol/L Mg-ATP in the pipette. Acute application of drugs was achieved by bath perfusion. Current recordings done under control conditions were repeated every 5 minutes up to a total of 15 minutes after drug superfusion. To ensure the purity of I_{Kr}, the amplitude was measured as dofetilide-sensitive currents. For group comparisons, data were expressed as current density calculated by dividing the current amplitude by the cell capacitance.

II-3-3-8 Data Analysis

Group data are expressed as mean ± S.E. Statistical comparisons (performed using ANOVA followed by Dunnett's method) were carried out, and paired or unpaired *t*-test was used, as appropriate, for single comparisons, using Microsoft Excel. A two-tailed $p < 0.05$ was taken to indicate a statistically significant difference. Nonlinear least square curve fitting was performed with CLAMPFIT in pCLAMP 8.0 or GraphPad Prism.

II-3-4 RESULTS

II-3-4-1 Role of I_{Kr}/HERG in $[K^+]_o$-↑ Induced by Low-perfusion Ischemia or by Exogenous LPC-16

The K^+ concentration in the perfusate ($[K^+]_o$) was 4 mmol/L and the $[K^+]_o$ measured from the effluent collected 30 min after stabilization with normal perfusion rate (30 ml/min) was 3.9 ± 0.1 mmol/L. $[K^+]_o$ was elevated ($[K^+]_o$-↑) when the perfusion rate was reduced to mimic global myocardial ischemia and the elevation was greater with lower perfusion rate (Figure 1A). For instance, with a perfusion rate of 10 ml/min (33% of the normal perfusion rate) for 10 min, $[K^+]_o$ was increased to 5.7 mmol/L and when the perfusion was decreased to 17% (5 ml/min) of the normal perfusion $[K^+]_o$ was further increased to 9.3 mmol/L which is similar to the published data.[3,7] In the subsequent experiments, a perfusion rate of 5 ml/min was used as the global acute ischemia model.

$[K^+]_o$-↑ developed rapidly and reached a steady-state (10.5 mmol/L) within 10 min of ischemia, as shown in Figure 1B. Administration with dofetilide (20 nmol/L) did not significantly alter $[K^+]_o$ under control conditions, but prevented ischemic $[K^+]_o$-↑ (Figure 1C). By comparison, blockers for I_{Ks} and K_{ATP}, 293B (50 μmol/L), HMR (100 nmol/L) and GA (glibenclamide, 10 μmol/L), respectively, all failed to affect the ischemic $[K^+]_o$-↑. The efficacies of these blockers were validated in isolated single canine ventricular myocytes; 293B at 50 μmol/L and HMR at 100 nmol/L produced 92% and 96% reduction of I_{Ks}, respectively, and GA at 10 μmol/L abolished K_{ATP} (data not shown).

Coincidently, perfusion with LPC-16 produced concentration-dependent $[K^+]_o$-↑ similar to that seen with ischemia. The EC_{50} calculated with the Hill equation was 2.3 μmol/L (Figure 2A). Hence, 3 μmol/L LPC-16 was used for the rest of the Langendorff study. LPC-16 caused a time-dependent $[K^+]_o$-↑ with the maximum effect occurring 10 min after perfusion; $[K^+]_o$ was increased from 4 mmol/L under baseline control to 7.3 mmol/L in the presence of LPC-16 (Figure 2B). Application of I_{Kr} blocker dofetilide but not the blockers for I_{Ks} or K_{ATP} prevented the $[K^+]_o$-↑ induced byLPC-16 (Figure 2C-2F).

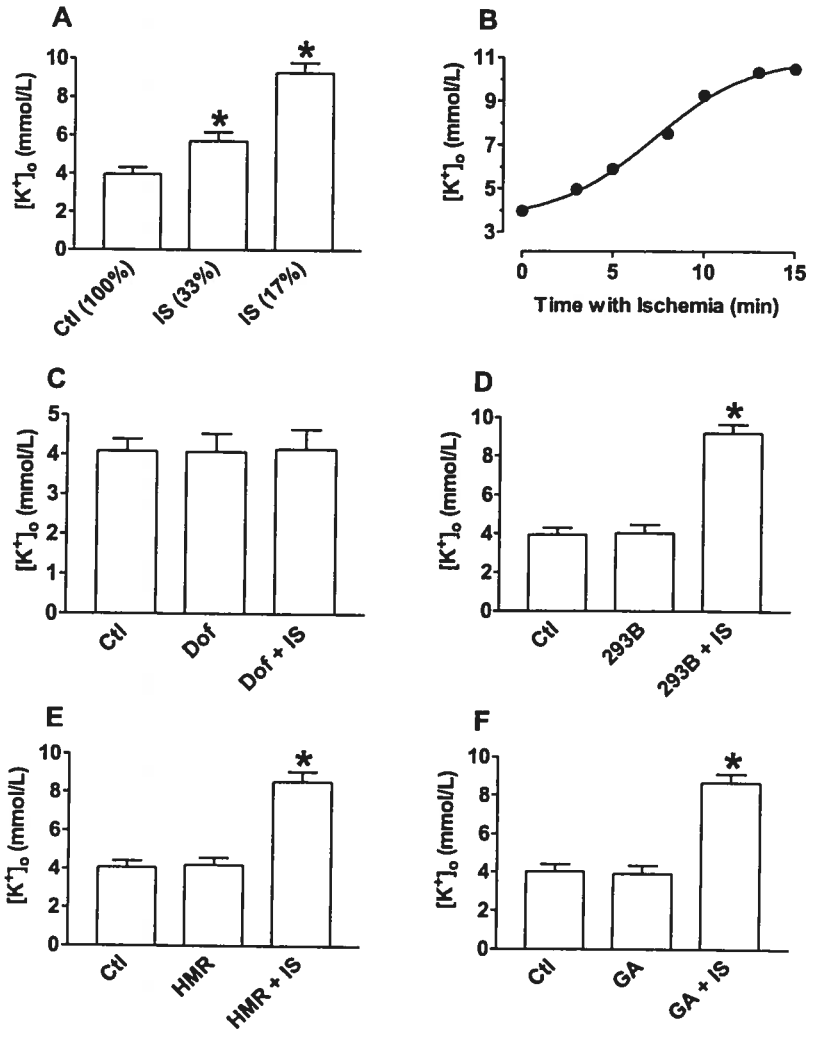

Figure 1. Increases in extracellular K^+ concentration ($[K^+]_o$-↑) during global ischemia and potential role of I_{Kr}. $[K^+]_o$ was measured with a K^+-sensitive electrode from the effluent of Langendorff-perfused rabbit hearts. (**A**) $[K^+]_o$-↑ when perfusion rate was reduced to 33% and 17% of the normal perfusion rate (30 ml/min), respectively. (**B**) $[K^+]_o$-↑as a function of time. Perfusion rate was 5 ml/min, 17% of normal control perfusion rate. (**C-F**) Effects of K^+ channel blockers on ischemic $[K^+]_o$-↑. Dof: dofetilide (20 nmol/L, I_{Kr}/HERG inhibitor); 293B: chromanol 293B (50 μmol/L, I_{Ks} inhibitor); HMR: (100 nmol/L, I_{Ks} inhibitor); GA: glibenclamide (10 μmol/L, K_{ATP} inhibitor). Note that $[K^+]_o$-↑ was prevented only in the hearts pretreated with Dof but not with other blockers studied. *$p<0.05$ *vs.* Control (Ctl); n=3-4 hearts for each group.

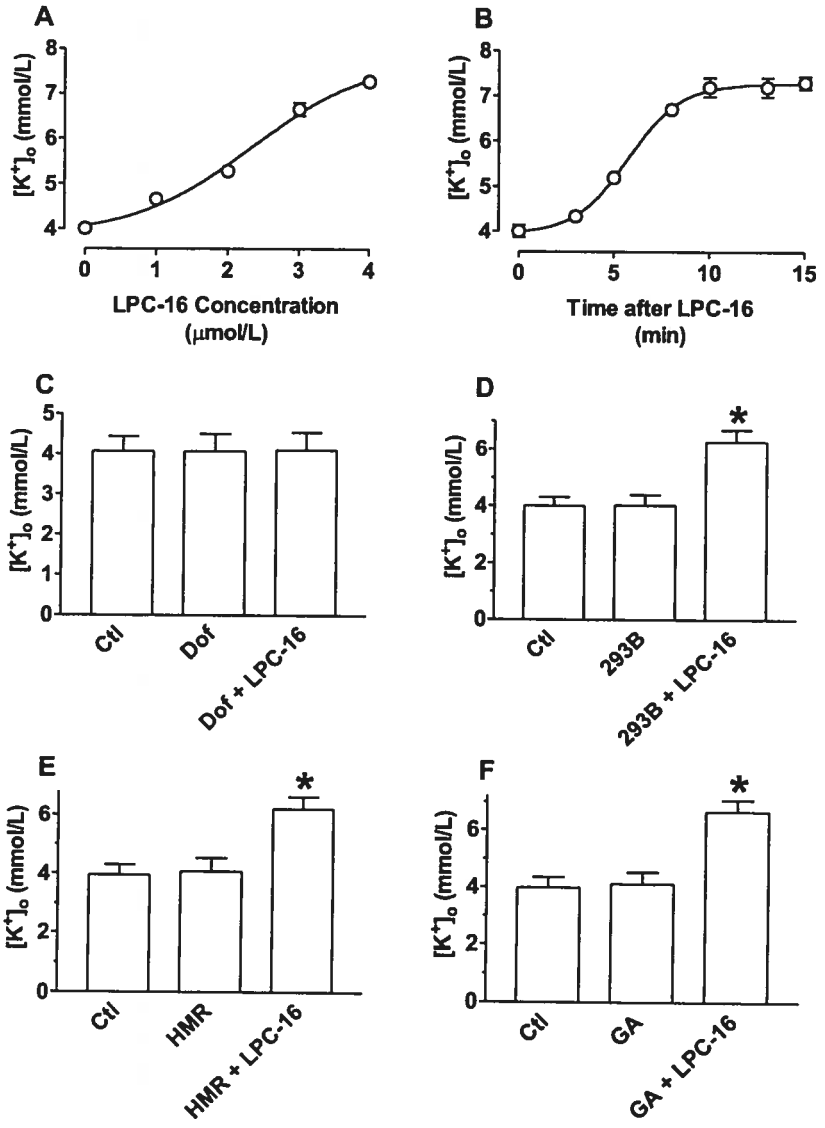

Figure 2. **$[K^+]_o$-↑ induced by lysophosphatidylcholine (LPC-16).** (**A**) Concentration-dependent $[K^+]_o$-↑ induced by LPC-16 with normal perfusion rate. (**B**) $[K^+]_o$-↑ as a function of time in the presence of LPC-16 (3 μmol/L). (**C-F**) Effects of K^+ channel blockers on $[K^+]_o$-↑ induced by LPC-16. Dof: dofetilide (20 nmol/L); 293B: chromanol 293B (50 μmol/L); HMR: (100 nmol/L); GA: glibenclamide (10 μmol/L). Note that $[K^+]_o$-↑ was prevented only in the hearts pretreated with Dof but not with other blockers studied. *$p<0.05$ *vs.* Control (Ctl); n=3-4 hearts for each group.

II-3-4-2 Role of I_{Kr}/HERG in QTc-↓ Induced by Low-perfusion Ischemia or by Exogenous LPC-16

Parallel to the $[K^+]_o$-↑ during ischemia or in the presence of exogenous LPC-16, QT interval and the heart-rated corrected QT interval (QTc) were consistently shortened during ischemia or with LPC-16 in the perfusate (Figures 3-5). The extent of QTc-↓ depended upon degree of ischemia or concentration of LPC-16. Intriguingly, QTc-↓ failed to reach a steady-state within 15 min low-rate perfusion (5 ml/min, Figure 4B) or 15 min with LPC-16 perfusion (Figure 5B). Perfusion with 20 nmol/L dofetilide prevented the QTc-↓ induced by either ischemia (Figure 4C) or LPC-16 (Fig. 5C). In contrast, blockers of I_{Ks} or K_{ATP} did neither affect the ischemic QTc-↓ (Figure 4D-4F) nor LPC-16-induced QTc-↓ (Figure 5D-5F).

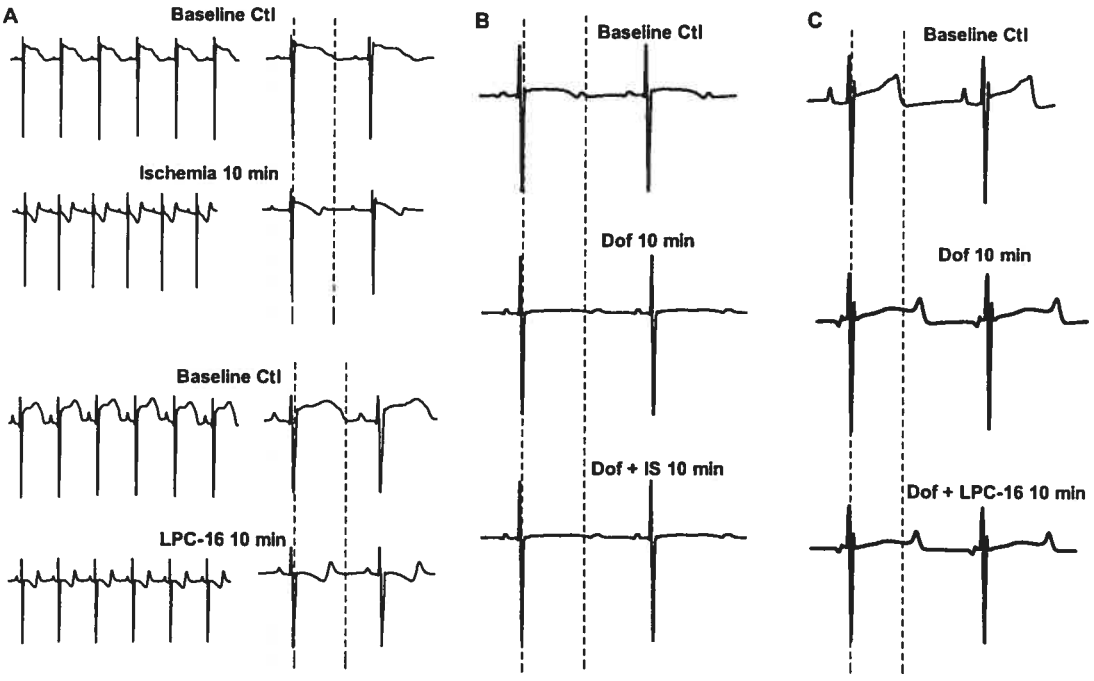

Figure 3. Shortening of QT interval (QT-↓) induced by global ischemia or by LPC-16. Shown are typical examples of ECG recordings showing QT-↓ during ischemia (slow perfusion rate, 5 ml/min) or in the presence of LPC-16 (3 μmol/L) and abolition of QT-↓ by dofetilide (Dof, 20 nmol/L).

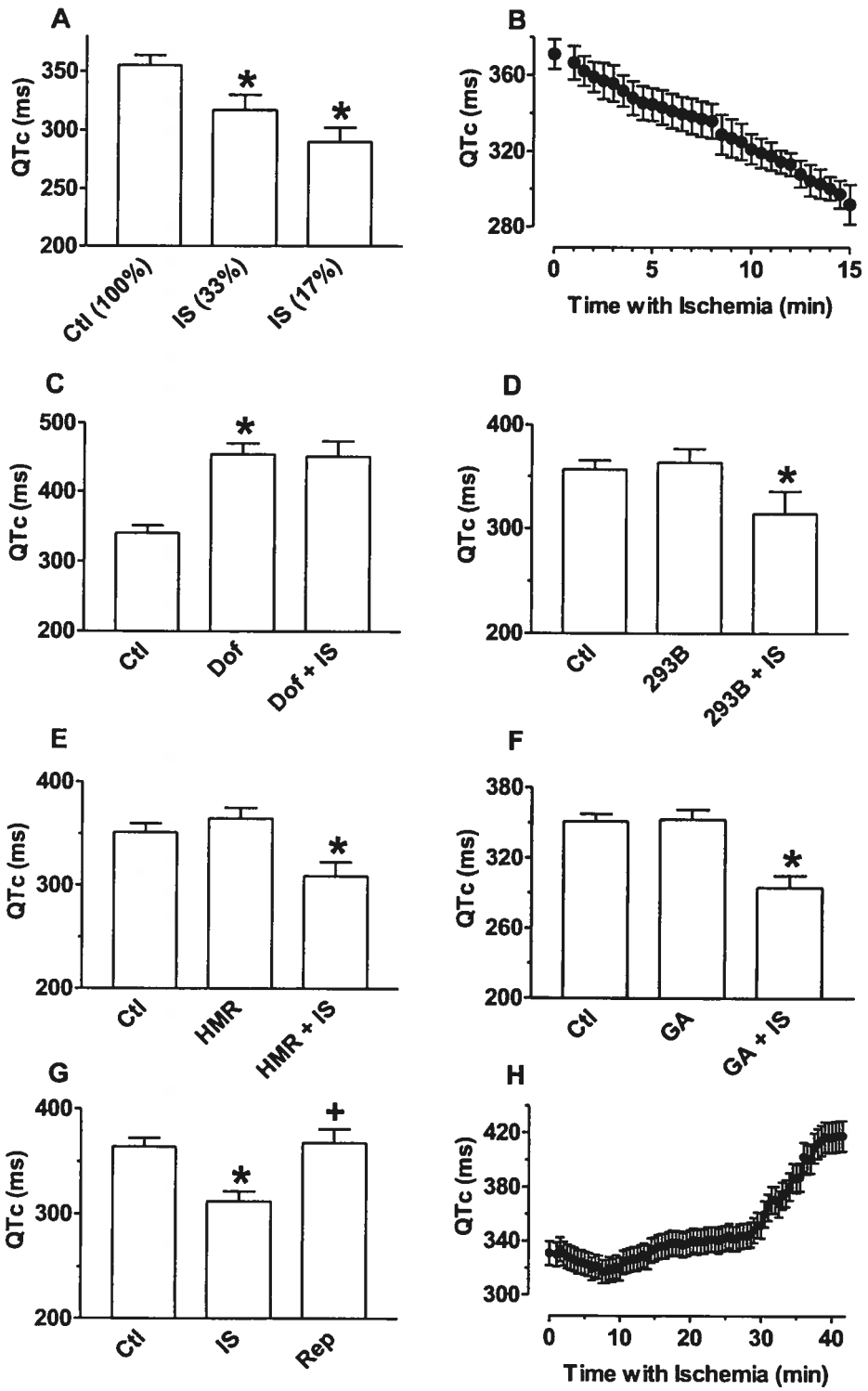
A
QTc (ms)
350
300
250
200
*
*
Ctl (100%)
IS (33%)
IS (17%)
B
QTc (ms)
360
320
280
0
5
10
15
Time with Ischemia (min)
C
QTc (ms)
500
400
300
200
*
Ctl
Dof
Dof + IS
D
QTc (ms)
400
300
200
*
Ctl
293B
293B + IS
E
QTc (ms)
400
300
200
*
Ctl
HMR
HMR + IS
F
QTc (ms)
380
320
260
200
*
Ctl
GA
GA + IS
G
QTc (ms)
400
300
200
*
+
Ctl
IS
Rep
H
QTc (ms)
420
380
340
300
0
10
20
30
40
Time with Ischemia (min)

Figure 4. Shortening of heart rate-corrected QT interval (QTc-↓) during global ischemia (IS) and potential role of I_{Kr}. (**A**) QTc-↓ with 33% and 17% of the normal perfusion rate (30 ml/min): 10 ml/min and 5 ml/min, respectively. (**B**) QTc-↓ as a function of time under ischemia (5 ml/min, 17% of the normal perfusion rate). (**C-F**) Effects of K^+ channel blockers on ischemic QTc-↓. Dof: dofetilide (20 nmol/L, I_{Kr}/HERG inhibitor); 293B: chromanol 293B (50 μmol/L, I_{Ks} inhibitor); HMR: (100 nmol/L, I_{Ks} inhibitor); GA: glibenclamide (10 μmol/L, K_{ATP} inhibitor). Note that QTc-↓ was prevented only in the hearts pretreated with Dof but not with other blockers studied. (**G**) QTc-↓ during ischemia and recovery upon reperfusion (10 min), indicating QTc-↓ being reversible. (**H**) QTc-↓ as a function of time under 33% slowing (10 ml/min) of normal control perfusion rate. Note the initial QTc-↓ within the first 10 min of ischemia and subsequent pronounced QTc *$p<0.05$ *vs.* Control (Ctl); n=3-4 hearts for each group.

The ischemic QTc-↓ was reversible upon reperfusion (resuming the normal perfusion rate of 30 ml/min). When the degree of ischemia was set less severe by reducing the perfusion rate to 33% (10 ml/min) of the normal rate, QTc demonstrated biphasic changes with time: initial shortening within the first 10 min followed by subsequent lengthening up to 40 min.

II-3-4-3 I_{Kr}/HERG Blocker Suppresses Arrhythmias Induced by Low-perfusion Ischemia or by Exogenous LPC-16

In our experimental model, either global ischemia (low perfusion rate of 5 ml/min) or LPC-16 (3 μmol/L) caused arrhythmias, mainly ventricular tachycardia (VT), defined as runs of at least five consecutive QRS complexes on ECG. VT was observed in all 4 hearts that underwent global ischemia and in 3 out of 4 hearts treated with LPC-16 (Figure 6). VT occurred primarily within the first 10 min after ischemia (average of 10 episodes) or LPC-16 perfusion (4 episodes), and within the next 10 min VT incidence considerably decreased with only one episode in ischemic hearts and none in LPC-16-treated hearts. When pretreated with dofetilide (20 nmol/L), only two out of four ischemic hearts had an average of one incidence of VT, and none of the five hearts treated LPC-16 showed any arrhythmias.

