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Modification of Ion Channel Auxiliary Subunits in Cardiac Disease

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Résumé

L'infarctus du myocarde (IM) survenant après l'obstruction de l'artère coronaire est la cause principale des décès cardiovasculaires. Après l'IM, le cœur endommagé répond à l'augmentation du stress hémodynamique avec une cicatrice et une hypertrophie dans la région non-infarcie du myocarde. Dans la région infarcie, la cicatrice se forme grâce au dépôt du collagène. Pendant formation de la cicatrice, les cardiomyocytes ventriculaires résidant dans la région non-infarcie subissent une réponse hypertrophique après l'activation chronique due au système sympathique et à l'angiotensine II. La cicatrisation préserve l'intégrité structurale du cœur et l'hypertrophie des cardiomyocytes apporte un support ionotropique.

Le canal $Ca_V1.2$ joue un rôle dans la réponse hypertrophique après l'IM. L'activation du $Ca_V1.2$ déclenche la signalisation dépendante de Ca^{2+} induisant l'hypertrophie. Cependant, il est rapporté que l'ouverture des canaux potassiques (K_{ATP}) ATP sensitifs joue un rôle sélectif dans l'expansion de la cicatrice après IM. Malgré leur expression dans les cœurs mâles, les K_{ATP} fournissent une cardioprotection sexe dépendante limitant l'expansion de la cicatrice chez les femelles.

L'administration de rapamycine aux rates ayant subi un infarctus produit l'expansion de la cicatrice, soutenant la relation possible entre la cible de rapamycine, mTORC1 et les K_{ATP} dans la cardioprotection sexe spécifique.

Effectivement, dans les cellules pancréatiques α, la signalisation mTORC1 était couplée à l'activation du K_{ATP}. Cependant, le lien entre mTORC1 et les canaux K_{ATP} dans le cœur reste inconnu. L'objectif de la thèse est d'examiner le rôle des canaux ioniques dans le remodelage cardiaque post-IM, surtout des canaux calciques dans l'hypertrophie et d'élucider la relation entre les K_{ATP} et mTORC1.

L'hypothèse première teste que l'hypertrophie médiée par le système sympathique des cardiomyocytes ventriculaires des rats néonataux (NRCM) produit une augmentation de l'influx calcique après une augmentation des sous-unités du $Ca_V1.2$. Le traitement de norépinéphrine (NE) quadruple l'amplitude du courant calcique type L et double l'expression protéique des sous unités de $Ca_V\alpha2\delta1$ et $Ca_V\beta3$. L'hypertrophie des NRCM au NE s'associe à une augmentation de la phosphorylation de la Kinase ERK 1/2. Le β1-bloqueur metoprolol et l'inhibiteur

de ERK1/2 diminuent l'effet de NE sur $Ca_V\alpha2\delta1$. Cependant, l'augmentation de $Ca_V\beta3$ et de la réponse hypertrophique persiste. Ainsi, le signal β1-adrenergique à travers ERK augmente les sous-unités $Ca_V\alpha2\delta1$ outre l'hypertrophie.

L'autre hypothèse examine la spécificité du sexe sur l'expansion cicatricielle médiée par rapamycine et l'influence de mTOR sur l'expression de K_{ATP}. Rapamycin augmente la surface de la cicatrice et inhibe la phosphorylation de mTOR chez les cœurs de femelles. Dans les cœurs des deux sexes, la phosphorylation de mTOR et l'expression de K_{ATP}, Kir6.2 et SUR2A sont similaires. Cependant, une grande inactivation de la tubérine et une faible expression de raptor sont détectées chez les femelles. Le traitement à l'ester de phorbol des NRCM induit l'hypertrophie, augmente la phosphorylation de p70S6K et l'expression SUR2A. Le prétraitement par Rapamycine atténue chacune des réponses. Rapamycin démontre un patron d'expansion cicatriciel sexe spécifique et une régulation de phosphorylation de mTOR dans IM. Aussi, l'augmentation de SUR2A dans les NRCM traités par PDBu révèle une interaction entre mTOR et K_{ATP}.

Mots-clés : Hypertrophie cardiaque, canaux calciques de type L, stimulation sympathique, kinases extracellulaires régulées par le signal ERK 1/2, infarctus du myocarde, canaux potassiques sensibles à l'ATP (canaux K_{ATP}), kinase cible de la rapamycine chez les mammifères (mTOR), cardioprotection.

Abstract

Myocardial infarction (MI) secondary to the obstruction of the coronary artery is the main cause of cardiovascular death. Following MI, the damaged heart adapts to the increased hemodynamic stress *via* formation of a scar and a hypertrophic response of ventricular cardiomyocytes in the non-infarcted myocardium. In the infarcted region, a scar is formed *via* the rapid deposition of collagen. With ongoing scar formation, ventricular cardiomyocytes in the non-infarcted myocardium undergo a hypertrophic response secondary to the chronic activation by the sympathetic system and angiotensin II. Collectively, scar formation and cardiomyocyte hypertrophy preserve the structural integrity of the heart and provide inotropic support, respectively.

$Ca_V1.2$ channels play a significant role in the hypertrophic response post-MI. Notably, the activation of $Ca_V1.2$ channel triggers Ca^{2+}-dependent signaling that induces hypertrophy. By contrast, the opening of ATP-sensitive potassium (K_{ATP}) channels was shown to partake in selective scar expansion following MI. Notwithstanding its expression in male hearts, K_{ATP} channels endow a sex-dependent cardioprotection limiting scar expansion selectively in females. Moreover, administration of the macrolide rapamycin to the infarcted female rat heart led to scar expansion, supporting the possible relationship between the target of rapamycin, mTORC1 and K_{ATP} channels in providing sex-specific cardioprotection. Indeed, in pancreatic-α cells, mTORC1 signaling was coupled to K_{ATP} channel activation. However, whether mTORC1 targets K_{ATP} channels in the heart remains unknown. Thus, the AIM of the thesis was to explore the role of ion channels in cardiac remodeling post-MI by specifically addressing the role of Ca channels in cardiomyocyte hypertrophy and elucidate the potential relationship between K_{ATP} channels and mTORC1 signaling.

The first study tested the hypothesis that hypertrophied neonatal rat ventricular cardiomyocytes (NRVMs) following sympathetic stimulation translated to an increase in calcium influx secondary to the augmentation of $Ca_V1.2$ channel subunits. NE treatment led to a 4-fold increase of L-type Ca^{2+} peak current associated with a 2-fold upregulation of $Ca_V\alpha2\delta1$ and $Ca_V\beta3$ protein subunits in hypertrophied NRVMs. The hypertrophic response of NNVMs to NE was associated with the increased phosphorylation of extracellular regulated kinase (ERK1/2). The β1-

blocker metoprolol and the ERK1/2 inhibitor suppressed NE-mediated protein upregulation of Cavα2δ1 whereas Cavβ3 upregulation and the hypertrophic response persisted. Therefore, sympathetic mediated β1-adrenergic signaling *via* ERK selectively upregulated the Cavα2δ1 subunit independent of NRVM hypertrophy.

The second study tested the hypothesis that rapamycin-mediated scar expansion was sex-specific and mTOR influenced K_{ATP} channel subunit expression. Rapamycin administration translated to scar expansion and inhibited mTOR phosphorylation exclusively in females. In normal adult male and female rat hearts, mTOR phosphorylation and protein levels of K_{ATP} channel subunits Kir6.2 and SUR2A were similar. However, greater tuberin inactivation and reduced raptor protein levels were detected in females. NRVMs treated with a phorbol ester induced hypertrophy, increased p70S6K phosphorylation and SUR2A protein levels and rapamycin pretreatment attenuated each response. Thus, rapamycin administration to MI rats unmasked a sex-specific pattern of scar expansion and highlighted the disparate regulation of mTOR phosphorylation. Moreover, rapamycin-dependent upregulation of SUR2A in PDBu-treated NRVMs revealed a novel interaction between mTOR and K_{ATP} channel subunit expression.

Keywords: Cardiac hypertrophy, L-type Calcium channels, sympathetic stimulation, extracellular signal-regulated kinases ERK 1/2, myocardial infarction, ATP-sensitive potassium channels (K_{ATP} channels), mammalian target of Rapamycin (mTOR), cardioprotection.

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List of abreviations

AVN	Atrioventricular node
ANP	Atrial natriuretic peptide
ARs	Adrenergic receptors
AC	Adenylyl cyclase
ATP	Adenosine triphosphate
AMPK	AMP-activated protein kinase
Ang-II	Angiotensin-II
α-MHC	α-myosin heavy chain
BNP	Brain natriuretic peptide
β-MHC	β-myosin heavy chain
cAMP	Cyclic adenosine monophosphate
CICR	Ca^{2+}-induced Ca^{2+} release
CCBs	Calcium channel blockers
CaMKII	Calmodulin kinase II
CAD	Coronary artery disease
DEPTOR	DEP domain-containing mTOR-interacting protein contain
DHP	Dihydropyridines
DAG	Diacylglycerol
DAMPs	Damage-associated molecular patterns
ER	Endoplasmic reticulum
ET-1	Endothelin-1
ERK	Extracellular-signal regulated kinases
ECM	Extracellular matrix proteins
ECG	Electrocardiography
GK	Guanylate kinase
GPI	Glycosylphosphatidylinositol

GABA	Gamma-aminobutyric acid
GATA4	GATA Binding Protein 4
GPCR	G-protein coupled receptors
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
GAP	Guanosine triphosphatase (GTPase)-activating protein
HEK 293	Embryonic kidney 293 cells
HVA	High voltage activated Ca^{2+} channels
HDAC4	Histone deacetylase 4
IP3	Inositol (1,4,5)-trisphosphate
IL-1	Interleukin-1
K_{ATP}	ATP-sensitive potassium
LVA	Low voltage activated Ca^{2+} channels
LV	Left ventricle
LTCC	Voltage-gated L-type Ca^{2+} channels
MI	Myocardial infarction
MIDAS	Metal ion-dependent adhesion site.
MAGUK	Membrane-associated guanylate kinase
MEF2	Myocyte enhancer factor
MMPs	Matrix metalloproteinases
mTOR	Mammalian target of rapamycin
mLST8	The mammalian lethal with Sec13 protein 8
mSin1	Mammalian stress-activated MAP kinase-interacting protein 1
NE	Norepinephrine
NFAT	Nuclear factor of activated T cells
NCX	Na^{+}/Ca^{2+} exchanger
NF-κB	Nuclear factor Kappa B
NRVMs	Neonatal rat ventricular cardiomyocytes

NBD	Nucleotide-binding domain
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLB	Phospholamban
PKA	Protein kinase A
PKC	Protein kinase C
PRAS40	Proline-rich Akt/PKB substrate 40 kDa
Protor	Protein observed with rictor
P-70S6K	p70 ribosomal protein S6 kinase
RyR	Ryanodine receptors
RGK	Rad/Rem/Rem2/Gem/Kir family of Ras-like GTPases
Rheb	Ras homolog enriched in brain protein
Rad	Ras associated with diabetes
ROS	Reactive oxygen species
RAPTOR	Regulatory-associated protein of mTOR
Rictor	Rapamycin-insensitive companion of mTOR
SERCA-2	Sarcoplasmic reticulum Ca^{2+} ATPase-2
SAN	Sinoatrial node
SR	Sarcoplasmic reticulum
SNS	Sympathetic nervous system
SERCA	Sarcoplasmic Ca^{2+} ATPase pump
SH3	Src homology 3 domain
siRNA	Small inhibitory RNA
S6K1	Ribosomal protein S6 kinases
SUR	Sulfonylurea receptor
TTCCs	Transient or T-type Ca^{2+} channels
TGF-β1	Transforming growth factor β1
TAC	Transverse aortic constriction

TLR	Toll-like receptors
Tti1-Tel2	Tel two interacting protein 1 complex
TSC1	Tuberous sclerosis 1
TSC2	Tuberin or tuberous sclerosis 2
UDMI	Fourth Universal Definition of MI
VWA	Von Willebrand factor A
4E-BP1	Eukaryotic translation initiation factor 4E (eIF4E)-binding protein-1

Dedication

This dissertation is dedicated to my loving parents, Nouhad and Faraj Al Katat.

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Chapter 1

1. Introduction

Myocardial infarction (MI) accounts for a significant portion of cardiovascular mortality. MI is caused by the death of cardiomyocytes due to prolonged ischemia resulting from an imbalance between coronary blood supply and myocardial demand [1,2]. Despite vast improvements in treatment strategies over the past decades, MI patients remain at high risk for adverse cardiovascular events [1,2]. Although MI is associated with adverse cardiovascular events, the subsequent pattern of cardiac remodeling is adaptive involving structural and functional modifications[3,4]. The loss of cardiomyocytes following MI induces molecular and interstitial alterations and triggers an inflammatory response essential for scar formation and subsequent healing[3,5,6]. In parallel, as the infarct expands, the surviving myocytes (non-infarcted tissue) undergo hypertrophy to compensate for the loss of cardiac tissue, leading to increased myocardial wall stress and altered loading conditions [3,5,6]. Thus, cardiac remodeling post-MI encompasses two phases: scar formation (fibrosis) and non-infarct LV hypertrophy[3,5,6]. Scar formation is an adaptive reparative response essential to provide and preserve the structural integrity of the left ventricle by preventing cardiac rupture[7–9]. Scar formation and healing requires the deposition of cross-linked fibrillar collagen secondary to neurohumoral and sympathetic activation[7–9]. Furthermore, following chronic sympathetic system stimulation, the prolonged release of neurohumoral factors plays a parallel seminal role in the hypertrophic response of ventricular cardiomyocytes residing in the non-infracted myocardium[3,6].

Intrinsic responses to cardiac remodeling modify ion channel expression and function, leading to the alteration of cardiac action potential[10]. In particular, $Ca_V1.2$ and K_{ATP} channels were shown to take part in cardiac remodeling following MI[11–13]. Following hemodynamic load, $Ca_V1.2$ channels are activated, promoting Ca^{2+} influx into the cytosol, which triggers Ca^{2+}-dependent signaling cascade such as the Calcineurin-NFAT pathway, known to trigger a hypertrophic response[11]. Hence, modifications in Ca^{2+} handling contribute to myocardial hypertrophy following MI.

Likewise, K_{ATP} channels are recognized as indispensable elements for adaptation to physiological and pathological stress[12,14,15]. The heart possesses several internal protective mechanisms to minimize cardiac injury resulting from ischemia and to enhance cardiac recovery[16,17]. Among

these mechanisms is the activation of ATP-sensitive potassium (K_{ATP}) channels[12,13,18]. K_{ATP} channels serve as metabolic sensors that partake in cardioprotection against myocardial infarction[12,13,18]. Under conditions of severe metabolic stress, the activation of K_{ATP} channels have been shown to reduce infarct size, ameliorate functional recovery of the myocardium, and mimic the effects of ischemia preconditioning[12,19,20]. However, despite the presence of K_{ATP} channels in the male and female rodent heart, K_{ATP} channels provide sex-specific cardioprotection against scar expansion following MI exclusively in females[21]. Indeed, a pharmacological approach revealed that the inhibition of K_{ATP} channels abolished the sex-disparity in infarct size following myocardial infarction [21]. In this regard, could the sex-specific cardioprotection of K_{ATP} channels selectively in females be attributed to the more rapid recruitment of these channels in females versus males following MI? A previous study reported that administration of the macrolide immunosuppressant rapamycin to the myocardial infarcted female rat led to scar expansion recapitulating the data observed with pharmacological blockade of K_{ATP} channels in the female MI rodent heart[22]. In this regard, could the mTOR pathway targeted by rapamycin represent a sex-specific pathway translating to the more rapid recruitment or increased expression of K_{ATP} channel subunit expression in female rodents following myocardial infarction? Therefore, the general aim of the thesis is to investigate the regulation of ion channels following cardiac remodeling secondary to myocardial infarction. The first study will test the hypothesis that $Ca_V1.2$ subunit protein expression is significantly increased following sympathetic-mediated ventricular cardiomyocyte hypertrophy *in vitro*. The second study will test the hypothesis that the impact of rapamycin on scar expansion occurs exclusively in female rats following myocardial infarction and further test the thesis *via* an *in vitro* approach that mTOR signaling may directly upregulate protein expression of K_{ATP} channel subunits.

1.1. Myocardial Infarction: Definition

In the late 19th century, autopsy examinations revealed a potential tie-in between thrombotic blockage of coronary artery and myocardial infarction (MI)[23]. However, it was not before the 20th century that the clinical features associated with thrombus formation in a coronary artery were reported[24].

Myocardial infarction is delineated as the death of cardiomyocytes due to prolonged ischemia that results from an imbalance between coronary blood supply and myocardial demand[1,2]. This imbalance leads to an irreversible myocardial injury/damage due to a shortage in oxygen supply[1], [2]. Clinically, MI is identified as chest discomfort, heaviness, fatigue, and dyspnea[2]. ECG and cardiac biomarker analysis should be executed in patients with chest pain to substantiate a diagnosis of MI[1,2].

1.1.1. Etiology of MI

The principal cause of ischemia is reduced coronary blood flow to the heart[1,25]. Decreased coronary blood flow is multifactorial. It could be secondary to atherosclerotic plaque rupture and is thus referred to as type 1 myocardial infarction, or a consequence of a non-atherosclerotic etiology (example: sepsis and anemia) that triggers increased myocardial oxygen demand and/or reduced myocardial oxygen delivery and is known as type 2 myocardial infarction[1,25].

1.1.1.1. Type 1 myocardial infarction

According to the Fourth Universal Definition of MI (UDMI), type 1 MI results from disruption (rupture or erosion) of the atherosclerotic plaque formed in the interior walls of the coronary artery[2]. The formation of atherosclerotic plaques narrows the artery, which, during conditions of increased myocardial demand (example: exercise), results in reduced blood flow to the heart[1], [25]. A ruptured plaque emanates a thrombus in the coronary artery, contributing to reduced coronary blood flow to the heart and ultimately resulting in myocardial infarction[2]. Occlusions caused by atherosclerotic plaques account for 70% of fatal events and are thus considered the predominant cause of MI[1].

1.1.1.2. Type 2 myocardial infarction

In contrast to type 1 MI, type 2 MI does not involve atherothrombotic plaque disruption. It is rather a sequel to a mismatch of oxygen supply to myocardial demand[1,25]. The latter imbalance could be attributed to either insufficient blood flow to the myocardium such as in coronary vasospasm, coronary embolism, coronary artery dissection or in conditions where oxygen supply is reduced

due to bradycardia, anemia, or hypotension; or to increased myocardial oxygen needs as a consequence of hypertension or tachycardia[1,25].

1.1.2. Pathophysiology of MI

Myocardial infarction (MI) results from partial or complete blockage of coronary blood flow to a territory of the heart[25]. Since coronary arteries have specific territorial distributions, the decrease in blood supply results in ischemic cascade and cardiomyocytes death in the portion of the heart subserved by the blocked coronary artery[1]. If blood flow is not restored, lack of oxygen delivery depletes mitochondrial ATP formation, leading to necrosis of cardiomyocytes and ultrastructural changes such as myofibril relaxation and sarcolemma disruption, which occur as early as 10 to 15 minutes following obstruction of the coronary artery[26–28]. The necrosis of ischemic cardiomyocytes and myocardial injury stimulate an inflammatory reaction, recruiting multiple immune cell subtypes (neutrophils, macrophages, and lymphocytes) that function to digest and clear the wound from dead cardiomyocytes and extracellular matrix debris[29]. Early inflammatory activation is essential to initiate reparative changes, which entail fibroblast proliferation, collagen deposition, and scar formation[30,31]. The latter is termed reparative fibrosis, which is essential to preserve the cardiac structural integrity[30,31]. These changes lead to ventricular remodeling of the infarct and non-infarct zone of the myocardium, which is clinically displayed as dilatation and cardiac hypertrophy[3,32].

1.1.3. The biphasic complexion of ventricular remodeling post-MI

Post-MI ventricular remodeling is categorized into two phases: early phase that occurs within 72 hours and a late phase after 72 hours post-MI [3].

1.1.3.1. Early Remodeling

Temporal prolongation of the inflammatory response and infarct expansion trigger cardiac remodeling in the infarct border zone and non-infarcted myocardial segments[33]. The latter results in wall thinning and ventricular dilatation and elevates systolic and diastolic wall stress[33].

1.1.3.2. Late Remodeling

The increased wall stress and myocardial stretch trigger the release of neurohormones and paracrine/autocrine factors, promote expansion of the extracellular matrix to remote non-infarct region of the myocardium[6]. The latter leads to reactive fibrosis (also called interstitial fibrosis) along with the elevated LV pressure induce modifications of the non-infarct ventricular architecture, which involve formation of sarcomeres, resulting in cardiac hypertrophy[3]. Post-MI cardiac hypertrophy compensates for the infarcted zone of the myocardium and attenuates dilatation, preventing further contractile deterioration[3].

1.1.4. Cardiomyocyte death *via* necrosis and apoptosis

Cell death is a hallmark feature of myocardial infarction[34]. Occlusion of the coronary artery results in mitochondrial alterations, triggering the death of cardiomyocytes in the infarcted zone *via* apoptosis and necrosis[34]. Necrosis is an unprogrammed form of cell death characterized by plasma membrane rupture, cellular swelling, ATP depletion, and marked inflammation[34–36]. The lack of oxygen during ischemia results in anaerobic glycolysis and subsequent metabolic acidosis that generates ionic imbalance, initiates multiple destructive changes such as rendering the plasma membrane permeable to Na^+ and Ca^{2+}, and reduces ATP production by impeding oxidative phosphorylation[36–39]. Increased intracellular Na^+ concentration stimulates the reverse-mode function of Na^+/Ca^{2+} exchanger (NCX), resulting in a further increase of cytosolic Ca^{2+}[38]. A high mitochondrial Ca^{2+} concentration stimulates the opening of the mitochondrial permeability transition pore (mPTP), a pore in the inner mitochondrial membrane (IMM) [38,40]. Persistent opening of mPTP blocks ATP generation and enhances the influx of water and electrolytes into the mitochondria, ultimately leading to swelling and necrosis[38,40]. The mitochondrial membrane rupture and the leakage of several factors to the surrounding environment triggers an inflammatory response[38,40].

In contrast to necrosis, apoptosis is a highly regulated programmed form of cell death[34,35]. The process entails shrinkage of the cell and nucleus followed by fragmentation into apoptotic bodies that are then phagocytosed and cleared by macrophages[34,35]. Apoptosis is activated by permeabilization of the outer mitochondrial membrane (OMM), which results in the release of mitochondrial apoptogens into the cytoplasm, triggering the activation of caspase cascades and ultimately leading to apoptosis[34,35].

1.1.5. Infarct healing following MI

Because the adult mammalian heart lacks regenerative capacity, cardiac injury presents a considerable challenge for the body's reparative processes[41]. Hence, dead cardiomyocytes are replaced with a non-contractile collagen-formed scar that preserves the structural integrity of the heart, warding off deteriorating events (e.g.: cardiac rupture).[28,42].

Cardiac repair post-MI develops as a result of an orchestrated series of events. Infarct healing is divided into three phases: (i) Inflammatory phase, initiated by the release of damage-associated molecular patterns (DAMPs) from dead (necrotic) cardiomyocytes, serves to clear damaged cells and extracellular matrix debris[28,36,43]. (ii) Proliferative and reparative phase follows the inflammatory phase and is associated with the formation of a highly-vascularized microvascular network and suppression of pro-inflammatory factors[28,36]. At the same time, fibroblasts proliferate and transition into myofibroblast-like phenotype[28]. Activated myofibroblasts secrete collagen and extracellular matrix proteins that partake in fibrotic scar formation[28]. (iii) In the final stage of wound healing, referred to as the Maturation phase, the fibroblasts and reparative cells undergo apoptosis, and cross-linking of extracellular matrix is built, generating a collagen-based scar that is associated with neovascularization[36,44]. As the healing of the infarcted area progresses, the ventricle dilates to preserve cardiac output, while the remote non-infarcted zone undergoes remodeling resulting from pressure and volume overload[28,36].

1.1.5.1. Inflammatory phase

Necrotic and injured cells release intracellular danger signals, known as damage-associated molecular patterns (DAMPs) or alarmins, that induce an intense innate immune response. Besides DAMPs, the inflammatory response post-MI has been shown to be generated by two other main factors, namely the complement system and reactive oxygen species (ROS)[28,36,43,45].

Alarmins serve as a signal defense by binding to and activating the pattern recognition receptors, such as Toll-like receptors (TLRs) and receptors for advanced glycation end-products (RAGE) located in vascular cells, fibroblasts, leukocytes, and surviving border zone of cardiomyocytes, inducing synthesis of chemokines and cytokines and triggering an inflammatory response [28,43,45]. Several Alarmins have been identified post-MI, such as High mobility group box 1 (HMGB1), heat shock proteins (HSP), members of the S100 family, and interleukin 1-alpha (IL-

1α) [28,43,45]. HMGB1 is one of the most characterized alarmins. In humans as well as in rodents with MI, the serum level of HMGB1 was elevated early after ischemic injury[46–50]. Moreover, neutralization of HMGB1 in rodents with re-perfused MI reduced cytokine release and leukocyte infiltration, suggesting that early release of HMGB1 is a potent mediator of inflammation post-MI[47,48].

Complement activation is also prominent in the infarcted myocardium and contributes to the inflammatory response by stimulating the release of downstream pro-inflammatory cytokines such as IL-1 and Tumor Necrosis Factor (TNF)- α from leukocytes, fibroblasts, and endothelial cells[51]. Pro-inflammatory cytokines stimulate adhesion molecules which enhance adhesive interactions between endothelial cells and leukocytes, promoting the recruitment of neutrophils, leukocytes, and monocytes to the injured zone[52–54]. The importance of the complement system was evident in experimental studies showing that pharmacological or genetic inhibition of complement cascade minimized the infarct size, reduced cardiomyocyte necrosis, and blunted the inflammatory response[55–59].

Alongside activation of complement system, reactive oxygen species are also mediators of inflammation[60]. The normal myocardium possesses enough antioxidants to counterbalance the production of ROS. However, following myocardial infarction, antioxidant defense system is inundated, resulting in excess of ROS and radical products[28]. In the infarcted myocardium, ROS generation enhances the synthesis of cytokines and upregulates the expression of P-selectin, promoting leukocyte chemotaxis[61]. The role of ROS in myocardial infarction is enigmatic. Although it is essential to induce inflammatory mechanisms favoring cardiac repair, excessive ROS enhances extracellular matrix degradation and cell apoptosis[43].

Furthermore, DAMPs, complement cascade, and ROS partake in the inflammatory response following MI by acting on TLRs on leukocytes, which in turn activate nuclear factor Kappa B (NF-κB)[62]. NFKB is a transcription factor which, when stimulated, translocates to the nucleus and initiates the transcription of genes involved in inflammation and cell adhesion, such as pro-inflammatory cytokines and chemokines [63,64].

1.1.5.1.1. The role of extracellular matrix during the inflammatory phase

A mesh of extracellular matrix proteins (ECM) surrounds both cardiomyocytes and noncardiomyocytes[65]. In addition to their role in providing structural and mechanical support,

ECM proteins regulate inflammatory response by modulating the phenotype and function of multiple cell types following MI[66]. As early as 10 minutes following coronary occlusion, elevated levels of matrix metalloproteinases (MMPs) are detected in the myocardium[67]. MMPs are Ca^{2+}-dependent proteolytic enzymes implicated in ECM degradation[68]. High MMPs levels post-MI trigger degradation and fragmentation of ECMs, which activate pro-inflammatory signaling such as leukocyte recruitment[69,70]. Moreover, ECM degradation is replaced by a fibrin-based provisional matrix, indicating that MMPs also contribute to the structural changes required to transition from the inflammatory phase to the proliferative phase during scar formation[71,72].

1.1.5.2. The proliferative phase

Removal of dead cells and ECM debris from the infarcted area drives the transition from the inflammatory phase to the proliferative phase[36,44]. The latter transition is initiated by apoptotic neutrophils that favor resolution of the inflammation phase through the release of several mediators such as annexin and lactoferrin, which dampen further neutrophil recruitment and favor phagocytic uptake of dead neutrophils by macrophages[36,73]. Phagocytosis of apoptotic neutrophils stimulates the synthesis and release of anti-inflammatory cytokines, such as IL-10 and Transforming Growth Factor (TGF)-β[44].

Anti-inflammatory cytokines serve pleiotropic roles. In addition to their involvement in suppressing inflammation, anti-inflammatory cytokines also trigger tissue repair[36,44]. For example, TGF-β serves as a switch that initiates the transition from inflammation to fibrosis through several mechanisms, including formation of myofibroblasts from differentiated cardiac fibroblasts and synthesis of MMPs and ECMs required for scar formation[44].

The expansion of cardiac fibroblasts and transition into myofibroblasts represent a hallmark of the proliferative phase[36,44]. Cardiac fibroblasts are resident cells that predominate in the healing myocardium. When dead cells are removed from the infarct zone, cardiac fibroblasts undergo phenotypic modifications and differentiate into synthetic myofibroblasts[36,44]. Myofibroblasts reside mainly in the infarct region and are characterized by the expression of alpha-smooth muscle actin (αSMA) and the secretion of ECM proteins[36,44]. Hence, myofibroblasts contribute directly to scar formation post-MI by serving as the primary source of ECM and matricellular protein synthesis in the infarcted heart[36,44]. While deposition of structural ECM proteins at the infarct site maintains

the structural integrity of the myocardium, matricellular proteins (such as osteopontin and thrombospondin-1) bind to cytokines and transduce growth-factor-induced signals that promote activation of fibroblasts and inhibit MMP activation, warding off matrix degradation, and contributing to the plasticity of the infarct area[36,44]. The development of a network of structural ECM proteins marks the end of the proliferative phase and sets the stage for the maturation phase[36,44].

1.1.5.3. The maturation phase

During the maturation phase, the extracellular matrix is cross-linked, and a collagen-based scar is formed[36,44]. As the scar matures, the proliferative feature of fibroblasts is repressed, and the infarct myofibroblasts turn into quiescent cells[36,44]. Moreover, reparative cells become deactivated and undergo apoptosis[36,44]. Notably, reduced levels of matricellular proteins and fibrotic growth factors are documented in the myocardium weeks following myocardial infarction[36,44]. Despite the latter observations, the exact mechanism underlying scar maturation following MI remains obscure.

1.1.6. Remote non-infarcted part of myocardium after MI

Following MI, the elevated mechanical stress, together with persistent active myofibroblasts induce the release of TGF-β and other pro-fibrotic factors that expand to the remote non-infarct area of the LV[30]. The latter results in the accumulation of the extracellular matrix proteins and the deposition of collagen in the interstitial and intermuscular spaces, resulting in interstitial (reactive) fibrosis, which stiffens the LV and leads to systolic and diastolic dysfunction[30]. Besides, scar expansion leads to increased myocardial wall stress, triggering hypertrophic response of the non-infarcted cardiomyocytes to compensate for the damaged zone of the myocardium and preserve cardiac function[30].

1.2. Cardiac hypertrophy

1.2.1. Definition

Cardiac hypertrophy is an adaptive process that encounters hemodynamic load in an attempt to preserve cardiac output and enhance myocardial contractility[74,75]. Clinically, cardiac hypertrophy is characterized by increased cardiac mass. Since cardiomyocytes are terminally differentiated postnatally[76], this increase is attributed to the enlargement in the size of individual myocytes owing to the *de novo* synthesis of the sarcomeres[74–77]. In general, cardiac hypertrophy is associated with qualitative changes such as increased protein synthesis, change in gene expression, and assembly of sarcomeres in different patterns [74–77]. Moreover, cardiac hypertrophy is also accompanied by the release of growth factors, cytokines, and hormones such as catecholamines, which activate diverse downstream signaling cascades implicated in the hypertrophic response[74]. Depending on the upstream stimuli, hypertrophy is categorized into physiological and pathological hypertrophy **(Figure 1)** [74–77]. While both phenotypes initiate as an adaptive mechanism in response to cardiac stress, pathological hypertrophy is linked to adverse cardiac conditions that predispose to heart failure[78]. Pathologic cardiac hypertrophy is a result of pressure overload stimulation (e.g., hypertension, aortic stenosis, chronic neurohormonal activation) and is associated with cardiac dysfunction; however, physiological hypertrophy is a reversible mechanism accompanied by an enhanced cardiac function and occurs mainly during exercise or pregnancy[75,79,80]. Depending on the type of ventricular wall stress, both physiological and pathological hypertrophy are further divided into concentric and eccentric phenotypes **(Figure 1)** [78]. In response to diastolic wall stress, sarcomeres are added in a series pattern, which results in cardiomyocyte elongation, increased chamber dilatation (ventricular diameter) but reduced wall thickness, accounting for the eccentric phenotype[81]. In contrast, an elevated systolic wall stress, also known as pressure overload, triggers sarcomere addition in parallel pattern, which lead to increased wall thickness and reduced chamber size, depicting the concentric phenotype [75].

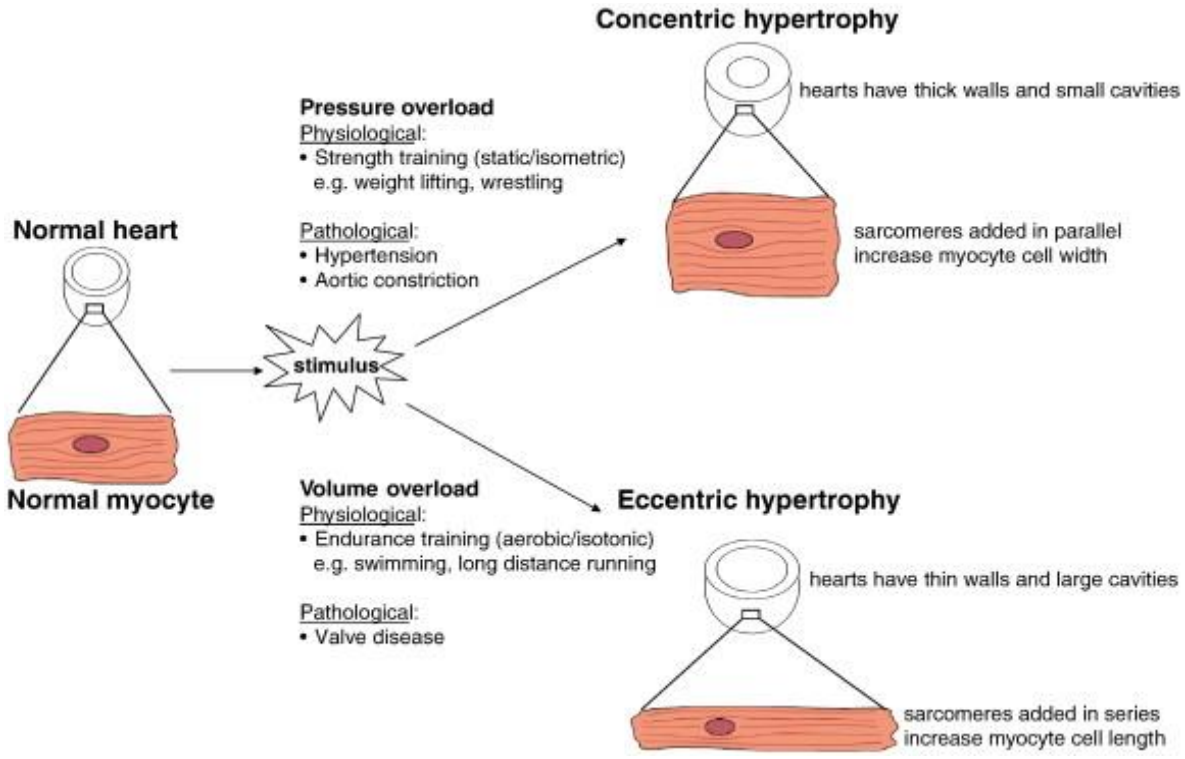

Figure 1 :Types and forms of cardiac hypertrophy.

Scheme demonstrating the development of concentric hypertrophy with the parallel addition of sarcomeres in pressure overload and eccentric hypertrophy with a series addition of sarcomeres in volume overload. Figure taken from Bernardo et *al*., 2010[82].

1.2.2. Physiological hypertrophy

Physiological hypertrophy occurs mainly during postnatal development, pregnancy, or intensive/repetitive exercise[74].

Physiological hypertrophy is a reversible mechanism and does not transition to heart failure. Hearts with physiological hypertrophy are characterized by preserved or enhanced cardiac function,

induced angiogenesis, and the absence of inflammatory response and fibrosis[74]. A characteristic hallmark of physiological hypertrophy is the absence of the fetal gene program re-expression that constitutes natriuretic peptides (ANP and BNP) and the cardiac contractile protein β-myosin heavy chain[82]. Moreover, genes encoding proteins involved in Ca^{2+} handling remain unaltered[82]. Imitsu et *al*. have documented that the mRNA expression of ANP and β-MHC were upregulated in hypertensive rats but not in swim-trained rats, indicating disparate alterations in the molecular features between physiological and pathological hypertrophy[83].

During postnatal development, cardiac myocytes lose their proliferative capacity and become terminally differentiated soon after birth [84]. Normal growth of the heart from birth to early adulthood is carried through an increase in the size of individual cardiac myocytes without a concomitant increase in their number in a process referred to as postnatal hypertrophy[75,84].

During pregnancy, cardiac output increases secondary to increased heart rate, expansion of the blood volume, and reduced systemic vascular resistance[85–87]. This increased demand reaches its peak from the third trimester to term, setting volume overload on the heart that ultimately leads to physiological hypertrophy of eccentric phenotype, and is usually reversed postpartum[86,87].

In the case of the athlete's heart, the type and intensity of the exercise impose a differential hemodynamic load on the heart[74]. Endurance training (also known as isotonic or dynamic training, e.g., running or swimming) augments cardiac output due to elevated heart rate and stroke volume and is often associated with reduced peripheral vascular resistance[88,89]. Prolonged isotonic exercise leads to increased ventricular filling time and facilitates venous return to the heart [89–96]. The latter elicits volume overload and eccentric hypertrophy, which is manifested as dilated chamber and series addition of sarcomeres[89–96]. In contrast, isometric exercise such as strength training (weightlifting or wrestling) generates brief periods of increased peripheral vascular resistance that elevates the aortic pressure, resulting in concentric hypertrophy [88]. Hearts of strength-trained athletes exhibit increased ventricular wall thickness attributed to the parallel addition of sarcomeres [74, 96]. Thus, the heart undergoes distinctive structural and functional adaptations depending on the imposed stimuli.

1.2.3. Pathological cardiac hypertrophy

Pathological hypertrophy is a maladaptive response to chronic hemodynamic pressure or volume overload, which occurs due to hypertension, valvular disease, diabetic cardiomyopathy, or myocardial infarction[74]. Pathological hypertrophy is associated with cardiac dysfunction, interstitial fibrosis, fibroblast proliferation, loss of myocytes, and extracellular matrix remodeling[75,97]. The loss of myocytes is replaced with collagen buildup, which stiffens the ventricles and results in impaired electric conduction and reduced cardiac contraction and relaxation, hence predisposing the myocardium to heart failure[74,75,97]. Chronic pressure overload-induced cardiac hypertrophy resulting from hypertension or aortic stenosis imposes elevated systolic wall stress, which induces parallel alignment of sarcomere formation, leading to concentric hypertrophy[98]. Morphologically, pressure-overload-induced concentric hypertrophy is characterized by increased wall thickness and reduced chamber radius[75,99]. A hallmark of pathological cardiac hypertrophy is the induction of the fetal gene expression, which includes the re-expression of the atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) mRNAs, and a shift of the myosin isozyme from the α-myosin heavy chain (α-MHC) to the β-myosin heavy chain (β-MHC) form [100–103]. The latter isoform shift has been shown to reduce ATPase activity, resulting in minimized ATP demand and lower cardiac contractility, hence representing an adaptive response that attempts to preserve ATP when increased workload and oxygen demand occur[104–106]. Similarly, the upregulation of ANP and BNP mRNA levels serves as a preventive mechanism to impede fibrosis and hypertrophy[107–110]. Several studies have demonstrated that diverse stimuli trigger distinct molecular features in cardiac hypertrophy[74]. For example, in a mice model subjected to transverse aortic constriction-induced pressure overload, hypertrophy was associated with upregulation of mRNA expression levels of ANP, BNP, and β-MHC[111]. In contrast, stimuli caused by aortic regurgitation or arteriovenous shunting set off volume overload, which leads to elevated diastolic wall stress[112, 113]. Increased diastolic wall stress triggers the formation of sarcomeres in a series pattern leading to elongation of cardiac myocytes. Increased myocyte length generates chamber enlargement and results in an eccentric pattern of cardiac hypertrophy[112–115]. Thus, pressure and volume overload-induced hypertrophy are linked to disparate myocytes phenotypes and differential ventricular morphology.

In addition to disease-mediated stimuli, the development of cardiac hypertrophy has been attributed to other factors, including growth-promoting molecules and neurohormones such as

angiotensin II, transforming growth factor β1 (TGF-β1), insulin-like growth factor-1, and circulating catecholamines[74].

1.2.4. Regulation of cardiac hypertrophy by the sympathetic nervous system

The sympathetic nervous system (SNS) is activated by various stressful stimuli, including physical activity, emotional change, physiological stress, or other disease-related stimuli (e.g., pheochromocytoma)[116]. The stress-induced activation of SNS promotes the release of catecholamines serving as a counter-regulatory adaptation mechanism to stressful conditions, which is referred to as the fight or flight response[117]. Catecholamines such as Noradrenaline (also referred to as norepinephrine) and adrenaline (or epinephrine) are released by the sympathetic nervous system and adrenal medulla, respectively, and partake in modulating a host of diverse physiological and pathophysiological processes[118]. The cell response to catecholamines is mediated *via* activation of α and β adrenergic receptors which are G protein-coupled receptors[118]. In the normal heart, three types of β-adrenergic receptors β1, β2, and β3 have been detected, with β1-adrenergic receptor being the major predominant type[119,120]. β-adrenergic receptors account for 90% of the total cardiac ARs, while $\boldsymbol{\alpha}_1$-ARs account for approximately 10% [121].

During the fight-or-flight response, the prompt release of catecholamines (norepinephrine and epinephrine) elevates heart rate (positive chronotropy) and enhances cardiac contractility (positive inotropy) and relaxation (lusitropy) through the β-adrenergic receptor-mediated activation of cyclic adenosine monophosphate/PKA (cAMP/PKA) signaling cascade[122–124] Clinically, exogenous administration of catecholamines has been employed as a lifesaving treatment in conditions such as hypotension and low cardiac output [125]. In a randomized trial, the administration of norepinephrine (NE) was shown to ameliorate the outcomes in patients with cardiogenic or septic shock[124,126].

In contrast to the short-term adaptive response, a prolonged increase of catecholamines is detrimental to the heart[127,128]. Increased sympathetic stimulation and elevated circulating catecholamines are the principal impetus that initiates and maintains cardiac hypertrophy [127,128]. Laks et *al.* demonstrated that chronic Norepinephrine (NE) infusion into dogs increased ventricular wall thickness and consequently elicited ventricular hypertrophy[129]. Moreover, mice

treated with NE exhibited increased ventricular wall thickness and induction of the fetal genes, indicating that hypertrophy was established[130]. The role of catecholamines was further confirmed in a study showing that genetically altered mice devoid of endogenous NE and epinephrine did not develop cardiac hypertrophy in response to transverse aortic constriction (TAC), a model of pressure-overload-induced hypertrophy[131], implying that catecholamines are required for the induction of hypertrophy. *In vitro*, neonatal ventricular cardiomyocytes treated with NE induced hypertrophy as depicted by increased myocytes surface area and elevated protein/DNA ratio[132].

First demonstrated by Simpson et *al*., NE was shown to induce cardiac hypertrophy *via* the α1-adrenergic receptor, a seven-transmembrane-spanning receptor coupled to heterotrimeric G proteins of the $G_{\alpha q/\alpha 11}$ subfamily [133–136]. Besides, NE also activate β-adrenergic receptors coupled to Gαs, which stimulate L-type Ca^{2+} currents resulting in elevated intracellular Ca^{2+}[136]. High cytosolic Ca^{2+} concentration initiate Ca^{2+}-dependent signaling pathways, including pro-hypertrophic cascades that induce hypertrophic genes such as myocyte enhancer factor (MEF2) and GATA Binding Protein 4 (GATA4)[137,138]. (Signaling pathways involving Ca^{2+}-dependent cascades in cardiac hypertrophy will be discussed in section 1.2.5.3.).

Besides NE, other humoral factors such as angiotensin-II (Ang-II) and endothelin 1 induce cardiac hypertrophy by acting through the α1-adrenergic receptor [139–143].

1.2.4.1. Regulation of hypertrophy by Angiotensin-II

In addition to the adrenergic system, hypertrophy is regulated by the renin-angiotensin system, *via* its main effector, angiotensin II (Ang-II) [144,145].

Several studies demonstrated that the hypertrophic effects of Ang-II are due to its hypertensive and vasoconstrictive actions. Besides, Ang-II has been shown to be locally synthesized in the heart, serving as an autocrine molecule[145–148]. Ang-II is released from neonatal cardiomyocytes subjected to mechanical stretch, thus activating the angiotensin type 1 receptor that is associated with several downstream protein kinases, ultimately leading to hypertrophy[149]. Similarly, treating neonatal cardiomyocytes with Ang-II stimulated the reactivation of fetal gene expression, enhanced protein synthesis, and promoted the formation of new myofibrils [146,147]. Moreover, mice subjected to pressure overload exhibited elevated cardiac Ang-II in the hypertrophied hearts, suggesting that hypertrophy was developed due to the local action of Ang-II[145,150]

1.2.4.2. Regulation of hypertrophy by endothelin 1

Endothelin-1 (ET-1) is a potent 21 amino acid vasoconstrictive peptide secreted by endothelial cells and cardiomyocytes[151]. It acts by binding to two disparate receptors: ET_AR and ET_BR, which show extensive tissue distribution, including the myocardium[151]. It has been demonstrated that locally produced ET-1 partakes in cardiac remodeling in an autocrine/paracrine way[152]. Archer et *al.* showed that infusion of endothelin-1 in adult rats *in vivo* induced cardiac hypertrophy as depicted by increased mRNA expression of fetal genes[141]. Similarly, treating neonatal cardiomyocytes with ET-1 increased myocyte surface area and enhanced protein synthesis[141]. Moreover, aortic-banded rats exhibited elevated levels of ET-1 associated with cardiac hypertrophy[153,154].

1.2.5. Signaling pathways implicated in the development of cardiac hypertrophy

Although the molecular cascades behind cardiac hypertrophy have been extensively investigated, several unchartered territories still need to be examined to uncover mechanisms by which their therapeutic targeting could translate to the treatment of cardiac hypertrophy and heart failure. Studies unveiled several signaling pathways that take part in the induction of cardiac hypertrophy including, but not limited to, Gq-phospholipase C-diacylglycerol (DAG)/ Protein Kinase C (PKC)[155–157], insulin-like growth factor-I (IGF-I), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT), Ca^{2+}-dependent pathways that involve the calmodulin kinase CaMKII and its downstream target histone deacetylase 4 (HDAC4) and MEF2, and a cascade involving the Ca^{2+}calmodulin-dependent serine/threonine phosphatase, and its downstream target, nuclear factor of activated T cells (NFAT)[158,159]. Neonatal cardiomyocytes were used as the mainstay model system to study signaling mechanisms involved in cardiac hypertrophy. However, a better understanding of the complexities linked to the hypertrophic response has been acquired from genetically modified animal models.

1.2.5.1. Gq-phospholipase C-diacylglycerol (DAG)/ Protein Kinase C (PKC)

Hormones and neurotransmitters act by binding to membrane receptors coupled to heterotrimeric G proteins, known as G-protein coupled receptors (GPCR). G proteins, consisting of three

subunits, α, β, and γ, possess GTPase activity and serve to convert the external stimuli into intracellular signaling pathways [160]. Stimulation of GPCRs by external stimuli induces the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) [160]. This results in the dissociation of Gα from Gβγ, stimulating the downstream targets[160].

Among the G proteins, Gαq-coupled receptor has been shown to mediate signaling events implicated in the development of pathological cardiac hypertrophy[161,162]. Adam et *al.* demonstrated that adenovirus-based overexpression of Gαq receptor in neonatal cardiomyocytes resulted in enlarged cardiomyocytes surface area and elevated ANP mRNA levels[161]. Furthermore, sustained cardiac-specific overexpression of the Gαq receptor *in vivo* led to cardiac hypertrophy and subsequent decompensation to heart failure as manifested by cardiomyocytes apoptosis[161,163]. In support of these data, transgenic mice with cardiac-specific constitutively active α1 adrenergic receptors developed cardiac hypertrophy that was depicted by increased heart-to-body weight ratio and elevated ANP mRNA levels[164].

The downstream signaling cascades involved in Gαq-mediated cardiac hypertrophy include PKC and Ca^{2+}. Gαq activates phospholipase C, which fragments phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG)[165]. While DAG stimulates the serine/threonine PKC, IP3 binds to IP3R on the sarcoplasmic reticulum resulting in the massive release of Ca^{2+} into the cytosol[166,167]. Increased cytosolic Ca^{2+} concentration activates the calcineurin-NFAT pathways, which ultimately results in the development of cardiac hypertrophy[158,168,169]. In a study pinpointing the downstream proteins that mediate Gq-stimulated hypertrophy, it was discerned that the mRNA and protein expression level of PKC was elevated[162,170]. Stimulating cardiomyocytes with α1-adrenergic agonists (NE or ET-1) triggered the translocation and activation of PKC[171]. PKC is a serine/threonine kinase of diverse isoforms divided into conventional Ca^{2+}-dependent isoforms (PKCα, βI, βII, and γ) and Ca^{2+}-independent novel (ε, θ, η, and δ) and atypical (ζ, ι, ν, and μ) isoforms [93]. Although several isoforms (e.g., PKCβ and PKCγ) were detected in the myocardium[172–174], PKCα was shown to be the major predominant isozyme in the mouse and rabbit heart[175, 176]. PKC is implicated in cardiac contractility and Ca^{2+} cycling[174]. Transgenic mice overexpressing PKCα exhibited ventricular dysfunction associated with altered Ca^{2+} homeostasis attributed to PKC-dependent modifications in the phosphorylation of sarcoplasmic reticulum Ca^{2+} ATPase-2 (SERCA-2) and phospholamban (PLB)[177]. Likewise, pharmacological inhibition of PKCε enhanced cardiac

contractility and reduced hypertrophy and excessive fibrosis in rats with hypertension-induced cardiac hypertrophy[176,178]. Clinically, PKCα was upregulated in the left ventricle of patients with aortic stenosis[179]. Altogether, these data indicate that PKC is implicated in cardiac contractility and Ca^{2+} handling/homeostasis, which, when altered, lead to cardiac hypertrophy that eventually transitions to heart failure.

1.2.5.2. Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway

PI3K is a serine/threonine kinase that phosphorylates and regulates multiple physiological responses, including proliferation, trafficking, and cell growth and survival[180]. P110α isoform of PI3K, which couples to tyrosine kinase receptor (e.g., insulin and insulin-like growth factor I (IGF-I) receptors), is implicated in physiological hypertrophy and is shown to play a cardioprotective role *via* its downstream effector serine-threonine kinase, Akt (or Protein Kinase B)[181]. In contrast, the p110γ isoform of PI3K is activated by the GPCR and mediates pathological cardiac hypertrophy[180,182]. In the left ventricles of mice subjected to TAC-induced cardiac hypertrophy, elevated PI3K(p110γ) activity was detected[183]. By contrast, PI3K(p110α) activity remained unaffected, suggesting that PI3K(p110γ) isoform mediates pathological hypertrophy[183]. Consistent with these data, homozygous knockout of PI3K (p110γ) attenuated pathological hypertrophy and reduced fibrosis in mice subjected to isoproterenol-induced hypertrophy[184].

Initial evidence of the role of PI3K(p110α) in physiological hypertrophy emanated from a study demonstrating that mice with cardiac specific-constitutively active PI3K(p110α) exhibited enlarged heart size associated with normal cardiac function and absence of fibrosis[185]. Employing a transgenic approach, mice with homozygous knockout of PI3K(p110α) displayed hypertrophy in response to pressure overload (TAC) but not swimming exercise training[180]. The latter hypertrophic response was associated with cardiac dysfunction manifested by reduced fractional shortening and the development of interstitial fibrosis[180].

1.2.5.3. The Ca^{2+}-dependent pathways: Calcineurin-NFAT and CaMKII-HDAC4-MEF2 pathways

Normal intracellular Ca^{2+} cycling and homeostasis are essential to preserve cardiac excitability and contractility[186,187]. However, an imposed hemodynamic load (hypertension) alters Ca^{2+}

cycling and results in increased intracellular Ca^{2+} that induces a Ca^{2+}-dependent pathway, ultimately leading to pathological cardiac hypertrophy[187]. $Ca_V1.2$ channel is the main pathway of Ca^{2+} entry into the cytosol[188,189]. The origin of Ca^{2+} to activate Ca^{2+}- dependent hypertrophic cascades was presumed to be LTCC as increased $Ca_V1.2$ current density in transgenic mice overexpressing α1C was sufficient to develop cardiac hypertrophy that eventually progressed to heart failure[190–192]. It was then hypothesized that $Ca_V1.2$ channels located exclusively in the caveolae represent the Ca^{2+} source that initiates the hypertrophic response[193,194]. However, an opposing study revealed that caveolae-specific overexpression of LTCC was incapable of developing a hypertrophic response[195]. Until now, the source of Ca^{2+} initiating the hypertrophic signaling is still disputable.

Despite the controversial outcomes regarding the source of Ca^{2+} initiating the hypertrophic cascades, elevated intracellular Ca^{2+} binds to calmodulin to activate two major Ca^{2+}-dependent signaling pathways: Ca^{2+}/calmodulin-calcineurin-NFAT and the Ca^{2+}/calmodulin-dependent kinases-MEF2[159,196]. Both pathways are involved in the transcriptional regulation of cardiac genes implicated in the induction of pathological cardiac hypertrophy[197].

1.2.5.3.1. Calcineurin-NFAT pathway

Calcineurin is a Ca^{2+}-calmodulin-activated serine/threonine phosphatase activated by Ca^{2+}-calmodulin **(Figure 2)** [198,199]. Calcineurin dephosphorylates and activates its downstream target, NFAT, which translocates from the cytosol to the nucleus[198,199]. NFAT, in turn, associates and work with GATA4, a cardiac-restricted zinc finger transcription factor, to induce the transcription of hypertrophic genes (e.g., fetal gene program), hence initiating pathological hypertrophy[198,199]. The pro-hypertrophic role of calcineurin and NFAT was reported *in vivo* as transgenic mice overexpressing cardiac-specific constitutively active calcineurin, or NFAT exhibited cardiac hypertrophy associated with interstitial fibrosis and fetal genes expression **(Figure 2)** [158]. However, these features were reversed upon treatment with the calcineurin inhibitor, cyclosporin[158]. Likewise, knockout of the NFAT gene inhibited the hypertrophic response in mice subjected to pressure overload hypertrophy induced by TAC, indicating that NFAT is an essential mediator of calcineurin-induced hypertrophy[200]. These data were in conformity with *in vitro* results demonstrating that adenovirus-mediated inhibition of calcineurin attenuated α1-adrenergic agonist-induced cardiac hypertrophy in neonatal cardiomyocytes[201].

Clinically, calcineurin activity and protein expression were elevated in the myocardium of patients with pressure-overload hypertrophy secondary to aortic valve stenosis[202]. These findings imply that the calcineurin-NFAT pathway is a key contributor to the initiation of pathological cardiac hypertrophy, and its inhibition might be a promising therapeutic target to treat patients with cardiac hypertrophy.

1.2.5.3.2. CaMKII-HDAC4-MEF2 pathway

Calmodulin kinase II (CaMKII) is a serine-threonine kinase that phosphorylates and modulates proteins implicated in Ca^{2+} cycling and homeostasis such as phospolamban[203], L-type Ca^{2+} channels[204,205], and ryanodine receptor[206,207]. Four distinct genes encode CaMKII, giving rise to four CaMKII isoforms (α, β, δ, γ), with CaMKIIδ being the predominant isoform in the heart[208–211]. Once activated by Ca^{2+}/Calmodulin, CaMKII phosphorylates the transcriptional repressor HDAC4, triggering its dissociation from MEF2, a transcription factor known to repress transcription activity **(Figure 2)** [212]. In the nucleus, HDAC4 binds to and prevents MEF2 activation [212–214]. Phosphorylation of HDAC4 by CaMKII induces its nuclear export to the cytosol, hence relieving MEF2 repression and enhancing transcriptional activation **(Figure 2)** [212–214]. CaMKII has been shown to take part in pathological hypertrophy[209–211]. Genetic deletion of CaMKIIδ attenuated the development of hypertrophy and maladaptive remodeling in mice subjected to pressure overload stress secondary to transverse aortic constriction[211,215]. Likewise, pharmacological inhibition of CaMKII reversed ET1-induced hypertrophy in neonatal cardiomyocytes[216]. Furthermore, in studies employing a transgenic approach, mice overexpressing constitutively active CaMKII exhibited elevated hypertrophic markers associated with alterations in Ca^{2+} cycling[214,217]. These data indicate that CaMKII is a prominent modulator of cardiac hypertrophy.

Despite the efforts, treating cardiac hypertrophy by targeting a specific molecular mechanism would be a challenging aspect given its pleiotropic effects on all body organs.

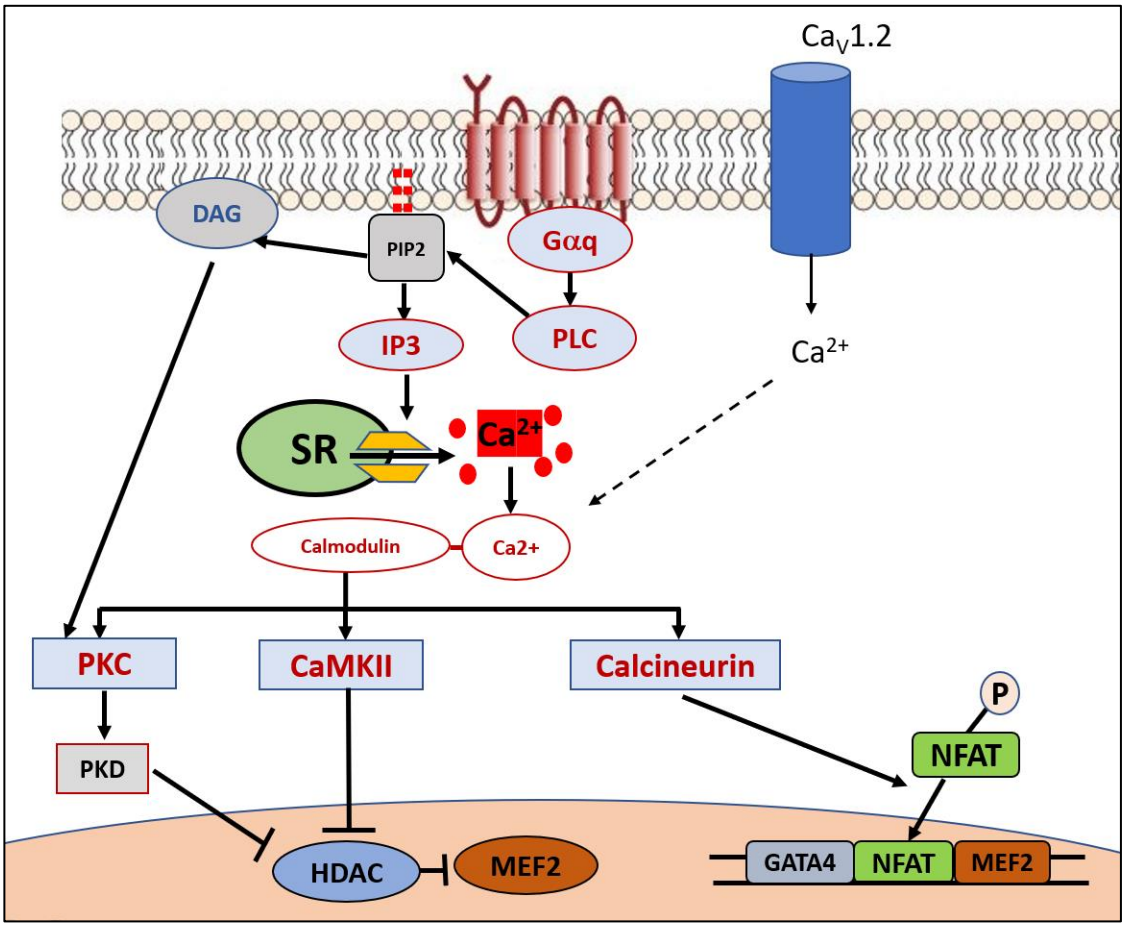

Figure 2: Signaling pathways implicated in cardiac hypertrophy.

1.3. Ca^{2+} channels

1.3.1. The physiological role of Ca^{2+} in the body

Calcium is a major second messenger that regulates diverse biological processes including, but not limited to, gene transcription, cell motility, muscle contraction, and exocytosis[218,219]. In the heart, normal intracellular Ca^{2+} cycling homeostasis is crucial to preserve cardiac excitability, contractility, and gene expression regulation[186]. Ca^{2+}-dependent signaling is highly modulated and determines the force of cardiac muscle contraction [186].

1.3.2. Cardiac action potential

The concatenation and synchronous contraction of the atria and ventricles necessitate the prompt activation of specialized subtypes of cardiac cells namely the pacemaker cells and non-pacemaker cardiac muscle cells[220]. Two major processes occur in the heart: cardiac conduction and cardiac contraction. Cardiac conduction is initiated by the spontaneous electrical activity of the pacemaker cells located in sinoatrial (SAN) node and atrioventricular (AVN) node[221]. The automaticity of the pacemaker cells engenders an action potential that propagates throughout the heart, ultimately resulting in ventricular contraction[221]. Action potentials are generated by the passive flow of ions across the membrane *via* ion channels, which results in membrane potential changes[222]. The cardiac action potential encompasses five phases (**Figure 3**) that represent the differences in the ion currents flowing in each phase during the cardiac cycle[220].

A cardiac action potential is initiated when the pacemaker cells generate an action potential that passes down the conduction system to reach the cardiomyocytes[220]. This electrical impulse stimulates the opening of the voltage-gated Na^+ channel, resulting in sodium influx that generates a rapid depolarization phase, **phase 0**, with a membrane potential increasing from -90 mV to +30 mV[220]. Once the positive membrane potential is reached, **phase 1** is initiated wherein the K^+ channels (transient outward K^+ currents) are activated while the previously opened voltage-gated sodium channels are inactivated, resulting in partial repolarization of the membrane, which triggers the initiation of phase 2[220]. **In phase 2**, also termed the plateau phase, voltage-gated L-type Ca^{2+} channels ($Ca_V1.2$) open, prompting Ca^{2+} influx which counterbalances the K^+ efflux, thus maintaining the positive depolarization membrane potential at around +50mV[220]. Accelerated repolarization then follows in **phase 3** due to Ca^{2+} channels inactivation and the dominance of K^+ efflux through the opening of the voltage-gated K^+ channels (delayed rectifiers; IKs, IKr, and IKur) [220,223]. **Phase 4** depicts the resting membrane potential during diastole in non-nodal cells wherein K^+ ions remain to flow out of the cell *via* the rapid (inward) rectifying K^+ channels (Kir)

[220]. Ion fluxes become balanced due to the activity of Na^+/K^+ ATPase, ultimately generating a negative potential of -90 mV[220].

Schematic diagram showing the 5 phases of the ventricular action potential, highlighting the depolarization and repolarization phases as well as the ion currents dominating in each phase[224]

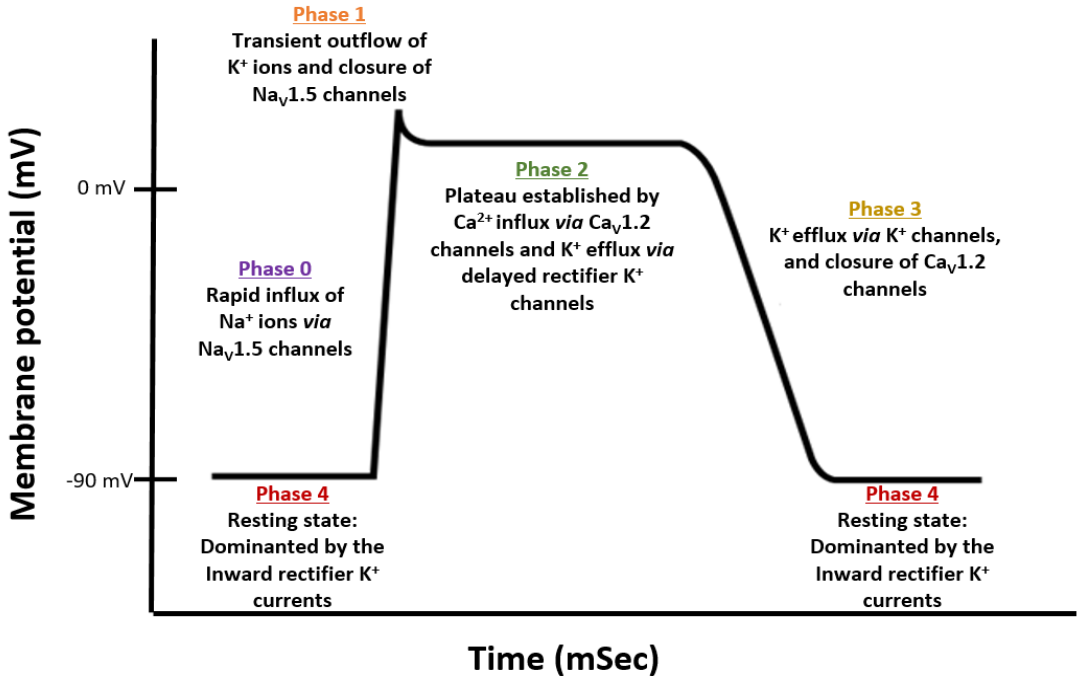

Figure 3: Action Potential in Cells

1.3.3. Role of Ca^{2+} in Cardiac excitation-contraction coupling

Excitation-contraction coupling is a mechanism where electrical excitation of the sarcolemma is converted into cardiac muscle contraction, which generates the force required to pump blood to the body[186,225]. This process is initiated when the membrane depolarization produced by an action potential triggers the opening of the voltage-gated L-type Ca^{2+} channels (LTCC), resulting in extracellular Ca^{2+} influx into the cell **(Figure 4)**[186,226]. This Ca^{2+} entry stimulates Ca^{2+}

release *via* the opening of the ryanodine receptors (RyR) in the sarcoplasmic reticulum (SR) through a mechanism termed Ca^{2+}-induced Ca^{2+} release (CICR)[186,226,227]. The combination of Ca^{2+} entry and Ca^{2+} release results in the increase of the cytosolic Ca^{2+}, which binds to the myofilament protein troponin and activates the contractile machinery[186,226,227]. Cardiac cells, also known as cardiomyocytes, are composed of long, fibrous proteins termed myofibrils **(Figure 5)**[228]. Myofibrils consist of repeated units named sarcomeres, which are the main contractile component of the cardiomyocyte, granting the cardiac muscle its unique striated appearance[228]. Sarcomeres are made up of the thick myosin filaments and the thin filament that consists of actin, troponin (troponin C, I, and T), and tropomyosin **(Figure 5)** [228]. When Ca^{2+} binds to Troponin-C, it triggers a conformational change of the troponin complex, exposing the active sites on actin and making it accessible to the myosin head[229]**.** Myosin then binds to actin *via* cross-bridging and, in the presence of Adenosine triphosphate (ATP), pulls actin towards the center of the sarcomeres, resulting in sarcomere shortening and cardiac contraction[229]. After contraction, intracellular Ca^{2+} concentration is removed from the cytosol through either the uptake to the SR *via* the Sarcoplasmic Ca^{2+} ATPase pump (SERCA) or expulsion to the extracellular *via* the sodium/ Ca^{2+} exchange (NCX) and sarcolemma Ca^{2+} ATPase[186,230]. The decrease in Ca^{2+} concentration enables Ca^{2+} to dissociate from troponin, resulting in the filaments reinstating to their initial position, ultimately relaxing the cardiac muscle[186].

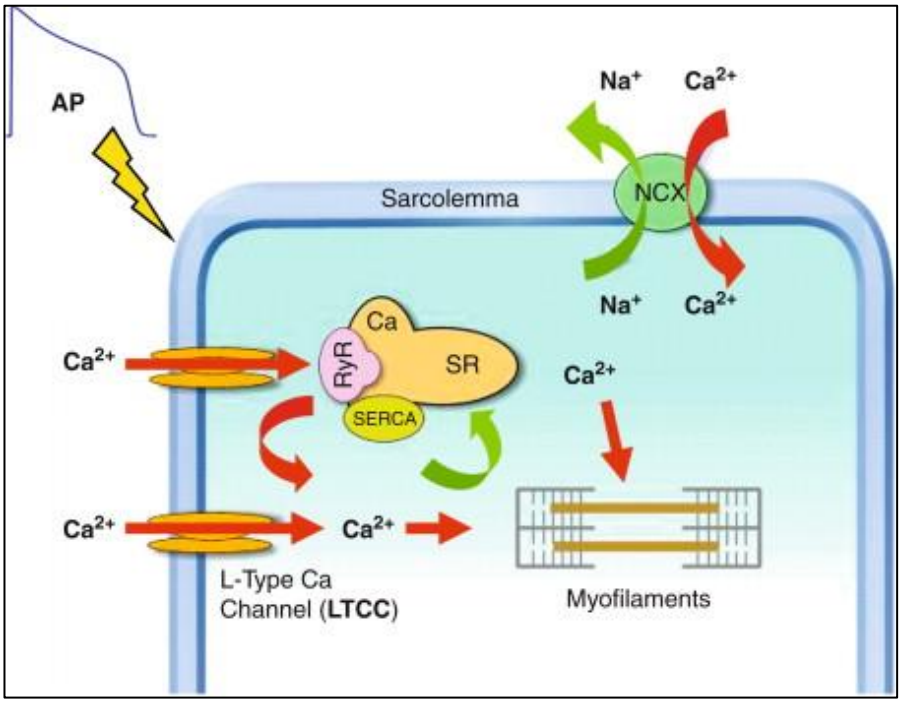

Figure 4: Excitation-contraction coupling in ventricular cardiomyocytes.

The activation of the voltage-gated L-type Ca^{2+} channels at the T-tubules following depolarization of the sarcolemma favor Ca^{2+} influx into the cytosol. Ca^{2+} entry triggers further Ca^{2+} release from the sarcoplasmic reticulum to the cytosol through the Ryanodine receptors 2 (RYR2). In turn, intracellular Ca^{2+} binds to troponin and initiates cardiac contraction. During relaxation, removal of Ca^{2+} is facilitated by SERCA2a, NCX and PMCA, resulting in Ca^{2+} dissociation from Troponin and muscle relaxation.