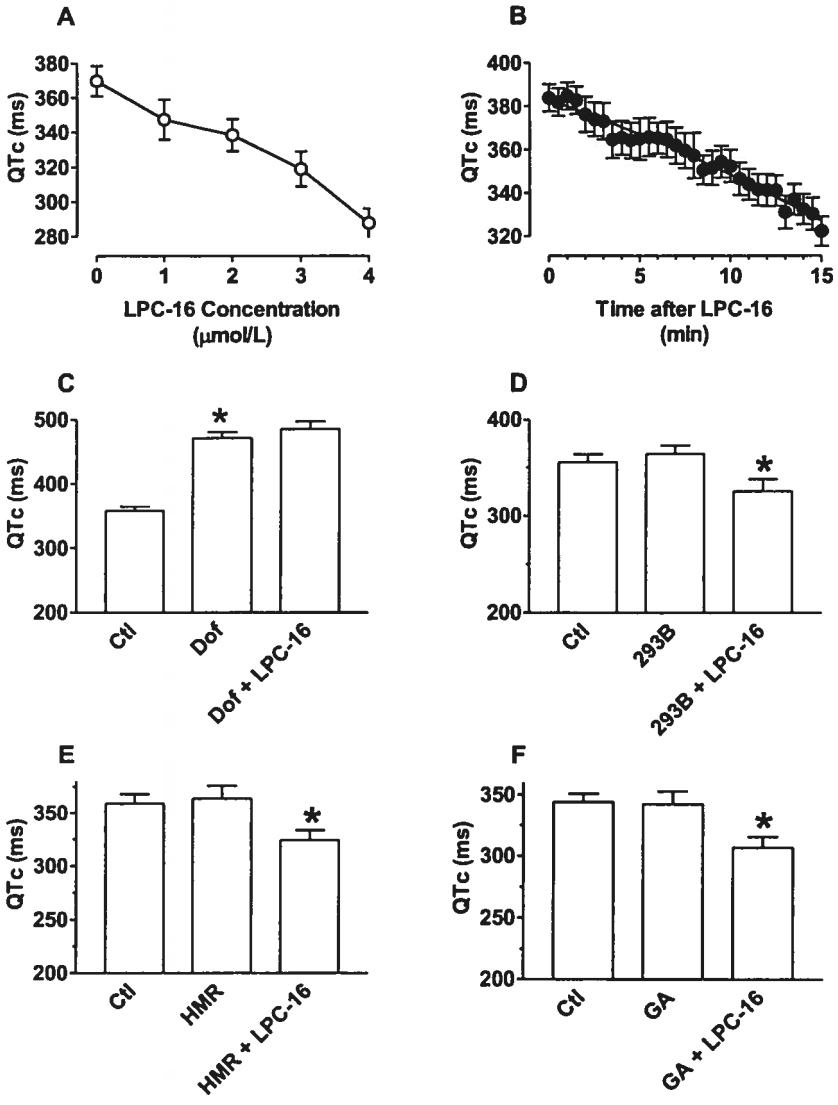

Figure 5. Shortening of QTc interval (QTc-↓) induced by LPC-16 and potential role of I_{Kr}. (**A**) QTc-↓ in the presence of various concentrations of LPC-16. (**B**) QTc-↓ as a function of time with 3 μmol/L LPC-16. (**C-F**) Effects of K^+ channel blockers on LPC-16-induced QTc-↓. Dof: dofetilide (20 nmol/L, I_{Kr}/HERG inhibitor); 293B: chromanol 293B (50 μmol/L, I_{Ks} inhibitor); HMR: (100 nmol/L, I_{Ks} inhibitor); GA: glibenclamide (10 μmol/L, K_{ATP} inhibitor). Note that QTc-↓ was prevented only in the hearts pretreated with Dof but not with other blockers studied. *$p<0.05$ *vs.* Control (Ctl); n=3-4 hearts for each group.

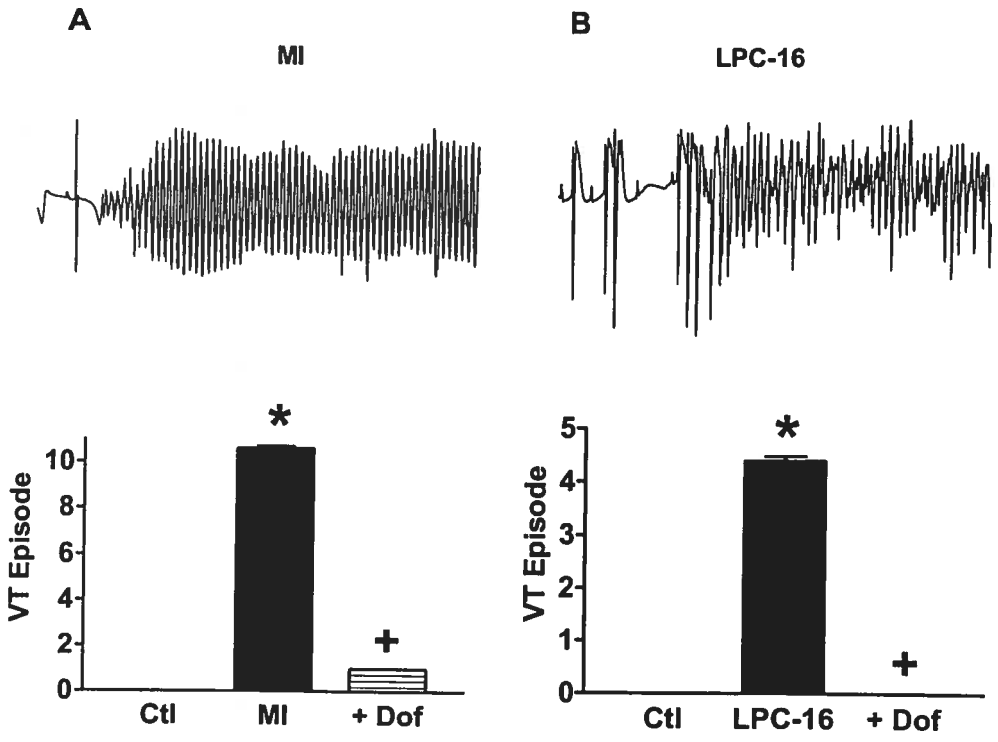

Figure 6. Arrhythmias induced by ischemia or LPC-16 (3 μmol/L) and the effects of I_{Kr}/HERG blocker dofetilide (20 nmol/L). Ventricular tachycardia (VT), defined as runs of multiple extra-excitations of >10 s in duration, occurring within the first 10 min of low rate perfusion (5 ml/min) (**A**) or LPC-16 application (**B**). Upper panels are examples of VT during ischemia or in presence of LPC-16 and lower panels show averaged data of VT episodes. Note that in the hearts pretreated with dofetilide (Dof), arrhythmias were abolished. *p<0.05 *vs.* Control (Ctl); +p<0.05 *vs.* ischemia or LPC-16; n=3-4 hearts for each group.

II-3-4-4 Enhancement of I_{Kr}/HERG by LPC-16 Accounts for APD-↓

To investigate the cellular mechanisms for the observed QTc-↓, we assessed the effects of LPC-16 on single cell action potential (AP) in isolated rabbit ventricular myocytes. As illustrated in Figure 7, LPC-16 (3 μmol/L) applied to the superfusion solution substantially shortened action potential duration (APD) and the shortening was prevented by co-application with dofetilide (20 nmol/L) that when applied alone caused striking prolongation of APD.

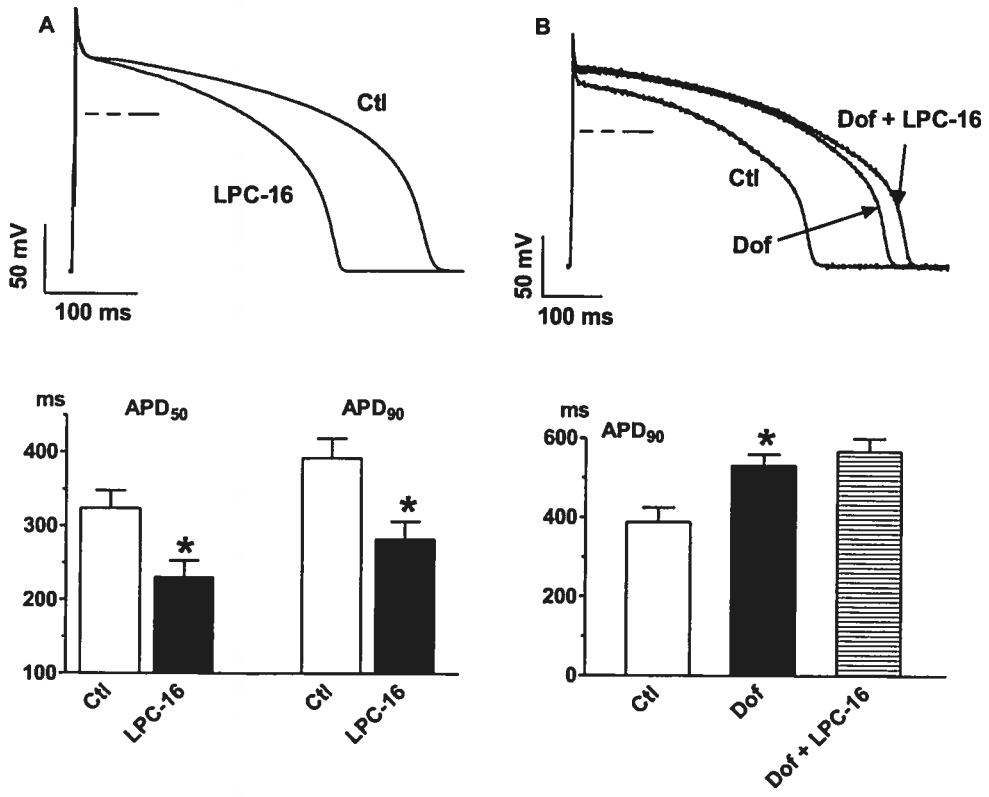

Figure 7. Shortening of action potential duration (APD-↓) induced by LPC-16 and potential role of I_{Kr}. Action potentials were recorded in isolated single ventricular myocytes from left endocardial wall of rabbits. (**A**) APD-↓ induced by LPC-16 (3 μmol/L). Measurements made under baseline control conditions (Ctl) were repeated 10 min after superfusion with LPC-16. Upper panels: typical examples of action potentials; lower panels: averaged data from 10 cells. APD_{50} and APD_{90}: action potential duration at 50% and 90% repolarization, respectively. (**B**) Effect of dofetilide (Dof, 20 nmol/L) on APD-↓ induced by LPC-16 (3 μmol/L). Cells were pretreated with Dof for 10 min before co-application with LPC-16. Note Dof alone prolonged APD and prevented APD-↓ induced by LPC-16. Dash lines indicate 0 mV potential. *p<0.05 *vs.* Ctl.

The above data suggest that LPC-16 shortens APD by enhancing the function of I_{Kr}/HERG. To examine this notion, we performed whole-cell action potential-clamp (AP-clamp) experiments in HERG expressing HEK293 cells. AP waveforms, recorded in myocytes from various regions of the canine heart, including ventricular endocardium, mid-myocardium and right atrium, were used as command pulses to elicited HERG

current (I_{HERG}). As depicted in Figure 8A-8C, LPC-16 (3 μmol/L) increased I_{HERG} with various AP waveforms. Noticeably, however, the effect was to the greatest extent with atrial AP with an order of atrial AP > endocardial AP > mid-myocardial AP. Intriguingly, APD is shortest in atrial AP in the order of atrial AP < endocardial AP < mid-myocardial AP. This implies that the shorter the APD, the greater the enhancement of I_{HERG} by LPC-16. If it is true then more pronounced I_{HERG} increase is expected with briefer APD of APs from the same cell type. This was indeed verified by applying two endocardial APs in tandem, one recorded at 2.5 Hz with shorter APD and the other at 0.5 Hz with longer APD. As shown in Figure 8D, I_{HERG} enhancement was markedly greater with shorter AP than with longer AP. This point is further addressed by the data in Figure 8E where APD_{50} and APD_{90} (APD to 50% and 90% full repolarization, respectively) and percent increases in I_{HERG} are shown. Correlation between I_{HERG} increase and APD was analyzed by plotting the former as a function of the latter and by the linear regression. The goodness of fit was 0.997 for APD_{50} and 0.992 for APD_{90}, with a 95% confidence range of 41 to 85 for APD_{50} and of 25 to 141 for APD_{90}.

Enhancement of I_{Kr} by LPC-16 was verified in native myocytes isolated from guinea pig ventricle. Guinea pig was used because as it is well known, our current techniques allowed us to record larger I_{Kr} in this species than in any other species, for more accurate analysis of pharmacological properties. A series of voltage steps ranging from -40 mV to +60 mV were delivered to inactivate I_{Kr}, followed by a repolarizing pulse to -40 mV to reactivate I_{Kr} so as to observe I_{Kr} tail currents. The dofetilide-sensitive current when plotted as a function of inactivation pulses (depolarizing prepulses) shows characteristics of I_{Kr} (Figure 9A and 9B). The current reached a steady-state (maximum) level from potentials positive to -10 mV. Inclusion of LPC-16 (3 μmol/L) in the bathing solution for 10-15 min caused a pronounced increase in the tail current that was reversible upon subsequent washout of LPC-16. When the I_{Kr} tail currents were normalized to the maximum value to obtain conductance (G) curve, an approximately 5 mV negative shift of I_{Kr} activation in the presence of LPC-16 was revealed. This negative shift resulted in greater increases in I_{Kr} at more negative potentials or at the potentials closer to resting membrane potentials in ischemic myocardium.

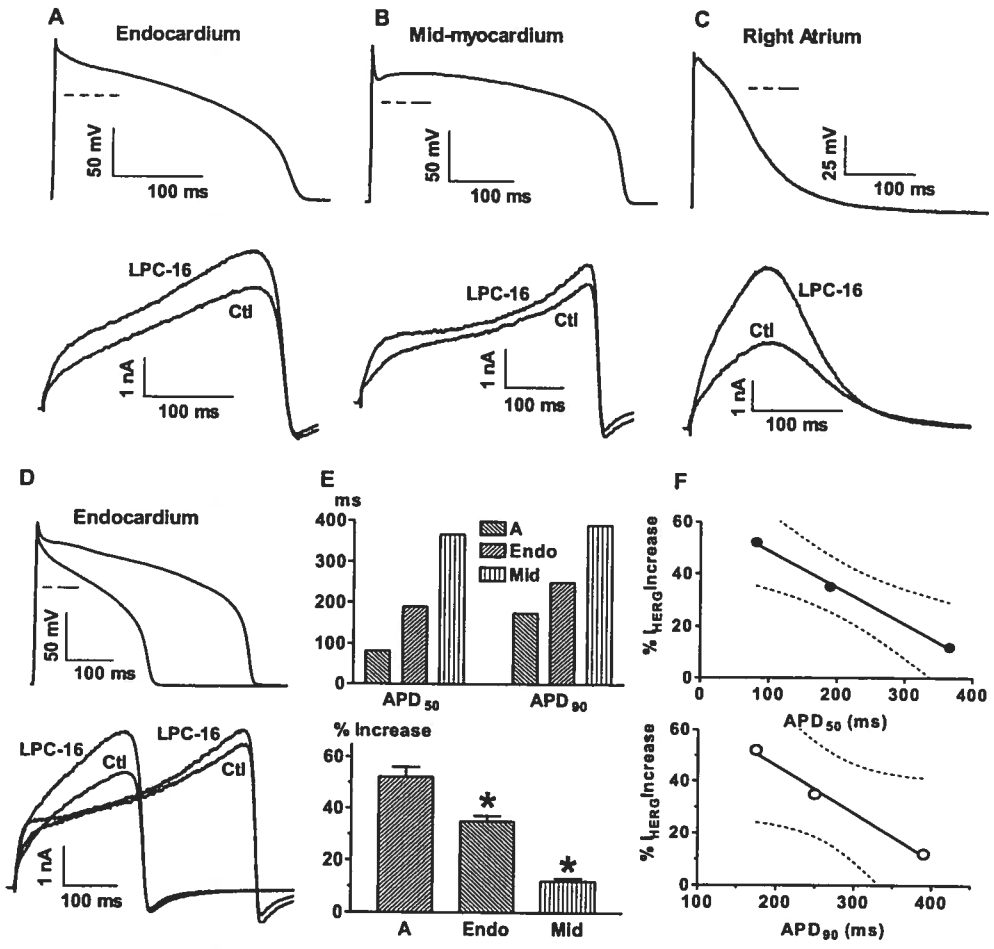

Figure 8. Enhancement of HERG current (I_{HERG}) by LPC-16 in HERG-expressing HEK293 cells. I_{HERG} was recorded by action potential (AP)-clamp techniques with AP waveforms obtained from single canine myocytes of different regions. (**A-C**) Typical examples of APs and the respective I_{HERG} from endocardium (Endo), mid-myocardium (Mid) and atrium (A), showing enhancement of I_{HERG} by LPC-16 (5 μmol/L). Note LPC-16 increases I_{HERG} more with AP waveforms of shorter duration. (**D**) Direct comparison of LPC-16-induced I_{HERG} enhancement between short and long APs. Note LPC-16 increases I_{HERG} more with shorter APD. (**E**) Comparison between APD_{50} and APD_{90} (Upper) and I_{HERG} enhancement (Lower). *$p<0.05$ *vs.* atrium (A); n=5 cells. (**F**) Correlation between I_{HERG} enhancement by LPC-16 and APD_{50} (Upper) or APD_{90} (Lower).

For example, at -30 mV, LPC-16 produced 136% enhancement of I_{Kr}, but the effect decreased to only 35% at +20 mV. The same pattern of actions of LPC-16 was consistently seen with the cloned HERG channel expressed in HEK293 cells (Figure 9C): negative shift of activation and voltage-dependent increase with markedly more pronounced enhancement at the potentials near the resting potential of ischemic heart (145% increase at -40 mV *vs.* 34% increase at +20 mV).

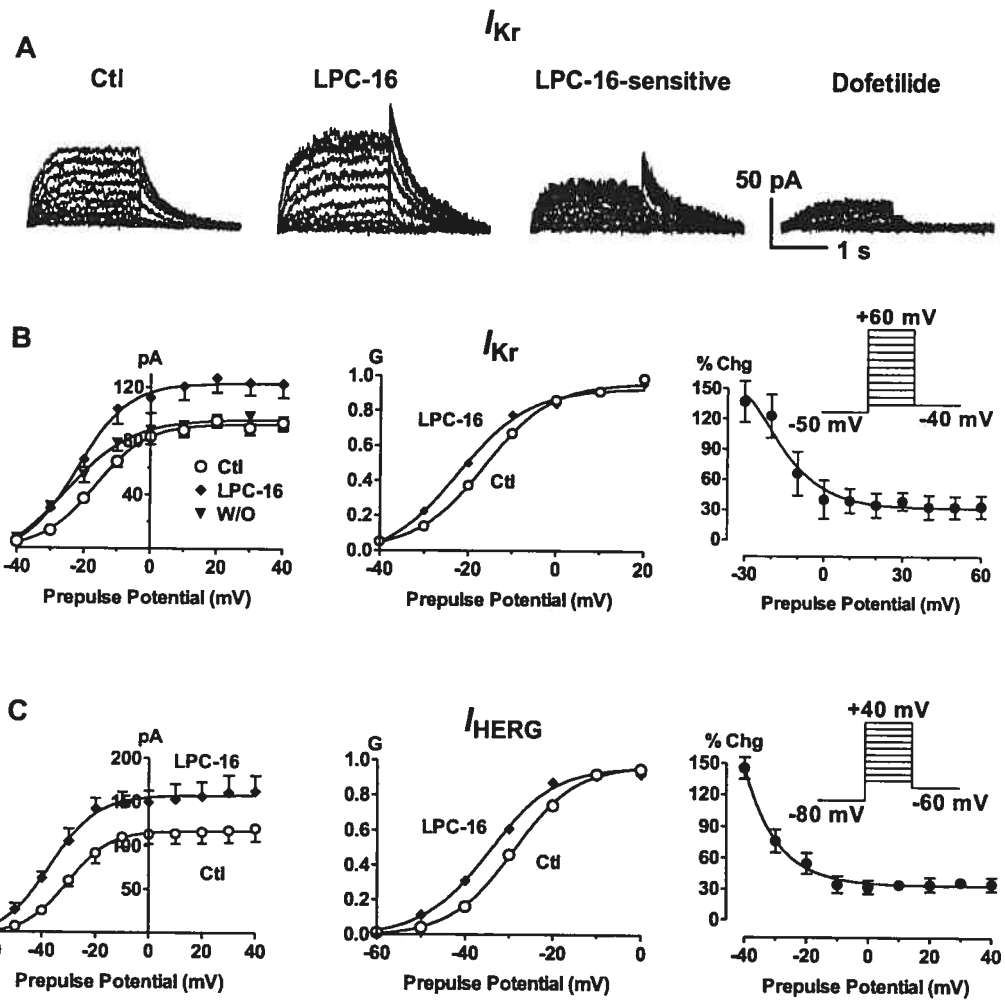

Figure 9. Enhancement of native rapid delayed rectifier K^+ current (I_{Kr}) in left ventricular endocardial myocytes isolated from guinea pigs, and cloned I_{HERG} in HEK293 cells, by LPC-16. I_{Kr} was elicited by the voltage protocol shown in the inset. (**A**) Typical examples of I_{Kr} traces recorded under control conditions, in presence of LPC-16 (5 μmol/L), and in presence of LPC-16 + dofetilide (Dof, 20 nmol/L). The enhancement of I_{Kr} by LPC-16 is revealed by the LPC-16-sensitive currents obtained by digital subtraction between the current with and without LPC-16. Dofetilide, a specific I_{Kr}/HERG blocker was used to verify the native I_{Kr}. (**B**) Averaged data (n=8 cells) showing the reversible increase in I_{Kr} tail amplitude (left panel), the negative shift of activation curves (middle panel), and the voltage-dependence of percent increase in I_{Kr} (right panel), by LPC-16. The activation curves were constructed by plotting the conductance *G* as a function of depolarizing potentials. *G* was calculated by normalizing the tail currents at -40 mV by dividing the amplitude of the tail currents measured at various antecedent depolarizing potentials by that of the tail current at +60 mV. Symbols are mean of experimental data and lines represent the Boltzmann fit: $G/G_{max}=1/\{1+\exp[(V_{1/2}-V)/k]\}$, where G_{max} represents the maximal conductance at +60 mV, $V_{1/2}$ is a half-maximal activation voltage, and *k* is a slope factor. W/O: washout. $p<0.05$ LPC-16 *vs.* Ctl at potentials from -30 mV to +60 mV. (**C**) Averaged data (n=6 cells) showing the increase in I_{HERG} tail amplitude (left panel), the negative shift of activation curves (middle panel), and the voltage-dependence of percent increase in I_{HERG} (right panel), by LPC-16. $p<0.05$ LPC-16 *vs.* Ctl at potentials from -40 mV to +40 mV.