NCX: Na^{+}/Ca^{2+} exchanger; SR: sarcoplasmic reticulum; LTCC: L-type Ca^{2+} channel; RyR2: Ryanodine receptor 2; SERCA2a: Sarcoplasmic reticulum Ca^{2+} ATPase 2. Figure taken from Shiels et *al*., 2011[231]**.**

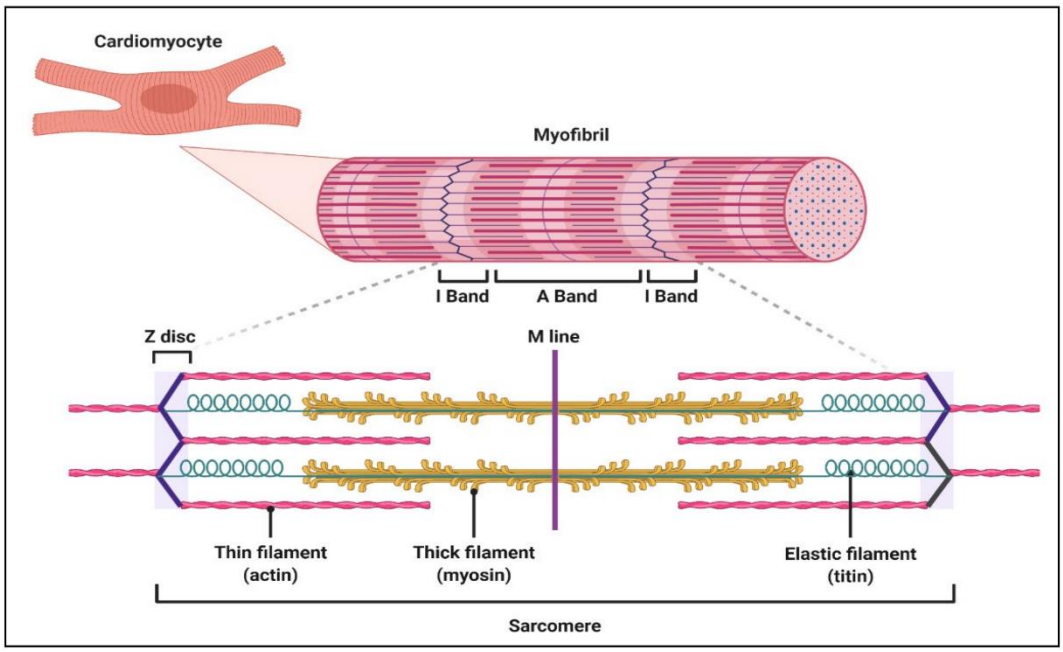

Figure 5: A schematic diagram depicting the structure of an individual sarcomere.

Sarcomeres is the functional unit of cardiomyocytes and is represented as the region between two Z-lines. Each sarcomere contains thin (actin) and thick (myosin) filaments and titin. Figure taken from Santiago et *al.*, 2010[232].

1.3.4. Voltage-gated Ca^{2+} channels

In resting condition, free intracellular Ca^{2+} concentration in the cytosol lies at ~100 nM [233,234]. Upon membrane depolarization, voltage-gated Ca^{2+} channels open [235] and enable Ca^{2+} influx into the cell, thus increasing the cytosolic Ca^{2+} concentration to the micromolar range[234,236]. Increased cytosolic Ca^{2+} concentration serves as a second messenger that triggers diverse physiological events such as muscle contraction, cell excitability, neurotransmitter release, and gene expression [234,237,238]. In such wise, voltage-gated Ca^{2+} channels serve as signal transducers by transforming the electrical signal of the depolarizing signals and action potential to cytosolic Ca^{2+} transients[234,237,238]. Voltage-gated Ca^{2+} channels are transmembrane proteins located in excitable cells (e.g., neuron and muscle cells)[234,235] and non-excitable cells (e.g., epithelial cells, red blood cells, and some endocrine cells)[239,240]. Voltage-gated Ca^{2+} channels are categorized according to their pharmacological and electrophysiological characteristics such as the activation/inactivation threshold, as well as their amino acid sequence identity, which engender two categories: Low-voltage activated (LVA) and High-voltage activated (HVA) Ca^{2+} channels [235,241]. LVA channels activate at a more hyperpolarized potential range (less than -

40 mV) [242], inactivate rapidly [235,243], and are insensitive to Ca^{2+} channel blockers (CCBs)[235,243]. In contrast, HVA channels open at potentials more positive than -40 mV [234] and exhibit slow voltage-dependent inactivation [235,243].

Owing to the amino acid sequence identity of the pore-forming subunit, $Ca_V\alpha1$, HVA channels are further subdivided into Ca_V1 (L-type) and Ca_V2 (N, P, Q, and R types), while LVA Ca^{2+} channels encompass only Ca_V3 (T-type) Ca^{2+} channels (**figure 6)**[241,243].

In the heart, two types of Ca^{2+} channels predominate L-type Ca^{2+} channels (LTCCs) and T-type Ca^{2+} channels (TTCC)[244,245]. L-type Ca^{2+} channels form the Ca_V1 category. They are termed Long-lasting or L-type Ca^{2+} channels because of their slow voltage-dependent inactivation and are long-lasting when barium is employed as the charge carrier[244,245].

The Ca_V1 family encompasses four isoforms: $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$, and $Ca_V1.4$ channels. $Ca_V1.1$ is expressed primarily in the skeletal muscle, where it partakes in skeletal excitation-contraction coupling[246–248], while the expression of $Ca_V1.4$ is limited to the retina[249,250]. $Ca_V1.3$ channels are expressed in most cells that also express $Ca_V1.2$, such as pancreatic β cells, brain, and adrenal glands[248,251]. In the heart, the expression of the $Ca_V1.3$ channel is restricted to the atrial myocytes, SAN, and AVN[252,253], where it partakes in the pacemaking function and automaticity[252,253]. $Ca_V1.2$ is expressed in endocrine cells[254], neuronal cells[255], cardiac[246,253] and skeletal muscle[256]. In the heart, the $Ca_V1.2$ channel predominates in the ventricles[253]. It serves as a determinant of the duration of the action potential plateau phase[253] and is responsible for initiating the cardiac excitation-contraction coupling by triggering the CICR mechanism[226,253]. $Ca_V1.2$ channels are the target of calcium channel blockers (CCBs), including dihydropyridines, phenylalkylamines, and benzothiazepines [235,243].

Ca_V2 Ca^{2+} channels are mainly located in the neurons. P/Q-type channels ($Ca_V2.1$) reside primarily in the Purkinje cells of the brain[235,257], cerebellar granule neurons[235], and presynaptic terminal of the neurons[234,258,259]. They are involved in neurotransmitter release, synaptic transmission[260], exocytosis stimulation[261,262], and neural excitability[259,263]. Neuronal or N-type Ca^{2+} channels ($Ca_V2.2$) are located predominantly in the central and peripheral neurons[264,265]. Ca^{2+} influx through $Ca_V2.2$ partake in synaptic transmission[265,266], neurotransmitter release[267], and spinal nociception[268]. While P/Q and N-type Ca^{2+} channels can be blocked by toxins[269,270], residual or R-type ($Ca_V2.3$) Ca^{2+} channels are distinguished

by their resistance to all types of Ca^{2+} channel blockers (dihydropyridines and toxins)[271,272]. Similar to P/Q and N-type channels, R-type Ca^{2+} channels also contribute to synaptic transmission and neurotransmitter release[273].

Transient or T-type Ca^{2+} channels (TTCCs) represent the Ca_V3 family, which includes $Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$ isoforms[274,275]. They are characterized by their insensitivity to CCBs and toxins[274,275]. TTCCs are found in neurons, endocrine secretory cells[276,277], kidney, heart[278], and brain[275].

In the heart, $Ca_V3.1$ and $Ca_V3.2$ are prominent in the SAN and Purkinje cells[279–281] and are hence crucial for maintaining conduction and pacemaker activity required for firing an action potential[282]. The activation of TTCCs at hyperpolarized potential (-60 mV) facilitates the diastolic pacemaker depolarization[275,283]. Moreover, TTCCs have been shown to be re-expressed in ventricles of animal models encountering cardiac diseases such as cardiac hypertrophy and myocardial infarction (MI) [284]. For example, T-type Ca^{2+} currents were detected in hypertrophied ventricular myocytes isolated from adult feline[285] and adult rat heart[286]. In studies employing myocardial infarction models, T-type Ca^{2+} currents reappeared during the healing process post-MI[287].

	Gene	Protein	α_1 name	Physiological name
HVA associate with β and $\alpha_2\delta$	*CACNA1S*	$Ca_V1.1$	α_1S	L-type
	CACNA1C	$Ca_V1.2$	α_1C	
	CACNA1D	$Ca_V1.3$	α_1D	
	CACNA1F	$Ca_V1.4$	α_1F	
	CACNA1A	$Ca_V2.1$	α_1A	P/Q-type
	CACNA1B	$Ca_V2.2$	α_1B	N-type
	CACNA1E	$Ca_V2.3$	α_1E	R-type
LVA	*CACNA1G*	$Ca_V3.1$	α_1G	T-type
	CACNA1H	$Ca_V3.2$	α_1H	
	CACNA1I	$Ca_V3.3$	α_1I	

ıd naming.

Scheme showing the subfamilies of Voltage-gated Ca^{2+} channels: Ca_V1 (L-types), Ca_V2 (neuronal types), and Ca_V3 (T-types). Adapted from Heyes et *al*., 2015[288].

1.3.5. L-type Ca^{2+} channels: $Ca_V1.2$

1.3.5.1. Role of $Ca_V1.2$ channels in the different body organs

$Ca_V1.2$ is an isoform of the Ca_V1 L-type Ca^{2+} channels family that are characterized by their high voltage activation threshold[246,253]. $Ca_V1.2$ Ca^{2+} channels are broadly expressed in endocrine cells[289–291], heart[246,253], and brain[292]. Studies have demonstrated that Ca^{2+} entry *via* $Ca_V1.2$ Ca^{2+} channels contributes to exocytosis and insulin secretion in pancreatic β cells [289–291]. Moreover, $Ca_V1.2$ Ca^{2+} channels have been shown to partake in several physiological processes such as synaptic transmission[293], dopamine release[294], and memory-related processes[292]. In smooth muscle cells, $Ca_V1.2$ Ca^{2+} channels are responsible for contraction such as in blood vessels, uterus, and intestine[295].

1.3.5.2. Distribution and physiological role of $Ca_V1.2$ channels in the heart

$Ca_V1.2$ is expressed throughout the heart, including the SAN[296], AVN[297], atria[297], and the ventricles[296–298]. Its expression is more abundant in the ventricles than in other compartments[297]. In the pacemaker cells, $Ca_V1.2$ contributes to the Ca^{2+}-dependent upstroke of the action potential[244,253].

In the ventricular myocytes, $Ca_V1.2$ is localized in the T-Tubules in close proximity to Ryanodine receptor 2 [299,300]. Ca^{2+} entry *via* $Ca_V1.2$ initiates the CICR mechanism responsible for stimulating cardiac contraction [299,300]. Thus, $Ca_V1.2$ channels on the T-tubules dictate the extent of the cardiac contraction[299].

Another set of $Ca_V1.2$ channels was shown to localize in the caveolae[193,301–303], a subdomain of invaginated plasma membrane lipid rafts known to play a role in endocytosis and cell signaling[304,305]. Coimmunoprecipitation and immunofluorescence studies have demonstrated that Caveolin and $Ca_V1.2$ are colocalized in ventricular cardiomyocytes[306,307]. The function of $Ca_V1.2$ in the caveolae remains unclear. Some findings indicated that caveolar $Ca_V1.2$ is involved in excitation-transcription coupling such as mediating Ca^{2+}-induced activation of nuclear factor of activated T cells (NFAT) in adult feline ventricular myocytes[193]. Moreover, Balijepalli et *al.* have shown that the localization of $Ca_V1.2$ in the caveolae is essential for its regulation by β2-adrenergic signaling[307]. However, whether the subcellular localization of $Ca_V1.2$ in the caveolae contributes to the excitation-contraction coupling is still unclear.

1.3.5.3. Structure and function of Cav1.2 subunits

LTCCs were first identified in the skeletal muscle tubules [308]. Biochemical analysis depicted them as oligomeric protein complexes formed by the $Ca_V\alpha1$ subunit which forms the pore, associated with the auxiliary subunits $Ca_V\alpha2\delta$ and $Ca_V\beta$ [298,309] **(Figure 7).**

In the heart, cardiac $Ca_V1.2$ constitutes of $Ca_V\alpha1C$, $Ca_V\beta$ (mainly $Ca_V\beta2$ and $Ca_V\beta3$), and $Ca_V\alpha2\delta1$[298,309].

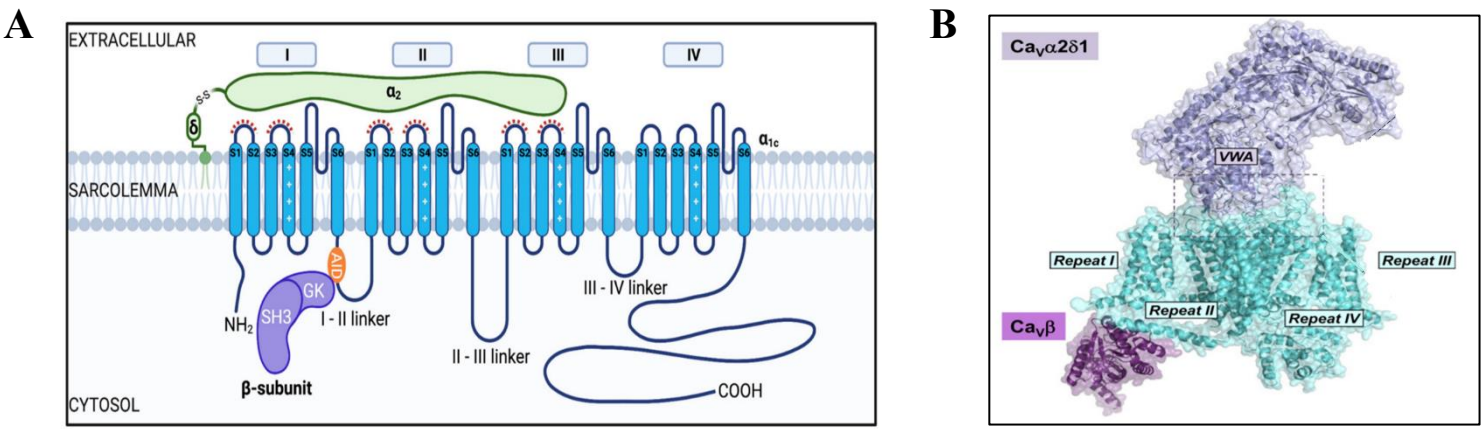

Figure 7: (A) Cartoon showing the structure of L-type $Ca_V1.2$ calcium channel. (B) Cartoon depicting the Cryo-EM structure of the $Ca_V1.1$ channel.

The pore-forming Cavα1 subunit is composed of four homologous repeat domains (I-IV), each containing six transmembrane segments (S1–S6)[310]. The voltage-sensing domain (VSD) is constituted by S1–S4 segments and the pore domain is formed by S5–S6 form. $Ca_V\beta$ subunit is composed of SH3, HOOK, and GK domain. Interaction of $Ca_V\beta$ with the $Ca_V\alpha1C$ occurs between the GK domain on $Ca_V\beta$ subunits and the alpha interaction domain (AID) on the I-II linker. $Ca_V\alpha_2\delta$ subunit represents the extracellular subunit of the channel[311]. Figure 7 (A) taken from Westhoff et *al*., 2021[299]. Figure 7 (B) adapted from Briot et *al*., 2017 [310]

1.3.5.3.1. Cavα1C

$Ca_V\alpha1C$ is the pore-forming subunit that accounts for Ca^{2+} influx and serves as the principal functional component of the $Ca_V1.2$ channel[254,312]. It is a 250KDa protein encoded by the *CACNA1C* gene[254,311,312]. $Ca_V\alpha1C$'s prominent role was demonstrated by a study showing that homozygous knock-out of *CACNA1C* gene resulted in lethality[313].

$Ca_V\alpha1C$ is formed by 4 homologous domains (I–IV), each having six-transmembrane segments (S1-S6)[254,312,314]. The domains are linked by intracellular loops and flanked by cytoplasmic N- and C-termini, which encompass sites for modulation with regulatory proteins such as protein kinases (PKA, PKC, and Calmodulin kinase II)[315]. Moreover, calmodulin binds to the isoleucine-glutamine "IQ" domain in the C terminus of the $Ca_V\alpha1C$, constituting a Ca^{2+}-sensing machinery that acts *via* a negative feedback mechanism to control the inactivation of $Ca_V1.2$ channel, referred to Ca^{2+}-dependent inactivation (CDI)[316,317].

In every domain (I–IV), S4 acts as a voltage sensor wherein the positively charged arginine and lysine residues discern the membrane depolarization and instigate $Ca_V1.2$'s conformational change, accounting for the channel's activation[318], [319]. Moreover, 4 glutamate residues in the P-loop linking S5-S6 segments constitute the Ca^{2+} selectivity filter[320,321].

$Ca_V\alpha1C$ is sensitive to Ca^{2+} channel blockers (dihydropyridines, phenylalkylamines, and benzodiazepines), which are among the most employed molecules for treating cardiovascular diseases such as angina pectoris and hypertension[322,323]. Besides, $Ca_V\alpha1C$ undergo alternative splicing wherein $Ca_V1.2a$ is the predominant isoform in the cardiac muscle including exons 1a/8a/_9*/32/33, whereas $Ca_V1.2b$ isoform predominates in the smooth muscle and contains exons1b/8/9*/32/33[324,325].

1.3.5.3.2.Cavβ

$Ca_V\beta$ subunit is a cytoplasmic protein with a molecular weight of ~55-65KDa[326]. Four $Ca_V\beta$ isoforms ($Ca_V\beta1$- $Ca_V\beta4$) emanate from four distinct genes (*CACNB1-CACNB4*), with some undergoing alternative splicing[326,327]. $Ca_V\beta1$ and $Ca_V\beta3$ were found in human and canine ventricles, while $Ca_V\beta4$ was detected specifically in young rat atrial myocytes[327,328]. $Ca_V\beta2$ is the predominant isoform in cardiomyocytes[326,329]. The importance of $Ca_V\beta2$ subunit in the heart was confirmed by studies showing that mice with homozygous knockout of the CACNB2 gene died at the embryonic stage due to cardiac impairment which was depicted by defective heart

tubes, reduced cardiac contractions, and pericardial effusion[330]. Moreover, mutations in Cavβ2 were linked to brugada syndrome [331–333] and detected in patients with short QT interval and elevated ST segment[334] associated with sudden cardiac death (SCD).

Cavβ subunit is recognized to be a part of the membrane-associated guanylate kinase (MAGUK) scaffolding proteins[326,335]. MAGUK proteins consist of the Src homology 3 domain (SH3) and guanylate kinase (GK) domain connected by the HOOK region and bordered by the N and C termini[336,337]. SH3 and GK domains form the conserved interior section of Cavβ, while the HOOK region, N-, and C termini are highly discrepant in length and amino acid constitution between the different Cavβ isoforms[336,337]. The GK domain contains a hydrophobic cleft known as the α1-binding pocket that binds with high affinity to the CaVα1-interacting domain (AID) in the I-II loop of the CaVα1 subunit[326,338,339]. Moreover, studies involving crystal structure showed that the GK domain of the Cavβ subunit encompasses sites that interact with other proteins besides CaVα1C, such as the Rad/Rem/Rem2/Gem/Kir (RGK) family of Ras-like GTPases that serve as potent inhibitors of LTCC[338,340]. Although structurally related, RGK proteins are subject to distinctive tissue expression. Rad (Ras associated with diabetes) and Rem are the predominant RGK member proteins in the heart[341,342]. Adenovirus-mediated overexpression of Rem or Rad blunted L-type Ca^{2+} currents (I_{Ca}) in embryonic[343] or adult[344] ventricular myocytes, resulting in reduced cardiac contractility[344].

1.3.5.3.3. Cavβ subunit is essential for normal channel function

Cavβ fine-tunes the channel's activity by enhancing the trafficking of CaVα1 subunit to the plasma membrane, hence fostering its cell surface expression and increasing the channel's current density (I_{Ca})[340,345,346]. Confocal imaging showed plasma membrane staining of CaV1.2 in embryonic kidney 293 cells (HEK293) transfected with CaVα1C and Cavβ2, but not CaVα1C alone [347]. In addition to that, Cavβ has been long recognized to modulate CaV1.2's gating properties[346]. In adult ventricular myocytes, adenovirus-mediated overexpression of Cavβ2 increased LTCC current density and enhanced voltage-dependent inactivation kinetics [348].

1.3.5.3.4. Cavα2δ1

Cavα2δ is a glycosylphosphatidylinositol (GPI)-anchored protein with a large extracellular domain composed of Cavα2 linked to the smaller Cavδ subunit by disulfide bonds[349,350]. First identified in the skeletal muscle, a single gene encodes the $\alpha_2\delta$ subunit that is subsequently translated into α_2 and δ[349,350]. In reducing conditions, the molecular weight of α2 and δ subunit was 150~KDa and 17-25 KDa, respectively[349]. By contrast, under non-reducing conditions, Cavα2δ1 migrates to 175 KDa, suggesting that Cavα2δ1 is bonded by disulfide bridges[349].

Four Cavα2δ isoforms (Cavα2δ1-Cavα2δ4) emanate from four mammalian genes (CACNA2D1–CACNA2D4) whose expression is identified in various cell types[351–354]. Cavα2δ4 is found in the retina[355] and endocrine cells[354]. Cavα2δ2 and Cavα2δ3 are widely expressed in the central nervous system[237,356]. Cavα2δ1 was first identified in the skeletal muscle[349–351]. It was then found that Cavα2δ1 is also expressed in endocrine cells[357], cardiac[358–360], and smooth muscle cells[361,362]. Indeed, Cavα2δ1 was detected as the predominant isoform in cardiomyocytes[359,363]. In the whole heart, mRNA expression of Cavα2δ1 is more abundant in the atrium than ventricles[364].

1.3.5.3.4.1. Role of Cavα2δ1 in regulating Cav1.2

In cardiac muscle, Cavα2δ1 is the main Cavα2δ isoform associated with Cav1.2[360,365]. Similar to the Cavβ subunit, Cavα2δ1 regulates the electrophysiological features of Cav1.2[366–368]. Studies exploiting recombinant HEK cells demonstrated that the co-expression of Cavα1C with Cavα2δ1 enhances the Cav1.2 peak current density by 5-10-fold and facilitates its activation at more physiological voltages[366–368]. The latter observation was further corroborated in a study employing mice with homozygous knockout of Cavα2δ1 gene[359]. Cardiomyocytes isolated from these mice exhibited~45% reduction in Cav1.2 current density and a positive shift in the activation potential[359]. The mechanism underlying Cavα2δ1-mediated facilitation of Cav1.2 channel activation was shown to be attributed to Cavα2δ1's action in elevating the energetic coupling of voltage-sensor domains I–III (VSD I–III) to the pore opening, ultimately resulting in a negative shift in the movements of VSD I–III[369]. Moreover, Bicer et *al.* showed that in mice subjected to Cavα2δ1 knockout, the myocardium displayed impaired cardiac contractions without signs of cardiac hypertrophy[359], indicating that Cavα2δ1 contributes to cardiac contractility[359].

1.3.5.3.4.2. Clinical importance of Cavα2δ1 in cardiac diseases

Human mutations in CACNA2D1 have been linked to cardiac dysfunction, particularly cardiac arrhythmias[370,371]. Three missense mutations were identified in patients with Brugada syndrome: S709N and Q917H located in the extracellular domain of Cavα2δ1, and D550Y residing in the CACHE domain[356,372]. The molecular mechanism by which the latter mutations result in short QT syndromes was unveiled in HEK293T cells[370]. Bourdin et *al.* demonstrated that the double mutant D550Y/Q917H decrease the trafficking of Cavα2δ1 to the plasma membrane, resulting in reduced L-type Ca^{2+} current density, which ultimately generates a short QT interval [370]. Moreover, a mutation (S956T) located in the extracellular domain of Cavα2δ1 was reported in patients with early repolarization syndrome associated with short QT interval [372].

1.3.5.3.4.3. Structure, post-translational modification, and non-canonical role of Cavα2δ1

The Cavα2δ subunit undergoes intricate post-translational modifications[349,373]. Cavα2δ gene is subjected to multiple processing events, including GPI-anchoring, glycosylation, and proteolytic translation into α2 and δ linked by disulfide bridge[349,373].

Cavα2δ1 subunit encompasses an N-terminal sequence that drives the subunit to the endoplasmic reticulum (ER), where the cleavage of the signal sequence occurs[311,349,374,375]. Cavα2δ1 also possesses a hydrophobic C-terminal domain that is cleaved and replaced by a GPI-lipid anchor in the ER lumen[311,349,374,375]. The phospholipid tail of the GPI endows mobility to Cavα2δ, allowing its anchorage to the ER membrane and subsequent trafficking to the Golgi and plasma membrane[349]. As the protein folds into its mature form in the ER, several disulfide bonds are formed between α2 and δ[349]. In the Golgi complex, glycosylation occurs on 16 asparagine residues[349,365,373]. The latter modification is essential for the trafficking of Cavα2δ1 to the cell surface. Localization of Cavα2δ1 at the cell surface is a requisite for fine-tuning LTCC peak current density[311]. Biochemical studies employing mutation approaches showed that substituting Arginine to Alanine residues at position 663 prevented the cell surface expression of Cavα2δ1 and abolished Cavα2δ1-mediated upregulation of L-type Ca^{2+} currents[373].

Through cryo-EM structure of Cav1.1 channel, deeper investigations into the structural intricacies of Cavα2δ1 has divulged the existence of a von Willebrand factor A (VWA) domain[376] and four CACHE domains[377] in the α2 segment of Cavα2δ1. VWA mediates extracellular protein-protein interaction *via* metal ion-dependent adhesion site (MIDAS) [374,376]. The latter interaction

involves divalent cation binding (Mg^{2+}, Mn^{2+}, or Ca^{2+}) by the five residues that form the MIDAS. Hence, in addition to their canonical role in regulating $Ca_V1.2$ channels, $Ca_V\alpha2\delta1$ serves a non-canonical role *via* interaction with several proteins such as thrombospondins, potassium channels, and **N**-methyl-D-aspartate (NMDA) receptors [357,358,378]. Indeed, $Ca_V\alpha2\delta1$ has been shown to partake in several physiological processes throughout the body *via* canonical and non-canonical functions, such as neuronal morphology, synaptic transmission, and insulin release[357,358]. Eroglu et *al*. showed that $Ca_V\alpha2\delta1$, *via* its VWA domain, interacts with thrombospondin 2 to enhance synaptogenesis[378]. A recent study set forth $Ca_V\alpha2\delta1$ as an interactive partner of low-density lipoprotein receptor-related protein 1, a protein that executes in a diverse range of processes such as protection against atherosclerosis, modulation of lipid metabolism, and protection against atherosclerosis[379, p. 1]. These studies identify $Ca_V\alpha2\delta1$ as a hub of interacting proteins that partake in various physiological processes.

In close proximity to VWA lies a gabapentin-binding site composed of 3 arginine residues[380,381]. Gabapentin is an anti-convulsant drug prescribed for multiple diseases such as neuropathic pain[380,381]. It has been thought that Gabapentin is a gamma-aminobutyric acid (GABA) analog[380,381]. However, further studies have shown that their anti-convulsant properties are not established through their interaction with GABA receptors but rather through binding to $Ca_V\alpha2\delta1$, which implies that $Ca_V\alpha2\delta1$ serves as a receptor of Gabapentinoids (Gabapentin and pregabalin), whose therapeutic targeting translates to pain relief[382,383].

1.3.6. Pathophysiology of $Ca_V1.2$

1.3.6.1. Role of $Ca_V1.2$ in cardiac pathophysiology

In response to membrane depolarization, $Ca_V1.2$ serves as the main pathway of Ca^{2+} entry into the ventricles, which determines the duration of the action potential plateau phase and is responsible for cardiac excitation-contraction coupling[315].

Owing to its prominent role in cardiac physiology, subtle alterations in the primary structure or function of $Ca_V1.2$ contribute to various cardiovascular diseases of either electrical phenotype, such as arrhythmias[361] and Timothy syndrome[384], or mechanical phenotype such as cardiac hypertrophy[385,386], and heart failure[39,298,387].

1.3.6.2. Arrhythmias

Cardiac arrhythmia is among the main causes of cardiac death[388,389]. It is caused by gain-of-function or loss-of-function mutations in the CACNA1C gene which encodes the $Ca_V\alpha1C$ subunit of $Ca_V1.2$ channel, resulting in $Ca_V1.2$ dysfunction[361]. Arrhythmia entails a broad scope of heart rate disorders and rhythm abnormalities, including, but not limited to, Brugada syndrome, short QT syndrome, and Timothy syndrome[390].

1.3.6.3. Timothy syndrome

Timothy syndrome is a multisystem disease characterized by prolonged QT interval, developmental delay, and syndactyly[391–394].. It is caused by mutations in *CACNA1C* gene, encoding for the $Ca_V\alpha1C$ subunit of $Ca_V1.2$ [391–394]. These mutations decelerate the inactivation of $Ca_V1.2$ channel, which leads to prolonged cardiac action potential[391–394].

1.3.6.4. Brugada syndrome

In contrast to Timothy syndrome, Brugada syndrome is caused by loss-of-function mutations in the CACNA1C (G490R, A39V, E1115K, R1880Q, V2014I, D2130N, E1829_Q1833, C1873Y, and E850del), CACNB2 (S481L), and CACNA2D (S709N, D550Y, Q917H) genes. These mutations reduce the L-type Ca^{2+} current, which generates a short QT interval[395,396]. Clinically, Brugada syndrome is delineated by elevated ST segment on the ECG and is linked to high risk of ventricular fibrillation[397].

1.4. Cardiac hypertrophy and Ca^{2+} channel regulation

1.4.1. Sympathetic nervous system acts *via* β1-adrenergic system to regulate LTCC

The sympathetic nervous system regulates excitation-contraction coupling through activation of β1-adrenergic receptors (β1-ARs), which are coupled to G_s-protein[398]. Following the binding of an agonist to β1-AR, $G\alpha_s$ is released from the G protein complex, which then binds to and activates adenylyl cyclase (AC)[398,399]. AC, in turn, catalyzes the conversion of **ATP** to cAMP, a second messenger that modulates several cellular functions such as energy metabolism, cell division, and cell proliferation[400]. In the heart, cAMP activates several downstream targets, including protein kinase A (PKA), exchange protein directly activated by cAMP (EPAC), and

phosphodiesterases (PDEs)[398,399,401]. The β1-adrenergic-mediated inotropic effects on the heart are achieved mainly through PKA by regulating proteins implicated in Ca^{2+} handling such as the $Ca_V1.2$ Ca^{2+} channel, ryanodine receptors, and phospholamban[398,402].

During the fight or flight response, activation of PKA augments L-type Ca^{2+} currents (I_{CaL}), hence mediating Ca^{2+} entry into the cardiomyocytes, which ultimately enhances cardiac contractility.

Multiple studies employing *in vivo* and *in vitro* models demonstrated that β1-adrenergic stimulation increases L-type Ca^{2+} currents ≈2-fold, mainly by increasing the channel's open probability associated with a decrease in closed time[403–406]. Furthermore, PKA activation renders the LTCC more sensitive to depolarization[315,407]. Mice treated with isoproterenol, a non-selective β-adrenergic agonist, increased I_{CaL} and was completely reversed following treatment with the PKA inhibitor, H89 [408]. Similarly, I_{CaL} was upregulated in adult cardiomyocytes treated with either isoproterenol or 8-Bromo-adenosine 3′5′-cyclic monophosphate (8-Br-cAMP), a membrane-permeable cAMP analog [409,410].

A long-established paradigm of PKA-mediated activation of $Ca_V1.2$ was a direct phosphorylation of $Ca_V\alpha1C$ or $Ca_V\beta$ subunits[410–413]. Nevertheless, the latter mechanism was questioned by studies showing that PKA activation was still able to increase $Ca_V1.2$ Ca^{2+} currents in genetically engineered mice possessing mutant PKA phosphorylation sites on $Ca_V\alpha1C$ and/or $Ca_V\beta$[414,408,409,415,416]. Recently, Liu et *al.* further tackled the underlying mechanism and discovered Rad as the "missing link" required for the regulation of $Ca_V1.2$ by PKA. Rad is part of the RGK family of small GTP-binding proteins[417]. RGK proteins bind to the $Ca_V\beta$ subunit and inhibit all Ca_V1 and Ca_V2 channels[418,419]. Adenovirus-mediated knockdown of Rad resulted in increased L-type Ca^{2+} currents and enhanced contractility in cultured cardiomyocytes[344]. Moreover, knockout of Rad rendered LTCC insensitive to β-adrenergic stimulation[420]. Using a recombinant system, Liu et *al.* demonstrated that Rad binds to $Ca_V\beta$ and inhibits LTCC at basal state[421]. Upon β-adrenergic stimulation, PKA phosphorylates Rad, stimulating its dissociation from $Ca_V\beta$ subunit, relieving its inhibitory action on the LTCC[421]. These data corroborate Rad as a new go-between for the regulation of LTCC by PKA following β1-adrenergic receptor stimulation. Although acute β1-adrenergic activation partakes in adaptive cardiac response (e.g., fight-or-flight response), chronic stimulation induces cardiac hypertrophy that can further progress to heart failure. The relationship between LTCC, β1-adrenergic system, and cardiac hypertrophy remains disputable despite decades of investigations.

1.4.2. Modification of calcium channel subunits in animal models of cardiac hypertrophy and heart failure

Although multiple lines of evidence revealed a direct relationship between Ca^{2+} mishandling and cardiac hypertrophy, the role of LTCC in hypertrophy and heart failure remains unresolved. Animal models of cardiac hypertrophy set forth controversial data with either increased[422], decreased[423,424], or unchanged LTCC currents[425–427]. Studies employing animal models subjected to TAC-induced pressure-overload hypertrophy showed that I_{CaL} density was unaltered[426,427]. However, opposing studies demonstrated that I_{CaL} density were reduced in hearts with advanced stages of hypertrophy[423,428] and in failing human hearts, perhaps due to decreased LTCC abundance[428]. Furthermore, an electrophysiological study using single-channel analyses demonstrated that single channel current was augmented in failing human myocardium that eventuated from increased open probability and number of functional channels[429]. The discrepancies in these results might be attributed to the different stages of hypertrophy development.

1.4.3. Impact of overexpression/downregulation of LTCC subunits on cardiac hypertrophy

Given the eminent role of each LTCC subunit on the channel's current density, researchers aimed to investigate the modifications in LTCC subunits at the protein level in models of cardiac hypertrophy and heart failure. Initial evidence of the role of LTCC subunits in cardiac hypertrophy emanated from a study showing that homozygous genetic deletion of the pore-forming subunit $Ca_V\alpha1C$, or the $Ca_V\beta2$ subunit resulted in embryonic lethality[313,330,430]. Clinically, Haase et *al.* reported upregulation of DHP receptors in patients with hypertrophied hearts[431]. Moreover, failing human heart exhibited increase in $Ca_V\beta2$ subunit protein expression without concomitant change in the protein expression of $Ca_V\alpha1C$ and $Ca_V\alpha2\delta1$[328], suggesting that modifications in the expression of LTCC subunits might account for I_{CaL} alterations observed in cardiac hypertrophy and heart failure.

To further investigate the role of LTCC subunits in cardiac hypertrophy, researchers employed transgenic models with overexpression or downregulation of LTCC subunits. Transgenic mice with cardiac-specific heterozygous expression of $Ca_V\alpha1C$ aggravated hypertrophy and reduced cardiac function following pressure overload stimulation[432], suggesting that a lower protein expression of $Ca_V\alpha1C$ associated with lower I_{CaL} is not cardioprotective. In support of these data, mice overexpressing $Ca_V\alpha1C$ developed cardiac hypertrophy that transitioned to heart failure as a function of age[192,433]. By contrast, cardiac-specific knockdown of $Ca_V\beta2$ gene reduced LTCC density and attenuated the hypertrophic response in rats subjected to TAC-induced pressure overload hypertrophy[434]. $Ca_V\alpha2\delta1$ is a steadfast contributor of LTCC [370,435]. Fuller-Bicer et *al.* employed mice with homozygous knockout of the CACNA2D1 to study its contribution in cardiac hypertrophy[359].. Diminished protein expression of $Ca_V\alpha2\delta1$ was associated with lower I_{CaL} density[359]. Although the deletion of the subunit was not lethal, the hearts of these mice exhibited impaired cardiac contractility but did not develop cardiac hypertrophy[359]. Altogether, these data suggest that the regulation of I_{CaL} by $Ca_V1.2$ subunits is involved in cardiac hypertrophy.

1.5.ATP-Sensitive potassium channels (K_{ATP} channels)

1.5.1. Discovery of K_{ATP} channels

The ATP-sensitive potassium channel (K_{ATP}) was initially identified by Noma in the cardiac muscle as an outward K^+ current that was activated by hypoxia or cyanide and blunted by intracellular injection of ATP [436]. K_{ATP} channels were later found in several other tissues and cells, including skeletal myocytes[437], vascular smooth muscle[438], pancreatic β cells[439], and the central nervous system[440], where they exhibit different properties and functions.

1.5.2. Role of K_{ATP} channels in the body: A general overview

ATP-sensitive potassium (K_{ATP}) channels are characterized as weak inward rectifying channels implicated in several physiological processes[441,442]. At the cellular level, K_{ATP} channels regulate the flow of K^+ ions across the plasma membrane, which changes the membrane resting potential in response to modifications in cellular metabolism[443]. Thus, K_{ATP} channels serve as molecular sensors that couple the cellular metabolic state to the electrical activity of the cell membranes[444]. In a multiplex interplay of mechanisms, K_{ATP} channel is activated by binding to

Mg^{2+}-bound nucleotides but blunted by interacting with ATP[445]. During normal conditions, the inhibitory effect of ATP dominates, and K_{ATP} channels are closed[446]. However, as the cellular metabolism dwindles, ATP/ADP ratio declines, resulting in the activation of K_{ATP} channels[446]. Depending on the tissue, K_{ATP} channels are associated with distinct functions. In the pancreatic β-cells, K_{ATP} channels activity is modulated as a function of blood glucose and accounts for insulin secretion[444,447]. By contrast, K_{ATP} channels regulate vascular tone blood flow in the vascular smooth muscle[448]. In the heart, K_{ATP} channels are activated during conditions of hypoxia, ischemia, or metabolic stress, promoting exit of K^+ ions to the extracellular[12,449,450]. K^+ efflux results in hyperpolarization of the membrane, which in turn, prevents opening of voltage-gated calcium channels and subsequent Ca^{2+} influx[12,449,450]. Limiting Ca^{2+} entry into the cell attenuates ischemic damage and its associated metabolic deterioration[12,449,450]. Mutations in the K_{ATP} channels have been associated with multiple diseases such as congenital hyperinsulinism, neonatal diabetes mellitus[451], dilated cardiomyopathy[452], and myocardial infarction[453]. The disparate functions of K_{ATP} channels in each tissue emanate from the distinct assembly of specific subunit combination of several Kir6.x and SURx subunits[444]. K_{ATP} channel is an octameric complex composed of four pore-forming Kir6 subunits, each coupled to one sulfonylurea receptor (SUR) subunit[454,[455]. Kir6.2/SUR1 channels are expressed in pancreatic β cells, whereas Kir6.2/SUR2A channels are mainly expressed in ventricular myocytes[454,455]. This tissue-specific subunit combination engenders various classes of K_{ATP} channels, each possessing their biophysical properties, sensitivities to pharmacological molecules, and function[444]. The scope herein will be limited to cardiac K_{ATP} channels.

1.5.3. Structure of cardiac K_{ATP} channels

K_{ATP} channel constitutes of the association of the pore-forming Kir6.2 subunit with the regulatory subunit SUR2A[454]. Kir6 subunit forms the channel pore and encompasses the binding site for ATP[454]. Kir6.x proteins belong to the Kir superfamily of proteins (Kir 1-7) [456]. The Kir channels are divided into strong inward rectifiers or weak rectifiers, which show greater outward currents upon membrane depolarization[456]. The majority of Kir channels are strong inward rectifiers that favor the inward flow of K^+ ions from the extracellular to the cytosol[457]. This directionality is attributed to the strong affinity of positively charged cations to the intracellular side of the channel, resulting in a physical occlusion that hinders K^+ entry[458,459]. By contrast,

Kir6.x proteins have weak binding for positively charged blockers, which prompt currents from the inside to the extracellular side of the cell[458,459]. Kir6.x channels are hence classified as weak inward rectifiers channels[458].

The regulatory portion of the channel encircling the pore is made up of four sulfonylurea receptor (SUR) subunits[12,15]. The SUR subunit belongs to the ATP-binding cassette (ABC) protein family and provides the sites for channel activation by MgADP and modulation by channel agonists (e.g., diazoxide, pinacidil, and cromakalim) and antagonists (e.g., glibenclamide) [12,15]. ABC proteins are a superfamily of membrane proteins that couple energy from ATP hydrolysis to the transportation of substrates across the membrane (export or import)[460,461].

Crystallographic research of eukaryotic Kir channels revealed a conserved structure of Kir channels[462]. The Kir subunit is formed of two transmembrane helices (M1, M2) linked by a pore-forming loop that governs K^+ ion selectivity through a glycine-phenylalanine-glycine motif[12,15]. The M1 and M2 domains are also flanked by cytoplasmic domains residing at the N- and C- terminal. These cytoplasmic domains are sites for inhibition by ATP **(Figure 8)** [12,15].

SUR subunit contains 3 transmembrane domains (TMDs) organized as such: two 6-helix TMDs, TMD1 and TMD2, and an additional unique 5-helix transmembrane domain (TMD0) at the N terminus of the SUR subunit[12,15]. TMD0 is linked to TMD1 by a cytoplasmic linker that is prominent for trafficking and gating of the Kir6 subunit[12,15]. Moreover, TMD1 and TMD2 encompass two cytoplasmic: one nucleotide-binding domain (NBD1) located between TMD1 and TMD2, and another nucleotide-binding domain (NBD2) that resides after TMD2 at the COOH terminus **(Figure 8)** [12,15]. Both NBD1 and NBD2 contain Walker A motif and Walker B motif. These motifs include Mg^{2+}-ADP binding sites required for nucleotide binding and harbor ATPase activity, granting the SUR subunit the capacity to regulate ATP-induced Kir6.2 inhibition[463–465]. The latter SUR-mediated catalysis contributes to the regulation of K_{ATP} channel gating[463–465]. Besides being a site for modulation by Mg^{2+}-ADP, SUR subunit is essential for the trafficking of K_{ATP} channel to the cell surface through masking the RKR endoplasmic reticulum retention signal[466–468]. The latter was confirmed in a study reporting that overexpression of SUR2A in ventricular cardiomyocytes generated increased number of K_{ATP} channels at the sarcolemma[449].

Thus far, two genes, *KCNJ8* and *KCNJ11,* have been identified that encode two isoforms Kir6.1 and Kir6.2, respectively[454]. Similarly, two genes, ABCC8 and ABCC9, encode two isoforms, SUR1 and SUR2, respectively[454,469]. Alternative splicing of SUR generates two splice variants: SUR2A and SUR2B, which vary by the amino acid residues of the COOH-terminal [469]. Ventricular K_{ATP} channels comprise of Kir6.2 and SUR2A subunits. Disruption of *KCNJ11* gene generates a Kir6.2-deficient condition characterized by lack of functional K_{ATP} channels in ventricular cardiomyocytes[470].

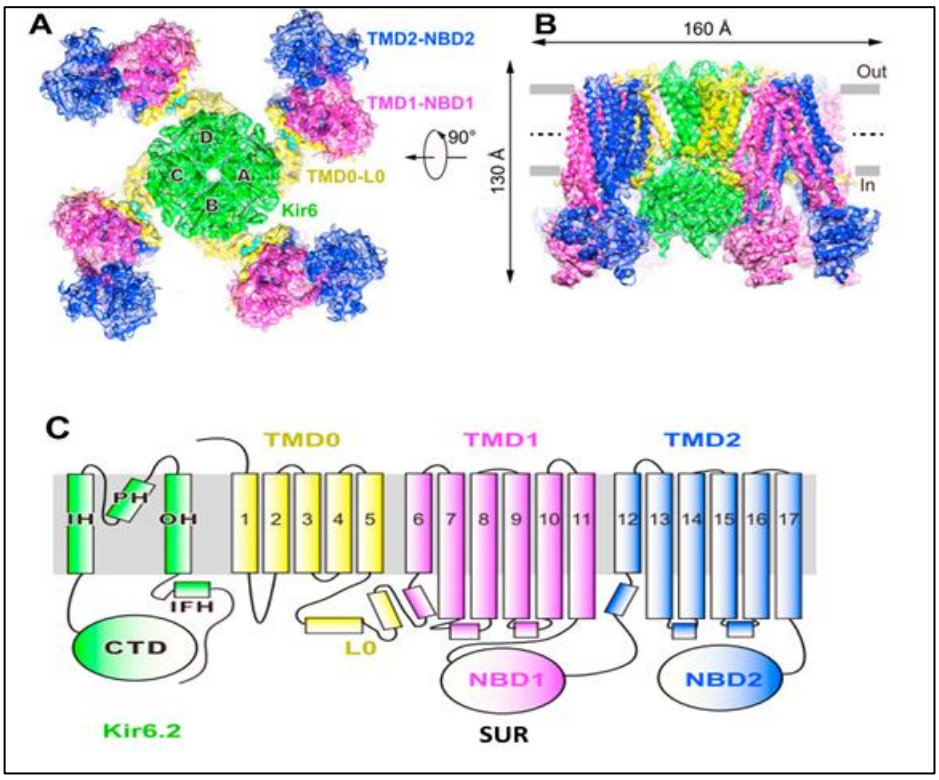

Figure 8: Scheme showing the structure of K_{ATP} channels.

The structure represents the octameric K_{ATP} channel consisting of Kir6.x and SURx subunits[443]. Kir6.x is the pore-forming subunit formed of two transmembrane helices (TMD1 and TMD2: green in figure C) connected by a loop and ending with cytoplasmic –NH2 and –COOH terminal

domains[443]. The SURx subunit consists of three transmembrane domains (TMD0, TMD1, and TMD2) and two nucleotide-binding domains (NBD1/NBD2) facing the cytoplasm[443].

(A) Cryo-EM structure of K_{ATP} channel shown from the intracellular side (Bottom view) [471].

(B) Side view of the Cryo-EM structure of K_{ATP} channel [471].

(C) Cartoon structure of the K_{ATP} channel. NBD, nucleotide-binding domain; TMD, transmembrane domain; L0, loop 0; CTD, cytoplasmic domain; IFH, interfacial helix; OH, outer helix; PH, pore helix; IH, inner helix[471].

Kir6.2 is shown in green, TMD0-L0 in yellow, TMD1-NBD1 in magenta, and putative glibenclamide in cyan. Figure adapted from Li et *al.,* 2017 [471].

1.5.4. Cardiovascular tissue distribution of K_{ATP} channel subunits

Different association of the subunits forms channels with distinct physiological and pharmacological properties. Kir6.1, Kir6.2, SUR2, and SUR1, as well as the splice variants, are all expressed in the heart[472–475]. In mice, SUR1 and Kir6.2 are the predominant isoforms forming the K_{ATP} channels in the atria, while SUR2A and Kir6.2 are the major constituents of K_{ATP} in the murine ventricles[476,477]. In a study employing neonatal rat ventricular myocytes (NRVMs), knockdown of SUR2 expression by antisense oligonucleotides reduced functional K_{ATP} currents[478]. Besides, dominant-negative expression of Kir6.2 subunits diminished ventricular K_{ATP} currents[479,480].

Nevertheless, in the human heart, both SUR1 and SUR2A subunits account for K_{ATP} channels in both ventricular and atrial myocytes, albeit SUR2A and Kir6.2 are the predominant isoforms in the human ventricles[481,482]. Moreover, Kir6.1, Kir6.2, and SUR2B subunits were found to be crucial for functional K_{ATP} channels in the pacemaker and conduction system, including atrioventricular nodes[483], Purkinje fibers[484], and sinoatrial node[485,486].

1.5.5. Regulation of K_{ATP} channels

The regulation of K_{ATP} channels is intricate and includes metabolites, hormones/enzymes, and neurotransmitters.

1.5.5.1. Physiological regulation by nucleotides

A hallmark feature of the K_{ATP} channel is its sensitivity to metabolic alterations of nucleotide levels, mainly ATP/ADP ratio[12,15]. Hence, a subtle balance in nucleotide concentrations in the channel's surroundings is the primary molecular determinant and modulator of K_{ATP} channel activities[12,15]. When ATP/ADP ratio is high, ATP binds to the nucleotide-binding sites located at the N and C-terminal of the Kir6.2 subunit, leading to inhibition of the channel's activity by reducing the mean channel open time[487–489].

Contrariwise, in response to a decline in the ATP to ADP ratio as occurs during metabolic stress, K_{ATP} channels are activated, resulting in K^+ efflux from the cell and membrane hyperpolarization[12,15]. Activation of K_{ATP} channels is triggered in two ways: 1) through the direct association of Mg-ADP to the NBD domain of the SUR subunit or 2) *via* binding and hydrolysis of Mg-ATP at the SUR binding domains[490–492]. In both cases, Mg^{2+} is indispensable for regulating the K_{ATP} channel's response to ADP[493,494]. In the latter way, SUR exploits the energy from Mg-ATP hydrolysis to stimulate a conformational change in the subunits, prolonging the channel's mean open time and, ultimately, resulting in channel activation[444,495,496]. Thus, the SUR subunit also serves as an enzyme through its intrinsic ATPase activity. Indeed, studies have reported that mutations in NBD2 reduce SUR's ATPase activity and generate K_{ATP} channels with increased sensitivity to inhibition by ATP[464].

The concentration at which ATP inhibits K_{ATP} channels varies according to the cell type, subunit combination, and species[15]. Given that normal concentration of intracellular ATP in the heart is in the millimolar range (1-10 mM) and varies slightly with metabolism, the ATP levels are thought to be sufficient to inhibit the channel activity in normal conditions[436]. In early studies carried out using patch-clamp on ventricular cardiomyocytes, the ATP concentration required to generate half-maximal inhibition (IC50) was 100 μM[436]. Therefore, nucleotides act as the key modulators that dictate the Kir6 channel activity.

1.5.6. The physiological role of K_{ATP} channels in the heart

Cardiac K_{ATP} channels, harnessing the capacity to sense modification in the cellular metabolic condition and translate it into changes in the electrical activity of the cell membranes, provide a liaison to preserve myocardial energy and ionic homeostasis[12,15]. Since ATP concentration in the cardiac tissue is high, K_{ATP} channels are closed under a physiological aerobic state. Accordingly, ventricular cardiomyocytes isolated from rodents with Kir6.2 gene knockout

exhibited action potential duration and contractile performance similar to those isolated from wild-type rodents[448,497,498]. Thus, it has been thought that cardiac K_{ATP} channels play a limited role in modulating cardiac excitability in the basal state[448,497,498]. Nevertheless, studies employing genetic rodent models underpin a broader interpretation of the ventricular K_{ATP} channel as a guarantor of ion and energy homeostasis in response to a spectrum of physiological or pathological stress, including exercise, fight or-flight response, or ischemia[12,15].

Acute exercise stimulates sympathetic stimulation that enhances cardiac contractility, heart rate, and cardiac output required to meet higher body demand[499]. The increased cardiac output imposes elevated metabolic load due to highly-energy demanding processes that result in massive Ca^{2+} influx into the cytosol[500]. To offset the Ca^{2+} overload, Kir6.2 channels open when the sustained increase of myocardium workload competes with the capacity of energetics to preserve contractile and electric stability[12,15]. Activation of the channel promotes K^+ efflux out of the cell, which shortens the action potential and attenuates Ca^{2+} influx into the cell, limiting actomyosin ATP consumption[12,15]. The latter serves as a compensatory mechanism to preserve energy stores and prevent Ca^{2+} overload.

The physiological action of K_{ATP} channels was underscored in studies demonstrating that mice with knockout of Kir6.2 channels exhibited diminished tolerance to exercise. Kir6.2$^{-/-}$ mice had less capacity in performing treadmill exercise compared to wild-type controls[501,502,479]. Moreover, Kir6.2$^{-/-}$ mice developed cardiac dysfunction and structural malformities that was associated with sudden death, due to Ca^{2+} overload [501,502,479].

Besides, when the heart rate is elevated such as in fight or flight response, K_{ATP} channels partake in adapting the action potential duration (APD), which is crucial to reduce refractoriness and avoid arrhythmias[503,504]. However, hearts subjected to cardiac-specific overexpression of dominant-negative Kir6 subunits or treated with K_{ATP} channel inhibitor (e.g.: glibenclamide) lack APD adaptation and exhibit ventricular arrhythmia (such as early afterdepolarization syndrome) in response to catecholamine challenge[503,505].

Altogether, these data indicate that K_{ATP} channel is crucial for cardiac adaptation to physiological or pathophysiological stress conditions, and further implicate K_{ATP} channel dysfunction in predisposing the heart to Ca^{2+} overload and cardiac diseases.

1.5.7. Cardioprotective role of K_{ATP} channels in the myocardial infarction

One of the early responses that occur secondary to a cessation of blood flow is the loss of K^+ ions from the ischemic heart [506–508]. Thereafter, studies reported that glibenclamide, a K_{ATP} channel inhibitor, reduced extracellular K^+ accumulation during ischemia in isolated rats, rabbits, and guinea pig hearts[509,510]. However, it is noteworthy that the inhibition of K_{ATP} channels prevented K^+ accumulation 10 minutes following ischemia, suggesting that K_{ATP} channels are involved in K^+ loss during early phases of ischemia[509,510].

K_{ATP} activation has been shown to be cardioprotective during ischemia because, secondary to stress, the opening of K_{ATP} channel partakes in action potential shortening, which limits intracellular calcium overload, reduces contraction, and preserves ATP stores. Indeed, genetic disruption of Kir6.2 gene developed poor recovery secondary to coronary hypoperfusion and minimized the protective effects of ischemic preconditioning[511–514]. In support of this premise, knockout of Kir6.2 (Kir6.2 KO) was associated with absence of action potential shortening in mice subjected to metabolic-deficient conditions[514]. Moreover, hearts from Kir6.2 KO-mice exhibited impaired contractility during ischemia, indicating that the lack of K_{ATP} channels renders the heart more susceptible to ischemic injury[514]. Conversely, cardiomyocytes from transgenic mice overexpressing SUR2A protein showed upregulation of K_{ATP} channel protein expression on the sarcolemma[449]. When subjected to ischemia, transgenic cardiomyocytes overexpressing SUR2A subunit exhibited shortened action potential duration and was associated with higher survival as compared to wild-type cardiomyocytes following hypoxia, indicating that a higher expression of sarcolemmal K_{ATP} channels produced a phenotype resistant to ischemia[449].

Besides transgenic approaches, K_{ATP} channel modulating molecules (openers and blockers) were used to further substantiate the channels' cardioprotective role during ischemia. Guinea pigs treated with pinacidil, a K_{ATP} opener, exhibited augmented ATP and energy pools during ischemia[515]. Similarly, ischemia-induced ATP depletion was mitigated in rat hearts following treatment with the K_{ATP} opener cromakalim[516]. By contrast, action potential shortening was abolished in hearts treated with the K_{ATP} channel blocker glibenclamide[517].

Besides their role in cardiac contraction, K_{ATP} channels also contribute to cardiac relaxation. Pharmacological inhibition or genetic disruption of Kir6.2 channel minimized cardiac

performance and impaired cardiac relaxation as depicted by elevated left ventricular end-diastolic pressure[518].

The extent of an infarct following a coronary artery blockage depends on the level of ischemic damage[519]. Investigations employing K_{ATP} channel openers such as nicorandil, aprikalim, cromakalim, and pinacidil demonstrated that activation of K_{ATP} channels reduced myocardial damage and minimized infarct development following cardiac ischemia[520–524]. On the other hand, K_{ATP} channel blockers such as glibenclamide expanded the infarct size following ischemia[521].

During ischemic preconditioning, blocking K_{ATP} channel activity attenuated the reduction of infarct size induced by preconditioning[525,526]. Moreover, Mizimura et *al.* corroborated the latter findings by demonstrating that treating ischemic dogs with Bimakalim, a K_{ATP} channel opener, mimicked the protective effects of preconditioning in reducing infarct size post-ischemia[527].

In addition to their protective role in ischemia, several studies have reported the anti-ischemic and antiarrhythmic effects of cardiac K_{ATP} channels in settings of ischemia reperfusion[514,528–531]. The majority of these studies reported that K_{ATP} channel openers minimized infarct size and improved the contractile recovery of the myocardium during postischemic reperfusion[532–537]. By contrast, the protective effects of the K_{ATP} channel openers were reversed in presence of K_{ATP} channel blockers[537–540]. Baczko *et al.* have demonstrated that the K_{ATP} channel opener pinacidil averted Ca^{2+} overload resulting from chemically-induced hypoxia/reoxygenation. The mechanism behind the latter protection was thought to be through K_{ATP} channel-mediated hyperpolarization of membrane potential, which favors the reverse mode of the Na^{+}/Ca^{2+} exchange[541]. Moreover, transgenic overexpression of SUR2A subunit in mice resulted in reduction of infarct sizes following ischemia reperfusion[449]. Therefore, these data indicate that K_{ATP} channels possess cardioprotective functions during ischemia and ischemia reperfusion.

1.6. Sex differences in cardiac physiology and diseases

1.6.1. Sex differences in normal cardiac structure and function

Sex-linked cardiac differences are evident even in healthy individuals. Women and men have disparate LV dimensions and functions. For example, men have greater ventricular mass due to the larger dimensions of LV chambers[542]. Moreover, women have a faster-resting heart rate compared to men[543]. In aging women, cardiac mass is more preserved than in aging men[543]. At the cellular level, the number of cardiomyocytes is similar between both sexes at birth; however, during development, women show an attenuated reduction in cardiomyocyte number compared to the declining number in men[544]. Accordingly, women are less prone to cardiac hypertrophy compared to men. At the molecular level, significant differences have been discerned in the calcium metabolism and handling[545,546], generation of ROS, and mechanisms of cardiac excitation-contraction coupling[547].

The apparent sex-related distinct features existing in the normal myocardium led to surmising that sex-based disparity also prevails in the response of the myocardium to various pathological stimuli, such as ischemic injury.

1.6.2. Sex differences in Myocardial infarction

Risk factors and genetic background predispose both men and women to myocardial ischemia. A clinical study named Multinational MONItoring was organized by The World Health Organization (WHO) in the early 1980's to prospectively monitor trends and determinants in the cardiovascular disease (MONICA) Project[548]. The study sample consisted of ten million men and women, ages 35-64, in 21 countries, for a span of ten years. Despite regional variations, men in western countries including North America and Europe were reported to have a higher prevalence of cardiovascular diseases compared with women[549,550].

Epidemiological studies showed that in premenopausal women, coronary artery disease (CAD) appears later and present with less severe atherosclerosis in the coronary arteries than men[551]. As a consequence, myocardial infarction occurs 10 years later in women than in men[551]. Nevertheless, the frequency of myocardial ischemia in women increases noticeably after menopause, suggesting that reduced estrogen level is the reason behind development of

ischemia[551]. Accordingly, hormone replacement therapies post menopause prevailed in preclinical research. However, randomized trials revealed that the incidence of ischemia increased in postmenopausal women receiving estrogen replacement therapy[552,553]. These data elucidate the complexity of sex-related disparity in cardiac diseases.

Sex disparities in coronary artery disease (CAD) and ischemia have been well documented[554–556]. According to the CONFIRM-long-term registry, CAD and MI are less prevalent in women (27%) than in men of the same age (43%). Moreover, men who presented with CAD suffered from obstructive form of CAD while women exhibited more often a non-obstructive CAD[557]. Sex-linked response following ischemia also appears during remodeling as men tend to have greater adverse remodeling than women[557]. In response to prolonged ischemia (chronic phase), cardiomyocytes undergo apoptosis and necrosis[27]. Even if patients were subjected to successful primary percutaneous coronary intervention, women still exhibited greater myocardial salvage as compared to men in a randomized trial[558]. In patients with MI, cardiomyocyte death in the failing heart was two-fold higher in men compared to women[559], indicating that the female heart is protected from necrosis and apoptosis.

Besides clinical data, several studies have employed animal models to examine sex differences observed in the initial response to MI as well as the complications associated with MI such as heart failure. In a mouse model of acute MI, males exhibited exacerbated structural remodeling as manifested by higher rate of cardiac rupture and larger infarct expansion within the first week of MI (acute phase)[560]. During twelve weeks post-MI (chronic), males showed progressive declines in contractile function associated with aggravated cardiac remodeling as displayed by dilatation, myocytes hypertrophy, and delayed myocardial healing[560]. By contrast, females displayed better cardiac contractility and delayed decompensation to heart failure[561]. Similarly, female adult mouse cardiomyocytes had a greater survival rate as compared to male following oxidative stress-induced cell death[562]. During the healing process, Wang et *al.* reported that female had less-exaggerated inflammation and was associated with increased reparative fibrotic response and neovascularization 1 week following coronary artery ligation[562]. Whereas males showed delayed cardiac healing, consequently leading to early myocardial rupture[560]. Surviving male mice after MI had poor cardiac performance and pronounced maladaptive ventricular remodeling[560]. It was postulated that greater matrix metalloproteinase activation and

inflammatory response in males might account to the sex-linked disparities in the response to MI[563].

Therefore, experimental and clinical studies have both documented that cardioprotection is favored in female sex during ischemic injury, adaptive response, and ventricular remodeling secondary to myocardial infarction. Despite controversies, the latter cardioprotection is attributed to several genomic and non-genomic factors.

1.7. The mammalian target of rapamycin (mTOR)

1.7.1. Background and overview of mTOR

The mammalian target of rapamycin (mTOR) was first isolated and detected as a target of the drug rapamycin or sirolimus, a macrolide originating from the Streptomyces Hygroscopicus bacteria [564,565]. Shortly afterward, mTOR was purified and identified in mammalian cells[566–568]. mTOR is a large (289KDa) serine/threonine kinase, belonging to the phosphoinositide 3-kinase (PI3K)-related kinase family[569,570]. mTOR regulates diverse fundamental cellular processes such as protein synthesis, cell survival and growth, and autophagy.

1.7.2. mTOR structure

mTOR exerts its action by binding to specific adaptors to generate two disparate complexes, mTORC1 and mTORC2[569–573]. The constituents of each protein complex are essential for either upstream modulation, complex formation, or downstream signal activation[569–573]. Common to both complexes are the catalytic mTOR subunit, DEP domain-containing mTOR-interacting protein contain (DEPTOR), the mammalian lethal with Sec13 protein 8 (mLST8), and the Tel two interacting protein 1 (Tti1-Tel2) complex **(Figure 9)** [569–573]. Unique to mTORC1 is the proline-rich Akt/PKB substrate 40 kDa (PRAS40) and regulatory-associated protein of mTOR (RAPTOR), whereas rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated MAP kinase-interacting protein 1 (mSin1), and the protein observed with rictor (Protor) are only part of mTORC2 **(Figure 9)** [569–573]. Most notably, however, mTORC2 is differentiated from mTORC1 by its resistance to inhibition with Rapamycin[569–573].

mTORC1 is the most characterized of the two mTOR complexes, and much more is known about the upstream regulators and downstream targets of mTORC1. The scope herein will be limited to mTORC1.

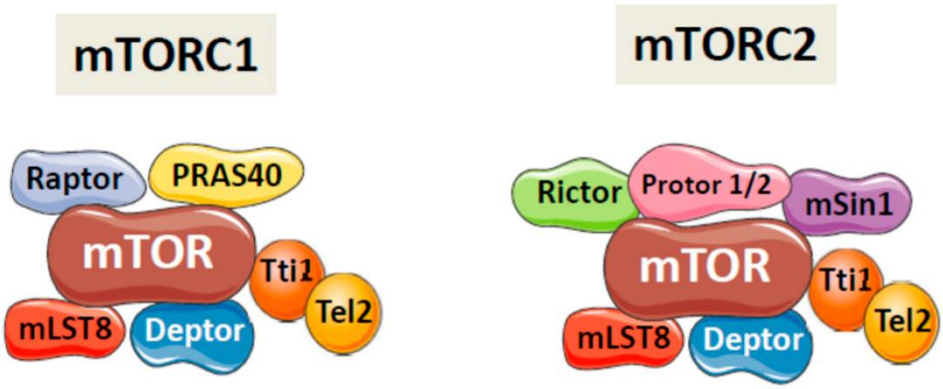

Figure 9: Structure of mTORC1 and mTORC2 complexes.

mTORC1 consists of the core mTOR machinery: mTOR, DEPTOR, Tti/Tel2, and mLST8; combined with Raptor and PRAS40. While mTORC2 consists of the core mTOR machinery associated with RICTOR, and mSIN1.

DEPTOR: DEP domain-containing mTOR-interacting protein; PRAS40: proline-rich Akt substrate 40; mLST8: mammalian lethal with sec-13 protein 8; Protor: protein observed with rictor 1/2; Raptor: regulatory-associated protein of mTOR; and RICTOR: rapamycin-insensitive companion of mTOR; and Tel1/2, Tel 2 interacting protein 1. Figure adapted from Sangüesa et *al.,* 2019 [582].

1.7.2.1. mTORC1

mTORC1 is constitutively composed of mTOR, mLST8, and RAPTOR, while the proline-rich Akt/PKB substrate 40 kDa (PRAS40) acts as a specific negative regulatory subunit of mTORC1 **(Figure 9)** [574,575]. Raptor, a 150-kDa protein, is an indispensable component of mTORC1 that acts as a scaffolding protein to facilitate recruitment and phosphorylation of specific downstream substrates of mTORC1[576–578]. Moreover, the interaction of Raptor with mTORC1 drives the complex to its appropriate subcellular localization[578,579]. Following a stimulus (example: amino acid deficiency), raptor binds directly to Rag small GTPases, recruiting mTORC1 complex to the lysosome membrane, which results in its activation[578,579]. Although several studies have disputed the functional role of mLST8 in modulating mTORC1, it was shown that, through its association with mTOR catalytic domain, mLST8 is required for the kinase activity of mTORC1[576,577,580]. DEPTOR and PRAS40 serve as the inhibitory components of the complex[578,581].

1.7.3. Regulators of mTORC1

1.7.3.1. Directly upstream of mTOR: TSC1/2 and Rheb

A renaissance in the mTOR domain prevailed following the finding that the tuberous sclerosis complex (TSC) acts as an upstream negative regulator of mTOR[574,580,583]. TSC is a heterodimer composed of hamartin, also known as tuberous sclerosis 1 (TSC1), tuberin or tuberous sclerosis 2 (TSC2), and TBC1 Domain Family Member 7 (**TBC1D7**) [574,580,583]. Hamartin acts as a stabilizing component of Tuberin, while Tuberin is a guanosine triphosphatase (GTPase)-activating protein (GAP) for Ras homolog enriched in brain protein (Rheb) [574,580,583]. Rheb is a small GTPase that, when bound to GTP, activates mTORC1[574,580,583]. In an unstressed heart, dephosphorylated Tuberin suppresses mTORC1 activity by converting the active GTP-bound Rheb into inactive GDP-bound Rheb[574,580,583]. In response to stimulation, Tuberin is phosphorylated at the threonine residue, which suppresses its GAP activity and renders the complex inactive, ultimately triggering mTORC1 activation[574,580,583].

1.7.3.2. mTOR stimuli: Growth factors, nutrients, hypoxia

The TSC 1/2 complex has been shown to receive inputs from signaling cascades following activation by various modulators, namely growth factors, neurohormones, hypoxia, ROS, energetic status, and amino acids[574,580,583]. The Akt and AMP-activated protein kinase (AMPK) pathways represent the most described regulators of mTORC1[574,580,583].

Neurohormones and growth factors (e.g., insulin and insulin growth factor 1) bind to tyrosine kinase or GPCRs and produce PIP3 through activation of PI3K, triggering Akt activation[574,580,583]. The active Akt/PKB pathway, in turn, activates mTORC1 activation through either minimizing the interaction of PRAS40 with mTORC1 or phosphorylating TSC2, which promotes its dissociation from TSC1 and relieves its suppression of Rheb-mediated activation of mTORC1[574,580,583].

TSC2 activity can also be modulated by AMPK[574,580,583]. Under conditions of low cellular energy, such as hypoxia and elevated ratio of AMP/ATP, active AMPK inhibits mTORC1 to inhibit ATP-consuming processes and preserve cellular energy pools[584].

1.7.4. Physiological role and downstream targets of mTORC1

mTOR regulates diverse cellular processes by serving as an integration point that transduces the above-mentioned signals to multiple downstream effectors[574,580,583]. mTORC1 partakes in regulation of cellular homeostasis, response to stress, and cell growth and proliferation[574,580,583]. In response to environmental and intracellular contexts, mTOR stimulates anabolic mechanisms such as protein, lipid, and glucose metabolism, while suppressing catabolic processes such as autophagy **(Figure 10)** [574,580,583].

1.7.4.1. Protein, lipid, and nucleotide Synthesis

mTORC1 stimulates protein synthesis through the phosphorylation of two primary substrates, p70S6 Kinase 1 (S6K1) and the eukaryotic translation initiation factor 4E (eIF4E)-binding protein-1 (4E-BP1) **(Figure 10)** [574,580,583]. The phosphorylation of S6K on its hydrophobic motif site Threonine 389 and 4EBP1 on Threonine 37 and Threonine 41 have been long employed as functional readouts of mTOR activity[574,580,583]. mTORC1 directly phosphorylates and activates ribosomal protein S6 kinases (S6K1). Following activation, S6K1 phosphorylates the ribosomal protein S6, initiating the mRNA translation process and subsequently activating protein synthesis[574,580,583].

In contrast, mTORC1 phosphorylates and inhibits 4E-BP1, an inhibitor of eIF4E, hence stimulating translation and promoting cell growth **(Figure 10)** [574,580,583].

Proliferating cells require ample lipids to form cellular membranes. mTORC1 stimulates *de novo* lipid synthesis by inducing transcription factors that trigger genes involved in cholesterol and fatty acid synthesis[574,580,583]. Moreover, mTORC1 also contributes to cell proliferation by inducing the activating transcription factor 4, which promotes ribosome and nucleotide biosynthesis and preserves the nucleotide pool required for DNA replication in growing and proliferating cells[585–588].

1.7.4.2. Glucose metabolism

The heart predominantly oxidizes fatty acids but switches to carbohydrate metabolism in response to neurohormonal, nutritional, or hypoxic stimuli[574,580,583]. In response to stress stimuli, mTORC1 enhances the expression of the hypoxia-induced transcription factor-1α, which stimulates a metabolic switch from fatty acid oxidation to glucose metabolism[574,580,583]. Hearts of mice subjected to raptor gene knockout depicted increased glucose metabolism associated with reduced expression of genes that partake in fatty acid oxidation[589].

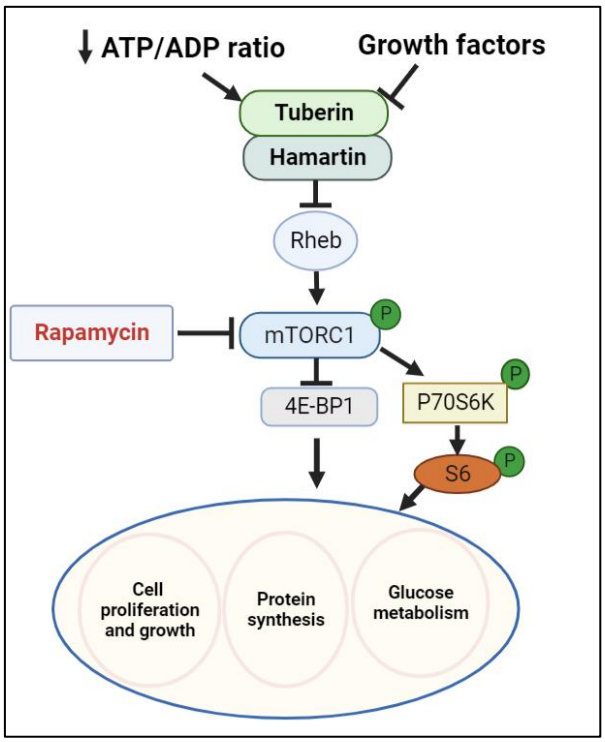

Figure 10: Scheme demonstrating the mTOR signaling axis.