II-3-5 DISCUSSION

II-3-5-1 Major Findings of the Study

Extracellular K^+ accumulation in myocardium and APD shortening due to abnormally enhanced K^+ efflux are the critical cause of electrical disturbance, including the associated ischemic arrhythmias, during the early stage of acute myocardial ischemia. Despite the paramount importance of understanding $[K^+]_o$-↑ and APD-↓ for the treatment of ischemic electrical disorders, the intrinsic pathways through which K^+ flows out of the cell remained poorly defined and the ionic mechanism for ischemic $[K^+]_o$-↑ and APD-↓ is still a puzzle to cardiologists and scientists. Similarly, metabolic perturbation of ischemic

myocardium is known to have great impact on electrical disturbance. Among the various metabolic insults, the rapid accumulation of phospholipid metabolite LPC in the ischemic myocardium is of pivotal importance. However, while LPC has long been recognized as a biochemical trigger of ischemic arrhythmias, presumably due to its ability to cause $[K^+]_o$-↑ and APD-↓,[17] how LPC causes $[K^+]_o$-↑ and APD-↓ remained a mystery. To shed some lights on these issues, we carried out the present study and made several important and novel findings. First, exogenous LPC-16 mimics the $[K^+]_o$-↑ and APD-↓/QTc-↓ seen in low-perfusion ischemia and the effects are reversed by specific I_{Kr}/HERG blocker dofetilide. Second, dofetilide is highly effective in preventing ventricular tachy-arrhythmias induced by low-perfusion ischemia or by exogenous LPC-16. Third, LPC-16 enhances I_{Kr}/I_{HERG} and the effects are greater with shorter action potential duration (APD) and at more negative potentials. These results indicate that enhancement of I_{Kr}/I_{HERG} by LPC-16 contributes to $[K^+]_o$-↑ and APD-↓/QTc-↓ and the associated arrhythmias during acute myocardial ischemia. In other words, HERG K^+ channel forms the pathway or one of the pathways, and LPC-16 acts as a trigger, for abnormal intracellular K^+ loss or K^+ efflux in ischemic myocardium. Therefore, in addition to the paired ATP depletion/K_{ATP} activation, LPC accumulation/HERG enhancement forms another link between metabolic trigger and ionic pathway for ischemic $[K^+]_o$-↑ and QTc-↓.

II-3-5-2 Current Knowledge of Mechanisms for Ischemic $[K^+]_o$-↑ and QTc-↓

Because of the importance of $[K^+]_o$-↑ along with APD-↓ to ischemic arrhythmias, mechanisms responsible for it have been a subject of intensive research. It is generally believed that $[K^+]_o$-↑ and APD-↓ are of multifactorial nature with 3 major determinants:[1,7] increased passive K^+ efflux, reduced active K^+ influx, and diminished extracellular space. Increased passive K^+ efflux is a prerequisite for ischemic $[K^+]_i$-↓ or $[K^+]_o$-↑ and APD-↓. Experimental studies support the hypothesis that K^+ efflux increases rapidly during acute myocardial ischemia; this increase is most likely due to a net increase in the conductance of K^+ channels. Reduced active K^+ influx is a warranty for ischemic $[K^+]_o$-↑ and it is achieved by reduction of the Na^+-K^+ pump activity. Ischemic conditions make a moderate inhibition of the pump plausible; metabolic stresses have been found to reduce pump activity.[1,28-30] Owing to the remarkable capacity of the pump

(150 pA or 3×10^{-15} mol/sec), small reduction of the activity can render dramatic changes of $[K^+]_o$. The normal reversal potential (E_{rev}) of the pump is –180 mV. It may however drop to –60 mV which is about the membrane potential of the cells during the plateau of $[K^+]_o$-↑ in ischemic myocardium. The pump, in other words, will stop operating at the potential expected under ischemic conditions.[1,30] Diminished extracellular space produces a concentrating effect on $[K^+]_o$-↑ caused by increased passive K^+ efflux and reduced active K^+ influx. Shift of water from extracellular to the intracellular space occurs as a consequence of increase in osmotically active particles in the cell, such as lactate and phosphate. It is estimated that the increase in osmotically active particles can results in a restriction of the extracellular space by 15% after 10 min of ischemia.[1,31,32] Apparently, increased passive K^+ efflux acts as an initiation mechanism for $[K^+]_o$-↑ and APD-↓, decreased active K^+ influx as a maintenance mechanism for $[K^+]_o$-↑, and shrinkage of extracellular space as an amplification mechanism for $[K^+]_o$-↑. However, despite the key importance of increased passive K^+ efflux, the ionic mechanisms are still uncertain and our understanding of pathways along which K^+ leaves the cells have been controversial for nearly two decades.

Cardiac K^+ channels play a pivotal role in defining resting potential, cell excitability and membrane repolarization and thereby the likelihood of arrhythmias. Cardiac cells possess several K^+ currents: inward rectifier K^+ current (I_{K1}),[33] transient outward K^+ current (I_{to}),[23,24] rapid delayed rectifier K^+ current (I_{Kr}),[27] slow delayed rectifier K^+ current (I_{Ks}),[27] ultrarapid delayed rectifier K^+ current (I_{Kur}),[34] ATP-sensitive K^+ current (K_{ATP}),[35] Na^+-activated K^+ current (I_{KNa}),[36] arachidonic acid-activated K^+ current (I_{KAA}),[37] etc. Some of these K^+ currents have been reported to be downregulated in their function and expression during acute ischemia; such as I_{to}, I_{K1} and I_{Ks}.[1,38] I_{Kur} is believed to be absent in ventricular cells of various species including humans, dogs, guinea pigs and rabbits. Hence, these channels as carriers of ischemic K^+ efflux can be excluded. K_{ATP} as a candidate for ischemic $[K^+]_o$-↑ has been extensively studied; yet the results are inconsistent. While either I_{KNa} or I_{KAA} has the potential to be a contributor to ischemic $[K^+]_o$-↑, the existing studies are sparse and do not allow us to reach a definite conclusion. The possible role of I_{Kr} remained yet to be investigated.

Since K_{ATP} is gated by intracellular ATP and regulated by a variety of cellular metabolites, K_{ATP} channel is naturally considered to be a sensor of the metabolic state of a myocardial cell, and activation of these channels to be an indicator of cellular metabolic compromise.[39-41] However, K_{ATP} channels do not open unless intracellular ATP level ($[ATP]_i$) drops down to less than 100 μmol/L; the IC_{50} for K_{ATP} inhibition is <100 μmol/L. This implies that K_{ATP} channels normally stay in closed state because the physiological $[ATP]_i$ is in the order of millimolar range (5 mmol/L). This is perhaps why the existing data regarding the role of K_{ATP} in ischemic $[K^+]_o$-↑ have been conflicting. Evidence exists that the K_{ATP} channel mediates ischemic K^+ efflux and APD-↓ and most of the evidence is from studies using K_{ATP} inhibitors or openers; K_{ATP} inhibitors such as glibenclamide have been found to effectively reverse ischemia/hypoxia APD-↓ and the rate of $[K^+]_o$-↑.[35,40-43] In addition, glibenclamide also exhibits potent antifibrillatory activity, abolishing irreversible ventricular fibrillation during regional ischemia and during global ischemia. However, there are also experimental findings against K_{ATP} channel as a mediator of ischemic $[K^+]_o$-↑ and APD-↓.[44-49] First, the measured $[ATP]_i$ at the time of metabolic blockade of acute ischemia is still in the millimolar range which is far too high to induce massive K_{ATP}.[50,51] Second, the time-course of K_{ATP} channel opening is far too slow to explain the start of $[K^+]_o$-↑ within the first minute of ischemia. Third, the results from K_{ATP} openers and inhibitors have been conflicting; early ischemic $[K^+]_o$-↑ and surface acidification are relatively insensitive to K_{ATP} channel inhibition or activation prior to the ischemic insult[48] and K_{ATP} opener pinacidil.[52] Finally, some blockers of voltage-gated K^+ channels are effective in preventing and reversing ischemic $[K^+]_o$-↑ and APD-↓; these include diltiazem.[53] Moreover, K_{ATP} was found to be inhibited by a variety of lipid metabolites such as lysophospholipids and arachidonic, linoleic, linolenic, and eicosatrienoic free fatty acids, which accumulate in ischemic myocardium.[38] The role of K_{ATP} channel in response to myocardial ischemia, therefore, has been questioned. It is thus conceivable that other mechanisms or more specifically other K^+ currents, in addition to K_{ATP}, may also contribute to $[K^+]_o$-↑ and APD-↓ in ischemic myocardium.

As already described in the introduction, as the major species of lysophosphatidylcholines that are overproduced and accumulate in ischemic myocardium, LPC-16 has been commonly regarded as a crucial triggering factor for arrhythmogenesis

of ischemic heart.[54-56] While the exact mechanisms have not yet been elucidated, it is generally accepted that disruption of the cytoplasmic membrane integrity by LPC-16 is responsible because of the ability of LPC-16 to incorporate into the membrane lipid bi-layer. If this is true then the deleterious effects of LPC-16 would be largely irreversible and thus many of the electrical perturbations of ischemic heart would be inconvertible.

II-3-5-3 I_{Kr}/HERG as an Ionic Determinant and LPC-16 as a Metabolic Trigger Together Contribute to Ischemic $[K^+]_o$-↑ and "Short QT Syndrome"

Based on the information described above and on our previous studies,[24,25] we propose that ischemic $[K^+]_o$-↑ and APD-↓ are an event consequent to combined metabolic stress and ion channel dysfunction. Specifically, I_{Kr}/I_{HERG} is abnormally enhanced during the early stage of acute myocardial ischemia and the enhancement is mainly caused by LPC-16 overproduction and accumulation. The enhancement of I_{Kr}/I_{HERG} by LPC-16 is a critical contributor to intracellular K^+ loss/$[K^+]_o$-↑ and APD-↓ thereby the associated ischemic arrhythmias. In other words, I_{Kr}/I_{HERG} is one of the important ionic pathways for ischemic K^+ loss and the associated APD shortening. The present study provides several lines of evidence in support of our hypothesis. First, LPC-16 produced qualitatively the same $[K^+]_o$-↑ and QTc-↓ as acute ischemia did. Second, the $[K^+]_o$-↑ and QTc-↓ induced by either ischemia or LPC-16 were prevented by I_{Kr}/HERG blocker dofetilide. Third, LPC-16 shortened APD and enhanced I_{Kr}/HERG function, effects which were again abolished by dofetilide.

One important point to be mentioned here is that the conductance of I_{Kr}/HERG is known to critically depend upon extracellular K^+ activity; conductance is strikingly enhanced with increasing extracellular K^+ activity.[57,58] It is therefore conceivable that increase in I_{Kr}/I_{HERG} in response to LPC-16 during ischemia results in increase in K^+ efflux which in conjunction with partial impairment of Na^+/K^+ pump function and shrinkage of extracellular space leads to $[K^+]_o$-↑ and APD-↓/QTc-↓. The increased extracellular K^+ activity as a result of enhanced I_{Kr}/I_{HERG} in turn promotes I_{Kr}/I_{HERG} by enhancing the channel conductance, a positive feedback mechanism.

Another interesting finding of this study concerns the mode of LPC-16 actions on I_{Kr}/HERG. Our data demonstrate that cardiac membrane repolarization facilitates the

I_{Kr}/HERG-enhancing effect of LPC-16. This notion is supported by the following data. First, the I_{Kr}/HERG-enhancing effect of LPC-16is ~2-fold greater with shorter AP or faster repolarization rate (Figure 8). Considering that APD is shortened during acute ischemia, or in the presence of LPC-16, relative to control conditions, the I_{Kr}/HERG-enhancing effect of LPC-16 is expected to be further promoted, another positive feedback mechanism. Second, also significant is the fact that the I_{Kr}/HERG-enhancing effect of LPC-16 is ~3-fold greater at negative or repolarized potentials near the resting membrane potential of the ischemic heart, relative to depolarized potentials positive to 0 mV (Figure 9). This would imply that LPC-16 can cause increase in K^+ efflux not only during an excitation but also during diastolic period. This latter effect might counteract with Na^+/K^+ pump so as to weaken the process of K^+ reuptake back into the cell, reinforcing $[K^+]_o$-↑ and APD-↓/QTc-↓. It is noted that the concentrations of LPC-16 used in this study are within the range of its level found in ischemic myocardium[20,21] and the effects of LPC-16 are readily reversible upon washout of LPC-16 or by co-application with dofetilide, at both whole heart level and cellular level. These facts are in favor of the idea that LPC-16 acts mainly on HERG channel to produce $[K^+]_o$-↑ and APD-↓/QTc-↓ and its potential permanent damage to the cytoplasmic membrane does not seem to operate, at least in the present ischemic model.

The third issue needed to be discussed is that administration with dofetilide prevents ischemic $[K^+]_o$-↑ but does not alter $[K^+]_o$ under control conditions (Figure 1C). On the other hand, dofetilide causes striking prolongation of APD and QTc, regardless of whether the hearts undergo low-perfusion ischemia or whether the superfusion solution contains LPC-16. The results suggest that under normal situations HERG channel albeit carrying K^+ efflux does not cause extracellular K^+ accumulation and only when HERG function is abnormally enhanced during ischemia or in the presence of LPC-16 does the excessive K^+ efflux leads to $[K^+]_o$-↑. This might be one of the explanations for the selective action of dofetilide towards diseased state.

We are therefore tempted to propose that there is a quick ionic remodeling process for ischemic $[K^+]_o$-↑ and APD-↓/QTc-↓. Enhanced function of HERG and other ion channels such as K_{ATP}, due to metabolic perturbations (such as accumulation of LPC) during acute ischemia, causes rapid ischemic $[K^+]_o$-↑ and APD-↓/QTc-↓, and the $[K^+]_o$-↑

and APD-↓/QTc-↓ in turn further enhance the function of HERG channel by directly increasing HERG conductance and by indirectly increasing the effect of LPC-16 on HERG amplitude. And this subsequent increase in HERG function will then go on to amplify $[K^+]_o$-↑ and APD-↓/QTc-↓.

II-3-5-4 Potential Implications and Possible Limitations of Our Findings

The present study is, to our knowledge, the first to document the role of I_{Kr}/HERG in ischemic $[K^+]_o$-↑ and QTc-↓ and the first to propose the ionic remodeling for ischemic $[K^+]_o$-↑ and QTc-↓ with a positive feedback mechanism for abnormal enhancement of HERG function. Hence, in addition to the paired ATP depletion/K_{ATP} activation, LPC accumulation/HERG enhancement may be considered as another link between metabolic trigger and ionic pathway, for ischemic $[K^+]_o$-↑ and QTc-↓. This notion provides an alternative explanation, or one of multiple mechanisms, for ischemic $[K^+]_o$-↑ and QTc-↓.

Our findings that LPC-16 acts as a metabolic trigger, and HERG as an ionic pathway, for ischemic $[K^+]_o$-↑ and QTc-↓ and that ischemic arrhythmias is preventable and convertible by I_{Kr}/HERG blocker dofetilide suggest that inhibition of LPC-16 production and accumulation and/or of I_{Kr}/HERG may be a promising therapeutic strategy to attenuate the incidence of lethal arrhythmias associated with ischemic heart disease.[54-56] Nonetheless, it should be mentioned that the initial rapid $[K^+]_o$-↑ and APD-↓ may be an adaptive mechanism of cardiac cells to minimize ischemic injuries because $[K^+]_o$-↑ may reduce cell excitability thereby contractility and energy consumption and APD-↓ can limit excessive Ca^{2+} entry so as to prevent intracellular Ca^{2+} overload and cell death. Unfortunately, this adaptation is at the cost of increased risk of cardiac arrhythmias, often lethal arrhythmias. This contradiction creates a difficulty of handling $[K^+]_o$-↑ and APD-↓. Obviously, restoration of $[K^+]_o$-↑ and APD-↓ alone may not be clearly beneficial to ischemic heart. Combination of I_{Kr}/HERG blocker and Ca^{2+} channel blocker might be the way to go because the approach can restore $[K^+]_o$-↑ and APD-↓ on one hand and diminish intracellular Ca^{2+} overload on the other hand. This notion warrants further investigation. It should also be noted that our study does not provide a definitive link between LPC-16 and I_{Kr}/HERG due to current lack of LPC-16 inhibitors.

It is known that inhibition of I_{Kr}/HERG function can generate long QT type of arrhythmias. Our present study revealed that enhancement of I_{Kr}/HERG function can also be proarrhythmic, causing short QT type of arrhythmias such as reentry and DAD. The latter concept has actually been proposed by a recent study that describes a form of inherited malignant ventricular arrhythmias associated with short QT syndrome.[59,60] Short QT syndrome is a genetic syndrome characterized by constantly short QT intervals, sudden death, short refractory periods, and inducible ventricular fibrillation. This short QT syndrome is caused by mutation in HERG gene, which increases HERG function and can be treated by agents that block I_{Kr}/HERG. Our data suggest that functional enhancement of HERG channel activity can produce acquired "short QT syndrome".

Our experiments with the I_{Ks} blockers chromanol 293B and HMR compound suggest that I_{Ks} is unlikely significantly involved in ischemic $[K^+]_o$-↑ and QTc-↓. In addition, we have also conducted experiments studying the effects of LPC-16 on I_{Ks} in guinea pig ventricular myocytes and K_VLQT1 transiently expressed in COS-7 cells and we found that LPC-16 did not alter I_{Ks} (data not shown). Therefore, contribution of I_{Ks} to ischemic $[K^+]_o$-↑ and QTc-↓ in our model can be excluded . Nevertheless, in *in vivo* system where sympathetic innervation is intact, enhanced adrenergic stimulation during acute ischemia may well serve to enhance I_{Ks} and in this case I_{Ks} may also contribute to ischemic $[K^+]_o$-↑ and QTc-↓.

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Chapter III

Sphingolipid Metabolite Ceramide Causes Metabolic Perturbation Leading to HERG K^+ Channel Dysfunction and Abnormal Slowing of Cardiac Repolarization

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III Sphingolipid Metabolite Ceramide Causes Metabolic Perturbation Leading to HERG K^+ Channel Dysfunction and Abnormal Slowing of Cardiac Repolarization*

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Running Title: **Cellular mechanisms of HERG depression by ceramide**

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III-1 SUMMARY

Ceramide, a sphingolipid metabolite, has emerged as a key second messenger molecule that mediates multiple cellular functions. Its *de nova* synthesis and accumulation in ischemic myocardium, and congestive heart failure and diabetic cardiomyopathy have been associated with the pathological processes including the abnormal QT prolongation (QT-↑) and increased risk of arrhythmias. To investigate how ceramide can be involved in modulating cardiac repolarization, we performed whole-cell patch-clamp studies on HERG current (I_{HERG}), a critical determinant of cardiac repolarization, expressed in HEK293 cells. Acute application (bath superfusion for 25 min) of membrane permeable ceramide (C2, 50 μM) did not alter I_{HERG}. Prolonged incubation with C2 for 10 hrs caused pronounced I_{HERG} inhibition in a concentration-dependent and voltage-independent fashion and positive shift of voltage-dependent HERG activation. The IC_{50} for I_{HERG} suppression was 19.5 μM. C2 did not affect the inactivation property and time-dependent kinetics of I_{HERG}. Similar effects were observed with production of endogenous ceramide catalyzed by sphingomyelinase. Tyrosine kinase inhibitors failed to reverse C2-induced suppression of HERG function, and PKA and PKC inhibitors only slightly reversed the I_{HERG} depression. Western blotting and immunocytochemical analyses indicate that C2 does not alter HERG protein expression on the cytoplasmic membrane. The inhibitory effect of C2 on I_{HERG} was reversed by antioxidants vitamin E or MnTBAP. C2 caused considerable production of intracellular reactive oxygen species (ROS), which was prevented by vitamin E or MnTBAP. We conclude that ceramide depresses I_{HERG} mainly via ROS overproduction and ceramide-induced I_{HERG} impairment may contribute to QT-↑ in prolonged myocardial ischemia, heart failure and diabetic cardiomyopathy.