Growth factor stimulation activate downstream targets that phosphorylate and inactivate the tuberous sclerosis (TSC) tumor suppressor protein complex. The inactivation of the TSC complex retains Rheb in the GTP-bound active form which, in turn, activates mTORC1 complex. mTORC1 activates cell proliferation, protein synthesis, and metabolism through the regulation of P70S6K and 4E-BP1.

Rheb: Ras homolog enriched in brain protein (Rheb). mTORC1: mammalian target of rapamycin complex 1; P70S6K: Ribosomal protein S6 kinase beta-1 (S6K1); 4E-BP1: eukaryotic translation initiation factor 4E (eIF4E)-binding protein-1.

1.7.5. The role of mTOR signaling in the regulation of cardiac homeostasis and physiological growth

Given its pleiotropic effects, mTORC1 regulates multiple physiological and pathological processes in the heart. mTORC1 was found to be indispensable for prenatal and postnatal cardiac development, normal cardiac homeostasis, and adaptation to cardiac hypertrophy[574,580,583]. The prominence of mTORC1 in the cardiac muscle was discerned in animal models with loss or gain of mTOR activity. Systemic knockout of mTOR and Raptor is embryonically lethal[590–592]. Moreover, embryos with cardiac-specific deletion of mTOR died in utero, while only 8% survived, indicating that mTOR is essential for embryogenesis[593]. Surviving embryos with cardiac-specific deletion of mTOR exhibited a significant reduction in cardiomyocyte proliferation associated with increased apoptosis[593]. As a result, the hearts displayed dilatation associated with fibrosis that, consequently, transitioned during development to heart failure[593]. Consistent with these data, adult mice with cardiac-specific ablation of mTOR or Raptor genes developed dilated cardiomyopathy that was characterized at the molecular level by apoptosis, mitochondrial dysfunction, and autophagy[594,589]. Similar results were observed in mice with cardiac-specific deletion of Rheb[595]. Deletion of Rheb reduced mTORC1 activity and resulted in embryonic lethality due to impaired sarcomere maturation, cardiomyocytes apoptosis, and cardiac dysfunction[595]. Altogether, these studies indicate that mTORC1 is involved in cardiac homeostasis and serves as an essential component for cardiac development during embryogenesis and postnatal growth.

1.7.6. The role of mTOR signaling in ischemic injury and myocardial infarction

Inhibition of mTORC1 preserves energy pools by reducing ATP-consuming processes and activating protective mechanisms such as autophagy, ultimately limiting myocardial infarction[574,580,583]. During ischemia and low energy conditions, mTORC1 activity is blunted through inhibition of Rheb by AMPK [66]. Forced reactivation of Rheb/mTOR cascade inhibited autophagy and stimulated cardiomyocyte death and endoplasmic reticulum stress in neonatal rat ventricular cardiomyocytes (NRVMs) subjected to glucose deprivation, leading to increased infarct size[596]. Consistent with these results, the pharmacological inhibition of mTORC1 by the

macrolide Rapamycin rescued nutrient-starved adult cardiomyocytes by activating autophagy[597], indicating that mTORC1 inhibition is cardioprotective during acute ischemia.

In clinical settings such as atherosclerosis, patients encounter prolonged periods of ischemia before reperfusion is restored[42,550]. In response to chronic ischemia, mTORC1 is activated in the remote region of the myocardium due to elevated load, and partakes in pathological ventricular remodeling following MI in rats[598,599]. Inhibition of mTORC1 by everolimus attenuated adverse LV remodeling and minimized infarct size following myocardial infarction in rats[598]. Mice with MI displayed cardiac dysfunction and structural malformations associated with apoptosis[600]. Interestingly, treating these mice with S6K1 or mTORC1 inhibitors reversed post-MI adverse remodeling features through the activation of Akt pathway[600]. Völkers et *al.* have demonstrated that the cardioprotective effects of mTORC1 inhibition following MI are based on a crosstalk between mTORC1 and mTORC2[599]. Indeed, PRAS40 overexpression inhibits mTORC1 and attenuates post-infarction remodeling and apoptosis by activating mTORC2-mediated activation of AKT cascade[599]. Accordingly, cardiac-specific deletion of Rictor deteriorated cardiac remodeling and aggravated cardiac dysfunction following MI[599]. These data confirm the cardioprotective effects of mTORC1 inhibition in MI and pinpoint the importance of both complexes in regulating cardiac adaptation in response to chronic ischemia.

Chapter 2: Hypothesis and Objectives

Following MI, the necrotic zone of the myocardium triggers ventricular remodeling, which is an adaptive response to preserve structural integrity and cardiac function. Cardiac remodeling is initiated by scar formation, also known as fibrosis, which emanates from the deposition of collagen. In parallel, several events are also associated with fibrosis namely chronic sympathetic activation and release of neurohumoral factors. As the scar expands, the loss of cardiomyocytes stimulates a hypertrophic response of the non-infarcted cardiomyocytes to compensate for the damaged zone of the myocardium and offset the increased hemodynamic load. The failure to normalize elevated wall stress leads to progressive dilatation and renders the ventricle prone to myocardial contractile dysfunction. Thus, scar formation and ventricular hypertrophy represent adaptive responses to stabilize cardiac contractility, attenuate progressive dilatation, and prevent cardiac rupture.

Ionic disturbance and modification of ion channel expression and function represent intrinsic responses to cardiac dysfunction during ventricular remodeling. Ca^{2+} and K^{+} flow *via* the voltage gated-L type Ca^{2+} channels and K^{+} channels, respectively, determine the cardiac action potential, which is critical for proper heart function. In addition to their electrophysiological role in cardiac contractility, Ca^{2+} channels and K_{ATP} channels play a prominent role in remodeling post-myocardial infarction. In particular, Ca^{2+} influx *via* $Ca_V1.2$ channels following sympathetic activation triggers Ca^{2+}-dependent signaling cascades involved in hypertrophic response. By contrast, the activation of ATP-sensitive potassium (K_{ATP}) channels is implicated in selective cardioprotection against scar expansion exclusively in females following MI. Despite its expression in male rodent heart, K_{ATP} channels limit scar expansion only in female heart following MI. Furthermore, a previous study reported that administration of the macrolide rapamycin to the infarcted female rat led to scar expansion, supporting the possible relationship between the target of rapamycin, mTORC1 and K_{ATP} channels in providing sex-specific cardioprotection. The latter relationship prospect was further underpinned by a study demonstrating that mTORC1 was coupled to K_{ATP} channel activation. However, whether mTORC1 targets K_{ATP} channels in the heart remains unknown. Over the past decade, a significant amount of work has been performed to investigate cardiac disease-induced remodeling of ion channels. However, little research has been directed to the mechanism of modulating L-type Ca^{2+} channels and K_{ATP} channel expression in

cardiac hypertrophy and myocardial infarction, respectively. **Thus, the AIM of the thesis was to investigate the modification of ion channels in cardiac remodeling post-MI by specifically addressing the role of Ca^{2+} channels in cardiomyocyte hypertrophy and elucidate the potential relationship between K_{ATP} channels and mTORC1 signaling in scar expansion following MI.** Hence, the following studies are based on several hypotheses and tackled by specific aims:

Specific hypotheses

Hypothesis I - Chapter 3

Hypertrophy of neonatal rat ventricular cardiomyocytes (NRVMs) following sympathetic stimulation translates to enhanced calcium influx due to the upregulation of $Ca_V1.2$ channel subunits.

Specific aims

- Examine the characteristics of $Ca_V1.2$ and its subunits at the level of protein expression and changes in localization and current in cardiac hypertrophy.
- Investigate the mechanism underlying the change/increase of the $Ca_V\alpha2\delta1$ subunit.

Hypothesis II - Chapter 4

The impact of the macrolide rapamycin on scar expansion of the infarcted adult rat heart was sex-specific and mTOR signaling directly influenced K_{ATP} channel subunit expression.

Specific aims

- Elucidate the differential protein expression of K_{ATP} channels and phosphorylated form of mTOR in male and female adult hearts.
- Investigate the impact of P-mTOR on K_{ATP} channels expression in NRVMs
- Test the effect of rapamycin on scar size and K_{ATP} channel subunit expression post-MI

Chapter 3: Article 1

Title: Sympathetic stimulation upregulates the Ca^{2+} channel subunit, $Ca_V\alpha2\delta1$, *via* the β1 and ERK 1/2 pathway in Neonatal Ventricular Cardiomyocytes

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Contribution of authors

Aya Al Katat: Conceptualization, performed the isolation of cardiomyocytes and treatments, performed immunoblotting and confocal imaging, analyzed the data, interpreted the results, carried out statistical analysis, prepared the figures, drafted the manuscript, edited, and revised the manuscript.

Juan Zhao: Performed patch-clamp experiments, analyzed the data, interpreted the electrophysiology results, and prepared figure 2.

Angelino Calderone: General supervision, Conceived and designed the experiments, interpreted the results, corrected the manuscript, and approved the final version of the manuscript.

Lucie Parent: General supervision, Conceived and designed the experiments, interpreted the results, corrected the manuscript, and approved the final version of the manuscript.

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Sympathetic Stimulation Upregulates the Ca^{2+} Channel Subunit, $Ca_V\alpha 2\delta 1$, via the β1 and ERK 1/2 Pathway in Neonatal Ventricular Cardiomyocytes

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Abstract: Intracellular Ca^{2+} overload secondary to chronic hemodynamic stimuli promotes the recruitment of Ca^{2+}-dependent signaling implicated in cardiomyocyte hypertrophy. The present study tested the hypothesis that sympathetic-mediated hypertrophy of neonatal rat ventricular cardiomyocytes (NRVMs) translated to an increase in calcium influx secondary to the upregulation of $Ca_V1.2$ channel subunits. Confocal imaging of norepinephrine (NE)-treated NRVMs revealed a hypertrophic response compared to untreated NRVMs. L-type $Ca_V1.2$ peak current density was increased 4-fold following a 24-h stimulation with NE. NE-treated NRVMs exhibited a significant upregulation of $Ca_V\alpha2\delta1$ and $Ca_V\beta3$ protein levels without significant changes of $Ca_V\alpha1C$ and $Ca_V\beta2$ protein levels. Pre-treatment with the β_1-blocker metoprolol failed to inhibit hypertrophy or $Ca_V\beta3$ upregulation whereas $Ca_V\alpha2\delta1$ protein levels were significantly reduced. NE promoted the phosphorylation of ERK 1/2, and the response was attenuated by the β_1-blocker. U0126 pre-treatment suppressed NE-induced ERK1/2 phosphorylation but failed to attenuate hypertrophy. U0126 inhibition of ERK1/2 phosphorylation prevented NE-mediated upregulation of $Ca_V\alpha2\delta1$, whereas $Ca_V\beta3$ protein levels remained elevated. Thus, β_1-adrenergic receptor-mediated recruitment of the ERK1/2 plays a seminal role in the upregulation of $Ca_V\alpha2\delta1$ in NRVMs independent of the concomitant hypertrophic response. However, the upregulation of $Ca_V\beta3$ protein levels may be directly dependent on the hypertrophic response of NRVMs.

Keywords: cardiac hypertrophy; L-type Ca^{2+} channels; sympathetic stimulation; adrenergic stimulation; ERK 1/2; cardiomyocytes

Abbreviations: NRVMs: neonatal rat ventricular cardiomycoytes; NE: Norepinephrine; ERK 1/2: Extracellular regulated kinase 1/2; LTCCs: voltage-gated L-type Ca^{2+} channels

Introduction

Normal intracellular Ca^{2+} cycling and homeostasis are required for cardiac excitability, contractility, and gene expression [1,2]. Several studies have reported that intracellular calcium overload secondary to a sustained hemodynamic stimulus contributed to the development of cardiac hypertrophy *via* recruitment of the nuclear factor of activated T cells (NFAT) pathway and calmodulin kinase-dependent signaling events [3,4,5]. Cardiac hypertrophy is an adaptive mechanism secondary to a sustained chronic hemodynamic overload [6,7,8]. In response to elevated mean arterial pressure, the heart develops a concentric pattern of cardiac hypertrophy [6,7,8]. During the hypertrophic response, new sarcomeres are added in a parallel fashion leading to an increase in the width of individual ventricular cardiomyocytes [6,7,8]. Morphologically, the latter response translates to increased ventricular wall thickness and reduced chamber diameter [6,7,8].

In ventricular cardiomyocytes, voltage-gated L-type Ca^{2+} channels (LTCCs) mediate extracellular Ca^{2+} entry initiating Ca^{2+}-induced Ca^{2+} release and triggering cardiac excitation-contraction coupling [1,9,10]. LTCC are oligomeric proteins composed of the pore-forming subunit $Ca_V\alpha1$ bound to the auxiliary subunits $Ca_V\beta$, calmodulin, and $Ca_V\alpha2\delta$ [11,12]. In ventricular cardiomyocytes, the major isoforms are $Ca_V\alpha1C$ [13,14], $Ca_V\beta2$ [14,15,16], and $Ca_V\alpha2\delta1$ [17,18]. $Ca_V\alpha1C$ is the pore-forming subunit responsible for Ca2+ selectivity, activation and inactivation kinetics [10,19]. $Ca_V\beta$ is a cytoplasmic chaperone protein that supports the trafficking of $Ca_V1.2$ channels to the plasma membrane [20,21,22]. $Ca_V\alpha2\delta1$ is a large extracellular Glycosylphosphatidylinositol (GPI)-anchored protein that facilitates channel activation at physiological voltages and stimulates (≈5-fold) peak current density [9,23,24,25]. In human embryonic kidney (HEK) cells, the co-expression of $Ca_V\alpha1C$ and $Ca_V\alpha2\delta1$ increased the peak current density of $Ca_V1.2$ [23].

Numerous studies have identified the sympathetic system as an essential homeostatic mechanism providing inotropic support, whereas chronic activation contributes in part to the progression of cardiac hypertrophy. Acute sympathetic stimulation of ventricular cardiomyocytes via the β1-adrenergic receptor activation increases voltage-gated L-type Ca^{2+} currents via protein kinase A phosphorylation which releases the Rad-mediated inhibition through a likely reduction in the affinity of Rad with the membrane and the $Ca_V\beta$-subunit of the channel [26]. The relationship

between chronic stimulation of the sympathetic system and Ca^{2+} overload remains unresolved. Therefore, the present study tested the hypothesis that sympathetic-mediated hypertrophy of neonatal rat ventricular cardiomyocytes (NRVMs) translated to an increase in Ca^{2+} influx secondary to the upregulation in the protein expression of $Ca_V1.2$ channel subunits.

Material and Methods

Animal Ethics Approval

The use and care of laboratory rats were according to the Canadian Council for Animal Care and approved by the Animal Care Committee of the Montreal Heart Institute.

Isolation of Neonatal Ventricular Cardiomyocytes

Neonatal rat ventricular cardiomyocytes (NRVMs) were isolated from 1-day old Sprague-Dawley rat pups (sacrificed by decapitation) (Charles River, Senneville, QC, Canada) as previously described [7,27,28]. Ventricular cells were plated at a density of 400 cells/mm2 in DMEM-low glucose (Hyclone Laboratories, Logan, UT, USA) supplemented with 7% heat-inactivated FBS and 1% penicillin-streptomycin for 24 h, subsequently washed and maintained in DMEM-low glucose containing insulin (5 μg/mL), transferrin (5 μg/mL), and selenium (5 ng/mL) (ITS; BD Bioscience, Bedford, MA, USA) for 24 h prior to the experimental protocol. In all experiments, neonatal rat ventricular cardiomyocytes were plated at a density of 400–500 cells/mm^2.

Treatment of Neonatal Ventricular Cardiomyocytes

Cardiomyocytes were treated with 1 μM norepinephrine (NE) for 1, 4, 16, 24, or 48 h. The untreated cardiomyocytes were subjected to the same procedure and plating duration as norepinephrine-treated cardiomyocytes. In parallel experiments, neonatal cardiomyocytes were pre-treated with 10 μM U0126 (CST, 9303S, Danvers, MA, USA) or 100 nM metoprolol tartrate (TOCRIS, 3251, Barton, UK) for 30 min to 1 h prior to the addition of 1 μM NE for 24 h.

Immunofluorescence and Surface Area Assessment

Neonatal rat ventricular cardiomyocytes (NRVMs) were plated on glass coverslips coated with poly-D-lysine in 12-well plates. Cardiomyocytes were fixed with 2% paraformaldehyde for 20 min. Immunofluorescence was performed as previously described [27]. Primary antibodies employed include mouse anti-Troponin-T (1:200; Abcam, Cambridge, UK; ab8295), rabbit anti-

CaVα2δ1 (1:200, Alomone, Jerusalem, Israel, ACC-015), and rabbit anti-CaVα1C (1:200, Alomone, ACC-003). Secondary antibodies employed include goat anti-mouse coupled to Alexa 555 (1:800; Invitrogen, Waltham, MA, USA; A21424) and donkey anti-rabbit coupled to Alexa 488 (1:800; Invitrogen; A21206). The nucleus was identified with 4′,6′-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA) staining, and 4′,6-diamidino-2-phenylindole DAPI (1:800). Images were captured by confocal microscope with 20× or 63× objective. Images were analyzed using Zeiss LSM image software. The cross-sectional area (μm2) of 250 Troponin-T+ mononucleated cardiomyocytes from 5 different images per condition was determined using Zeiss LSM image software. For the colocalization analysis, wheat germ agglutinin (WGA) antibody (1:200; Invitrogen; W32466) was added to cardiomyocytes prior to fixation.

Western Blot

Protein lysates of neonatal rat ventricular cells were prepared and subjected to SDS-electrophoresis, as previously described [27]. Lysates containing 30 μg of proteins were subjected to SDS-polyacrylamide gel (8%) electrophoresis and transferred to a nitrocellulose paper. Antibodies used were rabbit anti-Cavα2δ1 (1:1000, Alomone ACC-015), rabbit anti-Cavα1C (1:1000, Alomone ACC-003), rabbit anti-Cavβ2 (1:5000, Alomone ACC-105), rabbit anti-Cavβ3 (1:500, Alomone ACC-008), rabbit anti-Phospho-ERK 1/2 (Cell Signaling Technology, Danvers, MA, USA, 9101S), rabbit anti-Total ERK 1/2 (Cell Signaling Technology, 137F5) and rabbit anti-GAPDH (Jackson, West Grove, PA, USA, 111-035-144). Membranes were incubated with primary antibodies overnight at 4 °C followed by incubation with secondary anti-rabbit antibody (1:10,000, Jackson) for 2 h at room temperature. Signal was detected using enhanced chemiluminescence (ECL) substrate.

Patch-Clamp

Whole-cell voltage-clamp recordings were performed in isolated NRVMs. Patch-clamp experiments were carried out with the Axopatch 200-B amplifier (Molecular Devices, Union City, CA, USA). PClamp software Clampex 10.4 coupled to a Digidata 1440A acquisition system (Molecular Devices, San Jose, CA, USA) was used for on-line data acquisition and analysis. Electrodes were filled with a solution containing (in mM) 140 CsCl, 0.6 NaGTP, 3 MgATP, and 10 EGTA, 10 HEPES, titrated to pH 7.4 with KOH. Pipette resistance ranged from 3 to 5 megohms. The bath solution contained (in mM) 135 NMDG, 20 tetraethylammonium chloride, 2 CaCl2, 1

MgCl2, and 10 HEPES, titrated to pH 7.4 with HCI. The measurements were performed at room temperature (22–25 °C).

Following a 40 ms prepulse to −40 mV to inactivate Na+ channels, Ca^{2+} currents were elicited from a holding potential of −80 mV and were depolarized to potentials ranging from −80 to 50 mV in 5 mV increments lasting 450 ms for each step (protocol shown in the inset above the current traces). Ca^{2+} current densities (pA/pF) were obtained by dividing the peak currents by the cell capacitance. Average I-V curves were obtained by plotting peak current densities as a function of applied voltage. The I-V relationships were fitted to a Boltzmann equation. Patch-clamp data were analyzed using Clamp fit software 10.4 (Molecular Devices), Microsoft Excel 2016 (Microsoft, Redmond, WA. USA), and Origin 2020 (Northhampton, MA, US).

Statistics

Data were presented as the mean ± S.E.M., and n represents the number of rat litters employed. Data were evaluated by one-way ANOVA analysis followed by Dunnett's multiple comparison test or Tukey's multiple comparison test (GraphPad). A value of $p < 0.05$ was considered statistically significant.

Results

Norepinephrine Induced Neonatal Rat Ventricular Cardiomyocyte Hypertrophy and Increased $Ca_V1.2$ Peak Current Density

The temporal hypertrophic response of neonatal rat ventricular cardiomyocytes (NRVMs) in response to 1 μM norepinephrine (NE) was examined. As compared to untreated NRVMs, a significant hypertrophic response as depicted by the increase in cell surface area was detected after a 24-h stimulation with NE, and hypertrophy persisted after 48 h (Figure 1A, B). In parallel, electrophysiological recordings using the whole-cell patch-clamp technique was performed on NE-treated NRVMs to assess the $Ca_V1.2$ peak current density. Whole-cell Ca^{2+} current traces were detected in untreated NRVMs (−5.5 ± 0.6 pA/pF, n = 9, N = 2) (Figure 2A, B). In hypertrophied NRVMs secondary to a 24-h stimulation with 1 μM NE, a significant ($p < 0.01$) four-fold increase in peak current density was detected (−20 ± 1.0 pA/pF, n = 5, N = 2) (Figure 2A, B). By contrast, activation kinetics of $Ca_V1.2$ Ca^{2+} currents in untreated and hypertrophied NRVMs were similar as the analysis of the Ca^{2+} whole-cell conductance properties yielded a $V_{1/2}$ of −14 ± 1 mV for untreated and −16 ± 1 mV for hypertrophied NRVMs ($p = 0.13$) (Figure 2C).

$Ca_V\alpha2\delta1$ and $Ca_V\beta3$ Are Upregulated in Norepinephrine-Induced Hypertrophy of Neonatal Rat Ventricular Cardiomyocytes

The stimulation of NRVMs with 1μM NE led to a significant increase of $Ca_V\alpha2\delta1$ protein levels at 24 h and remained elevated at 48 h as compared to untreated NRVMs (Figure 3A, B). Furthermore, 1μM NE treatment of NRVMs for 24 and 48 h significantly increased $Ca_V\beta3$ protein levels (Figure 3E, F). The increase of $Ca_V\alpha2\delta1$ and $Ca_V\beta3$ protein levels coincided with the hypertrophic response elicited by norepinephrine (Figure 1A, B). By contrast, $Ca_V\alpha1C$ and $Ca_V\beta2$ protein levels were not significantly altered in NRVMs treated with NE as compared to untreated NRVMs (Figure 3C, D, G, H).

The Subcellular Localization of $Ca_V\alpha2\delta1$ or $Ca_V\alpha1C$ Was Not Altered in Hypertrophic Neonatal Rat Ventricular Cardiomyocytes

In the present study, NE stimulation of NRVMs failed to alter the protein levels of $Ca_V\alpha1C$ (Figure 4A, C). Nonetheless, additional experiments were performed to assess whether NE altered the subcellular distribution of the $Ca_V\alpha1C$ subunit. Cardiac staining was used to confirm that the

cells examined were cardiomyocytes (data not shown). NRVMs were stained with cardiac troponin-T (staining not shown), $Ca_V\alpha1C$ subunit, wheat germ-agglutinin (WGA), or 4′,6-diamidino-2-phenylindole (DAPI; a nuclear marker). $Ca_V\alpha1C$ localization was determined by Pearson's coefficient assessing the convergence of the immunofluorescent signal between WGA/$Ca_V\alpha1C$ and DAPI/$Ca_V\alpha1C$. Figure 4A depicts immunofluorescence confocal images of NRVMs co-stained with WGA (red) and $Ca_V\alpha1C$ (green). In untreated NRVMs, $Ca_V\alpha1C$ staining was detected predominantly on the plasma membrane and perinuclear region (Figure 4A). In NRVMs treated with 1 μM NE for 24 or 48 h, $Ca_V\alpha1C$ localization at the plasma membrane was not altered (Pearson's coefficient, $r \sim 0.85$) as compared to untreated NRVMs (Pearson's coefficient, $r \sim 0.83$; $p > 0.05$) (Figure 4A, C).

Figure 4B depicts immunofluorescence confocal images of NRVMs co-stained with DAPI (red) and $Ca_V\alpha1C$ (green). The co-staining of NRVMs with DAPI and $Ca_V\alpha1C$ confirmed the perinuclear localization (convergence depicted by yellow signal) of the pore-forming subunit (Figure 4B). In NE-treated NRVMs (24 and 48 h), $Ca_v\alpha1C$ localization at the perinuclear region persisted (Pearson's coefficient, $r \sim 0.26$) and was similar to that observed in untreated cardiomyocytes (Pearson's coefficient, $r \sim 0.3$; $p > 0.05$) (Figure 4B, D).

Previous studies reported that $Ca_V\alpha2\delta1$ was localized at the plasma membrane of neonatal mouse cardiomyocytes [9]. Immunofluorescence experiments were performed to assess whether the upregulation of $Ca_V\alpha2\delta1$ protein levels secondary to NE-induced cardiac hypertrophy was associated with a greater translocation to the plasma membrane. Therefore, the colocalization of $Ca_V\alpha2\delta1$ with WGA or DAPI was examined. Figure 5A depicts immunofluorescence confocal images of NRVMs co-stained with WGA (red) and $Ca_V\alpha2\delta1$ (green). In NRVMs treated with 1 μM NE for 24 or 48 h, $Ca_V\alpha2\delta1$ localization at the plasma membrane was not altered (Pearson's coefficient, $r \sim 0.8$) when compared to untreated NRVMs (Pearson's coefficient, $r \sim 0.79$; $p > 0.05$) (Figure 5A, C). The confocal staining experiments are supportive of plasma membrane localization.

Figure 5B demonstrates immunofluorescence confocal images of NRVMs stained with DAPI (red) and $Ca_V\alpha2\delta1$ (green). The co-staining of NRVMs with DAPI and $Ca_V\alpha2\delta1$ in untreated NRVMs revealed a perinuclear and nuclear signal (convergence depicted by yellow signal) of the calcium subunit (Pearson's coefficient, $r \sim 0.51$) (Figure 5B). As seen, $Ca_V\alpha2\delta1$ localization at the plasma

membrane was not altered when comparing untreated NRVMs (Pearson's coefficient, r ~ 0.79)) with NRVMs treated with 1 μM NE for 24 or 48 h (Pearson's coefficient, r ~ 0.8); p > 0.05 (Figure 5A, C). Figure 5B demonstrates immunofluorescence confocal images of NRVMs stained with DAPI (red) and $Ca_V\alpha2\delta1$ (green). The co-staining with DAPI and $Ca_V\alpha2\delta1$ in untreated NRVMs revealed the presence of the subunit in perinuclear and nuclear regions, depicted by a yellow signal (Pearson's coefficient, r ~ 0.51) (Figure 5B). $Ca_V\alpha2\delta1$ localization in the perinuclear and nuclear regions persisted and was non-significantly reduced in NRVMs treated with 1 μM NE for 24 h or 48 h, (Pearson's coefficient, r ~ 0.34; p > 0.05; n = 3) (Figure 5B, D). In NRVMs treated with 1 μM NE for 24 h or 48 h, $Ca_V\alpha2\delta1$ localization in the perinuclear and nuclear regions persisted and was non-significantly reduced (Pearson's coefficient, r ~ 0.34; p > 0.05; n = 3) (Figure 5B, D).

β1-Adrenergic Receptor-Mediated NE-Induced Upregulation of $Ca_V\alpha2\delta1$ via Recruitment of the Downstream Signaling Kinase ERK 1/2

The pre-treatment of NRVMs with the selective β_1-adrenergic receptor antagonist metoprolol (100 nM) failed to inhibit NE-mediated hypertrophy (Figure 6A, B). However, NE-mediated upregulation of the $Ca_V\alpha2\delta1$ subunit in NRVMs after a 24-h stimulation was significantly attenuated following the pre-treatment with metoprolol (Figure 7A, B). By contrast, metoprolol pre-treatment did not inhibit NE-mediated upregulation of $Ca_V\beta3$ subunits (Figure 7C, D). The temporal pattern of ERK1/2 activation in NRVMs in response to NE was examined. A transient pattern of phosphorylation was observed as ERK1/2 phosphorylation was significantly increased 1 h after NE treatment (Figure 8A,B). Thereafter, ERK1/2 phosphorylation returned to baseline levels 4 and 16 h after NE treatment (Figure 8A,B). However, in hypertrophic NRVMs, ERK1/2 phosphorylation was significantly increased 24 h after NE treatment and remained elevated at 48 h (Figure 8A,B). In the presence of the β_1-adrenergic receptor antagonist metoprolol (100 nM), NE-mediated ERK1/2 phosphorylation was inhibited (Figure 9A,B). The pre-treatment with 10 μM U0126, a selective inhibitor of MEK1/2 (upstream activator of ERK1/2), suppressed NE-mediated phosphorylation of ERK1/2 (Figure 9A,B) but failed to attenuate the hypertrophic response (Figure 6A,B). Moreover, the pre-treatment with U0126 attenuated NE-mediated upregulation of $Ca_V\alpha2\delta1$, whereas $Ca_V\beta3$ protein levels remained elevated (Figure 7A,B).

Discussion

Numerous in vitro and in vivo studies have delineated the role of calcium-dependent signaling events linking various stimuli to ventricular cardiomyocyte hypertrophy [29,30,31]. However, the relationship between increased intracellular Ca^{2+} and the expression of the voltage-gated L-type Ca^{2+} channels in response to a hypertrophic stimulus remains unresolved. To address the latter paradigm, neonatal rat ventricular cardiomyocytes (NRVMs) were treated with norepinephrine as numerous studies have previously established a hypertrophic role of the sympathetic neurotransmitter. As demonstrated in previous studies, the exposure of NRVMs to sympathetic stimulation for 24 and 48 h led to a significant increase in the cell surface area as compared to untreated NRVMs. In parallel, L-type $Ca_V1.2$ peak current density was significantly elevated in NRVMs secondary to NE-induced cardiomyocyte hypertrophy. These data provided the impetus to assess the individual role of the subunits forming the oligomeric $Ca_V1.2$ channel in the increased Ca^{2+} influx in NE-induced hypertrophied NRVMs.

Activation of voltage-gated L-type Ca^{2+} channels in ventricular cardiomyocytes in response to acute sympathetic stimulation occurs predominantly via β-adrenergic receptor-mediated recruitment of protein kinase A and subsequent channel phosphorylation [32]. The present study further revealed that sympathetic system stimulation of NRVMs for a period of 24 h significantly increased voltage-gated L-type Ca^{2+} channel activity. The increase in the L-type CaV1.2 peak current density in NE-treated NRVMs was associated with a significant upregulation of $Ca_V\alpha2\delta1$ with a more modest increase in the protein expression of $Ca_V\beta3$. In contrast, the protein levels of the pore-forming $Ca_V\alpha1C$ and the accessory $Ca_V\beta2$ were unchanged. Previous work from our lab revealed that co-expression of $Ca_V\alpha1C$ and $Ca_V\alpha2\delta1$ subunits upregulated by 5- to 10-fold the peak current density and facilitated the opening of the L-type CaV1.2 activity at physiological voltages [9]. The more modest increase in $Ca_V\beta3$ protein levels in NE-induced hypertrophy of NRVMs was reported to be insufficient to promote on its own a change in the activity of $Ca_V1.2$ [9]. The upregulation of $Ca_V\alpha2\delta1$ protein levels in NE-treated NRVMs was blunted by pre-treatment with the selective β1-blocker metoprolol, whereas $Ca_V\beta3$ subunit upregulation remained unchanged. Moreover, metoprolol did not inhibit NE-mediated hypertrophy, which was in part consistent with the predominant role of the α1-adrenergic receptor in cardiomyocyte hypertrophy [33]. Collectively, these data highlight the novel finding that β1-adrenergic receptor-mediated upregulation of $Ca_V\alpha2\delta1$ protein levels in response to NE may have contributed in part to increased

L-type Ca^{2+} channel activity independent of the concomitant hypertrophic response. By contrast, upregulation of $Ca_V\beta3$ protein levels may be directly dependent on the hypertrophic response of NRVMs secondary to sympathetic stimulation. These data are supportive of β1-adrenergic receptor-mediated NE-induced upregulation of $Ca_V\alpha2\delta1$, but other regulators of adrenergic receptors need to be added to support the conclusion.

The upregulation of the $Ca_V\alpha2\delta1$ protein expression was not accompanied by a significant change in its plasma membrane localization. Immunofluorescence confocal images of untreated and NE-treated NRVMs co-stained with WGA and $Ca_V\alpha2\delta1$ revealed that localization of the subunit at the plasma membrane was similar. Moreover, the co-staining of DAPI and $Ca_V\alpha2\delta1$ in perinuclear and nuclear regions was not significantly modified in NE-treated NRVMs. Similarly, we failed to observe any significant change in the localization of $Ca_V\alpha1C$ at the plasma membrane in NE-hypertrophied NRVMs. It remains to be seen if the perinuclear/nuclear distribution of $Ca_V\alpha2\delta1$ and $Ca_V\alpha1C$ is exclusive to NRVMs or is conserved in adult cardiomyocytes after postnatal development.

A previous study reported that EGF stimulation of GH3 pituitary cells increased $Ca_V\alpha2\delta1$ protein levels via recruitment of the Ras/MEK/ERK 1/2 signaling pathway [34]. In NRVMs, sympathetic stimulation of the β1-adrenergic receptor is known to promote ERK 1/2 phosphorylation [35]. NE treatment of NRVMs translated to a biphasic pattern of ERK 1/2 phosphorylation and was elevated at 24 h coincident with cardiac hypertrophy. The β1-adrenergic receptor antagonist metoprolol suppressed NE-mediated ERK 1/2 phosphorylation. Furthermore, pharmacological inhibition of the upstream activator MEK with U0126 suppressed NE-mediated ERK 1/2 phosphorylation and the concomitant upregulation of $Ca_V\alpha2\delta1$ protein levels. By contrast, U0126 failed to inhibit NE-mediated NRVM hypertrophy and upregulation of $Ca_V\beta3$ protein levels. The absence of an anti-hypertrophic effect of U0126 after NE treatment of NRVMs was consistent with previous data demonstrating that recruitment of the ERK 1/2 signaling pathway alone was insufficient to promote cardiomyocyte hypertrophy in response to various stimuli [36].

The present study has revealed that in addition to the acute increase in Ca^{2+} influx via activation of the L-type $Ca_V1.2$ channel after sympathetic discharge [10], chronic NE treatment of NRVMs translated to a sustained increase in Ca^{2+} channel activity. The latter response required β1-adrenergic receptor-mediated recruitment of the tyrosine kinase ERK 1/2 translating to the

increased expression of the Cav1.2 auxiliary subunit, Cavα2δ1. β1-adrenergic receptor-mediated upregulation of the Cavα2δ1 subunit was independent of the hypertrophic response. Upregulation in the protein expression of the Cavα2δ1 subunit secondary to sympathetic hyperactivity may hence contribute to intracellular Ca^{2+} overload with or without hypertrophy [37].

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Institutional Review Board Statement: The animal study protocol was approved by the Animal Ethics Committee of the Montreal Heart Institute (protocol number 2018-44-02 initially approved on November 6, 2018, and #2019-2434 renewed on November 6, 2020).