Keywords: ceramide, HERG, reactive oxygen species, vitamin E, MnTBAP, QT prolongation

[1]The abbreviations used are: Bis, bisindolylmaleimide; C2, membrane permeable ceramide; C2, N-acetyl-D-erythro-sphingosine; CM-H2DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; Dihydro-C2, dihydro-, N-acetyl-D-erythro-

sphingosine; Gen, genestein; HA, herbimycin A; MnTBAP, Mn (III) tetrakis (4-benzoic acid) porphyrin chloride; $O_2^{\cdot-}$, superoxide anion; OA, okadiac acid; SMase, sphingomyelinase; VitE, vitamin E.

III-2 INTRODUCTION

Ceramide, a long-chain sphingolipid generated intracellularly on hydrolysis of sphingomyelin catalyzed by sphingomyelinase (SMase), has emerged as a key second messenger molecule that is capable of mediating multiple physiological effects including regulation of cellular differentiation, proliferation, and apoptosis by activating a variety of signaling cascades, including those triggered by cytokines, growth factors, and stress (1-3). Ceramide generation can be triggered by diverse stimuli and *de nova* ceramide synthesis and accumulation takes place in the myocardium under various pathological conditions such as myocardial ischemia (MI) or hypoxia, congestive heart failure (CHF), and diabetic cardiomyopathy (DCM) (4-11). In these situations, ceramide is involved in triggering cardiomyocyte apoptosis, cardiac pump failure and cardiac arrhythmias. In particular, prolongation of action potential duration (APD) and QT interval is a common feature with regard to cardiac electrical disturbances in chronic MI (12-13), CHF (14-15) and DCM (16-17).

Upon receiving an incoming impulse, cardiac cells show a rapid membrane depolarization followed by a relatively slow repolarization process. Repolarization disorders, either excessive slowing or accelerating of the rate of repolarization, can cause cardiac electrical perturbations or arrhythmias. The rate of repolarization is determined by several ion currents, of which the rapid delayed rectifier K^+ current (I_{Kr}) has a crucial role, particularly to the plateau phase of action potentials (18-19). The major molecular component of native I_{Kr} has been identified as the human *ether-a-go-go*-related gene (HERG), which generates I_{Kr}-like current when expressed in heterologous systems (20). Impairment of I_{Kr}/HERG can cause excessive prolongation of APD and QT interval, which is commonly believed to be a mechanism for both genetic and drug-induced long QT syndromes. It is important to know whether the sphingolipid metabolite ceramide that is excessively generated in MI, CHF or DCM can interact with HERG K^+ channels, so as to have better understanding of the metabolic and ionic mechanisms for the APD/QT

prolongation in these diseased settings. To this end, we performed studies to investigate the effects of ceramide on HERG K^+ channel function and to elucidate the potential signaling mechanisms. Our data identified HERG as a target for ceramide action and intracellular reactive oxygen species (ROS) as a mediator for ceramide-induced impairment of HERG function.

III-3 EXPERIMENTAL PROCEDURES

III-3-1 Cell Culture

HEK293 cells stably expressing HERG (a kind gift from Drs. Zhou and January) (21) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 200 μM G418, 100 units/ml penicillin, and 100 μg/ml streptomycin, as previously described in detail (22-23).

III-3-2 Whole-Cell Patch-Clamp Recording

Patch clamp recording of HERG K^+ current (I_{HERG}) has been described in detail elsewhere (22-23). The Currents were recorded in the whole-cell voltage-clamp mode with an Axopatch-200B amplifier (Axon Instruments). Borosilicate glass electrodes had tip resistances of 1–3 megohms when filled with the internal solution containing (mM) 130 KCl, 1 $MgCl_2$, 5 Mg-ATP, 10 EGTA, and 10 HEPES (pH 7.2). The extracellular (Tyrode) solution contained (mM) 136 NaCl, 5.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4). Experiments were conducted at 36 ± 1 °C. Junction potentials were zeroed before formation of the membrane-pipette seal. Series resistance and capacitance were compensated, and leak currents were subtracted.

III-3-3 Drugs and Treatment

N-acetyl-D-sphingosine, synthetic membrane permeable ceramide (C2), sphingomyelinase (SMase, the neutral form with long-lasting activity), forskolin (FSK, PKA activator), H89 (PKA inhibitor), Phorbol 12, 13-Dl-Decanoate (PDD, PKC activator), bisindolylmaleimide (Bis; PKC inhibitor), okadiac acid (OA), and vitamin E (VitE) were purchased from Sigma (Oakville, ON). Dihydro-, N-acetyl-D-erythro-

sphingosine (dihydro-C2, an inactive analogue of C2), herbimycin A (HA, PTK inhibitor) and genistein (Gen, PTK inhibitor) were purchased from Calbiochem (La jolla, CA). PDD, Bis, C2, dihydro-C2, HA, Gen, and FSK were dissolved in DMSO. OA was dissolved in phosphate-buffered saline (PBS). SMase was prepared in a working solution of 50% glycerol containing 50 mM Tris-HCl. All agents were prepared as 1000 x stock solutions and stored at –20°C.

For acute exposure, drugs were added to the bathing solution 10 min after baseline recording and the current amplitudes before and after drugs were compared. For prolonged exposure, cells were incubated in normal culture medium containing agents at the desired final concentrations for 10 hrs prior to patch-clamp recordings and group comparison between treated and untreated cells (treated with the same vehicles as for drug treatment groups) was made. Step I_{HERG} was defined as the current amplitude at the end of the 2.5-sec depolarizing voltage steps and tail I_{HERG} was measured as the peak value of the decaying tail currents upon repolarization to –50 mV. To have more rational comparisons between groups, I_{HERG} density was calculated by dividing the current amplitude by the capacitance of the same cell. For experiments involving protein kinase inhibitors, cells were pre-incubated with the drugs for 2 hrs before exposure to C2.

III-3-4 Western Blot

The procedures for measuring HERG protein levels were essentially the same as described previously (24). The primary antibody, polyclonal anti-HERG raised in rabbit against highly purified peptide (CY) EEL PAGAPELPQD GPT, corresponding to residues 1118-1133 of human HERG was purchased from Alomone Labs (Jerusalem, Israel). Bound antibodies were detected using the chemiluminescent substrate (Western Blot Chemiluminescence Reagent Plus, NEN Life Science Products, Boston, USA) and quantified using a Phosphorimager (Bio-Rad). The presence of HERG channel proteins was verified by the presence of a prominent band with expected molecular mass in the range of previous reports and by elimination of the band in preparations preincubated with the antigenic peptide. Coomassie staining was performed to verify equal protein loading for each sample.

III-3-5 Immunocytochemistry

The procedure was the same as previously described in detail (24). The anti HERG antibody was the same as used for Western blot analysis. The cells were examined under a Laser scanning confocal microscope (Zeiss LSM 510).

III-3-6 Intracellular Reactive Oxygen Species (ROS) Measurement

The procedures have been described in detail previously (25). CM-H2DFDA from Molecular Probes was used to detect oxidative activity in living cells. Cells were divided into 5 groups: control group, C2 10-hrs group, C2 20-min group, C2+VitE, and C2+MnTBAP group. For experiments involving VitE or MnTBAP, the cells were pretreated with VitE (100 μM) or MnTBAP (5 μM) for 2 hrs and then incubated in the culture medium containing C2 in the continual presence of VitE or MnTBAP. The percentage of positively stained cells and the fluorescence intensity of staining were determined by densitometric scanning with LSM software (Zeiss).

III-3-7 Data Analysis

Group data are expressed as mean ± s.e.m. Comparisons among groups were made by analysis of variance (*F*-test) and Bonferroni-adjusted *t*-tests were used for multiple group comparisons and paired or unpaired *t*-test was used, as appropriate, for single comparisons. A two-tailed $p < 0.05$ was taken to indicate a statistically significant difference. Nonlinear least square curve fitting was performed with CLAMPFIT in pCLAMP 8.0 or GraphPad Prism.

III-4 RESULTS

III-4-1 Effects of membrane permeable ceramide on I_{HERG} expressed in HEK293 Cells

Depolarizing voltage steps from –60 mV to +40 mV elicited delayed rectifier type of outward currents with characteristic inward rectification that manifests at stronger depolarization. Upon repolarization back to –50 mV, slowly decaying tail currents are recorded. Application of the membrane permeable ceramide (C2) to the superfusate at a

concentration of 25 μM for up to 20 min did not significantly alter the HERG currents (Figure 1A), suggesting that ceramide does not directly act on the HERG channels.

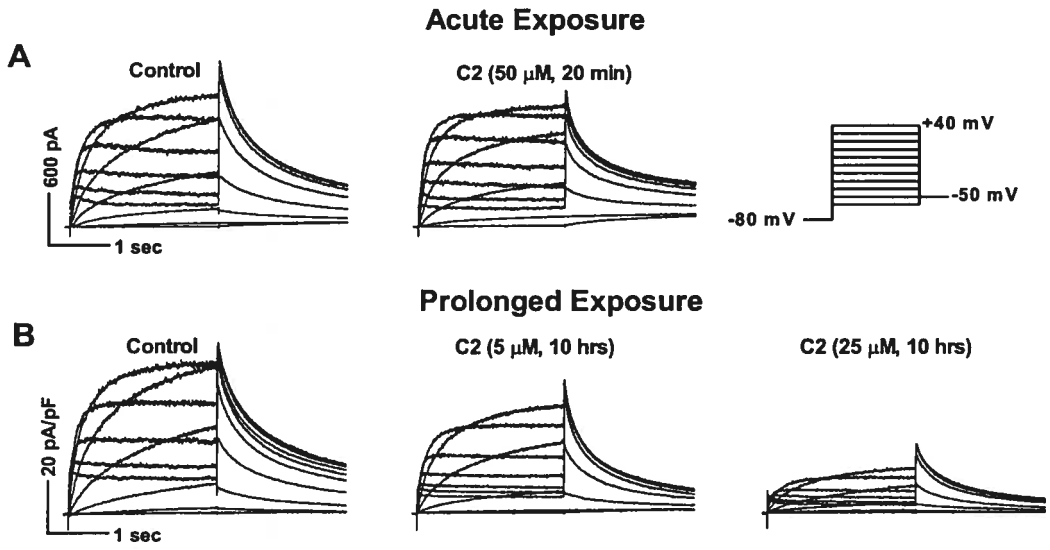

Figure 1. Analog data showing the effects of membrane permeable ceramide (C2) on HERG current (I_{HERG}) expressed in HEK293 cells. For acute exposure (**A**), C2 was applied to the Tyrode solution for patch-clamp recordings and currents recorded up to 20 min superfusion with C2 were taken to measure the drug effect as compared with the before-drug values. For prolonged exposure (**B**), HEK293 cells were preincubated with C2 in the normal culture medium for 10 hrs before patch-clamp studies and the current amplitude was normalized to cell capacitance to minimize the inter-cell variations of cell size for group comparison with untreated cells. I_{HERG} was elicited by 2.5-s depolarizing pulses to voltages ranging from –60 to +40 mV to record the step current, followed by 2.5-s repolarization step to –50 mV to record the tail current, as shown with the voltage protocol shown in the inset.

In contrast to the acute exposure, preincubation of cells with C2 (1-50 μM) in the culture medium for 10 hrs caused consistent changes of I_{HERG} in two different ways. First, I_{HERG} density was decreased by C2 at a concentration-dependent (Figure 1B and Figure 2) and voltage-independent (Figure 2D) manner. The IC_{50} of C2 for I_{HERG} suppression was calculated by the Hill equation to be 19.5 μM. Second, the steady-state voltage-dependent activation of HERG channels was appreciably shifted by C2 towards depolarizing voltages (Figure 2B). For example, the voltage for half maximum activation ($V_{1/2}$) was changed from –27.0 ± 0.5 mV (n=27) to –24.2 ± 1.0 mV (n=13, $p<0.05$ vs. control) and –22.0 ± 1.1 mV (n=17, $p<0.05$) by 1 and 5 μM C2, respectively, with no changes in the slope factor (k). Intriguingly, the IC_{50} for the $V_{1/2}$ shift was around 1.1 μM, some 20-fold lower than the effect on the current density. Higher concentrations of C2 (10, 25 and 50 μM) did not produce further shift of I_{HERG} activation. Dihydro-C2 (25 μM), the inactive analog of C2, failed to cause any appreciable alterations of I_{HERG} (Figure 2A). No effects of C2 on HERG kinetics and inactivation properties were observed (data not shown).

III-4-2 Effects of endogenous ceramide generated by sphingomyelinase on I_{HERG}

To verify the modulation of I_{HERG} by ceramide and the potential pathophysiological implication, we studied the effects of sphingomyelinase (SMase) that catalyzes production of endogenous ceramide on I_{HERG}. Qualitatively the same effects were observed with SMase as with exogenously applied C2. SMase significantly decreased I_{HERG} in a concentration-dependent but voltage-independent manner (Figure 3). SMase also produced positive shifts of I_{HERG} activation voltage; $V_{1/2}$ was changed from -30.6 ± 0.6 mV (n=25) for control to -27.3 ± 0.8 mV (n=20, $p<0.05$) and -24.4 ± 0.6 mV (n=13, $p<0.05$) in the presence of 0.2 U/ml and 0.6 U/ml SMase, respectively. SMase did not affect the activation and deactivation kinetics and inactivation properties of HERG channels (data not shown).

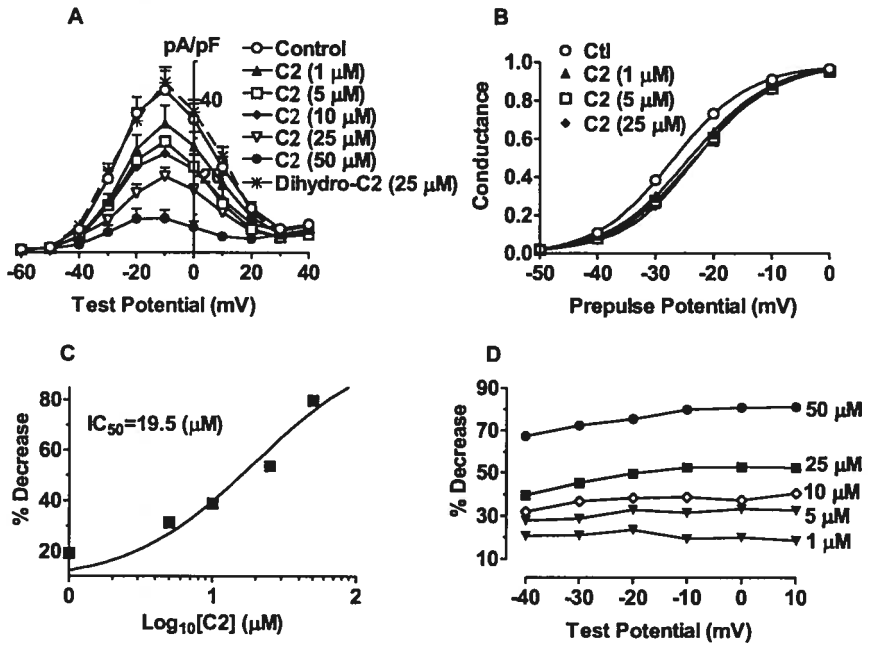

Figure 2. Characterization of I_{HERG} with prolonged exposure to ceramide (C2). (**A**) current density-voltage relationships. Shown are mean values from n=13 cells for control (Ctl), n=12 for 1 μM C2, n=14 for 5 μM, 10 μM, 50 μM C2, and 25 μM for dihydro-C2 (the inactive analogue of C2), respectively. (**B**) Steady-state voltage-dependent activation curves. The activation curves were constructed by plotting the conductance *G* as a function of depolarizing potentials. *G* was calculated by normalizing the tail currents at -50 mV by dividing the amplitude of the tail currents measured at various antecedent depolarizing potentials by that of the tail current at +40 mV. Symbols are mean of experimental data and lines represent the Boltzmann fit: $G/G_{max}=1/\{1+\exp[(V_{1/2}-V)/k]\}$, where G_{max} represents the maximal conductance at +40 mV, $V_{1/2}$ is a half-maximal activation voltage, and *k* is a slope factor. Note that C2 produces positive shift of HERG activation. (**C**) Concentration-dependent block of I_{HERG} by C2. Symbols are experimental data and the curve represents the fit to the Hill equation: $Y=B+(T-B)/(1+10\text{^}((\text{Log}IC_{50}-[C2]_o)*n))$, where *Y* is the percentage of I_{HERG} inhibition, *B* is the minimum inhibition and *T* the maximum inhibition observed, IC_{50} represents the concentration of C2 $[C2]_o$ for half-maximal inhibition of I_{HERG}, and *n* represents the Hill coefficient. (**D**) Percent decrease in I_{HERG} produced by various concentrations of C2. $^*P<0.05$ *vs.* Ctl.

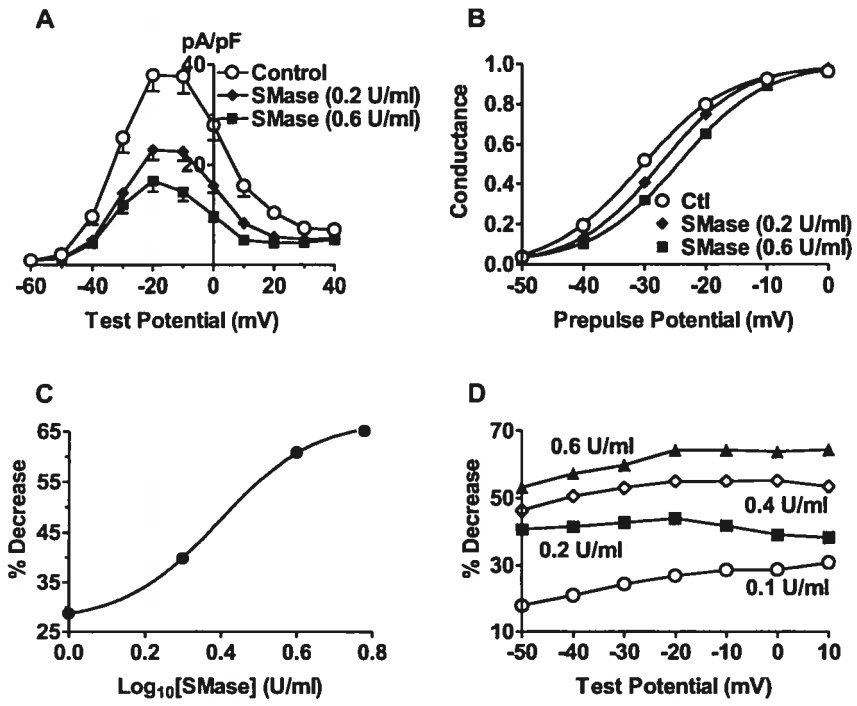

Figure 3. Characterization of I_{HERG} depression caused by sphingomyelinase (SMase). (**A**) Current density-voltage relationships. Shown are mean values from n=19 cells for control (Ctl), n=9 cells for 0.1 U/ml SMase, n=17 cells for 0.2 U/ml SMase, n=8 cells for 0.4 U/ml SMase and n=13 cells for 0.6 U/ml SMase. (**B**) Steady-state voltage-dependent activation curves. Note that similar to C2, SMase also produces positive shifts of HERG activation. (**C**) Concentration-dependent block of I_{HERG} by SMase. Symbols are experimental data and the curve represents the fit to the Hill equation. (**D**) Percent decrease in I_{HERG} produced by various concentrations of SMase. *P<0.05 *vs.* Ctl.

III-4-3 Effects of inhibitors to PTK, PKA or PKC on I_{HERG} modulation by Ceramide

It has been shown that ceramide inhibits T lymphocyte voltage-gated potassium channel via tyrosine phosphorylation of the channel proteins (26). On the contrary, the I_{HERG}-like current has been found to be enhanced by protein tyrosine kinase (PTK)

activation in MLS-9 rat microglia cell line (27). To investigate whether this mechanism also applies to HERG modulation by ceramide, we evaluated the effects of two PTK inhibitors herbimycin A (HA) and genistein (Gen). As illustrated in Figure 4A, preincubation of cells with HA (5 μM) alone for 2 hrs remarkably suppressed I_{HERG} (~66% at 0 mV) to a similar extent as the I_{HERG} inhibition caused by 25 μM C2 (~58% at 0 mV). Co-incubation of cells with HA and C2 caused a slightly further decrease in I_{HERG} (by ~77%). Similarly, Gen alone also reduced I_{HERG} albeit to a less extend compared with C2. Pretreatment with Gen (80 μM) did not alter the effects of C2 on I_{HERG} density (Figure 4B).

Ceramide is characterized by its ability to simulate activation of the atypical protein kinase C (PKCξ) (28). PKCξ can be inhibited by a phospholipase C inhibitor okadiac acid (OA). Our experiments demonstrated that OA (1 μM) alone significantly diminished I_{HERG} and pretreatment of cells with OA failed to prevent I_{HERG} reduction induced by C2; instead, further decreases in I_{HERG} were found in the presence of both OA and C2 (Figure 4C).

PKA phosphorylation of HERG channels was shown to cause reduction of current amplitude due to positive shift of HERG activation voltages (29-30). To investigate the potential involvement of PKA pathway in mediating ceramide-induced I_{HERG} depression, we assessed the effects of a PKA inhibitor H89. As illustrated in Figure 5A, incubation of cells with H89 (1 μM) alone for 10 hrs produced a marked shift of the HERG I-V relationship and activation curve towards more negative potentials. The maximum step I_{HERG} was unaltered despite that because of the positive shift, I_{HERG} was increased at potentials negative to –10 mV and decreased thereafter. The maximum tail I_{HERG}, however, was reduced by approximately 11%. Pretreatment with H89 only slightly reduced the degree of C2-induced I_{HERG} diminishment (Figure 5A).

PKC is another target of ceramide action. To investigate the possible involvement of PKC in mediating I_{HERG} modulation by ceramide, we evaluated the effects of a PKC inhibitor bisindolylmaleimide (Bis, 0.1 μM) on C2-induced I_{HERG} depression. Cells treated with Bis alone for 10 hrs had significantly smaller I_{HERG} density (30.5 ± 2.2 pA/pF, n=22, $p<0.05$) compared with control untreated cells (39.8 ± 2.8 pA/pF, n=22) (Figure 5B). The I_{HERG} activation property was not different between Bis and control groups. Bis

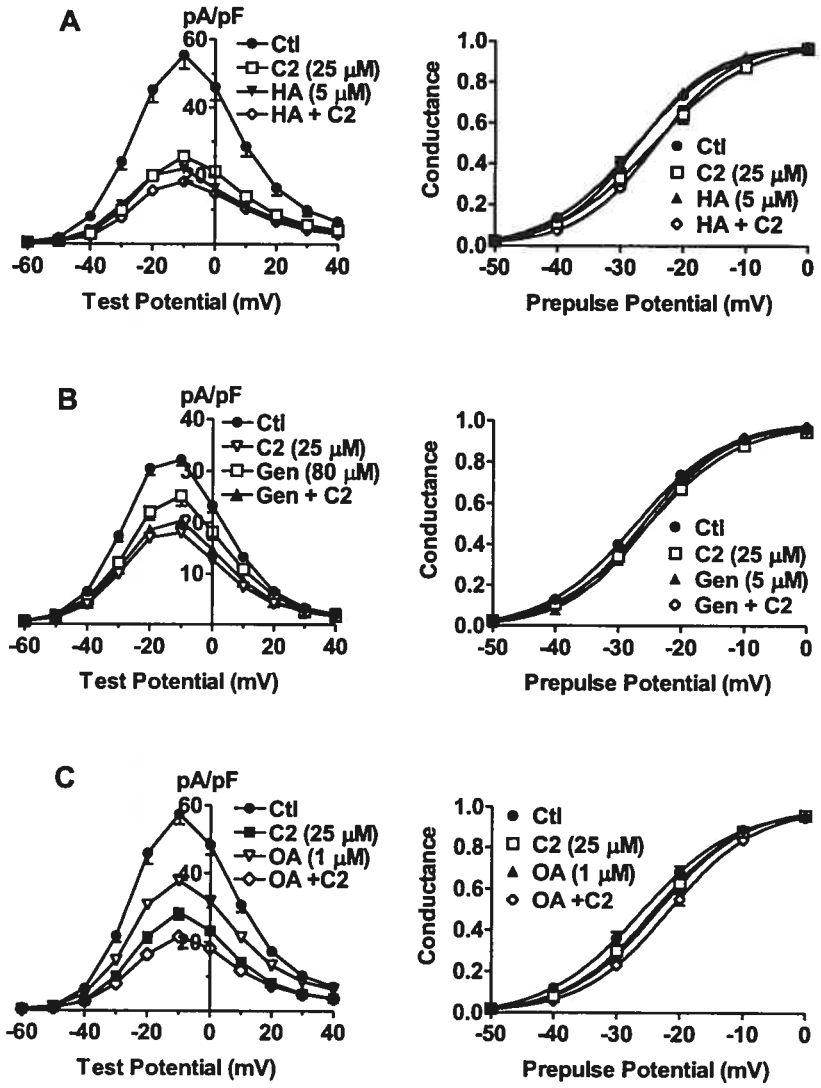

Figure 4. Effects of inhibitors of tyrosine protein kinases (TPKs) and atypical protein kinase C (PKCξ) on I_{HERG} modulation by ceramide (C2). Left panels: current density-voltage relationships; right panels: steady-state voltage-dependent activation curves. PTK inhibitors herbimycin A (HA, 5 μM) and genestein (Gen, 80 μM); PKCξ inhibitor okadiac acid (OA, 1 μM). Comparisons were made among 4 groups: Ctl-control untreated cells (Ctl), C2-cells treated with C2 alone, cells treated with an inhibitor alone, and cells co-incubated with an inhibitor and C2 for 10 hrs after 2-hrs preincubation with the inhibitor alone. *P<0.05 *vs.* Ctl.

pretreatment partially reduced C2-induced I_{HERG} depression (Figure 6A). Similarly, pretreatment with another PKC inhibitor cherylethrine (1 μM) partly restored C2-induced I_{HERG} reduction and cherylethrine alone also significantly decreased I_{HERG} (data not shown).

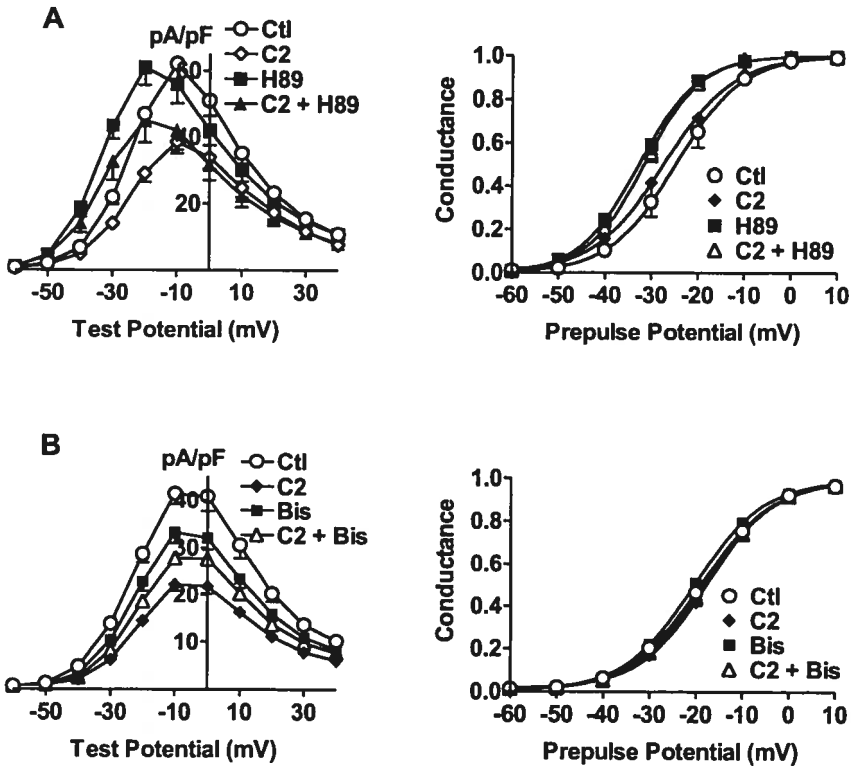

Figure 5. Effects of inhibitors of protein kinase A (PKA) or protein kinase C (PKC) on I_{HERG} modulation by ceramide (C2). (**A**) Effects of PKA inhibitor H89 (1 μM) on C2-induced I_{HERG} depression. Left panels: current density-voltage relationships; right panels: steady-state voltage-dependent activation curves. For experiments involving co-application of H89 and C2, the cells were preincubated with H89 for 2 hrs and then incubated with C2 (5 μM) in the presence of H89 for 10 hrs before patch-clamp recordings. **P*<0.05 *vs.* Ctl. (**B**) Effects of PKC inhibitor bisindolylmaleimide (Bis) on C2-induced I_{HERG} depression. Left panels: current density-voltage relationships; right panels: steady-state voltage-dependent activation curves. For experiments involving co-application of Bis and C2, the cells were preincubated with Bis for 2 hrs and then incubated with C2 (5 μM) in the presence of Bis for 10 hrs before patch-clamp recordings. **P*<0.05 *vs.* Ctl.

III-4-4 Lack of effects of ceramide on HERG protein expression level

There is a possibility that the decrease of I_{HERG} in presence of ceramide was due to down-regulation of HERG channel protein expression. To test this notion, we carried out Western blot analysis with membrane protein samples extracted from HERG-expressing HEK293 cells to compare the HERG protein levels between control cells and C2-treated cells. As shown in Figure 6A, anti-HERG antibody recognized a band of ~135 kDa, consistent with the molecular mass of HERG protein (21, 24), and the band disappeared if the antibody had been pretreated with its antigenic peptide. HERG protein levels were comparable between the C2-treated cells and control untreated cells.

This result was further reinforced by our immunocytochemical analysis demonstrating similar pattern and intensity of HERG staining on the surface membrane between C2-treated cells and control untreated cells (Figure 6B).

III-4-5 Role of reactive oxygen species (ROS) in I_{HERG} modulation by ceramide

It has been well established by numerous studies that ceramide can act on mitochondria and stimulate production of ROS, particularly superoxide anion ($O_2^{\cdot-}$) (2, 31-32). We have previously demonstrated that $O_2^{\cdot-}$ causes impairment of HERG K^+ channel function (25). It is quite plausible that C2-induced depression of I_{HERG} is related to increased ROS generation. We tested this notion by preincubating the cells with vitamin E (VitE, 100 μM) alone for 2 hrs and then incubating the cells with C2 (25 μM) in the continual presence of VitE for another 10 hrs. As shown in Figure 7A, VitE substantially weakened the depressing effects of C2, but VitE alone did not produce any appreciable effects, on I_{HERG}. For example, I_{HERG} density was ~50% smaller in control than in the cells pretreated with C2 alone, but it was only ~24% smaller in the cells pretreated with both VitE and C2. In other words, I_{HERG} density in the VitE+C2 group was approximately 52% greater than in the group with C2 alone (48.2 ± 3.2 pA/pF, n=19, *vs.* 31.7 ± 2.0 pA/pF, n=16, $p<0.05$), at 0 mV (Figure 7A). Furthermore, Mn (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), a superoxide dismutase (SOD) mimic, resembled the effects of VitE; it reversed C2-induced I_{HERG} reduction (Figure 7B).

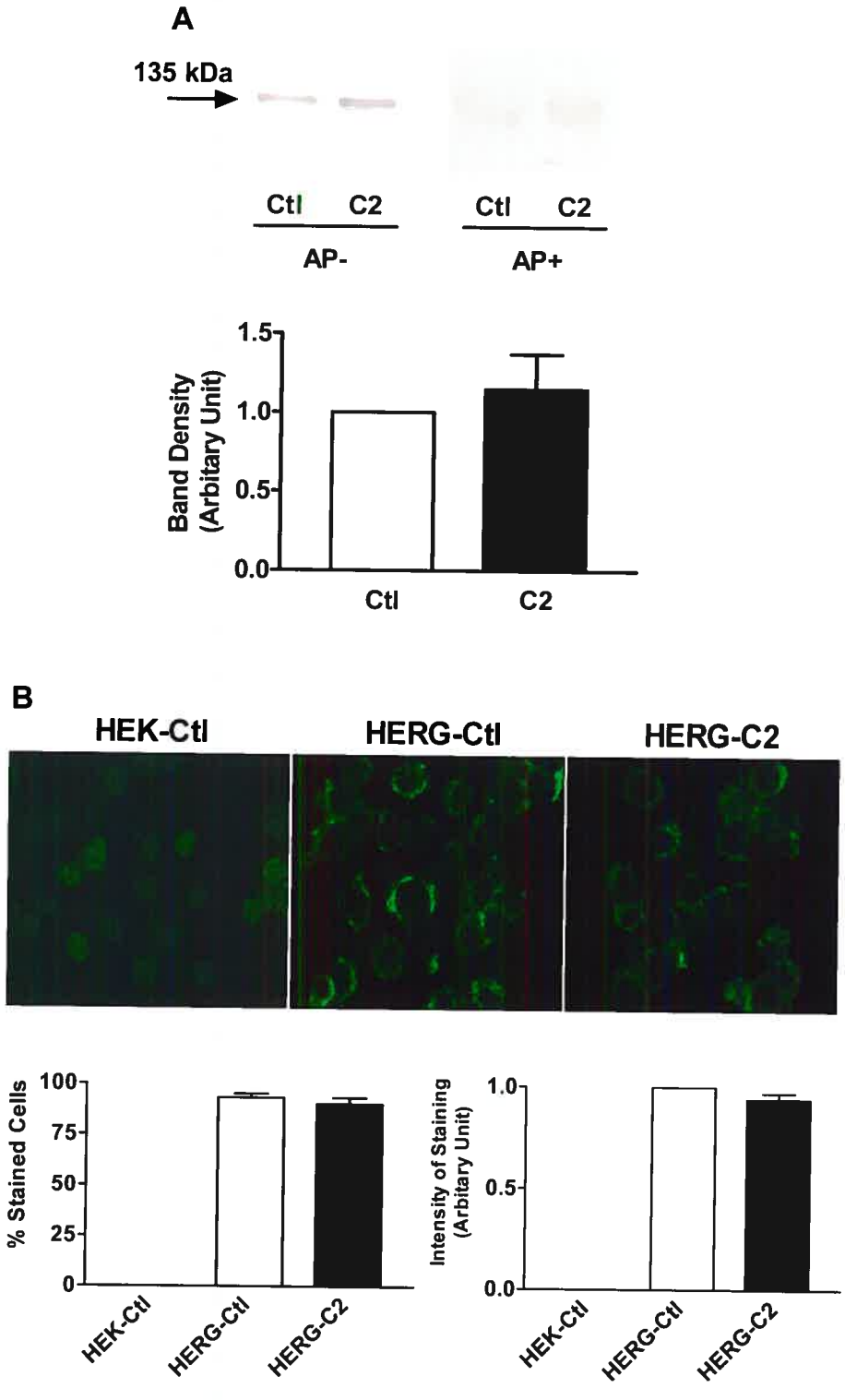
A
135 kDa
Ctl
C2
Ctl
C2
AP-
AP+
Band Density (Arbitary Unit)
1.5
1.0
0.5
0.0
Ctl
C2
B
HEK-Ctl
HERG-Ctl
HERG-C2
% Stained Cells
100
75
50
25
0
HEK-Ctl
HERG-Ctl
HERG-C2
Intensity of Staining (Arbitary Unit)
1.0
0.5
0.0
HEK-Ctl
HERG-Ctl
HERG-C2

Figure 6. Expression level of HERG protein determined by immunoblotting with membrane protein preparations extracted from HERG-expressing HEK293 cells. (**A**) Upper panel: examples of immunoblotting bands of ~135 kDa, corresponding to the molecular mass of HERG protein, from C2 treated (C2) and untreated cells (Ctl). Cells were incubated with C2 (25 μM) for 10 hrs in the culture medium prior to the procedures for membrane protein extraction. Lower panel: mean band density from 3 independent protein samples. AP-, without antigenic peptide pretreatment for anti-HERG antibody; AP+ with antigenic peptide pretreatment for anti-HERG antibody. (**B**) Upper panel: examples of immunocytochemical staining of HERG proteins on the cytoplasmic membrane. Lower panel: averaged data from four independent experiments, showing % cells with positive staining with anti-HERG antibody and intensity of staining.

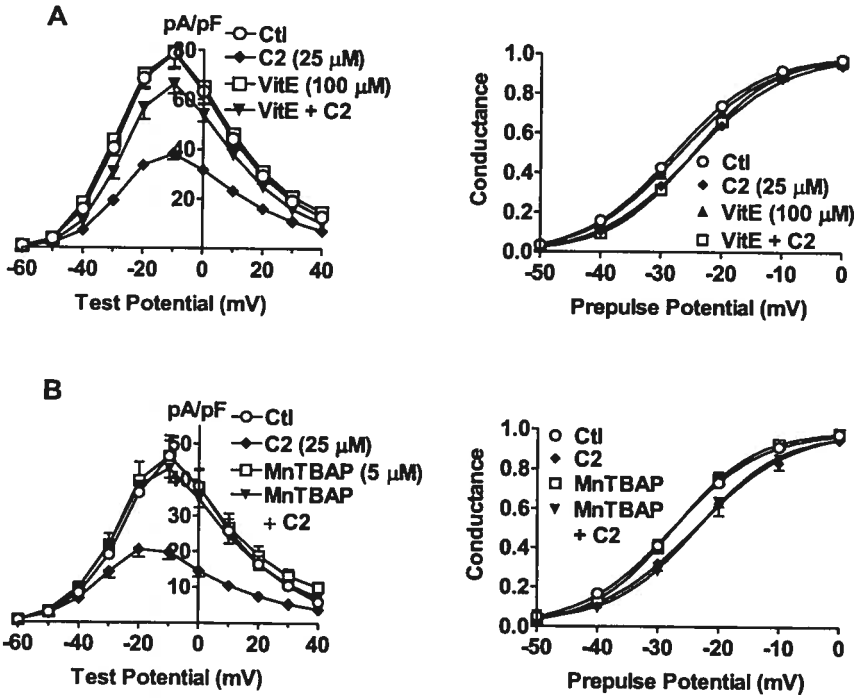

Figure 7. Role of reactive oxygen species (ROS) on I_{HERG} modulation by ceramide (C2). (**A**) Effects of vitamin E (VitE) on C2-induced I_{HERG} depression. (**B**) Effects of MnTBAP, a superoxide dismutase mimic, on C2-induced I_{HERG} depression. Left panels: current density-voltage relationships; right panels: steady-state voltage-dependent activation curves. The cells were preincubated with VitE (100 μM) or MnTBAP (5 μM) for 2 hrs and then incubated with C2 (25 μM, n=16 cells) in the presence of VitE (n=16 cells) or MnTBAP (n=16 cells) for 10 hrs before patch-clamp recordings. **P*<0.05 *vs.* Ctl (n=14 cells).

To confirm that ROS production was indeed increased by C2 and the C2-induced ROS was mainly of $O_2^{\cdot-}$, we proceeded to measure the intracellular ROS levels using CM-H2DCFDA fluorescence dye. The ROS level was measured in cells incubated with the culture medium containing 25 μM C2 for 10 hrs. The cells showing fluorescence intensity ≥5 times the background were defined as positive staining, and the number of cells with positive staining was pooled from 5 fields for each batch of cells (a total of 4 independent batches of cells for each group were studied). The intensity of staining by the fluorescent probe for ROS was analyzed by densitometric scanning using the LSM program, and cells with positive staining were taken for analysis, and the data were normalized to the control values. Under control conditions, cells with positive staining were sparse. Yet in the cells treated with C2, the number of the cells with positive staining, as well as the intensity of staining, was consistently higher. If pretreated with VitE or MnTBAP prior to exposure to C2, the cells had significantly lower ROS levels, as indicated by the smaller number of positively stained cells and the weaker intensity of staining (Figure 8A and 8B). Since 20 min superfusion of cells with C2 failed to affect I_{HERG}, we also conducted CM-H2DCFDA experiments with cells treated with C2 for only 20 min. In sharp contrast with 10-hrs exposure, 20 min treatment did not alter the level of intracellular ROS.

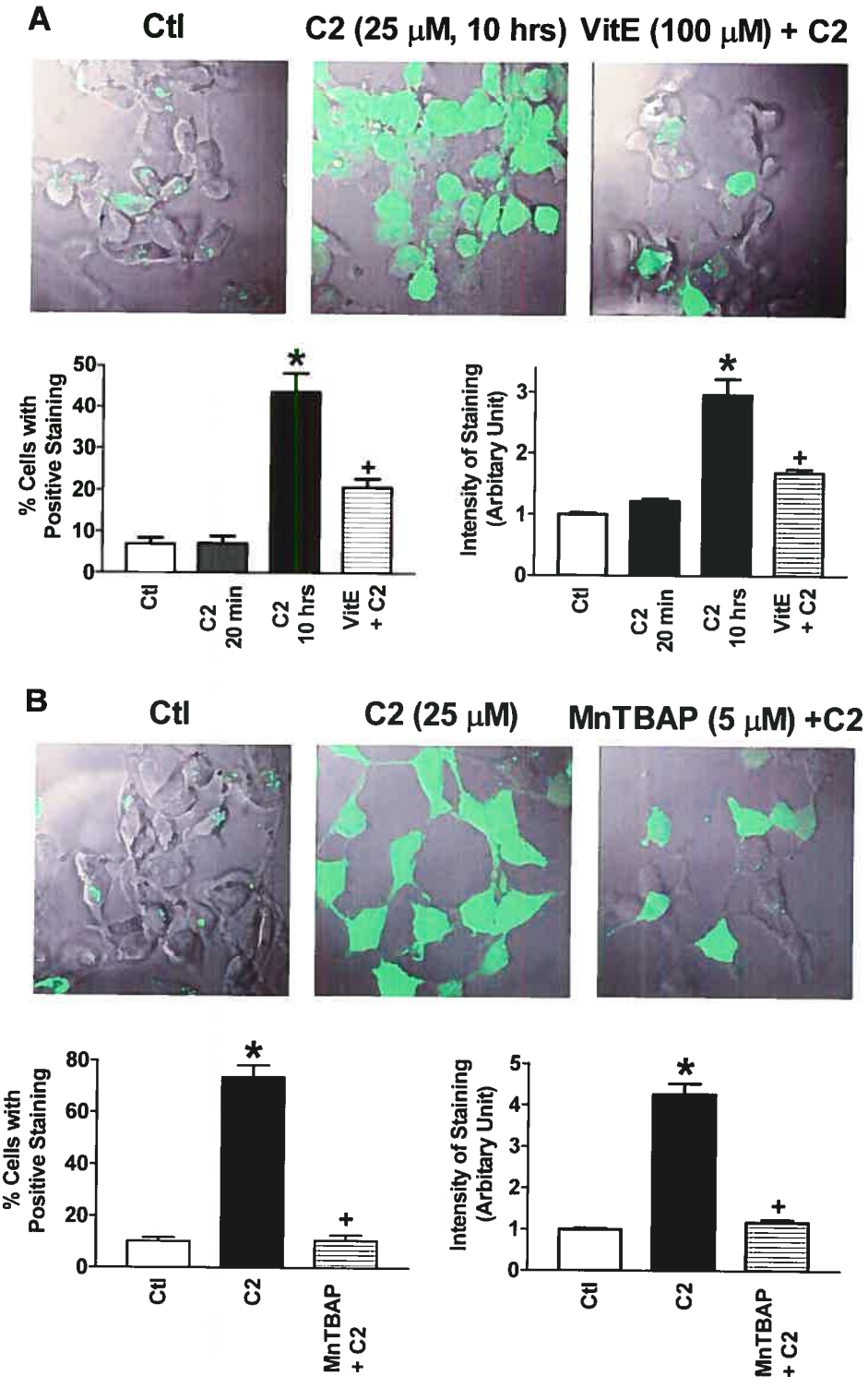
A
Ctl
C2 (25 μM, 10 hrs)
VitE (100 μM) + C2
% Cells with Positive Staining
Intensity of Staining (Arbitary Unit)
Ctl
C2 20 min
C2 10 hrs
VitE + C2
B
Ctl
C2 (25 μM)
MnTBAP (5 μM) +C2
% Cells with Positive Staining
Intensity of Staining (Arbitary Unit)
Ctl
C2
MnTBAP + C2

Figure 8. Effects of vitamin E (VitE) or MnTBAP (an SOD mimic) on intracellular levels of ROS measured by CM-H2DCFDA fluorescence dye. (**A**) and (**B**) Upper panels: laser scanning confocal microscopic images of CM-H2DCFDA staining reflecting the intracellular ROS levels. Lower panels: percentage of positively stained cells (mean ± S.E.), obtained from 5 fields of 4 experiments by counting the cells with staining intensity ≥ 5 times the background and averaged intensity of CMH-2DCFDA fluorescence measured from the positively stained cells. Data were obtained from control untreated cells (Ctl), cells treated with C2 (25 μM for 20 min or 10 hrs), and cells pretreated with VitE (100 μM) or MnTBAP (5 μM). **P*<0.05 *vs.* Ctl; +*P*<0.05 vs. C2.