Informed Consent Statement: Not applicable

Data Availability Statement: The datasets used and/or analyzed during the study are available from the first and the corresponding author upon reasonable request

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Figure legends

Figure 1. (A) Neonatal rat ventricular cardiomyocytes (NRVMs) in basal condition, treated with 1 μM norepinephrine (NE) for 4 h, 24 h or 48 h. Cardiomyocytes were fixed with 2% PAF for 20 min at room temperature. Cardiomyocytes were then permeabilized with 0.2% Triton-X. Primary antibody: mouse anti-Troponin-T was added for 90 min at room temperature followed by overnight incubation at 4 °C. This is followed by incubation of secondary antibody: Goat anti-mouse Alexa 555; and DAPI for 90 min at room temperature in the dark. Images were captured by microscope with 20× objective. Images were analyzed using ZEN software, and the surface area of 250 cells was measured by marking the borders of each cell. (**B**) Dot plot showing the surface area of each cardiomyocyte. The red line represents the mean surface area of 250 cells. * $p < 0.01$ vs. Basal (5 cardio preparation). Statistical analysis was performed using one-way ANOVA.

Figure 2. (**A**, **B**) Representative whole-cell Ca^{2+} current traces were recorded from neonatal rat cardiomyocytes under basal conditions (in red) or were treated with 1 μM norepinephrine (NE) for 24 h (in blue). In all cases, the cells were bathed in a saline physiological solution containing 2 mM Ca^{2+} in the absence of NE. The pulse protocol is shown above the traces. Currents were elicited from a holding potential of −80 mV and recorded at potentials ranging from −80 to 50 mV in 5 mV increments. Na^{+} currents were suppressed by applying a 40 ms prepulse to −40 mV as shown in the inset above the current traces. (**C**) Mean current-voltage relationships of whole-cell Ca^{2+} currents recorded from neonatal rat cardiomyocytes (NRVMs). Current densities were obtained by normalizing whole-cell current amplitudes to the membrane capacitance and were plotted versus applied voltages. The Boltzman analysis of Ca^{2+} channel activation voltage yielded a mid-point of activation at $V_{1/2} = -14 \pm 1$ mV (mean ± S.E.) (n=9, N= 2 repetitions) under basal conditions, and a mid-point of activation at $V_{1/2} = -16 \pm 1$ mV (n=5, N= 2 repetitions) for NE-treated NRVMs ($p = 0.13$). Experiments were carried out with 2 distinct cell preparations, and the total number of cells that were patched were pooled. (**D**) Bar graph of the average peak current density of basal and NE-treated groups. The NE-treated group displays a 4-fold increase in the peak current density compared with that of the basal currents (−20 ± 1 pA/pF for NE-treated vs. −5.5 ± 0.6 pA/pF for basal, * $p < 0.01$).

Figure 3. Expression of $Ca_V\alpha2\delta1$ (**A**), $Ca_V\alpha1C$ (**C**), $Ca_V\beta3$ (**E**), and $Ca_V\beta2$ (**G**) in neonatal rat cardiomyocytes. Total proteins were extracted from neonatal rat ventricular cardiomyocytes. Proteins were separated on an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-$Ca_V\alpha2\delta1$, anti-$Ca_V\alpha1C$, anti-$Ca_V\beta3$, anti-$Ca_V\beta2$, and anti-GAPDH antibodies overnight and then incubated with HRP-conjugated goat anti-rabbit secondary antibody. Lanes were loaded with 30 µg of proteins. NE: cardiomyocytes treated with 1 µM norepinephrine. Graph showing the total protein expression of $Ca_V\alpha2\delta1$ (**B**), $Ca_V\alpha1C$ (**D**), $Ca_V\beta3$ (F) (**E**), and $Ca_V\beta2$ (H) (**F**) normalized to GAPDH. * $p < 0.01$ vs. Basal. Statistical analysis was performed using one-way ANOVA.

Figure 4. Neonatal rat cardiomyocytes in basal condition or treated with NE for 24–48 h. WGA-Alexa 647 was added to live cardiomyocytes at room temperature followed by fixation with 2% PAF for 20 min at room temperature. Cardiomyocytes were then permeabilized with 0.2% Triton-X. Primary antibody: mouse anti-Troponin-T and rabbit anti-Ca_Va1C were added for 90 min at room temperature, followed by overnight incubation at 4 °C. This was followed by incubation of secondary antibodies: goat anti-mouse Alexa 555; Donkey anti-rabbit Alexa 488, and DAPI for 90 min at room temperature in the dark. Images were captured by microscope with 20× objective. Images were analyzed using ZEN software. (**A**) Images showing colocalization of WGA and $Ca_V\alpha1C$. Green: $Ca_V\alpha1C$; Red: WGA; Yellow: Overlap between $Ca_V\alpha1C$ and WGA. (**B**) Images showing colocalization of DAPI and $Ca_V\alpha1C$. Green: $Ca_V\alpha1C$; Red: DAPI; Yellow: Overlap between $Ca_V\alpha1C$ and WGA. (**C**) Graph showing Pearson's correlation coefficient of the overlap between WGA and $Ca_V\alpha1C$. (**D**) Graph showing Pearson's correlation coefficient of the overlap between DAPI and $Ca_V\alpha1C$. Pearson's correlation coefficient of the overlap is determined using ImageJ.

Figure 5. Neonatal rat cardiomyocytes in basal condition or treated with NE for 24–48 h. WGA-Alexa 647 was added to live cardiomyocytes at room temperature, followed by fixation with 2% Paraformaldehyde for 20 min at room temperature. Cardiomyocytes were then permeabilized with 0.2% Triton-X. Primary antibody: mouse anti-Troponin-T and rabbit anti-$Ca_V\alpha2\delta1$ were added for 90 min at room temperature followed by overnight incubation at 4 °C. This was followed by incubation of secondary antibodies: Goat anti-mouse Alexa 555; Donkey anti-rabbit Alexa 488,

and DAPI for 90 min at room temperature in the dark. Images were captured by microscope with 20× objective. Images were analyzed using ZEN software. (**A**) Images showing colocalization of WGA and $Ca_V\alpha2\delta1$. Green: $Ca_V\alpha2\delta1$; Red: WGA; Yellow: Overlap between $Ca_V\alpha2\delta1$ and WGA. (**B**) Images showing colocalization of DAPI and $Ca_V\alpha2\delta1$. Green: $Ca_V\alpha2\delta1$; Red: DAPI; Yellow: Overlap between $Ca_V\alpha2\delta1$ and WGA. (**C**) Graph showing Pearson's correlation coefficient of the overlap between WGA and $Ca_V\alpha2\delta1$. (**D**) Graph showing Pearson's correlation coefficient of the overlap between DAPI and $Ca_V\alpha2\delta1$. Pearson's correlation coefficient (r) of the overlap is determined using ImageJ.

Figure 6. (**A**) Neonatal rat cardiomyocytes in basal condition, treated with NE for 24 h, or pre-treated with either 10μM U0126 or metoprolol tartrate (β1-blocker) for 1 h followed by treatment with NE for 24 h. Cardiomyocytes were fixed with 2% PAF for 20 min at room temperature. Cardiomyocytes were then permeabilized with 0.2% Triton-X. Primary antibody: mouse anti-Troponin-T was added for 90 min at room temperature followed by overnight incubation at 4 °C. This is followed by incubation of secondary antibody: Goat anti-mouse Alexa 555; and DAPI for 90 min at room temperature in the dark. Images were captured by microscope with 20× objective. Images were analyzed using ZEN software, and the surface area of 250 cells was measured by marking the borders of each cell. Statistical analysis was performed using one-way ANOVA. (B) Dot plot showing the surface area of each cardiomyocyte. The red line represents the mean surface area of 250 cells. *$p < 0.01$ vs. Basal. ($n = 4$). Statistical analysis was performed using one-way ANOVA.

Figure 7. Expression of Cavα2δ1 (A) and CaVβ3 (C) in neonatal rat cardiomyocytes. Total proteins were extracted from neonatal rat ventricular cardiomyocytes. Cells were treated with 1 μM Norepinephrine (NE) for 24 h or pre-treated with metoprolol tartrate (β1-blocker) for 1 h followed by treatment with NE for 24 h. Proteins were separated on an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-Cavα2δ1, anti-Cavβ3, and anti-GAPDH antibodies overnight and then incubated with HRP-conjugated goat anti-rabbit secondary antibody. Lanes were loaded with 30 μg of proteins. NE: cardiomyocytes treated with 1 μM norepinephrine. 24 h + metoprolol tartrate: Cardiomyocytes pre-treated with metoprolol tartrate (β1-blocker) for 1 h, followed by treatment with NE for 24 h. (B) Graph showing the total protein

expression of Cavα2δ1 normalized to GAPDH. * $p < 0.01$ vs. Basal untreated NRVMs; † $p < 0.01$ vs. NE 24 h. (4 cardio-preparations). (D) Graph showing the total protein expression of CaVβ3 normalized to GAPDH. * $p < 0.01$ vs. Basal untreated NRVMs (4 cardio-preparations). Statistical analysis was performed using one-way ANOVA.

Figure 8. (A) Expression of p-ERK 1/2 in neonatal rat cardiomyocytes. Total proteins were extracted from neonatal rat ventricular cardiomyocytes. Proteins were separated on an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-P-ERK 1/2 and anti-total-ERK antibodies overnight, and then incubated with HRP-conjugated goat anti-rabbit secondary antibody. Lanes were loaded with 30 µg of proteins. NE: cardiomyocytes treated with 1 µM norepinephrine. (B) Graph showing the total protein expression of P-ERK 1/2 normalized to total-ERK 1/2. *$p < 0.01$ vs. Basal; *$p < 0.01$ vs. Basal; (4 cardio-preparations). Statistical analysis was performed using one-way ANOVA.

Figure 9. (A) Expression of P-ERK 1/2 in neonatal rat cardiomyocytes. Total proteins were extracted from neonatal rat ventricular cardiomyocytes. Cells were treated with 1 µM Norepinephrine (NE) for 4–24 h or pre-treated with either U0126 (P-ERK1/2 inhibitor) or metoprolol tartrate (β1-blocker) for 1 h followed by treatment with NE for 24 h. Proteins were separated on an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-P-ERK, anti-total-ERK antibodies overnight and then incubated with HRP-conjugated goat anti-rabbit secondary antibody. Lanes were loaded with 30 µg of proteins. NE: cardiomyocytes treated with 1 µM norepinephrine, 24 h + U0126: Cardiomyocytes pre-treated with U0126 for 1 h followed by treatment with NE for 24 h. 24 h + β1-blocker: Cardiomyocytes pre-treated with metoprolol tartrate (β1-blocker) for 1 h followed by treatment with NE for 24 h. (B) Graph showing the total protein expression of P-ERK normalized to total-ERK. * $p < 0.01$ vs. Basal; † $p < 0.01$ vs. NE 24 h. (3 cardio-preparations). Statistical analysis was performed using one-way ANOVA.

Figure 1

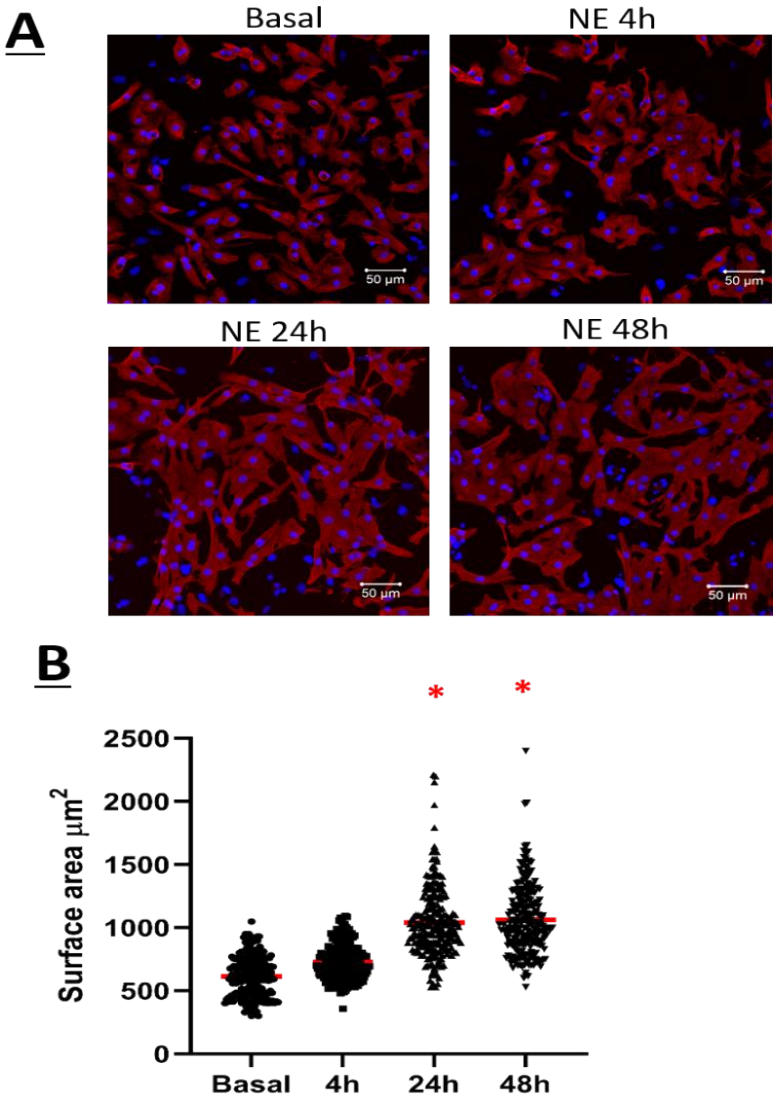

Figure 1: Confocal imaging of Neonatal rat ventricular cardiomyocytes in basal condition or treated with norepinephrine (NE).

Figure 2

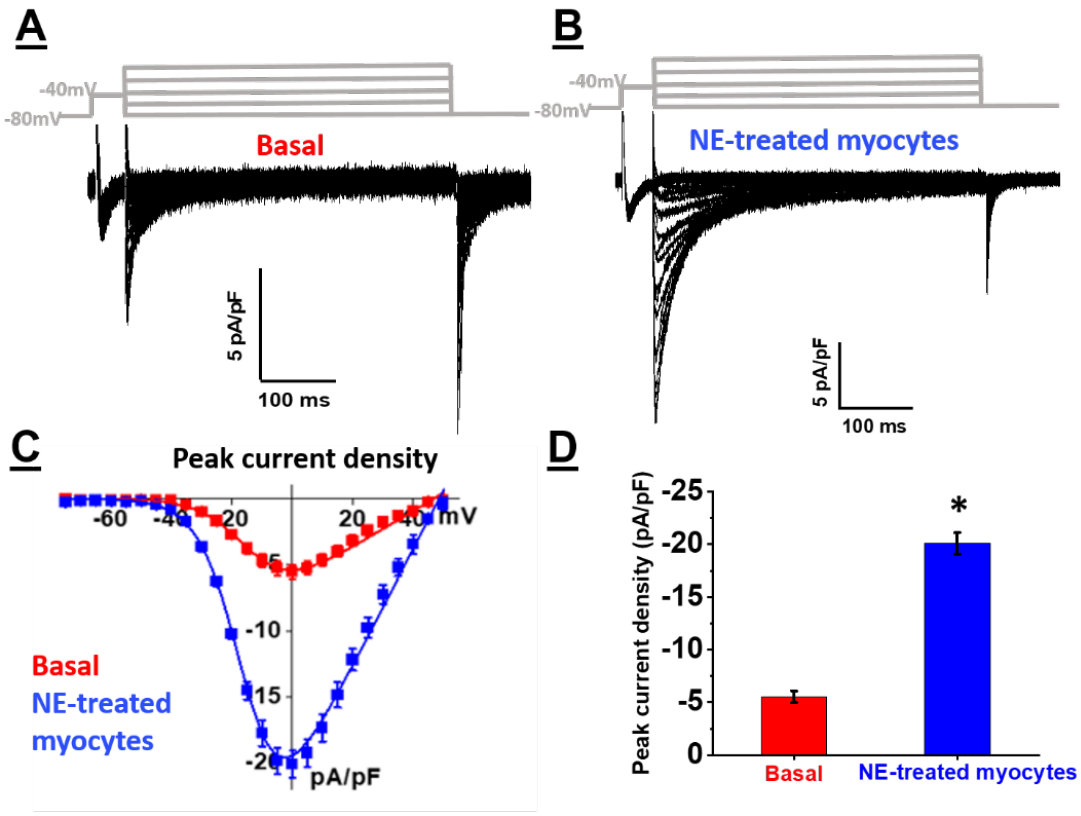

Figure 2: Whole-cell Ca^{2+} current traces recorded from neonatal rat cardiomyocytes under basal conditions or treated with NE

Figure 3

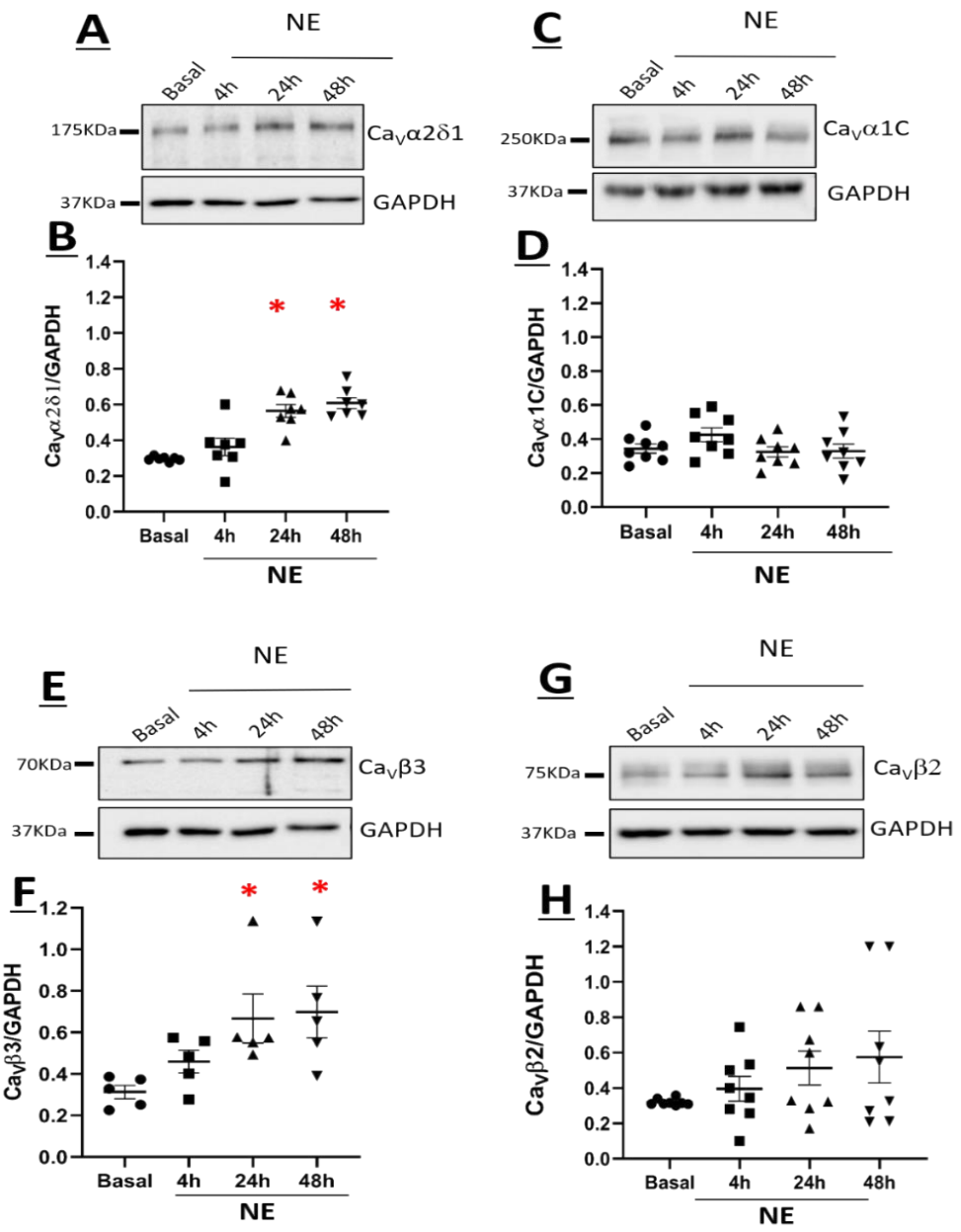

Figure 3: Expression of Cavα2δ1 (A), Cavα1C (C), Cavβ3 (E), and Cavβ2 (G) in neonatal rat cardiomyocytes treated with NE.

Figure 4

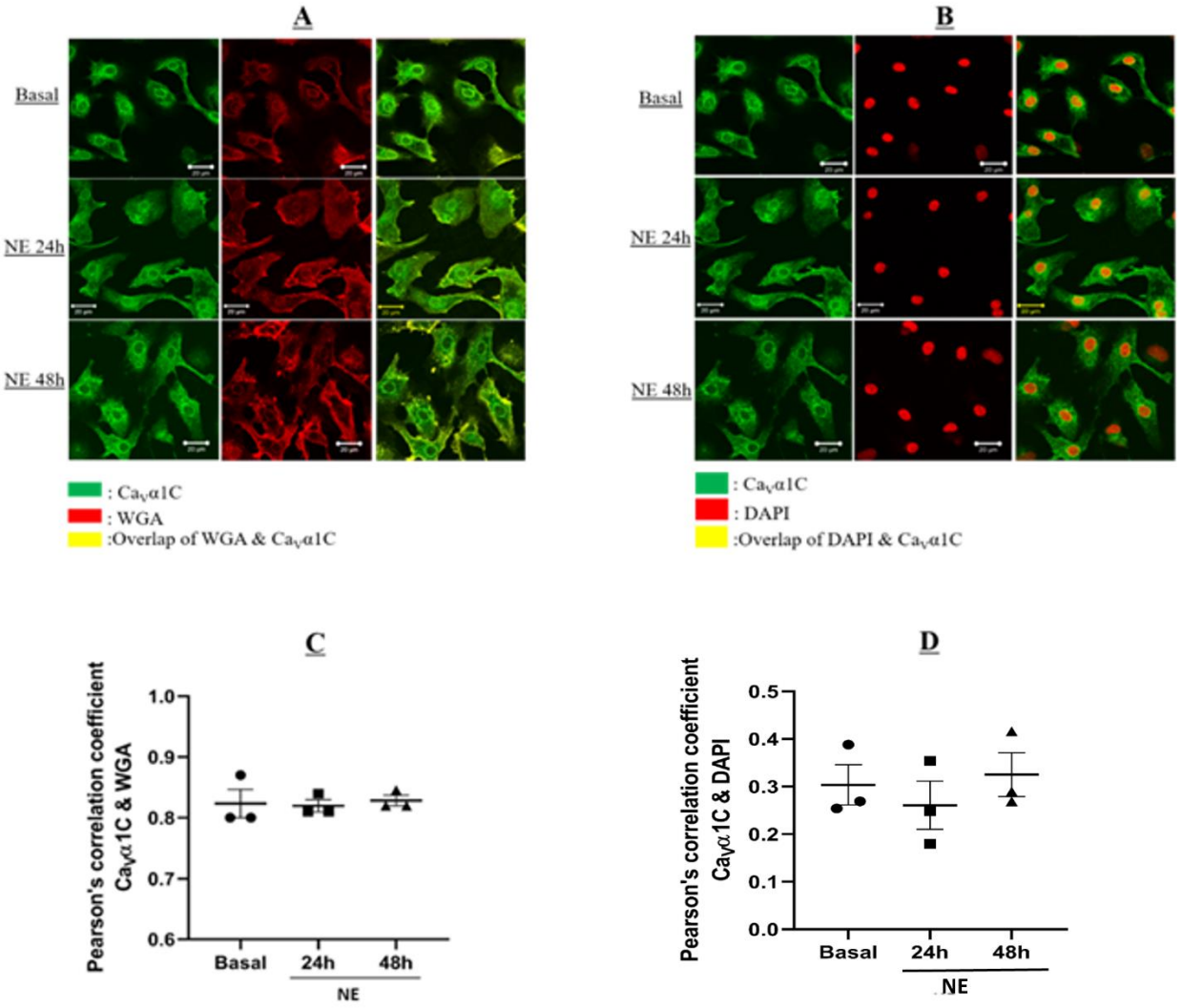

Figure 4: Colocolization of Cava1C and WGA or DAPI in Neonatal rat cardiomyocytes in basal condition or treated with NE for 24–48 h

Figure 5

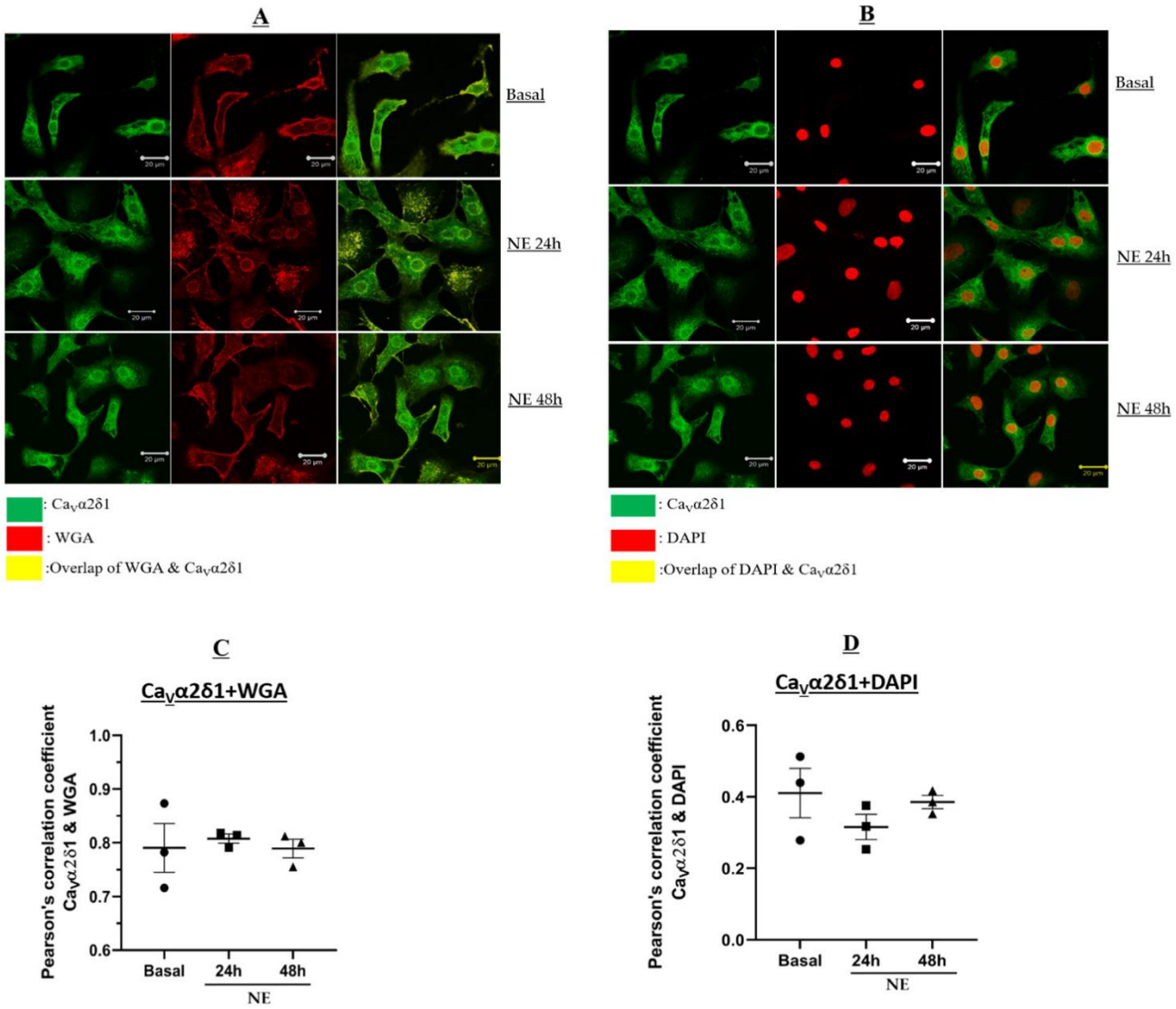

Figure 5: Colocolization of Cavα2δ1 and WGA or DAPI in neonatal rat cardiomyocytes in basal condition or treated with NE for 24–48 h.

Figure 6

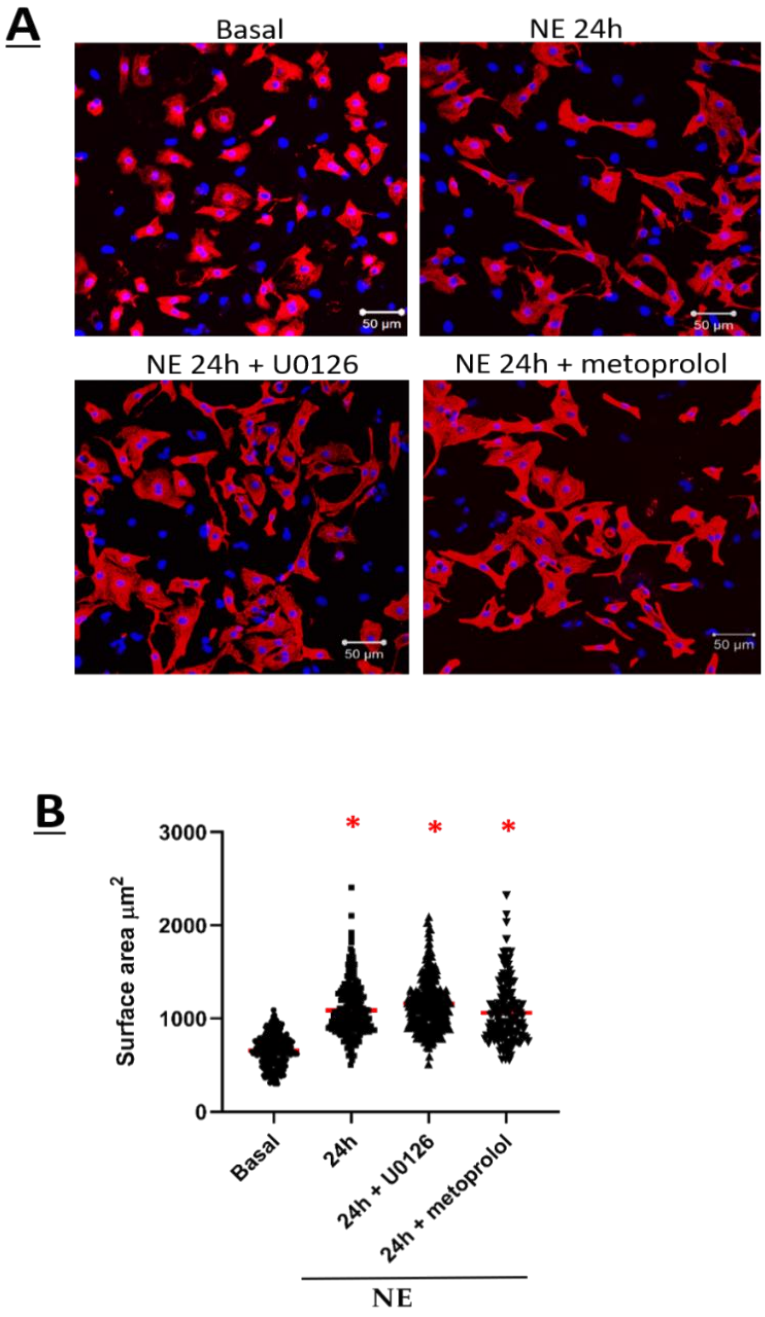

Figure 6: Confocal imaging of neonatal rat cardiomyocytes in basal condition, treated with NE, or pre-treated with either U0126 or metoprolol tartrate followed by NE.

Figure 7

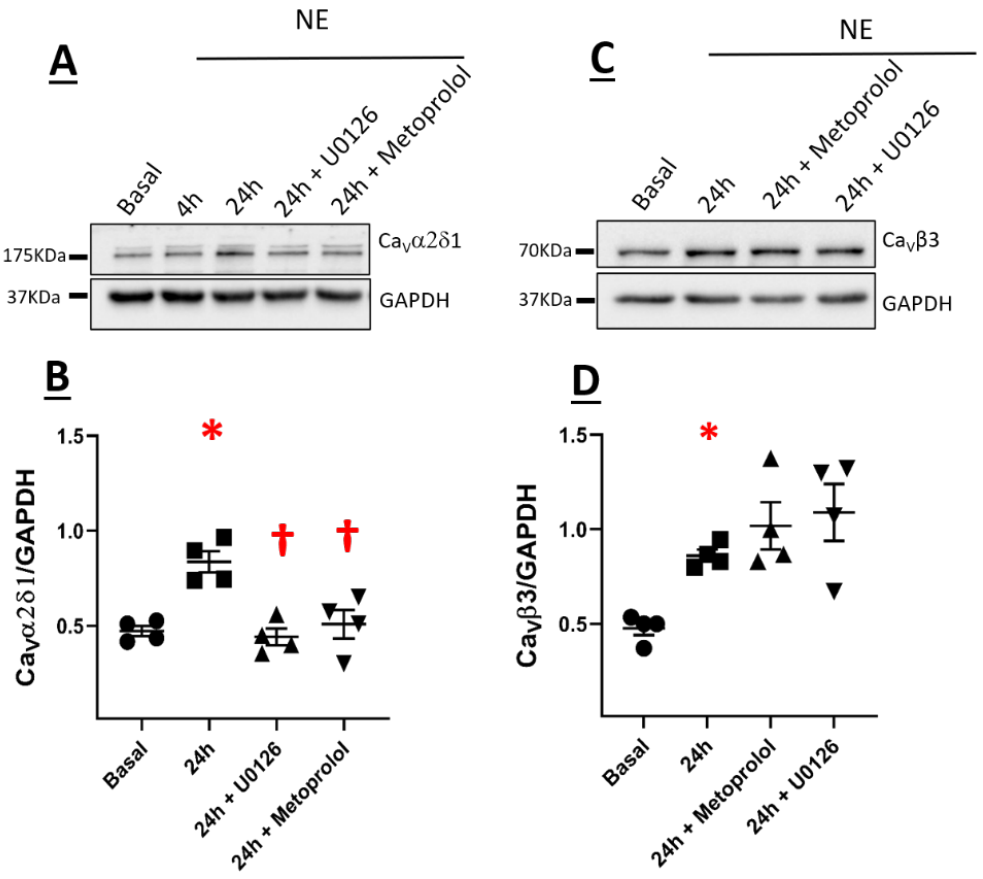

Figure 7: Expression of Cavα2δ1 (A) and Ca$_V$β3 (C) in neonatal rat cardiomyocytes in presence of U0126 or metoprolol tartrate.

Figure 8

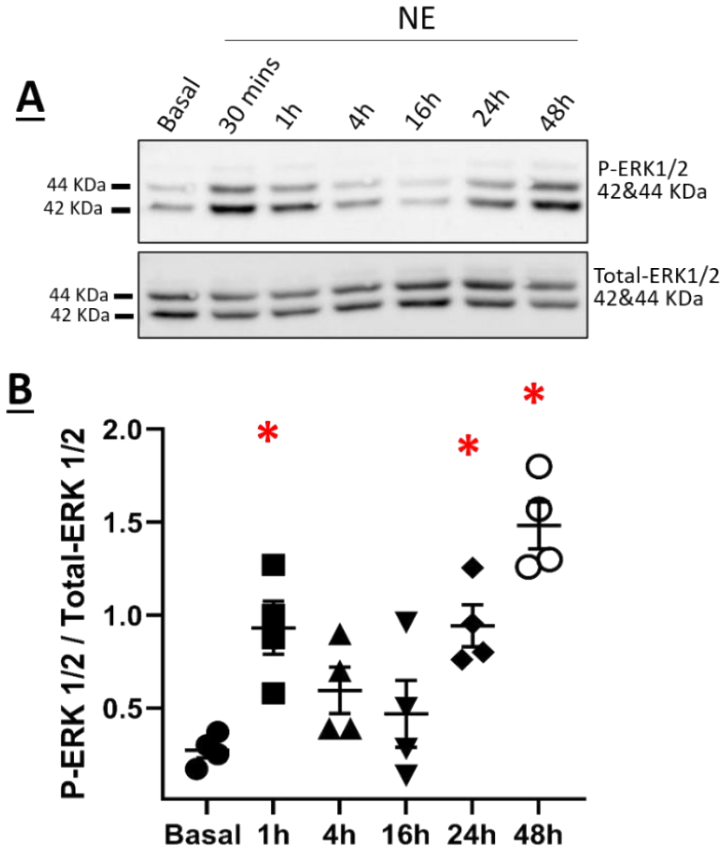

Figure 8: Expression of P-ERK 1/2 in neonatal rat cardiomyocytes treated wih NE.