III-5 DISCUSSION

The present study identifies HERG K^+ channel as a new target for ceramide action. The major findings of the study are: (1) prolonged exposure (chronic incubation), but not brief exposure (acute superfusion), of cells to C2 significantly impairs HERG K^+ channel function and (2) stimulation of intracellular reactive oxygen species (ROS) by C2 mediates the depressing effects of C2 on HERG K^+ channel function. In view of the critical role of HERG K^+ channel in regulating cardiac repolarization and the fact that ceramide is overproduced in MI, CHF or DCM, it is possible that HERG impairment by ceramide contributes to the electrical disturbances in these pathological conditions.

It has been reported that the HERG-equivalent K^+ current in rat microglia MLS-9 cell (27) is significantly reduced by inhibitors of PTK, indicating the role of PTK in maintaining HERG function. Consistent with these findings, our data demonstrated that PTK inhibitors herbimycin A and genestein alone both markedly depressed I_{HERG} amplitude, indicating a role of basal PTK activity in maintaining HERG function. Co-application of these inhibitors with ceramide did not alter ceramide-induced I_{HERG} depression, or if anything, caused further reduction of I_{HERG}. The latter suggests that ceramide has the ability to enhance HERG function and partially offsetting its inhibitory effect by stimulating PTK activities, but failed to do so in the presence of PTK inhibitors. Thus, PTKs do not account for ceramide-induced I_{HERG} depression. Involvement of PKA or PKC in ceramide-induced I_{HERG} depression appears to be minimal, if any. This conclusion was drawn on the basis that neither PKA inhibitor H89 nor PKC inhibitor Bis

significantly reversed the effects of C2, despite that these compounds themselves when applied in the absence of C2 produced direct effects on I_{HERG}. Obviously, these direct effects of H89 and Bis are unlikely related to ceramide action on I_{HERG}, but the positive shift of HERG activation may be caused by PKA because H89 completely abolished ceramide-induced shift.

A growing body of evidence is emerging indicating that oxidative stress and ceramide generation are intimately connected in cell death signaling. The major source for ROS in most cell types is probably the electron leakage from the mitochondrial electron transport chain, which results in the formation of superoxide anion ($O_2^{\cdot -}$). Experimental evidence indicates that cell-permeable ceramide analogs elicit a direct effect on mitochondria, ranging from inhibition of the respiratory chain and ROS overproduction to induction of mitochondrial permeability transition in intact cells and cytoplasts (33-34). Several studies have identified the mitochondrial ubiquinone pool of complex III (a protein complex that links proton translocation to electron transfer from ubiquinone to cytochrome c) as a ceramide target to produce ROS. Indeed, it has been reported that MnTBAP, an SOD mimic, inhibits ceramide-induced apoptosis in neuronal cells (35). The present study confirmed that C2 stimulates intracellular production of ROS as indicated by the increased ROS level and the increase was attenuated by VitE. Furthermore, our data suggest that C2-induced generation of ROS was mainly of $O_2^{\cdot -}$ because MnTBAP nearly abolished the ROS increases. These data are consistent with the electrophysiological results that VitE or MnTBAP reversed the C2-induced reduction of I_{HERG} amplitude. The fact that 20 min superfusion of the cells with C2 failed to affect I_{HERG} in our experiments (Figure 1A) may well be due to failure of C2 to increase ROS within such a short time frame and this notion is indeed supported by the data shown in Figure 8A. In other words, the reason why prolonged exposure (>2.5 hrs) was required for C2 to produce effects on I_{HERG} is that sufficient level of ROS needs to be raised with time in the presence of C2 to affect I_{HERG}. The ability of ceramide to induce ROS production in cultured neonatal rat ventricular myocytes has indeed been recently confirmed by Suematsu *et al* (36). Noticeably, neither VitE nor MnTBAP altered C2-induced positive shift of HERG activation, indicating that ROS is not responsible for ceramide-induced shift.

The present study has several important pathophysiological implications. First, it is known that ischemic myocardium demonstrates characteristic bi-phasic changes of electrophysiology with APD shortening during early phase of acute ischemia and subsequent APD lengthening after prolonged ischemic period, which are associated with different types of arrhythmias (12-13). However, the ionic mechanisms underlying these sequential alterations of APD remained unresolved. This study together with our previous work seems to provide an explanation. It is well recognized that altered lipid metabolism is an important deleterious factor in ischemic myocardial injury; some lipid metabolites such as lysophosphatidylcholines (LPCs) are overproduced rapidly within the first 10 min of acute myocardial ischemia, while others like ceramide accumulate progressively in the late phase (30 min after) of ischemia (1-3, 7). We have recently reported that LPCs produce pronounced enhancement of HERG function (22-23). This finding may explain in part the APD shortening and the associated arrhythmias occurring in the early phase of ischemia. On the other hand, i*n vitro* ischemia leads to a progressive accumulation of ceramide in cardiomyocytes. The content of ceramide in ischemic area was found to be elevated to 155% baseline levels after 30 min, and to 330% after 210 min, of ischemia. Ischemia (30 min) followed by reperfusion (180 min) increased the ceramide level to 250% in the ischemic area (4). In another study, the total basal ceramide concentration in the myocardium was 135 nmol/g tissue, and it was increased by 14.1% and 48.4% in 30 min global ischemia and 30 min ischemia/30 min reperfusion groups, respectively (5). Cordis *et al* (6) showed a 50% decrease of sphingomylein both during ischemia and subsequent reperfusion with a corresponding increase in ceramide. Another study suggests that a longer time period (>30 min) may be needed to alter ceramide mass in tissues in response to ischemic insult (7). Intriguingly, the cardiac AP is abnormally lengthened after prolonged ischemia (12-13). In lieu of the critical role of HERG K^+ channel in cardiac repolarization and the ability of ceramide to inhibit I_{HERG}, it is tempting to speculate that I_{HERG} depression produced by ceramide that accumulates during the late phase of myocardial ischemia might contribute to the observed prolongation of APD and the associated arrhythmias.

The second implication of our study is related to arrhythmias in CHF. One characteristic electrophysiological alteration in CHF is abnormally prolonged APD at the

cellular level and QT interval as reflected in electrocardiogram (15, 37). This prolongation provides an electrophysiological substrate for early afterdepolarizations (EADs) to occur, which can result in ventricular tachycardias that often predispose to ventricular fibrillation leading to sudden cardiac death (14). Polymorphic ventricular tachycardias, likely related to arrhythmogenic EADs, are common in CHF (14). The ionic mechanisms underlying APD prolongation in CHF have not been precisely defined. Intriguingly, *de novo* ceramide synthesis has been found to be increased in heart failure (8). Increased ceramide level in cardiomyopathy can cause impairment of HERG function and result in excessive APD prolongation. More importantly, our data demonstrate that ROS mediates the inhibitory effect of ceramide on I_{HERG} and antioxidants can prevent the I_{HERG} depression. This explains at least partly the benefits of antioxidants in preventing and suppressing arrhythmias in ischemic myocardium and failing hearts (38-39). Indeed, Tsuji *et al.* (40) showed I_{Kr}, measured as E-4031-sensitive tail current, to be ~36% smaller in rabbits with ventricular tachypacing-induced CHF than in healthy rabbits. Lodge and Normandin (41) demonstrated earlier that I_{Kr}, measured as dofetilide-sensitive tail current, reduced by ~45% in the BIO TO-2 strain of cardiomyopathic hamster of 10 months old, derived from the BIO 53.58 animals and providing a model of dilated low output heart failure, compared with the 10-month-old control (BIO F1B) hamsters. Moreover, simulations of cellular electrophysiology predict I_{Kr} inhibition to cause EADs in failing, but not nonfailing, myocytes (42).

Finally, the results of this study also have implications in diabetic cardiomyopathy (DCM). DCM is characterized by electrical remodeling with aberrant electrophysiology, metabolic remodeling with malignant biochemical processes and anatomical remodeling with progressive loss of cardiomyocytes, which result in impaired cardiac contractile and increased risk of lethal arrhythmias. QT prolongation and QT dispersion have been suggested as the predictor of mortality in both type I and type II (16-17, 43). It has been documented that *de nova* synthesis and accumulation of ceramide in diabetic myocardium contributes to the development of DCM (8, 10-11). In addition, oxidative stress is also known to be a critical deleterious factor for DCM (44-45). Moreover, we have recently found that I_{Kr}/HERG function is impaired in the rabbit model of DCM (46). Taken

together, it seems plausible that ceramide-induced HERG depression may be one of the multiple determinants for diabetic QT prolongation.

III-6 ACKNOWLEDGEMENTS

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CHAPTER IV

Impairment of HERG K^+ Channel Function by Tumor Necrosis Factor-α:

Role of Reactive Oxygen Species as a Mediator

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IV Impairment of HERG K^+ Channel Function by Tumor Necrosis Factor-α:

Role of Reactive Oxygen Species as a Mediator*

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[1] The abbreviations used are: TNF-α, tumor necrosis factor-α; CHF, congestive heart failure; APD, action potential duration; EAD, early afterdepolarization; ROS, reactive oxygen species; CM-H2DFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; VitE, vitamin E; MnTBAP, Mn(III) tetrakis(4-benzoic acid) porphyrin chloride; X/XO, xanthine/xanthine oxidase.

IV-1 ABSTRACT

Congestive heart failure (CHF) is associated with susceptibility to lethal arrhythmias and typically increases levels of tumor necrosis factor-α (TNF-α) and its receptor, TNFR1. CHF down-regulates rapid delayed-rectifier K^+ current (I_{Kr}) and delays cardiac repolarization. We studied the effects of TNF-α on cloned HERG K^+ channel (human *ether-a-go-go*-related gene) in HEK293 cells and native I_{Kr} in canine cardiomyocytes with whole-cell patch clamp techniques. TNF-α consistently and reversibly decreased HERG current (I_{HERG}). Effects of TNF-α were concentration-dependent, increased with longer incubation period, and occurred at clinically relevant concentrations. TNF-α had similar inhibitory effects on I_{Kr} and markedly prolonged action potential duration (APD) in canine cardiomyocytes. Immunoblotting analysis demonstrated that HERG protein level was slightly higher in canine hearts with tachypacing-induced CHF than in healthy hearts, and TNF-α slightly increased HERG protein level in CHF but not in healthy hearts. In cells pretreated with the inhibitory anti-TNFR1 antibody, TNF-α lost its ability to suppress I_{HERG}, indicating a requirement of TNFR1 activation for HERG suppression. Vitamin E or MnTBAP (Mn (III) tetrakis (4-benzoic acid) porphyrin chloride), a superoxide dismutase mimic) prevented, whereas the superoxide anion generating system xanthine/xanthine oxidase mimicked, TNF-α-induced I_{HERG} depression. TNF-α caused robust increases in intracellular reactive oxygen species, and vitamin E and MnTBAP abolished the increases, in both HEK293 cells and canine ventricular myocytes. We conclude that the TNF-α/TNFR1 system impairs HERG/I_{Kr} function mainly by stimulating reactive oxygen species, particularly superoxide anion, but not by altering HERG expression; the effect may contribute to APD prolongation by TNF-α and may be a novel mechanism for electrophysiological abnormalities and sudden death in CHF.

IV-2 INTRODUCTION

TNF-α is a potent inducible cytokine with pleiotropic biological effects (1). Up-regulation of TNF-α is a consistent finding in clinical (2) and experimental CHF (3).

Circulating concentrations of TNF-α and soluble TNFα receptors are independent predictors of mortality in CHF (4).

Patients with CHF are at increased risk of sudden death due to cardiac arrhythmias. CHF increases action potential duration (APD) (5), leading to early afterdepolarizations (EADs) and lethal ventricular tachyarrhythmias (6). Polymorphic ventricular tachycardias, likely related to arrhythmogenic afterdepolarizations, are common in CHF (6, 7). The molecular mechanisms underlying APD prolongation in CHF remain incompletely understood.

The rapid delayed rectifier K^+ current (I_{Kr}) is crucial in cardiac repolarization. The human *ether-a-go-go*-related gene (HERG) encodes the pore-forming α-subunit of I_{Kr} and congenital or drug-induced abnormalities in HERG protein function are a common cause of the long QT syndrome. Simulations of cellular electrophysiology predict I_{Kr} inhibition to cause EADs in failing, but not nonfailing, myocytes (8). A recent study demonstrated that transgenic mice overexpressing TNF-α with heart failure had significantly prolonged APD (9). It is unknown whether TNF-α affects cardiac K^+ channels. We therefore examined the hypothesis that TNF-α might affect HERG/I_{Kr}, thereby potentially contributing to CHF-related repolarization abnormalities.

IV-3 EXPERIMENTAL PROCEDURES

IV-3-1 Cell Disposition

HEK293 cells stably expressing HERG were a kind gift from Drs. Zhou and January. Cell culture and handling procedures have been described previously (10). Cardiomyocytes were isolated from healthy adult mongrel dogs as described in detail previously (11, 12). The procedures for animal use were in accordance with institutional guidelines.

IV-3-2 Whole-cell Patch Clamp Recording

Patch clamp techniques have been described in detail elsewhere (13–16). Experiments were conducted at 36 ± 1°C. For current recordings in canine cardiomyocyte

studies, the followings were included in the bath solution to block contaminating currents: $CdCl_2$ (200-μmol/liter, L-type Ca^{2+} current), 4-aminopyridine (1 mmol/liter, transient outward K^+ currents), glyburide (10 μmol/liter, ATP-sensitive K^+ current), and 293B (10 μmol/liter, slow delayed-rectifier K^+ current). Action potentials were recorded in the current clamp mode with Tyrode solution free of ion channel blockers. TNF-α was either added to the extracellular solution 10 min after formation of whole-cell configuration (acute studies), or cells were incubated with TNF-α in the medium for 10 h before patch clamp recording (long term exposure).

IV-3-3 Western Blot

The procedures were similar to those described previously (15). Polyclonal anti-HERG raised in rabbit against highly purified peptide (CY) EEL PAGAPELPQD GPT, corresponding to residues 1118–1133 of human HERG was purchased from Alomone Laboratories (Jerusalem, Israel).

IV-3-4 Intracellular Reactive Oxygen Species (ROS) Measurement

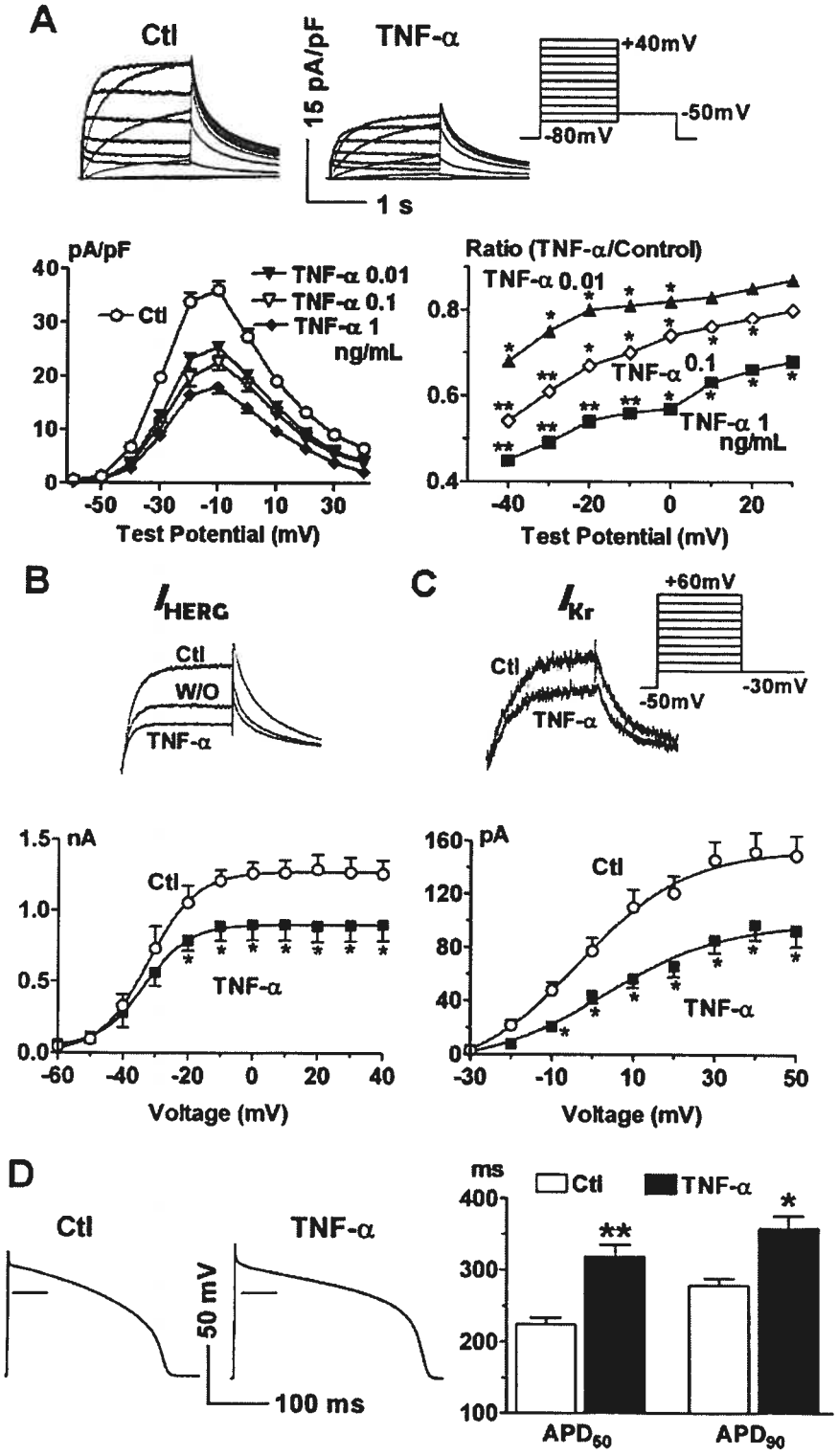
5-(and-6)-Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DFDA) from Molecular Probes was used to detect oxidative activity in living cells as described details previously (16).

IV-3-5 Data Analysis

Group data are mean ± S.E. Paired *t* tests were used for single comparisons. Kinetics was analyzed with CLAMPFIT (pCLAMP 8.0) or Graphpad Prism.

IV-4 RESULTS

I_{HERG} was elicited by 2-s depolarization followed by 2-s repolarizing steps (Figure 1, *inset*). Currents were recorded immediately after formation of whole-cell configuration and series resistance compensation. Comparisons were made between control cells (without TNF-α) and cells incubated for 10 h with various TNF-α concentrations from 0.01 to 10 ng/ml, which are within the pathophysiological range of TNF-α levels (~0.1 ng/ml) (17–19).


A
Ctl
TNF-α
15 pA/pF
+40mV
-50mV
-80mV
1 s
pA/pF
TNF-α 0.01
TNF-α 0.1
TNF-α 1
ng/mL
Ctl
Test Potential (mV)
Ratio (TNF-α/Control)
TNF-α 0.01
TNF-α 0.1
TNF-α 1
ng/mL
Test Potential (mV)
B
I HERG
Ctl
W/O
TNF-α
C
I Kr
+60mV
-50mV
-30mV
Ctl
TNF-α
1.5 nA
Ctl
TNF-α
Voltage (mV)
160 pA
Ctl
TNF-α
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D
Ctl
TNF-α
50 mV
100 ms
ms
Ctl
TNF-α
APD60
APD90

Figure 1. Impairment of HERG function by TNF-α. A, effects of 10-h exposure to various concentrations of TNF-α (in ng/ml) on I_{HERG} stably expressed in HEK293 cells. Original recordings are shown in the *upper panels*, mean current-voltage relations in the *lower left panel* and mean ratios of I_{HERG} in TNF-α-treated to untreated cells in the *lower right panel.* n = 15 for control; n = 13, 17, and 15 for TNF-α 0.01, 0.1, and 1 ng/ml, respectively. For 0.01 ng/ml TNF-α, $p < 0.05$ for voltages from –40 to 0 mV; for 0.1 ng/ml TNF-α, $p < 0.01$ at –40 and –30 mV and $p < 0.05$ from –20 to +20 mV; for 1 ng/ml TNF-α, $p < 0.01$ at –40 and –10 mV and $p < 0.05$ from 0 to +40 mV. **B,** effects of acute exposure to TNF-α (100 ng/ml) on I_{HERG} in HEK293 cells. Shown are mean data (n = 5) from the tail I_{HERG} recorded at –50 mV with original recordings (test pulse: 0 mV) in the *inset.* **C,** effects of bath application of TNF-α (100 ng/ml) on I_{Kr} in dog atrial myocytes, elicited with voltage protocol shown in the *inset.* Shown are mean (n = 4) I_{Kr} tail currents elicited at a repolarizing voltage of –30 mV with original recordings (test pulse: 0 mV) in the *inset.* **D,** effects of TNF-α (10 ng/ml, 6-h incubation at 4 °C) on action potential duration in canine ventricular cells (n = 15 for each group). The *horizontal lines* within the action potential recordings indicate the zero voltage level. *, $p < 0.05$ and **, $p < 0.01$ *versus* control (*Ctl*).