Figure 9

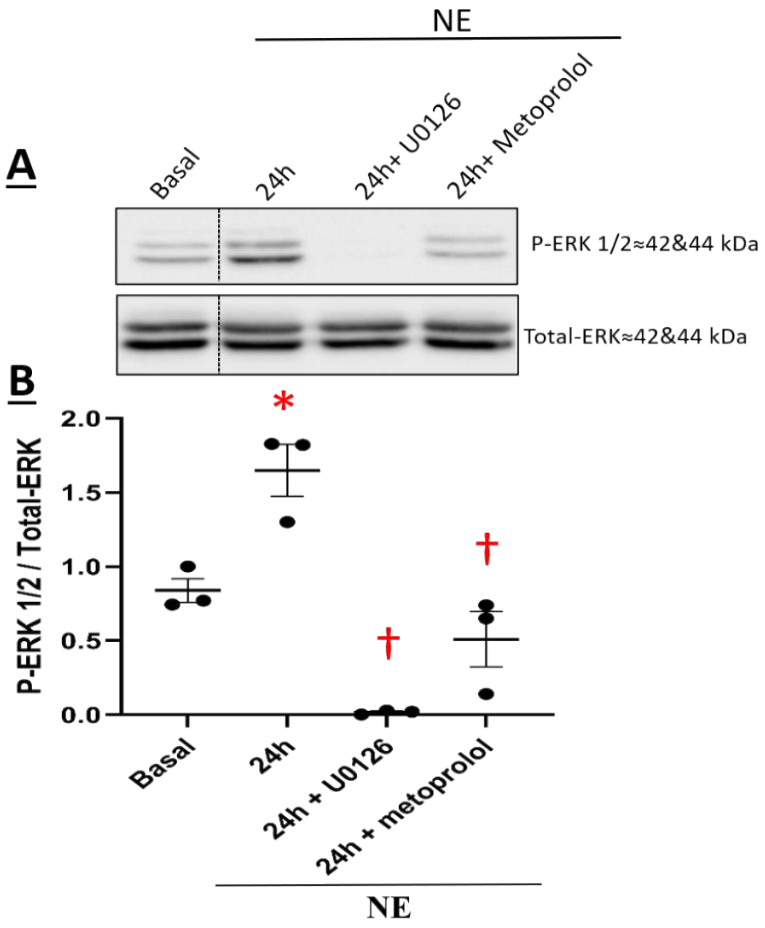

Figure 9: Expression of P-ERK 1/2 in neonatal rat cardiomyocytes in presence of U0126 or metoprolol tartrate.

Chapter 4: Article 2

Title: Rapamycin treatment unmasks a sex-specific pattern of scar expansion of the infarcted rat heart; potential interaction between mTOR and K_{ATP} channel

Aya Al Katat: Conceptualization, performed the isolation of cardiomyocytes, performed immunoblotting, analyzed the data, interpreted the results, and carried out statistical analysis.

Alexandre Bergeron: Assisted the animal facility technician in performing MI animal models.

Lucie Parent: General supervision and approved the final version of the manuscript.

Maxime Lorenzini: Performed patch-clamp experiments and analyzed the data.

Céline Fiset: Designed the patch clamp experiments, analyzed and interpreted patch-clamp data, and approved the final version of the manuscript.

Angelino Calderone: General supervision, Conceived and designed the experiments, analyzed the results, interpreted the results, wrote the manuscript, edited, and approved the final version of the manuscript.

Rapamycin treatment unmasks a sex-specific pattern of scar expansion of the infarcted rat heart; potential interaction between mTOR and K_{ATP} channel

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Keywords; *sex, myocardial infarction, mTOR, rapamycin, K_{ATP} channels*

Running Title; *Rapamycin and sex-dependent scar expansion*

ABBREVIATIONS

mTOR; mammalian target of rapamycin
PDBu; phorbol 12,13-dibutyrate
NNVMs; neonatal rat ventricular cardiomyocytes
MI; myocardial infarcted or myocardial infarction
ATP; adenosine triphosphate
AKT; protein kinase B
p70S6K; ribosomal p70 S6 Kinase

DECLARATIONS

Ethics Approval

The use and care of laboratory rats was according to the Canadian Council for Animal Care and approved by the Animal Care Committee of the Montreal Heart Institute (protocol: 2022-2993).

Consent for Publication

All authors have approved the final version of the manuscript, are accountable for all aspects of the data, each author qualifies for authorship and provide consent to publish.

Data Availability

Data will be made available upon a reasonable request

Competing Interests

All authors declare that there are no competing interests or conflicts

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Author Contribution

Aya Al-Katat; acquisition and analysis of data for the study

Alexandre Bergeron; acquisition and analysis of data for the study

Lucie Parent; conception of the study

Maxime Lorenzini; acquisition and analysis of data for the study

Celine Fiset; conception of the study and writing of the manuscript

Angelino Calderone; conception of the study and writing of the manuscript

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ABSTRACT

Inhibition of the mammalian target of rapamycin (mTOR) with the macrolide rapamycin or pharmacological suppression of K_{ATP} channel opening translated to scar expansion of the myocardial infarcted (MI) adult female rodent heart. The present study tested the hypotheses that rapamycin-mediated scar expansion was sex-specific and mTOR signaling directly influenced K_{ATP} channel subunit expression/activity. Scar size was significantly larger in post-MI male rats as compared to the previous data reported in post-MI female rats. The reported scar expansion of rapamycin-treated post-MI female rats was not observed following administration of the macrolide to post-MI male rats. Protein levels of the K_{ATP} channel subunits Kir6.2 and SUR2A and phosphorylation of the serine^{2448} residue of mTOR were similar in the normal heart of adult male and female rats. By contrast, greater tuberin inactivation characterized by the increased phosphorylation of the threonine^{1462} residue and reduced raptor protein levels were identified in the normal heart of adult female rats. Rapamycin pretreatment of phorbol 12,13-dibutyrate (PDBu)-treated neonatal rat ventricular cardiomyocytes (NNVMs) suppressed hypertrophy, inhibited p70S6K phosphorylation and attenuated SUR2A protein upregulation. In the presence of low ATP levels, K_{ATP} channel activity detected in untreated NNVMs was significantly attenuated in PDBu-induced hypertrophied NNVMs via a rapamycin-independent pathway. Thus, rapamycin administration to post-MI rats unmasked a sex-specific pattern of scar expansion and mTOR signaling in PDBu-induced hypertrophied NNVMs significantly increased SUR2A protein levels. However, the biological advantage associated with SUR2A protein upregulation was partially offset by an mTOR-independent pathway that attenuated K_{ATP} channel activity in PDBu-induced hypertrophied NNVMs.

INTRODUCTION

Clinical studies have established that adult men were at a higher risk of myocardial infarction and greater infarct expansion as compared to pre-menopausal women.[1-3] The latter paradigm extended to rodents as a smaller infarct size was identified in females compared to males in experimental models of ischemic injury.[4-8] The smaller infarct was not attributed to the presence of estrogen and progesterone as ovariectomized adult female rodents subjected to complete coronary artery ligation did not translate to scar expansion.[9-12] Moreover, experimental studies

performed in rodents reported that recruitment of cardiac sarcolemma K_{ATP} channels provided sex-specific cardioprotection to the female adult rodent heart in response to ischemic injury.[4,5] The cardiac K_{ATP} channel is composed of the pore-forming subunit Kir6.2 and the auxiliary sulfonylurea receptor subunit SUR2A and channel activity rapidly adjusted membrane excitability in response to changes in the energetic status of the cell.[13] With regard to a secondary increase in metabolic demand after an ischemic insult, ATP depletion in cardiomyocytes opened K_{ATP} channels leading to potassium efflux and hyperpolarization.[13] The outward K_{ATP} current shortened the cardiac action potential duration by limiting calcium entry and hyperpolarization prolonged the diastolic interval supporting myocardial relaxation leading to reduced metabolic activity.[13] Despite the selective cardioprotective phenotype in females, the absence of an analogous paradigm in males was not secondary to the absence of K_{ATP} subunit protein expression.[4,5] In this regard, the sex-specific cardioprotection identified in the female rodent heart may be related in part to a selective and/or more rapid activation of K_{ATP} channel activity and/or upregulation of one or both subunits. Two independent studies reported that the serine/threonine kinase mammalian target of rapamycin (mTOR) directly recruited K_{ATP} channel function and inactivation of the kinase led to downregulation of channel subunits.[14,15] Work from our lab revealed that p70S6K phosphorylation (downstream target of mTOR) was increased in the non-infarcted left ventricle of the female rat heart after complete coronary artery ligation and administration of the macrolide rapamycin suppressed phosphorylation of the kinase and concomitantly led to a two-fold increase in scar size after complete coronary ligation.[16] The latter data highlighted a cardioprotective role of mTOR signaling in adult female rats secondary to ischemic injury and further supported the potential interaction between rapamycin-dependent signaling events and K_{ATP} channel activity and/or subunit expression. Thus, to confirm that rapamycin-mediated cardioprotection was sex-dependent, *in vivo* experiments tested the hypothesis that rapamycin administration to the ischemically damaged adult male rat heart following complete coronary artery ligation did not translate to scar expansion. Thereafter, additional *in vitro* experiments tested the hypothesis that mTOR signaling directly influenced K_{ATP} channel subunit expression and/or activity in neonatal rat ventricular cardiomyocytes.

METHODS

ETHICAL APPROVAL

The use and care of laboratory rats was according to the Canadian Council for Animal Care and approved by the Animal Care Committee of the Montreal Heart Institute (protocol: 2022-2993).

Myocardial Infarction

With regard to the scar weight and surface area of untreated and rapamycin-treated infarcted female rats, these data were previously published in the Canadian Journal of Pharmacology and Physiology.[16] As the senior author of the paper, the journal permits the reuse of the data for non-commercial use. A transmural myocardial infarct was induced in adult male and female Sprague–Dawley rats (9–11 weeks old; Charles Rivers, Canada), as previously described.[16] Prior to surgery, rats were anesthetized with isoflurane (2%). Thereafter, a thoracotomy was performed and the heart was subjected to complete coronary artery ligation or a sham operation in which the coronary artery was not ligated. Buprenorphine (0.5 mg/kg) was administered once prior to surgery and repeated three times at 6 hours intervals after the surgery was completed. Twenty-four hours after surgery, rapamycin (1.5 mg/kg) dissolved in 0.25% DMSO was administered intraperitoneal for a period of 6 days.[16] In parallel, the vehicle (0.25% DMSO) alone was administered to sham and myocardial infarcted rats daily for a period of 6 days. One-week after surgery, rats were anesthetized with isoflurane (2%) and mean arterial pressure and left ventricular function were determined as previously described.[16] Thereafter, the heart was removed, the scar excised and weighed and the scar surface area determined by planimetry as images were captured with the Olympus QICAM color video camera interfaced with an Olympus CKX41 microscope and determined with the Olympus Stream Basic Image Analysis Software (Center Valley, PA).

Neonatal rat ventricular cardiomyocytes were isolated from a litter of 1-day old Sprague-Dawley rat pups (10-12 pups/litter; sex undetermined) (Charles River, Canada) as previously described.[17] Ventricular cardiomyocytes were plated at a density of 400 cells/mm^2 in DMEM-low glucose (Hyclone Laboratories, Logan, UT) supplemented with 7% heat-inactivated FBS and 1%

penicillin-streptomycin for 48 hours, subsequently washed and maintained in DMEM-low glucose containing insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (5 ng/ml) (ITS; BD Bioscience, Bedford, MA) for 36 hours prior to the experimental protocol. In the experimental protocol, phorbol 12, 13-dibutyrate (PDBu, 10^{-7} M, CAS#37558-16-0; Sigma-Aldrich) was added for a period of 24 hrs. In parallel, rapamycin (50 nM; LC Laboratories, USA) was added 15 minutes prior to the addition of PDBu and the experiment continued for a period of 24 hrs. In the present study, a single preparation of NNVMs was used to concomitantly assess the impact of PDBu on cardiomyocyte hypertrophy, regulation of the mTOR signaling pathway, expression of K_{ATP} channel subunits and channel activity in the absence or presence of rapamycin. To assess the latter parameters, n=4 independent litters of neonatal rat pups were employed.

WESTERN BLOT

Protein lysates (30 μg) of the left ventricle of normal adult male rats, the non-infarcted left ventricle of myocardial infarcted male rats and NNVMs were prepared and subjected to SDS-electrophoresis, as previously described.[16,17] Furthermore, protein lysates derived from our initial study performed in sham and myocardial infarcted female rat hearts treated with vehicle or rapamycin were used to assess mTOR content and phosphorylation status depicted in the present study. Antibodies used include a rabbit polyclonal antibody directed against phosphorylated threonine^{389} of p70S6K (1:1000, RRID:AB_330944; Cell Signaling Technology, Danvers, MA, USA); a rabbit polyclonal antibody directed against p70S6K; recognizes non-phosphorylated and phosphorylated p70S6K) ((1:1000, C-18; Santa Cruz Biotechnology, USA); a rabbit polyclonal antibody directed against the phosphorylated serine^{473} of AKT (1:500, RRID:AB_329825, Cell Signaling Technology) a rabbit polyclonal antibody directed against the phosphorylated threonine^{308} of AKT (1:500, RRID:AB_329828; Cell Signaling Technology); a rabbit polyclonal antibody directed against non-phosphorylated AKT (1:500, RRID:AB_329827; Cell Signaling Technology); a rabbit polyclonal antibody directed against the phosphorylated serine^{2448} of mTOR (1:500, RRID:AB_330944; Cell Signaling Technology); a rabbit polyclonal antibody directed against non-phosphorylated mTOR (1:500, RRID:AB_330978; Cell Signaling Technology); a rabbit polyclonal antibody directed against the phosphorylated threonine^{1462} of tuberin (1:500, RRID:AB_329855; Cell Signaling Technology); a rabbit polyclonal antibody directed against non-phosphorylated tuberin (1:500, RRID:AB_22078044; Cell Signaling Technology); a mouse

monoclonal antibody directed against ABCC9 (SUR2A) (1:500, RRID:AB_2923035; Abcam Inc, Toronto, Ontario); a rabbit polyclonal antibody against Kir6.2 directed against (1:500, RRID:AB_1640795; Abcam Inc); a rabbit monoclonal antibody against PKC-α (1:2000, catalogue #59745; Cell Signaling Technology); rabbit monoclonal antibody against PKC-ε (1:2000, catalogue #2683; Cell Signaling Technology); a rabbit monoclonal antibody against raptor (1:500, RRID:AB_561245; Cell Signaling Technology) and a mouse monoclonal anti-GAPDH (1:10,000, RRID:AB_437392; Ambion, Austin, TX). Following overnight incubation at 4°C, the appropriate secondary antibody-conjugated to horseradish peroxidase (1:10,000, Jackson Immunoresearch, West Grove, PA) was added and bands visualized utilizing the ECL detection kit (Perkin Elmer, Waltham, MA). Films were scanned with Image J software® and the target protein signal was depicted as arbitrary light units.

IMMUNOFLUORESCENCE

To assess the hypertrophic response of NNVMs, an immunofluorescence approach was employed to determine the cross-sectional surface area. NNVMs were plated on glass coverslips coated with poly-D-lysine and at the end of the experimental protocol fixed with 2% paraformaldehyde.[17] Primary antibody used was a mouse monoclonal anti-cardiac troponin-T (1:200, RRID:AB_306445; Abcam Inc). The nucleus was identified with 4',6'-diamidino-2-phenylindole (DAPI, 1.5 μM; emission wavelength, 460 nm; Sigma-Aldrich) staining. Secondary antibody used was a goat anti-mouse IgG conjugated to Alexa-555 (1:600-800; Life Technologies, Carlsbad, CA). Non-specific staining was determined following the addition of the conjugated secondary antibody alone. The Zeiss LSM Image Browser was used to calculate the cross-sectional surface area of troponin-T-immunoreactive mononucleated neonatal rat ventricular cardiomyocytes.

K_{ATP} CURRENT RECORDING in NEONATAL RAT VENTRICULAR MYOCYTES

Individual neonatal rat ventricular cardiomyocytes were obtained from n=4 independent litters of neonatal rat pups. Whole-cell K_{ATP} currents (I_{KATP}) were recorded using an Axopatch 200B amplifier and pCLAMP 10.2 software (Molecular Devices, Sunnyvale, CA, USA). Data was acquired at a sampling rate of 2 kHz and low pass filtered at 1 kHz. NNVMs were perfused with the following bath solution (in mM): NaCl 135, KCl 5.4, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10, and

glucose 10, with pH adjusted to 7.4 with NaOH. Patch pipettes had resistance in the range of 1.5-3MΩ when filled with the internal solution containing (in mM) potassium aspartate 110, KCl 20, NaCl 8, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10, K_2ATP 0.25, and EGTA 10 and the pH adjusted to 7.2 with KOH. The currents were elicited by a 500-ms ramp protocol from +40 mV to -120 mV, with a holding potential of -45 mV. The ramp protocol was run three times and the average used for analysis. All currents were normalized to the cell capacitance and expressed as current densities (pA/pF) and were corrected for a -10-mV junction potential to compensate for the patch pipette-bath liquid junction potential (K^+-aspartate). Current recordings were conducted at room temperature (20-22ºC).

STATISTICS

Data presented as mean ± SE, and (n) represents the number of rats per group or independent litters of neonatal pups used for the *in vitro* experiments. In the *in vivo* study, left ventricular contractile function, cardiac morphology, body weight and western blot analysis were evaluated by a two-way ANOVA and significant difference was determined by the Fisher LSD post hoc test (OriginLab Corporation, Northampton, MA, USA). Scar weight and/or surface area of 1-week myocardial infarcted male and female rats and rapamycin-treated myocardial infarcted adult rat hearts were evaluated by a Students' unpaired t-test and a value of $p<0.05$ considered statistically significant. Protein content/phosphorylation of normal adult male and female rat hearts was evaluated by a Students' unpaired t-test. Neonatal rat ventricular cardiomyocyte hypertrophy, protein content/phosphorylation were determined by a one-way ANOVA and significant difference was determined by the Fisher LSD post hoc test (OriginLab Corporation). K_{ATP} channel activity was determined by a one-way ANOVA and significant difference was determined by the Tukey post hoc test (GraphPad Software, San Diego, CA, USA).

RESULTS

Sexually dimorphic response of the adult rat heart to ischemic damage and rapamycin treatment

Scar surface area and weight of a transmural infarct of adult male Sprague-Dawley rat hearts were compared to a previously published study of female rat hearts subjected to complete

coronary artery ligation and the data depicted in Figures 1A and 1B.[16] Scar weight and surface area were significantly larger in myocardial infarcted male rat hearts following complete coronary artery ligation as compared to myocardial infarcted adult female rat hearts (Figure 1A). Furthermore, work from our lab previously reported that rapamycin administration to adult female Sprague-Dawley rats 24 hours after complete coronary artery ligation of the heart translated to a two-fold increase in scar size and surface area depicted in Figure 1B.[16]

In one-week myocardial infarcted male rat hearts, significant scar formation was associated with a marked reduction of left ventricular weight normalized to body weight (LV/BW) and the concomitant decrease of mean arterial pressure, left ventricular systolic pressure (LVSP), the rate of left ventricular contraction (+dP/dt) and relaxation (-dP/dt) as compared to vehicle-treated sham male rats (Tables 1 & 2). Rapamycin administration to sham and myocardial infarcted male rats significantly reduced body weight (Table 1). Furthermore, rapamycin treatment of myocardial infarcted male rats improved MAP, LVSP, +dP/dt whereas -dP/dt was partially improved but did not reach statistical significance compared to vehicle-treated myocardial infarcted male rats (Table 2). Despite the amelioration of contractile indices of rapamycin-treated myocardial infarcted male rats, LV/BW ratio was analogous to vehicle-treated myocardial infarcted male rats and significantly reduced compared to vehicle/rapamycin-treated male rats (Table 1). In contrast to myocardial infarcted female rats, scar weight and surface area were not significantly different in rapamycin-treated myocardial infarcted rats versus vehicle-treated myocardial infarcted adult male rats (Figure 1B & Table 1).

The scar expansion observed in female infarcted female rat heart following Rapamycin injection was associated with the published observation that rapamycin concomitantly abolished the increased phosphorylation state of the mTOR downstream target p70S6K in the non-infarcted left ventricle of myocardial infarcted female rat hearts.[16] To complement the latter *in vivo* signaling data, experiments performed in the present study revealed that the total protein content of mTOR in the non-infarcted left ventricle of myocardial infarcted female rat hearts was significantly elevated as compared to sham rats (Figure 2A). Furthermore, phosphorylation of the serine^{2448} residue of mTOR and subsequent normalization to higher mTOR content revealed that the phosphorylation status of the serine/threonine kinase in the non-infarcted left ventricle of the myocardial infarcted female rat heart was similar to vehicle-treated female rats (Figure 2A).[18] Rapamycin treatment of sham and myocardial infarcted female rats significantly reduced total

mTOR protein levels as compared to vehicle-treated groups (Figure 2A). Furthermore, phosphorylation of the serine^{2448} residue of mTOR in the non-infarcted left ventricle of rapamycin-treated myocardial infarcted female rats was nearly abolished and the latter effect extended to the left ventricle of sham female rats treated with the macrolide. (Figure 2A).

Likewise, phosphorylation of the serine^{2448} residue of mTOR in the non-infarcted left ventricle of myocardial infarcted male rats was similar to vehicle-treated male rats (Figure 2B). Furthermore, total mTOR content was similar between the left ventricle of vehicle-treated male rats and non-infarcted left ventricle of myocardial infarcted male rats (Figure 2B). Rapamycin administration to sham and myocardial infarcted male rats significantly reduced total mTOR protein levels as compared to vehicle-treated groups (Figure 2B). In stark contrast to sham and myocardial infarcted female rats, phosphorylation of the serine^{2448} residue of mTOR in the non-infarcted left ventricle persisted in 1-week rapamycin-treated sham and myocardial infarcted male rats (Figure 2B). Moreover, normalization of phosphorylated mTOR to total mTOR content revealed that phosphorylation of the serine/threonine kinase was higher in rapamycin treated sham and myocardial infarcted male rats as compared to vehicle-treated groups (Figure 2B).

Sex-dependent regulation of raptor expression and tuberin activity in the adult rat heart was not associated with a disparate expression of K_{ATP} channel subunits

Several studies reported a hyperactivated state of mTOR in female tissue as compared to males.[19-21] To assess whether mTOR signaling in the adult rat heart was sex-dependent, total protein content of raptor and phosphorylation of the serine^{2448} residue of mTOR were examined.[18] In lysates isolated from the left ventricle of normal adult male and female Sprague-Dawley rats, raptor protein levels were significantly lower in normal adult female rats whereas phosphorylation of the serine^{2448} residue of mTOR normalized to total mTOR protein was similar (Figures 3A, 3B). The upstream inhibitor GTPase tuberin inhibited mTOR activation and phosphorylation of the threonine^{1462} residue of the GTPase suppressed tuberin activity.[18,22] In lysates isolated from the left ventricle of normal adult male and female Sprague-Dawley rats, tuberin protein content was similar (Figure 3C). However, phosphorylation of the threonine^{1462} residue of tuberin normalized to total tuberin protein content was higher in the heart of normal adult female rats compared to the heart of adult male rats highlighting a greater intrinsic suppression of GTPase activity in normal adult female rats (Figure 3C).

Several studies have reported that the greater resistance of the female rodent heart to ischemic damage was related to the recruitment of K_{ATP} channels.[4,5] Western blot analysis revealed that expression of SUR2A and KIR6.2 channel subunits of the K_{ATP} channel in cardiac lysates of normal adult male and female Sprague-Dawley rats were similar between sexes (Figure 4). Thus, the established protective role of K_{ATP} channels in the female rat heart secondary to ischemic damage may be related to a selective activation and/or more rapid recruitment of K_{ATP} channel activity and/or upregulation of one or both subunits compared to the heart of normal adult male rats.

mTOR-dependent signaling in neonatal rat ventricular cardiomyocytes upregulated protein levels of the K_{ATP} channel subunit SUR2A

To examine the relationship between mTOR signaling and K_{ATP} channel expression/ activity, neonatal rat ventricular cardiomyocytes (NNVMs) were treated with the protein kinase C (PKC) activator phorbol 12,13-dibuytrate (PDBu). The 24-hour treatment of NNVMs with PDBu (100 nM) induced a hypertrophic response characterized by increased cross-sectional surface area as compared to untreated NNVMs (Figure 5A). The hypertrophic response of NNVMs to PDBu was associated with the recruitment of the conventional isoform PKC-α (untreated NNVMs, 0.49±0.07 vs PDBu-treated NNVMs, 0.18±0.014; n=2 independent litters) and the novel isoform PKC-ε (untreated NNVMs, 0.42±0.02 vs PDBu-treated NNVMs, 0.06±0.026; n=2 independent litters) characterized by the protein downregulation of each isoform (Figure 5B).

To examine the role of mTOR signaling, NNVMs were treated with rapamycin (50 nM) 15 minutes prior to the addition of PDBu. Rapamycin treatment significantly attenuated PDBu-mediated hypertrophic response of NNVMs (Figure 5A) and protein downregulation of PKC-α (rapamycin-treated NNVMs, 0.53±0.05 vs PDBu/rapamycin-treated NNVMs, 0.15±0.035; n=2 independent litters) and PKC-ε isoforms (rapamycin-treated NNVMs, 0.39±0.03 vs PDBu/rapamycin-treated NNVMs, 0.045±0.018; n=2 independent litters) persisted (Figure 5B).

Phosphorylation of the threonine^{389} residue of the serine/threonine kinase p70S6K, a putative downstream target of mTOR, was significantly increased in hypertrophic NNVMs secondary to PDBu stimulation (Figures 6A & 6B).[18,21] The latter response occurred in the absence of increased phosphorylation of the serine^{2448} residue of mTOR 24 hours after PDBu treatment suggesting that recruitment/phosphorylation of the serine/threonine kinase represented an early transient event

(Figures 6A & 6C). Moreover, phosphorylation of the threonine^{1462} residue of tuberin in response to PDBu treatment for 24 hours was also not significantly different from untreated NNVMs (Figures 6A & 6D) suggesting that inactivation of the GTPase likewise represented an early transient event following protein kinase C stimulation. Despite the hypertrophic response of NNVMs, phosphorylation of the serine^{473} and threonine^{308} residues of AKT normalized to total AKT protein levels were significantly reduced 24 hours after PDBu stimulation compared to untreated NNVMs (Figures 6A, 6E & 6F).[18,23]

Consistent with the suppressive effect on hypertrophy, the treatment of NNVMs with rapamycin alone or following co-treatment with PDBu significantly reduced phosphorylation of the serine^{2448} residue of mTOR and abolished phosphorylation of the threonine^{389} residue of p70S6K (Figures 6A, 6B & 6C). By contrast, phosphorylation of the threonine^{1462} residue of tuberin was unaffected by rapamycin alone or following co-treatment with PDBu (Figures 6A & 6D). However, phosphorylation of the serine^{473} and threonine^{308} residues of AKT in the presence of rapamycin alone were significantly increased versus untreated NNVMs and the reduced phosphorylated state in response to PDBu treatment alone was reversed following rapamycin treatment (Figures 6A, 6E & 6F). Lastly, PDBu-mediated NNVM hypertrophy was associated with the significant upregulation of SUR2A protein levels whereas KIR6.2 protein expression was unchanged as compared to untreated NNVMs (Figure 7). Rapamycin pretreatment significantly attenuated PDBu-mediated SUR2A protein upregulation whereas no change in KIR6.2 protein levels was observed (Figure 7).

K_{ATP} channel activity attenuated in PDBu-induced hypertrophic NNVMs via a rapamycin-independent pathway

To recapitulate in part the *in vivo* phenotype of a reduced pool of energy phosphates of ischemically-challenged ventricular cardiomyocytes, K_{ATP} activity in neonatal rat ventricular cardiomyocytes was recorded in low ATP levels.[24] The ramp protocol was used to generate the ionic current of the K_{ATP} channel under conditions of low ATP levels which elicited inward and outward currents that reversed near the predicted equilibrium potential for potassium (Figures 8A & 8B). The specificity of I_{KATP} current was confirmed using the K_{ATP} channel blocker glibenclamide (50 μM) as the drug significantly reduced current density (Figure 8C). Using these recording conditions, the current density of I_{KATP} was compared between untreated NNVMs and

NNVMs treated for 24 hours with rapamycin (50 nM), PDBu (100 nM), and PDBu/rapamycin (Figures 8A & 8B). I_{KATP} current density was similar between untreated NNVMs and NNVMs incubated with rapamycin for a 24-hour period (Figure 8A). However, as shown in Figure 8B, I_{KATP} density at -40 mV was significantly decreased in PDBu-induced hypertrophic NNVMs as compared to untreated NNVMs. Rapamycin administration to PDBu-treated NNVMs failed to reverse reduced K_{ATP} channel function despite the attenuation of the hypertrophic response and the density of the current remained significantly lower compared to untreated and rapamycin-treated NNVMs (Figure 8B). Thus, the electrophysiological data indicate that in PDBu-induced hypertrophic NNVMs, K_{ATP} channel activity in low ATP levels was partially inhibited via a rapamycin-independent pathway.

DISCUSSION

The present study has revealed that scar weight and scar surface area of the myocardial infarcted male rat heart were significantly larger compared to the previously reported data of myocardial infarcted female rat hearts. Previous work from our laboratory also depicted in the present study reported that rapamycin administration to myocardial infarcted female rats led to a ~two-fold increase in scar weight and surface area. To explore whether the latter effect was sex-dependent, the present study examined the impact of rapamycin administered to adult male Sprague-Dawley rats after complete coronary artery ligation of the heart. Rapamycin administration significantly reduced body weight and improved left ventricular function of myocardial infarcted male rats. By contrast, scar size and surface area of rapamycin-treated myocardial infarcted male rats were not significantly different from myocardial infarcted male rats. A modest explanation for the disparate sex-dependent effect of rapamycin may be attributed in part to the premise that the macrolide could not further initiate scar expansion of myocardial infarcted male rats, as scar weight and surface area were significantly greater versus myocardial infarcted female rats. However, in the myocardial infarcted male rat population, scar size was diverse as small to medium infarcts were identified and the distribution pattern of scar weight and surface area persisted after rapamycin administration.

Rapamycin-mediated scar expansion of myocardial infarcted female rats may be attributed in part to a disparate sex-dependent effect of the drug on mTOR signaling. In the non-infarcted

left ventricle of myocardial infarcted female rat hearts, mTOR protein levels were significantly increased compared to sham female rats and rapamycin administration reduced protein levels and abolished phosphorylation of the serine^{2448} residue of mTOR in sham and myocardial infarcted rats. By contrast, phosphorylation of the serine^{2448} residue of mTOR of sham and myocardial infarcted male rat hearts after rapamycin administration persisted and was significantly increased secondary to the lower protein content of mTOR. Consistent with these data, several studies have reported a preferential suppressive effect of rapamycin on female rodents after *in vivo* administration.[19-21,25] The latter phenotype may be attributed in part to the significantly lower raptor protein levels in the heart of normal female rats versus male rats observed in the present study. Rapamycin mediated inhibition of mTOR signaling required the association of the macrolide with the FKBP12 binding protein and the complex thereafter facilitated the dissociation of raptor from mTOR.[26] In this regard, for a given dose of rapamycin, the pharmacological effects of the drug may be more extensive and sustained in female rats secondary to a lower expression of raptor. The latter mechanism may be further potentiated in females via the reported sex-dependent metabolism of rapamycin. In a study performed in 4-month-old male and female C57BL/6 mice, an equivalent dose of rapamycin administered for a period of 6 months revealed that plasma levels of rapamycin remained consistently and significantly higher in females.[25] Moreover, the latter phenotype was associated with a significantly greater change in the transcriptome in the liver of female mice.[25] Thus, previously published studies and data depicted in the present study highlight an important sex-dependent effect of rapamycin.[16,19-21,25]

The disparate effect of rapamycin on scar expansion of adult male and female Sprague-Dawley rats after complete coronary artery ligation provided the impetus to assess whether the paradigm was related in part to an inherent sex-dependent pattern of mTOR signaling in the normal adult rat heart. In the adult female rat heart, phosphorylation of the serine^{2448} residue of mTOR and mTOR protein levels were similar to the normal adult male heart. However, phosphorylation of tuberin, an upstream inhibitor of mTOR was disparate between the heart of male and female rats. The upstream activator of mTORC1 is the small GTP binding protein Rheb and activity is tightly controlled by the heterodimeric complex consisting of the tumor suppressor tuberin (TSC2) and hamartin (TSC1).[18,22,23] Dephosphorylated tuberin/hamartin complex acts as a GTPase converting Rheb-GTP to Rheb-GDP preventing mTORC1 activation.[18,22,23] In the present study, phosphorylation of the threonine^{1462} residue of tuberin was higher in the heart of adult female rats

compared to male rats highlighting a greater inhibition of basal GTPase activity. However, the latter phenotype failed to increase mTOR phosphorylation of the serine^{2448} residue in normal adult female rat hearts. Nonetheless, greater tuberin phosphorylation/inactivation in the adult female rat heart may translate to a more rapid recruitment/activation of mTOR signaling following the superimposition of the appropriate stimulus (e.g., ischemia) thereby providing a cardioprotective advantage. Lastly, the underlying mechanism implicated in the greater phosphorylated state of tuberin in the female rat heart remains unknown. Enhanced tuberin phosphorylation/inactivation was identified predominantly in young women in a rare multisystem disease referred to as lymphangioleiomyomatosis (LAM).[22,27] LAM is a low grade, destructive, metastasizing neoplasm and the incipient cause is an acquired mutational inactivation of tuberin leading to a hyperactivated state of mTORC1 and uncontrolled cell proliferation.[22,27] Thus, at least in females, greater tuberin inactivation during embryogenesis may lead to the progression of a predominantly sex-specific disease.