I_{HERG} density was reduced by TNF-α, with effects that were concentration-dependent and voltage-dependent, being larger at more negative potentials (Figure 1*A*). I_{HERG} kinetics was unaltered by TNF-α. Exposure to TNF-α for 15 min concentration-dependently decreased I_{HERG}. I_{HERG} amplitude was decreased by 9, 16, and 35% by TNF-α at 0,01, 0.1, and 1.0 ng/ml, respectively. Results at 100 ng/ml are shown in Figure 1*B*. Depression of I_{Kr} by TNF-α was reproduced in both dog atrial and ventricular myocytes (Figure 1*C*). APD_{50} and APD_{90}, duration at 50 and 90% repolarization, respectively, were both significantly longer in single ventricular cells preincubated with TNF-α at 10 ng/ml in Tyrode solution for 10 h relative to control cells (Figure 1*D*).

Western blot analysis of HERG protein levels in the membrane preparations extracted from HERG-expressing HEK293 cells and from the ventricular myocytes of healthy dogs or dogs with tachypacing induced CHF was performed. A band of around 135 kDa was identified by anti-HERG antibody, and the band was abolished after the

antibody had been neutralized by its antigenic peptide. TNF-α treatment neither significantly alter HERG protein level in HEK293 cells nor in healthy dogs. HERG protein level was slightly higher in CHF than in healthy dogs and was slightly increased by TNF-α in CHF dogs (Figure 2*A*).

To clarify whether TNF-α acts on I_{HERG} via activation of TNF receptor I (TNFR1), we incubated HEK293 cells with H389 (an inhibitory anti-TNFR1 antibody) for 1 h before patch clamp recording upon acute exposure to 100 ng/ml TNF-α or beginning 1 h before prolonged (10 h) exposure to 1 ng/ml TNF-α. H389 prevented suppression of I_{HERG} by subsequent acute or prolonged application of TNF-α. Data from prolonged exposure experiments are shown in Figure 2*B*.

Activation of TNFR1 can stimulate overproduction of intracellular ROS (20). To investigate whether ROS mediates TNF-α-induced HERG depression, we assessed the effects of TNF-α on I_{HERG} in cells pretreated with the antioxidant vitamin E (VitE). Pretreatment with VitE for 2 h prevented I_{HERG} reduction by TNF-α (Figure 2*C*). Another antioxidant Mn (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), a superoxide dismutase mimetic, produced similar preventive effects on TNF-α-induced HERG impairment (Figure 2*D*). By contrast, preincubation of cells with superoxide anion generating system xanthine/xanthine oxidase (X/XO) mimicked the inhibitory effect of TNF-α on I_{HERG} (Figure 2*E*).

To confirm that intracellular ROS production was indeed stimulated by TNF-α, we detected ROS level using CM-H2DFDA fluorescence dye to stain the cells. The cells stained with fluorescence intensity ≥5 times the background were defined as positive staining, and the number of cells with positive staining was pooled from five fields. The intensity of staining was analyzed by densitometric scanning using the LSM program, and the data were normalized to the control values without TNF-α (0.01 and 1 ng/ml) treatment (16). Under control conditions, cells stained by CM-H2DCFDA were sparse, and the staining was weak. Yet with TNF-α treatment, the number of the cells with positive staining was considerably higher and the cells were stained evenly throughout the cytoplasm. Pretreatment with VitE or MnTBAP drastically diminished the number and the intensity of staining (Figure 2*F*). Similar results were obtained with isolated canine

ventricular myocytes; TNF-α (0.1 ng/ml) markedly increased ROS level and co-application with VitE (100 μM) or MnTBAP (5 μM) prevented the effects of TNF-α (Figure 2*G*).

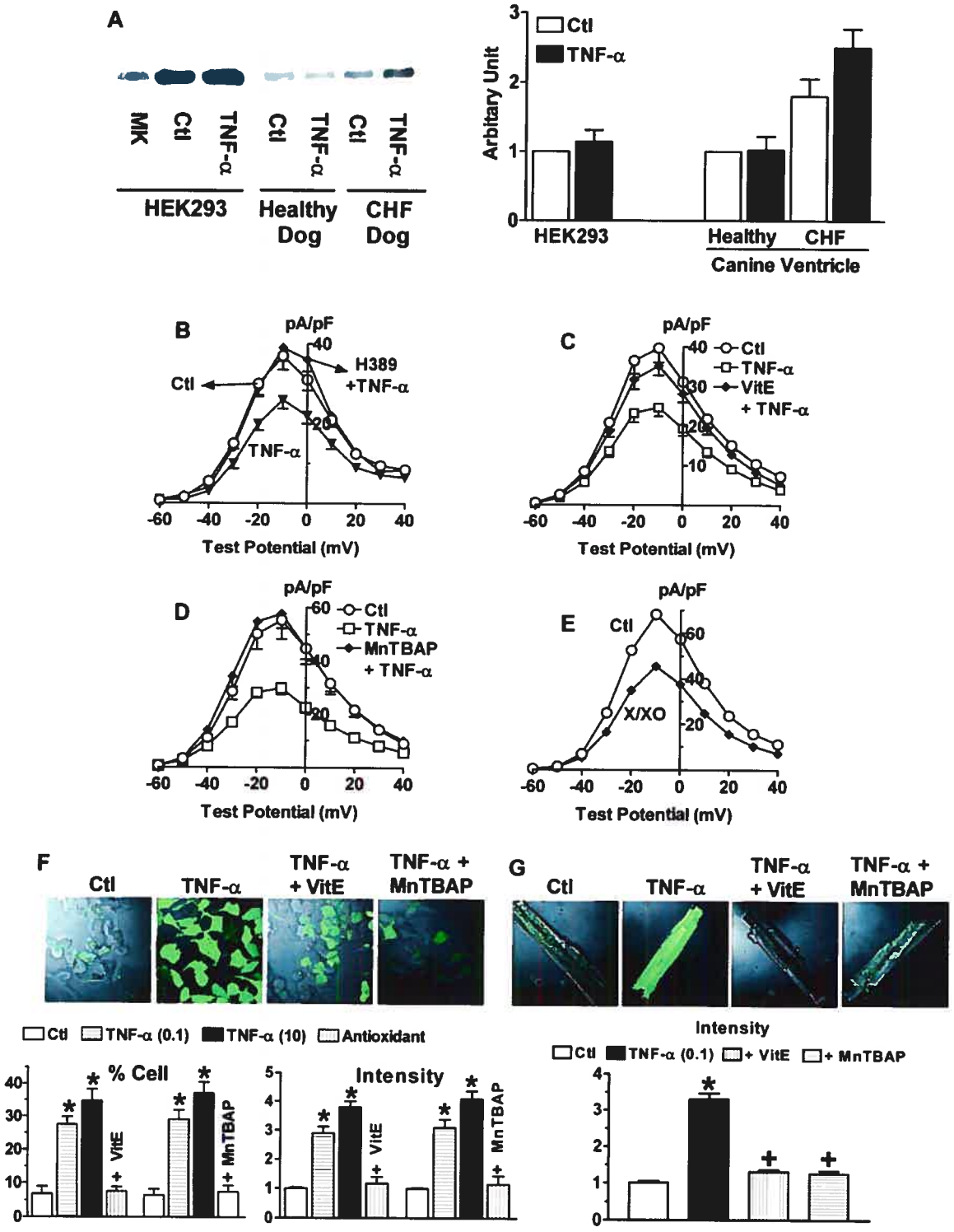

Figure 2. Mechanisms for HERG depression by TNF-α. A, Western blot analysis of HERG protein levels. Membrane protein samples were extracted from left ventricular myocytes isolated from healthy dogs and dogs with ventricular tachypacing-induced CHF and from HEK293 cells. HEK293 cells were treated with TNF-α (10 ng/ml) for 10 h in culture medium, and isolated myocytes were treated with TNF-α in Tyrode solution for 10 h. Mean data were calculated from a total of four independent samples for each group. *MK*, protein marker. *, $p < 0.05$ *versus* control (*Ctl*). **B,** inhibitory anti-TNFR1 antibody H389 prevents HERG depression by TNF-α. Cells were incubated with H389 (10 μg/ml) for 1 h before prolonged (10 h) exposure to TNF-α (10 ng/ml, n = 7). **C** and **D,** antioxidants VitE (100 μM, n = 14) or superoxide dismutase mimic MnTBAP (5 μM, n = 12) prevents HERG depression by TNF-α. *, $p < 0.05$ *versus* TNF-α alone. **E,** superoxide anion generating system X/XO (400 μM/5 units/ml, n = 16) mimicked the effects of TNF-α on I_{HERG}. **F,** alterations of the intracellular level of ROS by TNF-α (0.1 or 10 ng/ml) and antioxidants VitE (100 μM) and MnTBAP (5 μM), respectively, determined by CM-H2DFDA fluorescence dye staining (*green*) in HERG-expressing HEK293 cells. The *upper panels* show examples of confocal microscopic images, and the *lower panels* show the percentage of cells with positive staining (*% Cell*) and the intensity of positive staining (n = 4 batches of cells for each group). *, $p < 0.05$ *versus* Ctl; +, $p < 0.05$ *versus* TNF-α alone. **G,** increase in ROS by TNF-α (0.1 ng/ml) and reversal by VitE (100 μM) or MnTBAP (5 μM) in canine ventricular myocytes. The *upper panels* show examples of confocal microscopic images, and the *lower panels* show the intensity of positive staining (n = 3). *, $p < 0.05$ *versus* Ctl; +, $p < 0.05$ *versus* TNF-α alone.

IV-5 DISCUSSION

Heart failure is associated with APD and QT interval prolongation, believed to contribute to the occurrence of sudden cardiac death (6, 7). We show here that TNF-α suppresses I_{HERG} in HEK293 cells and I_{Kr} in dog cardiomyocytes and prolonged APD. Depression of I_{HERG}/I_{Kr}, as produced by TNF-α in this study, may contribute to delayed repolarization and associated malignant ventricular tachyarrhythmias with increased TNF-α level in patients with CHF.

Ionic remodeling in CHF has been studied (21). L-type Ca^{2+} current density appears to be unaltered (20). The inward-rectifier K^+ current is consistently reduced (5). The transient outward K^+ current (I_{to}) is also reduced, potentially causing APD prolongation (5, 22). However, inhibition of I_{to} reduces APD in human atrial cells (23), canine atrial cells (12), and dog Purkinje fibers (24). The effect of I_{to} on the AP depends largely on the magnitude of I_K (25). Tsuji *et al.* (26) showed I_{Kr}, measured as E-4031-sensitive tail current, to be ~36% smaller in rabbits with ventricular tachypacing-induced CHF than in healthy rabbits. Lodge and Normandin (27) demonstrated earlier that I_{Kr}, measured as dofetilide-sensitive tail current, reduced by ~45% in the BIO TO-2 strain of cardiomyopathic hamster of 10 months old, derived from the BIO 53.58 animals and providing a model of dilated low output heart failure, compared with the 10-month-old control (BIO F1B) hamsters. A recent study by London *et al.* (9) showed significant APD prolongation in transgenic mice that overexpressed TNF-α and developed heart failure. Our study suggests that TNF-α may be an important mediator of CHF-induced I_{Kr} reduction and is the first to demonstrate that TNF-α can modulate cardiac K^+ channels.

We further demonstrated that pretreatment with VitE or MnTBAP prevented, whereas X/XO mimicked, TNF-α-induced I_{HERG} depression. The effects of VitE and MnTBAP are likely due to their antioxidant actions because TNF-α increased the intracellular ROS level in a concentration-dependent manner in both HEK293 cells and canine ventricular myocytes, more specifically O_2^- level because VitE or MnTBAP effectively prevented the increase. In line with our finding, a recent study published during the course of this study clearly demonstrated the ability of TNF-α to stimulate mitochondrial production of ROS in cardiomyocytes (20). It has also been shown that ROS is one of the key deleterious factors in failing heart (28, 29). Our data therefore indicate that TNF-α-induced HERG depression occurs at the functional level, but not at the expression levels (TNF-α did not alter HERG protein content), and the functional impairment of HERG channels by TNF-α is mediated by ROS, particularly O_2^-.

Circulating TNF-α levels predict mortality in CHF, and therapies directed against TNF-α may limit the pathophysiologic consequences (1). In healthy human subjects, the TNF-α level is below 0.01 ng/ml, but in patients with heart failure, it can increase to over

0.1 ng/ml (17–19). TNF-α significantly inhibited I_{HERG} over this concentration range (*e.g.* by ~35% at plateau voltages from –10 to +10 mV in cells exposed to 0.1 ng/ml TNF-α for 10 h). Our study might have underestimated the effects of TNF-α on APD because the myocytes were incubated with TNF-α at 4 °C to maintain good quality of the cells. Our observations provide new insights into the potential molecular mechanisms underlying electrophysiological abnormalities and sudden arrhythmic death in patients with CHF.

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CHAPET V

OVERALL DISCUSSION & CONCLUSIONS

The aims of this chapter are to summarize the major findings in this study, to depict the significance and implications of the findings in the research areas, and to indicate the potential limitations involving in the whole process of study. Finally the conclusions will be drawn up for the overall research project.

V-1 Novel Findings, Significances and Implications

V-1-1 LPCs Enhance I_{Kr}/HERG: An Additional Mechanism Underlying $[K^+]_o$-↑ and APD-↓ During the Early Phase of Myocardial Ischemia

Ischemic heart disease remains the leading cause of mortality in the Western World, and is also a significant cause of death worldwide (Association, 2005). A partial reason is that our current insufficient understanding of the contributory factors for the myocardial ischemic injuries, especially myocardial ischemic arrhythmias, which account for the mainstream of the death in ischemic heart disease (Brunckhorst *et al.*, 2004; Nordlie *et al.*, 2005).

Enormous animal and human studies have revealed that $[K^+]_o$-↑ in myocardium and APD-↓ resulting from abnormally enhanced K^+ efflux are the critical causes of the ischemic arrhythmias during the early stage of acute myocardial ischemia. Cardiac K^+ channels, obviously, are major factors responsible for determining the $[K^+]_o$, thereby resting potential, cell excitability, membrane repolarization and ultimately the likelihood of arrhythmias. Among the assortment of cardiac K^+ channels, K_{ATP} as a most promising candidate for ischemic $[K^+]_o$-↑ has been proposed and extensively studied for the decades (Noma, 1983; Seino *et al.*, 2004; Seino *et al.*, 2003). However, the existing data regarding the role of K_{ATP} in ischemic $[K^+]_o$-↑ have been conflicting and the accumulating evidences have questioned the function of K_{ATP} channel in response to myocardial ischemic insult (Shivkumar *et al.*, 1997; Vanheel *et al.*, 1992; Venkatesh *et al.*, 1992; Wilde *et al.*, 1995). It is thus plausible that other mechanisms or more specifically other K^+ currents, in addition to K_{ATP}, may also contribute to $[K^+]_o$-↑ and APD-↓ in ischemic myocardium.

Correspondingly, metabolic perturbation of ischemic myocardium is known to have great impact on electrical disturbances. Among the various metabolic insults, the

rapid accumulation of phospholipid metabolite LPCs in the early stage of ischemic myocardium is of pivotal importance. However, while LPC has long been recognized as a biochemical trigger of ischemic arrhythmias, presumably due to its ability to cause $[K^+]_o$-↑ and APD-↓ (Goldhaber *et al.*, 1998), and how LPC causes $[K^+]_o$-↑ and APD-↓ remains an anonymity.

In our study we show that LPC-16 remarkably, reversibly and voltage-dependently increases the amplitude of I_{Kr}/HERG and shortens APD. The effects of LPC-16 on I_{HERG} are manifested with shorter APD and at more negative potential. I_{Kr}/HERG blocker dofetilide abolishes the I_{Kr}/HERG enhancing and APD shortening effects of LPC-16. Moreover, using the Langendorff heart method, we found that the most evident effects of LPC-16 are to produce prominent $[K^+]_o$-↑ and QT-↓, which are very parallel to those observed under the conditions of low-flow ischemia. Strikingly, the $[K^+]_o$-↑ and QT-↓ induced by either LPC-16 or ischemia are prevented by dofetilide, but not by the blockers chromanol 293B and HMR (I_{Ks} blockers), as well as glibenclamide (I_{KATP} blocker). Consistently, dofetilide efficiently abolishes the ventricular tachy-arrhythmias induced by LPC-16 or ischemia.

The findings described above are, to our knowledge, the first to decipher the role of I_{Kr}/HERG in ischemic $[K^+]_o$-↑ and QTc-↓ and to couple the ionic remodeling for ischemic $[K^+]_o$-↑ and QTc-↓ with a positive feedback mechanism for abnormal enhancement of I_{Kr}/HERG function. Hence, in addition to the paired ATP depletion/K_{ATP} activation, LPC accumulation/HERG enhancement may be considered as another association between metabolic trigger and ionic pathway for ischemic $[K^+]_o$-↑ and QTc-↓. This notion provides an alternative explanation, or one of multiple mechanisms, for ischemic $[K^+]_o$-↑ and QTc-↓. It resolves, in a certain extent, the puzzle and advances our understanding of the ionic and metabolic mechanisms of ischemic electrical disturbances and of how the interactions between the electrophysiological substrates and the biochemical substrates determine the ischemic arrhythmogenesis. In particular, it unravels some novel aspects of I_{Kr}/HERG K^+ channel function under metabolic stresses and provides a new guideline for better therapy of ischemic arrhythmias associated with management of I_{Kr}/HERG K^+ channel and LPC-16 production. Our findings that LPC-16 acts as a metabolic trigger, and HERG as an ionic pathway, for ischemic $[K^+]_o$-↑ and

QTc-↓ and that ischemic arrhythmias is preventable and convertible by I_{Kr}/HERG blocker dofetilide suggest that inhibition of LPC-16 production and accumulation and/or of I_{Kr}/HERG may be a promising therapeutic strategy to attenuate the incidence of lethal arrhythmias associated with ischemic heart disease.

In addition, we demonstrated in this study that LPCs enhance the I_{HERG} expressed in HEK293 cells. The current-enhancing effect was in large measure due to the acceleration of activation kinetics, as well as the substantial depolarizing shift in HERG channel inactivation, such that more current would be available at any given voltage within the activation range. Furthermore, we found that only the LPCs with 16 hydrocarbons such as LPC-16 and LPG-16 produced significant enhancement of I_{HERG} and negative shifts of HERG channel activation.

Given the critical roles of HERG K^+ channel in the etiology of inherited and acquired LQTs, the pharmacology of the HERG cardiac K^+ channel has been the subject of intense study for a decade (Abriel *et al.*, 2004; Kang *et al.*, 2005; Sanguinetti, 1999; Sanguinetti *et al.*, 1995; Tseng, 2001). It is now clear that a wide variety of drugs from numerous structural and pharmacological categories act as antagonists of the HERG K^+ channel (Pearlstein *et al.*, 2003; Redfern *et al.*, 2003). The channel has also been reported to be modulated by a number of intracellular molecules and pathways, including cAMP and PKA, as well as adrenergic pathway (Cui *et al.*, 2000; Kiehn *et al.*, 1998; Thomas *et al.*, 2004; Thomas *et al.*, 1999; Wei *et al.*, 2002), glucose and Akt (Zhang *et al.*, 2003a; Zhang *et al.*, 2003b), PKC (Thomas *et al.*, 2003), phospholipase C (Bian *et al.*, 2001; Bian *et al.*, 2004; Gomez-Varela *et al.*, 2003), and PTK (Cayabyab *et al.*, 2002). Increase of I_{HERG} can be induced by the substrate of phospholipase C, PIP_2 (Bian *et al.*, 2001; Bian *et al.*, 2004), activation of PTK (Cayabyab *et al.*, 2002), and probably via modulations of other unknown pathways. However, no natural metabolic substance can increase I_{HERG} has been reported before our investigation. Our studies revealed that HERG K^+ channel is not only a molecular target for long QT syndrome but also a target for "short QT syndrome". Thus, our studies also provided the first hint that activation of cardiac I_{Kr}/HERG could be beneficial in LQTS. It is therefore possible that LPCs, acting as HERG channel activators by modifying the molecular structure, could someday find

utilization in the treatment of repolarization disorders in the heart, including acquired long QT syndrome, congenital long QT syndrome, and heart failure.

V-1-2 Impairment of I_{Kr}/HERG by Ceramide or TNF-α in the Prolonged Ischemia and in Congestive Heart Failure Shares the Common Mechanism: Overproduction of ROS

The results from our study show that a sphingolipid metabolite ceramide or a proinflammatory cytokine TNF-α, which are both produced and accumulated drastically in the prolonged myocardial ischemia and congestive heart failure, impairs HERG K^+ channel function. Ceramide causes pronounced I_{HERG} inhibition in a concentration-dependent and voltage-independent fashion and positive shift of voltage-dependent HERG activation in the setting of prolonged incubation. We also demonstrated that TNF-α consistently and reversibly decreases I_{HERG} expressing in HEK293 cells. The Effects of TNF-α are concentration-dependent, increased with longer incubation period, and occurred at clinically relevant concentrations. Furthermore, TNF-α has similar inhibitory effects on I_{Kr} and markedly prolongs APD in canine cardiomyocytes. Intriguingly, either ceramide or TNF-α causes robust increases in intracellular ROS, and antioxidant VitE or MnTBAP abolishes the increases, in both HEK293 cells and canine ventricular myocytes, indicating the functional impairment of HERG channels and I_{Kr} by ceramide or TNF-α is mediated by overproduction of ROS. To our knowledge this is the first study to describe the effects of a sphingolipid metabolite or TNF-α on the HERG K^+ channel or I_{Kr} and to elucidate the underlying mechanisms.

Previously, it has been reported that ceramide regulates a number of potassium channels (Chik *et al.*, 2001; Ramstrom *et al.*, 2004; Wu *et al.*, 2001). Specifically, a stout inhibition within one minute of ceramide application (~70% with 10 μM C_2) was shown and a +10 mV shift of the activation curve in rat pituitary GH_3 cells (Wu *et al.*, 2001). Using HEK293 cells stably expressing HERG we found that acute application (bath superfusion for 25 min) of membrane permeable ceramide (C2, 50 μM) did not alter I_{HERG}. However, prolonged incubation with C2 for 10 hrs caused pronounced I_{HERG} inhibition. These differences probably emanate from the cellular systems used.

The inhibitory mechanism of ceramide on the K^+ channels was unresolved. It has been reported that the HERG-equivalent K^+ current in rat microglia MLS-9 cell is significantly reduced by inhibitors of PTK (Cayabyab *et al.*, 2002). Consistent with these findings, our data demonstrated that PTK inhibitors herbimycin A and genestein alone both markedly depressed I_{HERG} amplitude, indicating a role of basal PTK activity in maintaining HERG function. However, co-application of these inhibitors with ceramide did not alter ceramide-induced I_{HERG} depression. This suggests that ceramide has the ability to enhance HERG channel function and partially offsetting its inhibitory effect by stimulating PTK activities, but failed to do so in the presence of PTK inhibitors. Thus, PTKs do not account for ceramide-induced I_{HERG} depression. Involvement of PKA or PKC in ceramide-induced I_{HERG} depression appears to be minimal. This conclusion was drawn on the basis that neither PKA inhibitor H89 nor PKC inhibitor Bis appreciably reversed the effects of C2, despite that these compounds themselves when applied in the absence of C2 produced direct effects on I_{HERG}. Obviously, these direct effects of H89 and Bis are unlikely related to ceramide action on I_{HERG}. Interestingly, the inhibitory effect of ceramide on I_{HERG} is reversed by antioxidant VitE or MnTBAP. Consistent with this was the ceramide-induced inhibition of the ERG current in GH_3 cells was abolished by the reducing agent dithiothreitol (Wu *et al.*, 2001). Thus, ceramide depresses I_{HERG} mainly via ROS overproduction based on our study.

TNF-α, as a proinflammatory cytokine, is of important in the pathophysiology of myocardial ischemia/reperfusion injury, myocardial infarction, chronic heart failure and remodeling, myocarditis, cardiomyopathy (Aker *et al.*, 2003; McTiernan *et al.*, 2000; Meldrum, 1998; Sack, 2002; Schulz *et al.*, 2004). It has been well documented that myocardial TNF-α production is reinforced during acute myocardial ischemia with or without reperfusion (Irwin et al., 1999; Meldrum et al., 1998). It has also been demonstrated that the absence of TNF-α signaling results in enhanced myocardial apoptosis in response to a prolonged ischemic injury (Kurrelmeyer et al., 2000). TNF-α-mediated negative inotropic effect is believed to depend on a sphingosine-signaling pathway (Oral et al., 1997). TNF-α leads to degradation of cellular sphingomyelin with the formation of ceramide and its base sphingosine (Lecour *et al.*, 2003). Both have been demonstrated to be able to deteriorate myocardial force generation (Levade et al., 2001).

TNF-α also seems to play a role in deteriorating mechanical function through nitric oxide (NO) signaling (Finkel et al., 1992). Chronic myocardial ischemia and heart failure are associated with APD and QT interval prolongation, believed to contribute to the occurrence of sudden cardiac death (Marban, 2002; Nuss et al., 1999). However, it is unclear if TNF- α function on any cardiac potassium channels to give rise to APD and QT interval prolongation, if any, what is the mechanism underlying the function. We show here that TNF-α suppresses I_{HERG} in HEK293 cells and I_{Kr} in dog cardiomyocytes and prolonged APD. TNF-α consistently and reversibly decreases I_{HERG}. The Effects of TNF-α are concentration-dependent, increased with longer incubation period, and occurred at clinically relevant concentrations. Furthermore, TNF-α has similar inhibitory effects on I_{Kr} and markedly prolongs APD in canine cardiomyocytes. Antioxidant VitE or MnTBAP prevents, whereas the X/XO mimics, TNF-α-induced I_{HERG} depression. TNF-α causes robust increases in intracellular ROS, and VitE and MnTBAP abolishes the increases, in both HEK293 cells and canine ventricular myocytes, indicating the functional impairment of HERG channels and I_{Kr} by TNF-α is mediated by ROS.

We demonstrated that ceramide and TNF-α act on I_{HERG} in a similar manner with a similar mechanism: depression of amplitude via overproduction of ROS (Wang *et al.*, 2004a). Ceramide is known to be a downstream component in the TNF-α/TNF receptor-signaling pathway. Activation of TNF receptors stimulates ceramide generation and ceramide in turn mediates the cellular function of TNF-α (Perry & Hannun, 1998; Suematsu *et al.*, 2003). The data in this project in conjunction with other previous studies seem to provide the link: TNF-α enhances ceramide synthesis and ceramide damages mitochondria leading to ROS overproduction, thereby impairs the function of I_{Kr}/I_{HERG}.

However, it should be noted that there have been controversial reports as to the ROS modulation of I_{HERG} (Berube *et al.*, 2001; Taglialatela *et al.*, 1999). It was shown that ROS generation enhanced I_{HERG} in the manner of voltage dependence of activation and inactivation (Taglialatela *et al.*, 1997), whereas ROS was demonstrated to accelerate I_{HERG} deactivation (Taglialatela *et al.*, 1997). The acceleration of the HERG channel deactivation rate by ceramide may result from the action of ROS, or other effectors such as PKC (Thomas *et al.*, 2003). The determination of the components in the signaling

cascade initiated by ceramide and the resulting loss of surface HERG channels, as well as the alteration of deactivation, requires further investigation.

The cardiac APD is abnormally lengthened after prolonged ischemia (Boyden & Jeck, 1995; Carmeliet, 1999). In lieu of the critical role of HERG K^+ channel in cardiac repolarization and the ability of ceramide to inhibit I_{HERG}, it is conceivable to speculate that the depression produced by TNF-α/ceramide that accumulate during the late phase of myocardial ischemia might contribute to the observed prolongation of APD and the associated arrhythmias. Moreover, one characteristic electrophysiological alteration in CHF is abnormally prolonged APD at the cellular level and QT interval as reflected in electrocardiogram (Beuckelmann *et al.*, 1993; Nuss *et al.*, 1999). This prolongation provides an electrophysiological substrate for early afterdepolarizations (EADs) to occur, which can result in ventricular tachycardias that often dispose to VF leading to SCD. Polymorphic ventricular tachycardias, likely related to arrhythmogenic EADs, are common in CHF (Marban, 1999; Marban, 2002). More importantly, our data demonstrate that ROS mediates the inhibitory effect of TNF–α/ceramide on I_{HERG} and antioxidants can prevent the I_{HERG} depression. This explains at least partly the benefits of antioxidants in preventing and suppressing arrhythmias in ischemic myocardium and failing hearts (Walker *et al.*, 1998). Overall, the data obtained from this study provides new insights into the potential molecular mechanisms underlying electrophysiological abnormalities and sudden arrhythmic death in patients with CHF.

In synopsis, the present study has several important pathophysiological implications. First, it is known that ischemic myocardium demonstrates characteristic bi-phasic changes of electrophysiology with APD-↓ during early phase of acute myocardial ischemia and subsequent APD lengthening after prolonged ischemic period, which are associated with different types of arrhythmias (Boyden *et al.*, 1995; Carmeliet, 1999). However, the ionic mechanisms underlying these sequential alterations of APD remain unresolved. This study seems to provide an explanation. It is well recognized that altered lipid metabolism is an important deleterious factor in ischemic myocardial injury; some lipid metabolites such as LPCs are overproduced rapidly within the first 10 min of acute myocardial ischemia, while others like ceramide accumulate progressively in the late phase (30 min after) of ischemia (Kolesnick, 2002; Murase *et al.*, 2000). We have verified

that LPCs produce pronounced enhancement of HERG function. This may explain in part the APD-↓ and the associated arrhythmias occurring in the early phase of ischemia. It is therefore promising that LPCs, acting as HERG channel activators, could someday find use in the treatment of repolarization disorders in the heart, including acquired long QT syndrome, congenital long QT syndrome, and heart failure.

On the other hand, the prolonged ischemia leads to a progressive accumulation of ceramide in cardiomyocytes. The content of ceramide in ischemic area was found to be elevated drastically (Beresewicz *et al.*, 2002; Bielawska *et al.*, 1997); Murase *et al.*, 2000). Intriguingly, the cardiac AP is abnormally lengthened after prolonged ischemia (Boyden *et al.*, 1995; Carmeliet, 1999). In lieu of the decisive role of HERG K^+ channel in cardiac repolarization and the ability of ceramide to inhibit I_{HERG}, it is tempting to speculate that I_{HERG} depression produced by ceramide that accumulates during the late phase of myocardial ischemia might contribute to the APD prolongation and the associated arrhythmias.

The second implication of our study is related to arrhythmias in CHF. One characteristic electrophysiological alteration in CHF is abnormally prolonged APD at the cellular level and QT interval as reflected in electrocardiogram (Nuss *et al.*; 1999; (Beuckelmann *et al.*, 1993). This prolongation provides an electrophysiological substrate for EADs to occur, which can result in ventricular tachycardias that often predispose to ventricular fibrillation leading to SCD. Polymorphic ventricular tachycardias, likely related to arrhythmogenic EADs, are common in CHF (Marban, 2002). The ionic mechanisms underlying APD prolongation in CHF have not been precisely defined. Intriguingly, *de novo* ceramide synthesis has been found to be increased in CHF (Listenberger *et al.*, 2002). Increased ceramide level in cardiomyopathy can cause impairment of HERG function and result in excessive APD prolongation. More importantly, our data demonstrate that ROS mediates the inhibitory effect of ceramide on I_{HERG} and antioxidants can prevent the I_{HERG} depression. This explains at least partly the benefits of antioxidants in preventing and suppressing arrhythmias in ischemic myocardium and failing hearts (Walker *et al.*, 1998).

Furthermore, the results of this study also have implications in diabetic cardiomyopathy (DCM). DCM is characterized by electrical remodeling with aberrant

electrophysiology; metabolic remodeling with malignant biochemical processes; and anatomical remodeling with progressive loss of cardiomyocytes. Those remodeling interplay and result in impaired cardiac contractile and increased risk of lethal arrhythmias. The QT prolongation and QT dispersion have been suggested as the predictor of mortality in both type I and type II (Christensen *et al.*, 2000; Veglio *et al.*, 2004). It has been documented that *de nova* synthesis and accumulation of ceramide in diabetic myocardium contributes to the development of DCM (Hayat *et al.*, 2005; Listenberger *et al.*, 2002). In addition, oxidative stress is also known to be a critical deleterious factor for DCM (Johansen *et al.*, 2005; Maritim *et al.*, 2003). Moreover, we have recently found that I_{Kr}/HERG function is impaired in the rabbit model of DCM. Taken together, it seems plausible that ceramide-induced HERG depression may be one of the multiple determinants for diabetic QT prolongation.

Finally, correction of the electrical disorders is in no doubt an important step towards reducing cardiac death of lives suffering from myocardial ischemia, heart failure, diabetic cardiomyopathy, etc. To achieve the therapeutic goal, sufficient knowledge about the ionic mechanisms underlying ischemic electrical disorders is essential. Based on our findings, it appears conceivable that the HERG K^+ channel is a potential therapeutic target for the treatment of ischemic electrical disorders.

V-2 Limitations of the Study

V-2-1 No direct evidence provided on the direct LPC–I_{HERG} interactions

Our findings that LPC-16 acts as a metabolic trigger, and HERG channel as an ionic pathway, for ischemic $[K^+]_o$-↑ and QTc-↓ and that ischemic arrhythmias is preventable and convertible by I_{Kr}/HERG channel blocker dofetilide suggest that inhibition of LPC-16 production and accumulation and/or of I_{Kr}/HERG may be a promising therapeutic strategy to attenuate the incidence of lethal arrhythmias associated with ischemic heart disease (Corr & Yamada, 1995; Duan & Moffat, 1991; Giffin *et al.*, 1988). Nonetheless, it should be mentioned that the initial rapid $[K^+]_o$-↑ and APD-↓ may be an adaptive mechanism of cardiac cells to minimize ischemic injuries because $[K^+]_o$-↑ may reduce cell excitability thereby contractility and energy consumption and APD-↓ can

limit excessive Ca^{2+} entry so as to prevent intracellular Ca^{2+} overload and cell death. Unfortunately, this adaptation is at the cost of increased risk of cardiac arrhythmias, often-lethal arrhythmias. This contradiction creates a difficulty of handling $[K^+]_o$-↑ and APD-↓. Obviously, restoration of $[K^+]_o$-↑ and APD-↓ alone may not be clearly beneficial to ischemic heart. Combination of I_{Kr}/HERG blocker and Ca^{2+} channel blocker might be the way to go because the approach can restore $[K^+]_o$-↑ and APD-↓ on one hand and diminish intracellular Ca^{2+} overload on the other hand. This notion warrants further investigation. It should also be noted that our study does not provide definite link between LPC-16 and I_{Kr}/HERG due to lack of LPC-16 inhibitors currently. In addition, the possibility of that LPC-16/LPG-16 modulates HERG channel function *via* a third party is not completely excluded.

It is known that inhibition of I_{Kr}/HERG function can generate long QT type of arrhythmias. Our present study revealed that enhancement of I_{Kr}/HERG function can also be proarrhythmic, causing short QT type of arrhythmias such as reentry and DAD. The latter concept has actually been proposed by a recent study that describes a form of inherited malignant ventricular arrhythmias associated with short QT syndrome (Brugada *et al.*, 2004; Gaita *et al.*, 2004). Short QT syndrome is a genetic syndrome characterized by constantly short QT intervals, sudden death, short refractory periods, and inducible ventricular fibrillation. This short QT syndrome is caused by mutation in HERG gene, which increases HERG function and can be treated by agents that block I_{Kr}/HERG. Our data suggest that functional enhancement of HERG channel activity can produce acquired "short QT syndrome".

Our experiments with the I_{Ks} blockers chromanol 293B and HMR compound suggest that I_{Ks} is unlikely significantly involved in ischemic $[K^+]_o$-↑ and QTc-↓. In addition, we have also conducted experiments studying the effects of LPC-16 on I_{Ks} in guinea pig ventricular myocytes and KvLQT1 transiently expressed in COS-7 cells and we found that LPC-16 did not alter I_{Ks} (data not shown). Therefore, contribution of I_{Ks} to ischemic $[K^+]_o$-↑ and QTc-↓ in our model can be excluded. Nevertheless, in *in vivo* system where sympathetic innervations are intact, enhanced adrenergic stimulation during acute ischemia may well serve to enhance I_{Ks} and in this case I_{Ks} may also contribute to ischemic $[K^+]_o$-↑ and QTc-↓.

V-2-2 The lack of mechanistic link between ceramide/TNF–α and intracellular ROS Production

Another imperative limitation of the present study is the lack of evidence supporting the mechanistic link between ceramide/TNF–α and intracellular ROS production. We do not know what the source of ROS is, mitochondrial or cytosolic; neither do we answer how ceramide/TNF–α increase intracellular ROS. The second weakness of the work is that our study was carried out in HEK293 cells expressed only the pore-forming subunit of I_{Kr}, HERG, which may not be adequate to address the questions because it has been proposed that I_{Kr} is formed by co-assembly of HERG with mink (McDonald *et al.*, 1997) or with MirP1 (Abbott *et al.*, 1999). These auxiliary subunits might profoundly affect the effects of ceramide/TNF-α on I_{HERG}, although a recent study indicates that HERG itself is sufficient to account for the biophysical and pharmacological properties of the native I_{Kr} (Weerapura *et al.*, 2002).

V-3 Future Research Directions

To provide the direct evidence regarding the LPC–I_{HERG} interactions, further studies by *in vitro* protein-binding pull-down assays and *in vivo* coimmunoprecipitation experiments will be required to address whether LPC is physically associated with HERG channels; if so, to identify LPC binding region to HERG channels and to evaluate the functional consequences by disrupting LPC-HERG interaction, for instance, by mutation or/and deletion of binding sites. Further experiments are therefore necessary to delineate the potential unidentified pathways. To highlight the interaction between LPCs and I_{HERG}/I_{Kr}, particularly in the situation of acute myocardial ischemia, single channel recording in HEK293 cell expressing HERG should be conducted in the near future, and more feasibly, reconstitution of the sole HERG protein or association of HERG protein with the candidate auxiliary subunit such as MiRP1 or minK into lipid bilayer will be very promising (Ardehali *et al.*, 2004). If the lipid bilayer studies are performed in the setting of mimicked acute ischemia, such as acidosis and high $[K^+]$, it could provide direct evidence to explain the low LPCs affect the channel functions.

In the view of the mechanisms underlying $[K^+]_o$-↑ and APD-↓ in the early phase of acute myocardial ischemia, the future study should be directed to strive for a better

understanding of the behavior of other channels, including I_{Ks}, I_{to}, I_{KAA}, I_{KATP}, I_{KNa}, etc. at *in vitro* and *in vivo* cellular, tissue, and whole organ levels by combining classical electrophysiological techniques and molecular biology approaches. Especially, it is prospective that genetically modified animal models (GMAM) in which certain channels are either knockout (KO) or mutated or overexpressed will provide useful arrhythmia models. Similarly, to determine the role of LPCs in inducing the alteration of channel expression and functions we can also utilize the GMAM in which the enzymes that responsible for generation or deletion of LPCs are modified. For instance, phospholipase A2 (PLA2) is a key enzyme that initiates the generation of LPCs (Corr *et al.*, 1995); ischemia increases PLA2 activity, resulting in increased lysophospholipid and arachidonic acid production and contributing to arrhythmogenesis in IHD. It is therefore possible to minimize LPC production through KO of PLA2, or alternatively, through RNA interference (RNAi) techniques (Brummelkamp *et al.*, 2002;Miyagishi & Taira, 2002) in which small interfering RNAs (siRNAs) targeting plasmalogenase, an enzyme that directly catalyzes the reaction to generate LPCs will be employed to block the generation of LPCs (Tanaka *et al.*, 2003).

Another intriguing future direction regarding this project is to pay more efforts to chronic myocardial ischemia. Accumulating data have shown that next to acute modulation by electrical and metabolic disturbances, ion channels undergo changes on a longer time scale with which a variety of signaling cascades may result in multiple remodeling process, leading to the alterations in ion channel gene and protein expression, channel protein trafficking, electrical behavior, and architecture of heart. The investigation of these remodeling processes as well as the related signaling pathways involving in ischemic molecules such as sphingolipids, TNF–α, and ROS would provide the insights into the understanding of the changes occurring during chronic myocardial ischemia and accordingly rationally and improved therapeutic approaches in future.

V-4 Conclusions

1) Apart from being a well-recognized target for drug inhibition, I_{HERG} can also be enhanced by natural substances lysophospholipid LPCs. The increase in I_{HERG} might be best described by direct interactions between lysophospholipid molecules and

HERG proteins, which is consistent with lack of effects *via* membrane destabilization or modulation by intracellular signaling pathways. It is possible that LPCs, acting as HERG channel activators, could someday find utility in the treatment of repolarization disorders in the heart, including acquired and congenital long QT syndrome, and heart failure.

2) Association of LPC-16 accumulation, as the metabolic substrate, with I_{Kr}/HERG enhancement, as the electrical substrate possibly represents a additional mechanistic link between metabolic trigger and ionic pathway for ischemic $[K^+]_o$-↑ and QT-↓. Inhibition of LPC-16 production and accumulation and/or blockage of I_{Kr}/HERG may be a promising therapeutic strategy to attenuate the incidence of lethal arrhythmias associated with the early phase of ischemic heart disease.

3) Sphingolipid metabolite ceramide and TNF-α/TNFR1 system, mainly via ROS overproduction, depress I_{Kr}/HERG function in the prolonged incubation. The salient effects may contribute to QT prolongation in prolonged ischemia of myocardium and in failing heart. It is feasible that blockage of ceramide and TNF-α reduces ROS-induced QT prolongation by attenuating I_{Kr}/HERG impairment. Thus, the inhibition of ceramide and TNF-α/TNFR1 signaling may be an effective strategy to prevent not only the arrhythmogenesis but also the cardiac sudden death in chronic myocardial ischemia and CHF. On the other hand, the enhancement of ceramide and TNF-α/TNFR1 signaling can be a promising strategy for anticancer therapy.

V-5 References

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