Previous studies have reported that sarcolemma K_{ATP} channels selectively provided the female rodent heart greater resistance to ischemic damage.[4,5] In the present study, Kir6.2 and SUR2A protein levels were similar in the left ventricle of male and female adult Sprague Dawley rats. In this regard, the reported sex-protective effect of sarcolemma K_{ATP} channels in the infarcted female versus the infarcted male rodent heart may be attributed in part to their selective recruitment and/or greater expression following ischemic injury. A recent study reported that silencing of pro-opriomelanocorticotropic (POMC) neurons was attributed to a rapid activation and greater conductance of sarcolemma K_{ATP} channels via mTOR signaling.[14] Moreover, in pancreatic α-cells, mTORC1 recruited sarcolemma K_{ATP} channels leading to hyperpolarization and mTORC1 inactivation translated to K_{ir}6.2/SUR2A subunit downregulation.[15] Thus, to assess the potential relationship between mTOR and K_{ATP} channel expression/activity in the heart, we employed neonatal rat ventricular cardiomyocytes (NNVMs). In the present study, NNVMs were treated with the protein kinase C activator phorbol 12,13-dibutyrate (PDBu) as the novel protein kinase C isoform PKC-ε provided the female rat heart protection against ischemic damage.[28] The treatment of NNVMs with PDBu induced hypertrophy and recruited the conventional isoform PKC-α and the novel isoform PKC-ε depicted by significant protein downregulation.[29] Moreover, mTORC1 complex recruitment was observed as phosphorylation of the downstream target p70S6K was significantly increased in response to PDBu treatment of NNVMs and the enhanced

phosphorylated state was sustained during the hypertrophic response.[18,23] In parallel, phosphorylation of the serine^{473} and threonine^{308} residues of AKT were significantly reduced after PDBu treatment of NNVMs. Previous studies have reported that a second mTOR complex denoted mTORC2 was present in numerous cell types and consisted of mTOR and the protein rictor.[23] The primary biological role of the mTORC2 complex involved the phosphorylation of the serine^{473} residue of AKT.[23] Moreover, mTORC1 suppressed mTORC2 activity translating to a reduced phosphorylated state of the serine^{473} residue of AKT.[23] In this regard, the data presented in the present strongly supported the premise that reduced AKT phosphorylation in NNVMs may be attributed in part to PDBu-mediated mTORC1 suppression of mTORC2 activity. Lastly, coincident with p70S6K recruitment and the concomitant hypertrophic response, PDBu treatment led to the upregulation of the K_{ATP} channel subunit SUR2A whereas no change in Kir6.2 protein levels were observed. Increased SUR2A protein expression was reported to exert an important biological impact on cardiac function as selective upregulation of the subunit in the heart provided improved tolerance and endurance to stress by increasing the density of sarcolemmal K_{ATP} channels and presumably activity.[30] To confirm that increased SUR2A protein expression in response to PDBu proceeded via the mTORC1 complex, NNVMs were pre-treated with rapamycin. The macrolide attenuated PDBu-mediated hypertrophy, suppressed basal mTOR phosphorylation and inhibited the increased phosphorylation of the threonine^{389} residue of p70S6K.[18,23] Furthermore, rapamycin reversed the reduced phosphorylated state of the serine^{473} and threonine^{308} residues of AKT following PDBu treatment of NNVMs further supporting the established paradigm that mTORC1 inhibited mTORC2 activity. Lastly, rapamycin pretreatment attenuated PDBu-mediated SUR2A protein upregulation in NNVMs highlighting the novel observation that the mTORC1 complex directly influenced expression of the K_{ATP} channel subunit.

mTORC1-dependent SUR2A protein upregulation following PDBu treatment of NNVMs provided the impetus to assess the impact on K_{ATP} channel activity. In the present study, electrophysiological recordings of NNVMs were performed in low ATP levels as the incipient response secondary to an ischemic insult was the rapid reduction of endogenous energy phosphates translating in part to the loss of the inhibitory impact of the nucleotide on the opening of K_{ATP} channels.[24] Moreover, a previous study reported that in the presence of low ATP levels, protein kinase C-dependent pathways suppressed K_{ATP} channel activity.[31] In NNVMs, robust K_{ATP} channel activity was detected in the presence of low ATP levels and activity was significantly

attenuated in PDBu-induced hypertrophic NNVMs. The co-treatment with rapamycin failed to reverse reduced K_{ATP} channel activity in PDBu-treated NNVMs despite the inhibition of mTORC1 signaling and the concomitant hypertrophic response. Thus, reduced K_{ATP} channel activity following PDBu treatment was not directly related to hypertrophy and occurred via an mTORC1-independent pathway. Thus, it is tempting to speculate that the paradigm observed in NNVMs may be attributed in part to distinct PKC isoforms independently regulating K_{ATP} channel activity and mTOR-dependent SUR2A upregulation. Indeed, in the study by Bae and Zhang, the superior cardioprotective response reported in the adult female rat heart secondary to ischemic damage was associated with a greater recruitment of PKC-ε signaling as compared to the heart of male rats.[28] Thus, the potential biological advantage of mTOR-dependent upregulation of SUR2A protein levels on K_{ATP} channel activity in NNVMs in response to PDBu may have been in part overridden via recruitment of mTORC1-independent signaling events attenuating channel activity.

Perspective and Significance

Previous studies have reported that the adult female rodent heart was associated with a smaller scar compared to males following ischemic injury and the present study has reaffirmed the latter premise secondary to complete coronary artery ligation. However, in contrast to myocardial infarcted female rats, rapamycin administration did not lead to further scar expansion and failed to inhibit mTOR phosphorylation in the non-infarcted left ventricle of myocardial infarcted male rats. These data have unequivocally revealed that the sex-dependent greater resistance of the female rat heart to ischemic damage was attributed in part to the recruitment of a rapamycin-dependent pathway. Moreover, the disparate impact of rapamycin on scar expansion of myocardial infarcted male and female rats was concordant with the reported sex-dependent effect of K_{ATP} channel blockers on infarct formation following ischemic injury. In this regard, the relationship between mTOR and K_{ATP} channel subunit expression/activity was examined in NNVMs and the data revealed that protein kinase C-mediated recruitment of mTOR signaling selectively increased the protein levels of the SUR2A subunit. Thus, the greater inherent inactivation of the GTPase tuberin in the female rat heart may initiate a more rapid recruitment of mTOR signaling during the early phase of ischemic injury translating to K_{ATP} channel subunit SUR2A upregulation in ventricular cardiomyocytes. However, the latter cardioprotective mechanism may be partially compromised as protein kinase C activation of NNVMs attenuated K_{ATP} channel activity *via* an mTOR-

independent pathway. Thus, the latter response may in part offset the biological advantage associated with mTOR-dependent SUR2A protein upregulation in ischemically challenged ventricular cardiomyocytes.

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FIGURE LEGENDS

Figure 1. ***Scar weight of the myocardial infarcted heart of adult male and female rats 1-week after complete coronary artery ligation.*** (**A**) The scar surface area (*$p<0.01$) and weight (*$p<0.01$) of adult male rats were significantly higher compared to adult female rats following complete coronary artery ligation. (**B**) Following rapamycin treatment, scar surface area ($^{‡}p<0.001$) and weight ($^{‡}p<0.001$) were significantly elevated as compared to untreated post-MI female rats whereas no effect was observed in post-MI male rats. The female data was previously published by our lab (Lajoie et al., 2009). *$p<0.01$ vs. Females; $^{‡}p<0.001$ vs. MI of the same sex.

Figure 2. ***mTOR phosphorylation in the non-infarcted left ventricle of post-MI female and male rats.*** (**A**) Total mTOR protein levels (content normalized to GAPDH) were increased in the non-infarcted left ventricle of MI female rats (MI, n=4; †$p<0.01$) as compared to the left ventricle of untreated rats (SHAM, n=3). mTOR protein levels were significantly reduced in the left ventricle of rapamycin-treated sham rats (RAP, n=3; †$p<0.05$). mTOR protein levels were also significantly reduced in the non-infarcted left ventricle of rapamycin-treated MI rats (MI+RAP, n=4; $^{‡}p<0.01$). Phosphorylation of the serine^{2448} residue of mTOR (normalized to total mTOR content) in the non-infarcted left ventricle of untreated MI rats was similar to untreated sham rats. By contrast, phosphorylation of the serine^{2448} residue of mTOR was abolished in the left ventricle of rapamycin-treated female sham rats (n=3; †$p<0.01$) and the non-infarcted left ventricle of rapamycin-treated MI female rats (n=4; $^{‡}p<0.01$). (**B**) Total mTOR protein levels were significantly reduced in the left ventricle of rapamycin-treated sham male rats (RAP, n=4; †$p<0.05$) and the non-infarcted left ventricle of rapamycin-treated MI male rats (MI+RAP, n=4; $^{‡}p<0.05$). A significantly greater phosphorylated state of the serine^{2448} residue of mTOR normalized to total mTOR content was observed in the left ventricle of rapamycin-treated sham rats (RAP, n=4; †$p<0.01$) and non-infarcted left ventricle of rapamycin-treated MI male rats (MI+RAP, n=4; $^{‡}p<0.05$). †$p<0.01$ vs untreated sham of the same sex; †$p<0.05$ versus untreated sham of the same sex; $^{‡}p<0.01$ versus untreated MI; $^{‡}p<0.05$ versus untreated MI.

Figure 3. ***mTOR and tuberin phosphorylation in normal adult male and female rat hearts. (A)*** mTOR activity in the left ventricle of adult female rats (n=6) as depicted by the phosphorylation

state of the serine^{2448} residue of mTOR was similar to adult male rats (n=7). (**B**) Raptor protein levels were significantly lower (*p<0.01) in the left ventricle of adult female rats (n=8) as compared to the left ventricle of normal adult male rats (n=8). (**C**) A significantly greater inhibitory state of tuberin (*p<0.01) was identified in the left ventricle of adult female rats (n=7) depicted by the higher phosphorylation state of the threonine^{1462} of tuberin as compared to adult male rats (n=7). *p<0.01 vs sham male.

Figure 4. ***K_{ATP} channel subunit expression in normal adult male and female rat hearts.*** In the left ventricle of normal adult male (n=5-6) and female (n=6-7) rats, Kir6.2 and SUR2A protein levels were similar.

Figure 5. ***Rapamycin pretreatment*** **attenuated** ***Protein kinase C mediated hypertrophy of neonatal rat ventricular cardiomyocytes (NNVMs).*** (**A**) The 24-hour exposure of NNVMs to the protein kinase C activator phorbol 12,13-dibutyrate (PDBu; 100 nM, n=4) promoted a hypertrophic response characterized by a significant increase (* denotes p<0.001) in the cross-sectional area as compared to untreated NNVMs (n=4). Rapamycin (RAP; 50 nM; n=4) pretreatment significantly attenuated (** denotes p<0.001) PDBu-mediated increase in the cross-sectional surface area of NNVMs. (**B**) The 24-hour exposure of NNVMs to PDBu (100 nM; n=2) led to the recruitment of the conventional isoform PKC-α and the novel isoform PKC-ε as depicted by the significant downregulation of protein levels. In the presence of rapamycin (50 nM; n=2), PDBu-mediated downregulation of PKC-α or PKC-ε in NNVMs persisted.

Figure 6. ***Recruitment of mTOR-dependent signaling events in response to phorbol ester stimulation of neonatal rat ventricular cardiomyocytes (NNVMs).*** (**A & B**) Twenty-fours after the exposure of NNVMs (n=3-4 for each kinase examined) to the protein kinase C activator phorbol 12,13-dibutyrate (PDBu; 100 nM), phosphorylation of the threonine^{1462} residue of tuberin was unaffected and rapamycin (RAP, 50 nM) alone or in the presence of PDBu did not alter the phosphorylation status. Furthermore, 24 hours after exposure of NNVMs to PDBu, phosphorylation of the serine^{2448} residue of mTOR was similar to untreated cells. By contrast, phosphorylation of the serine^{2448} residue of mTOR was significantly reduced with rapamycin treatment alone (* denotes p<0.001 versus untreated NNVMs) or following the co-treatment of rapamycin and PDBu (* denotes p<0.01 versus PDBu-treated NNVMs). PDBu treatment of NNVMs significantly increased phosphorylation of the threonine^{389} residue of p70S6K (* denotes

p<0.05 versus untreated NNVMs) as compared to untreated NNVMs. Phosphorylation of the threonine^{389} residue of the p70S6K was abolished after rapamycin treatment alone of NNVMs (* denotes p<0.05 versus untreated NNVMs) or after PDBu and rapamycin co-treatment (* denotes p<0.01 versus PDBu-treated NNVMs). In PDBu-treated NNVMs, phosphorylation of the serine^{473} (* denotes p<0.01 versus untreated NNVMs) and threonine^{308} (* denotes p<0.05 versus untreated NNVMs) residues of AKT were significantly reduced as compared to untreated NNVMs. Rapamycin treatment alone increased the phosphorylation of the serine^{473} (* denotes p<0.05 versus untreated NNVMs) and threonine^{308} (* denotes p<0.01 versus untreated NNVMs) residues of AKT. The co-treatment of NNVMs with PDBu and rapamycin significantly reversed phosphorylation of the serine^{473} (* denotes p<0.01 versus PDBu treated NNVMs) and threonine^{308} (* denotes p<0.01 versus PDBu treated NNVMs) residues of AKT as compared to PDBU-treated NNVMs alone.

Figure 7. ***PDBu-mediated recruitment of mTOR signaling in neonatal rat ventricular cardiomyocytes (NNVMs) induced expression of the SUR2A subunit of the K_{ATP} channel.*** The 24 hour exposure of NNVMs to the protein kinase C activator phorbol 12,13-dibutyrate (PDBu; 100 nM;n=4) significantly increased the protein levels of the SUR2A subunit (* denotes p<0.01 versus untreated NNVMs) whereas protein expression of the Kir6.2 subunit was unchanged. Rapamycin (RAP; 50 nM) pretreatment (n=4) significantly attenuated PDBu-mediated upregulation of SUR2A protein levels (* denotes p<0.01 versus PDBu treated NNVMs) whereas Kir6.2 subunit protein expression remained unchanged.

Figure 8. ***K_{ATP} current recordings in NNVMs.*** **(A)** Typical K_{ATP} currents (I_{KATP}) recordings obtained in untreated (Basal) NNVMs, and NNVMs treated with rapamycin (50 nM), PDBu (100 nM) and Rapamycin-PDBu for a period of 24 hours. Whole-cell I_{KATP} were recorded under low ATP level conditions with the ramp protocol shown in inset. Corresponding mean current-voltage (I-V) relationships show that I_{KATP} density was similar in untreated NNVMs (n=20 individual NNVMs examined, N=4 independent preparation of NNVMs;) and rapamycin-treated cells (n=21, N=4). However, I_{KATP} density was significantly reduced in hypertrophied NNVMs induced by PDBu (n=18, N=4), and the response was not reversed by co-treatment with rapamycin (n=22, N=5). **(B)** Bar graphs report I_{KATP} measured at -40 mV and reveal that the current density was significantly reduced in PDBu-treated NNVMs as compared to untreated NNVMs. Rapamycin co-

treatment of PDBu-treated NNVMs failed to reverse reduced K_{ATP} channel activity (*** denotes $p<0.01$, ****$p<0.01$, one-way ANOVA with Tukey post-hoc test). **(C)** Exposure of untreated NNVMs to glibenclamide (50 μM), a K_{ATP} channel blocker significantly reduced current density (*$p<0.05$, paired Student t-test).

Table 1: The impact of rapamycin on the body weight and morphology of the heart of sham and myocardial-infarcted male Sprague-Dawley rats.

	BW (gram)	LV (mg)	LV/BW (mg/grams)	RV (mg)	RV/BW (mg/grams)
Sham (n=7)	316±7	499±25	1.59±0.07	160±9	0.51±0.03
Rapamycin (n=7)	274±4*	420±20	1.53±0.08	150±10	0.54±0.03
MI (n=9)	304±4	389±25*	1.27±0.06*	160±4	0.53±0.01
MI+Rapamycin (n=9-11)	270±3*	370±11*	1.36±0.04*	151±5	0.55±0.02

MI indicates myocardial infarction, BW, body weight, LV, left ventricle, LV/BW, left ventricle/body weight, RV, right ventricle, RV/BW, right ventricle/body weight. Data are presented as mean ± SEM, analyzed by a Two-way ANOVA followed by the Fisher LSD post hoc test and (*) represents $p<0,05$ versus sham and (n) number of rats examined.

Table 2: The impact of rapamycin on the contractile function of sham and myocardial-infarcted male Sprague-Dawley rats.

	MAP (mmHg)	LVSP (mmHg)	LVEDP (mmHg)	+dP/dT (mmHg/sec)	-dP/dT (mmHg/sec)
Sham (n=7)	126±4	147±8	10±2.0	7138±176	5995±224
Rapamycin (n=7)	123±5	132±7	10±2.0	7331±355	5069±385
MI (n=9)	101±4*	111±6*	17±2.0*	5305±195*	3949±136*
MI+Rapamycin (n=9-11)	121±6†	133±7†	14±2.0	6764±255†	4806±227

MI indicates myocardial infarction, MAP, mean arterial pressure, LVSP, left ventricular systolic pressure, LVEDP, left ventricular end-diastolic pressure, +dp/dt, rate of contraction; -dp/dt rate of relaxation. Data are presented as mean ± SEM, analyzed by a Two-way ANOVA followed by the Fisher LSD post hoc test and (*) represents $p<0,05$ versus sham, (†) $p<0,05$ versus MI and (n) number of rats examined.

Figures

Figure 1A

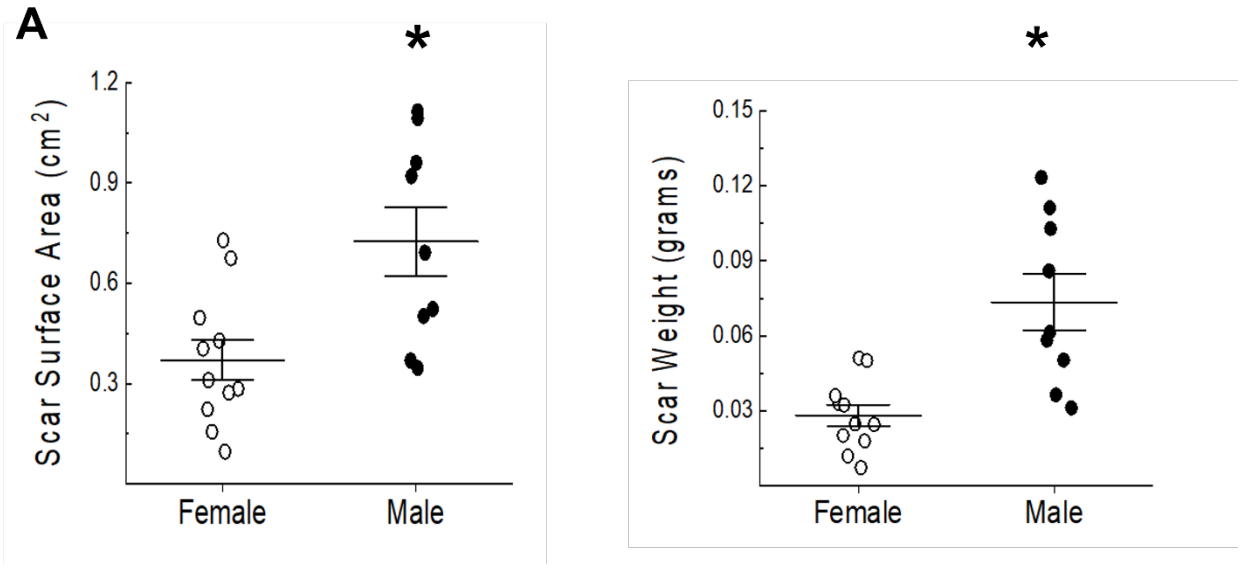


Figure 1B

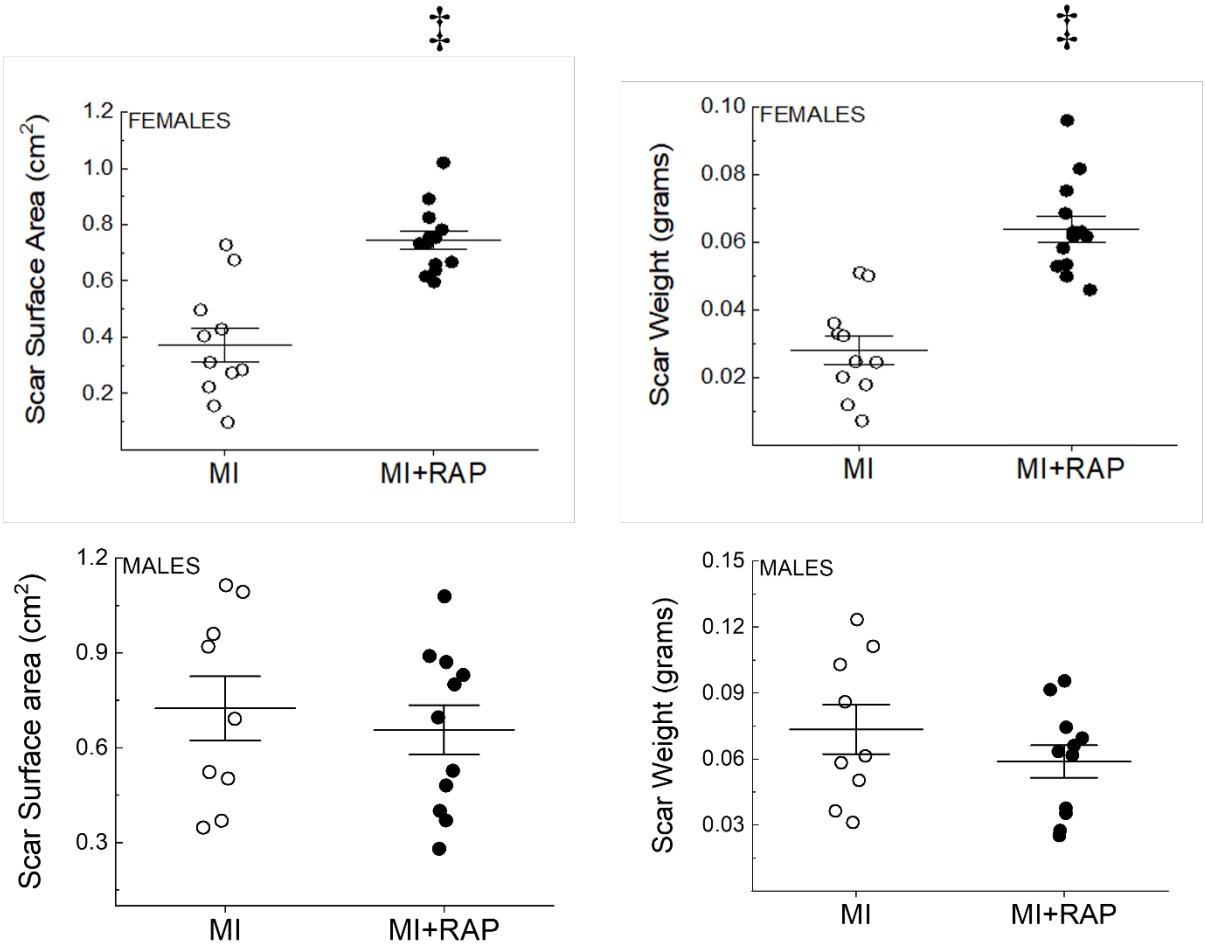
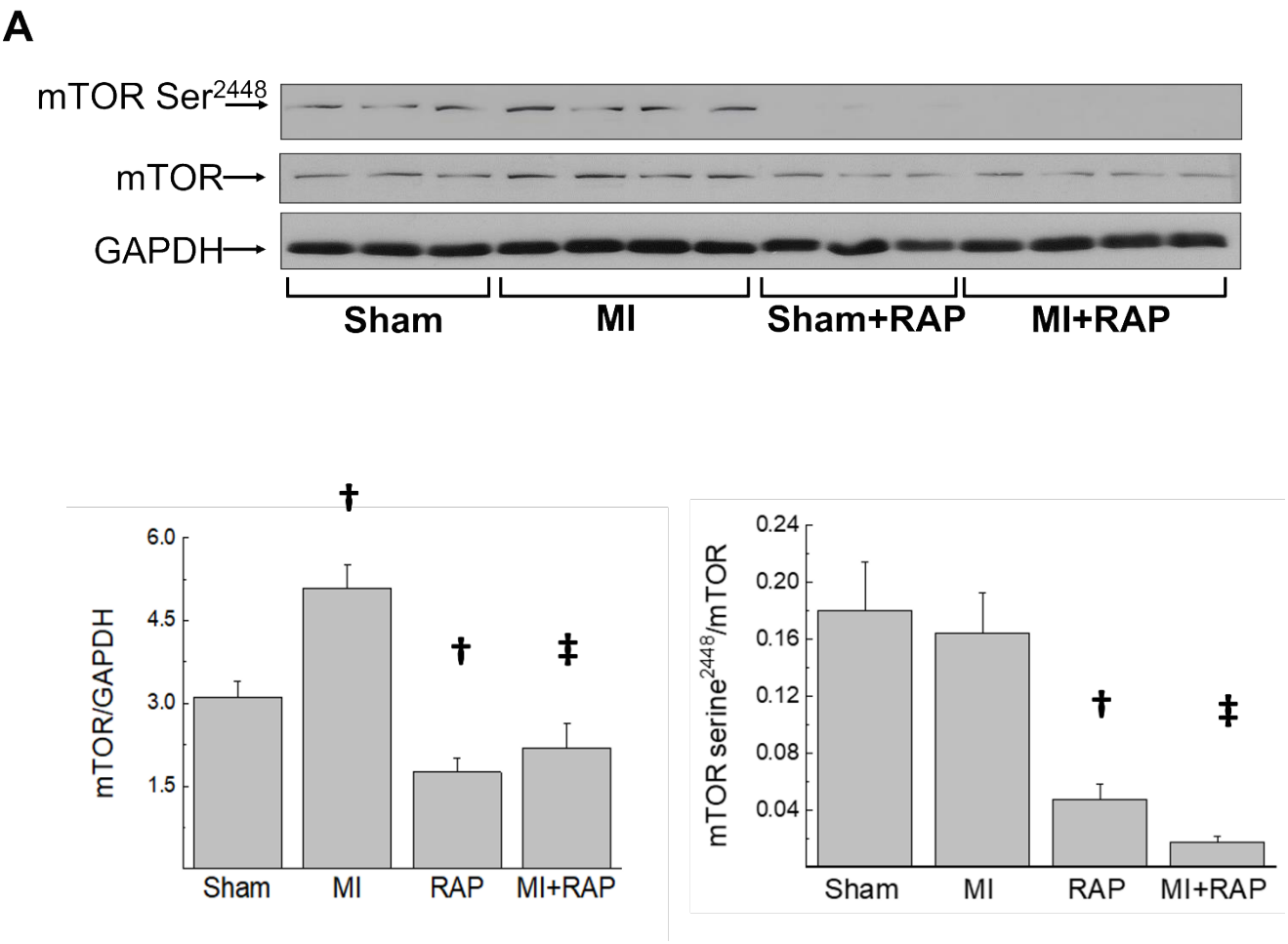


Figure 1

Figure 2


A
mTOR Ser2448
mTOR
GAPDH
Sham
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Sham+RAP
MI+RAP
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1.5
†
†
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RAP
MI+RAP
mTOR serine2448/mTOR
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MI+RAP

Figure 2

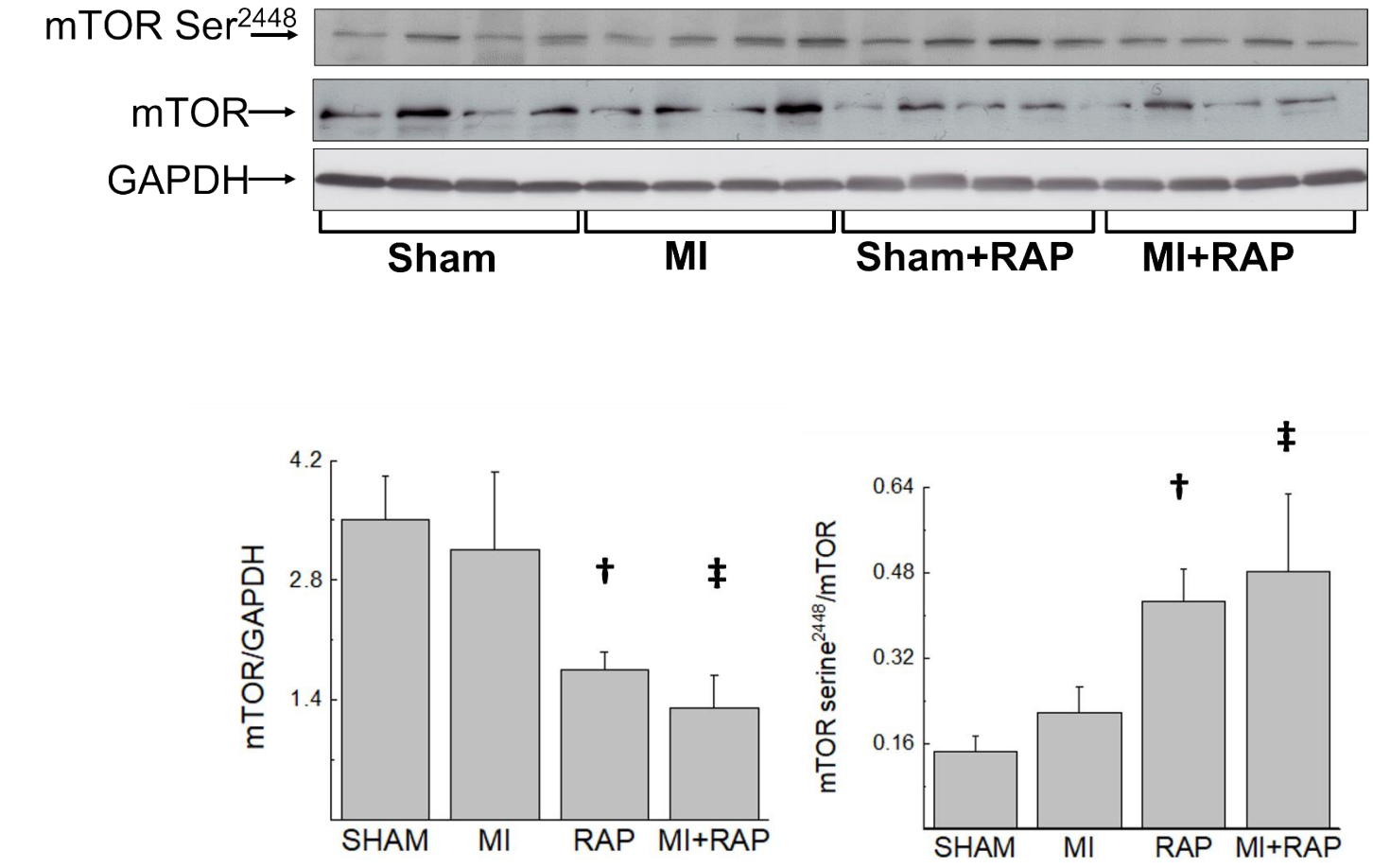
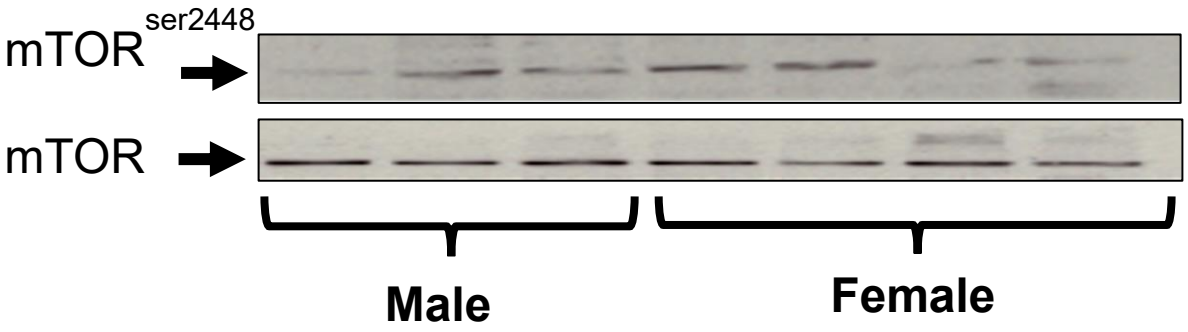


Figure 2

Figure 3A


mTOR ser2448
mTOR
Male
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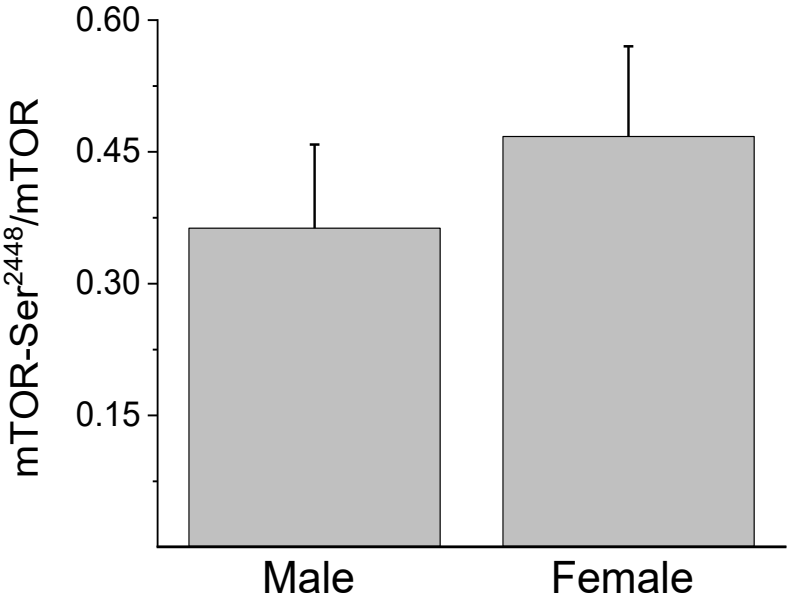
mTOR-Ser2448/mTOR
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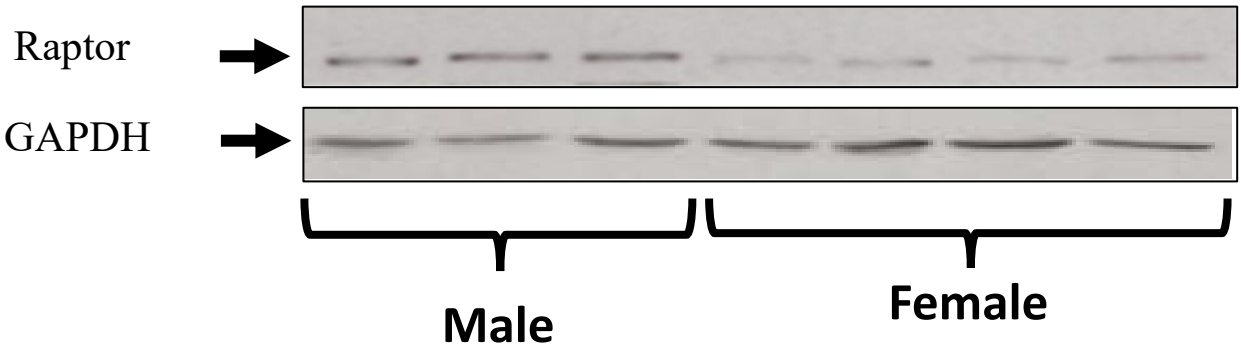
0.45
0.30
0.15
Male
Female

Figure 3B


Raptor
GAPDH
Male
Female

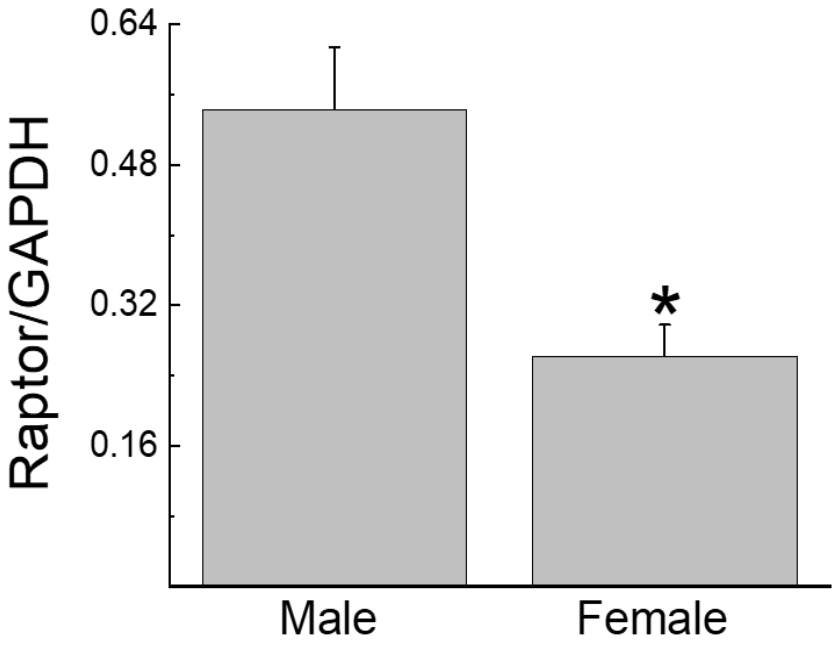
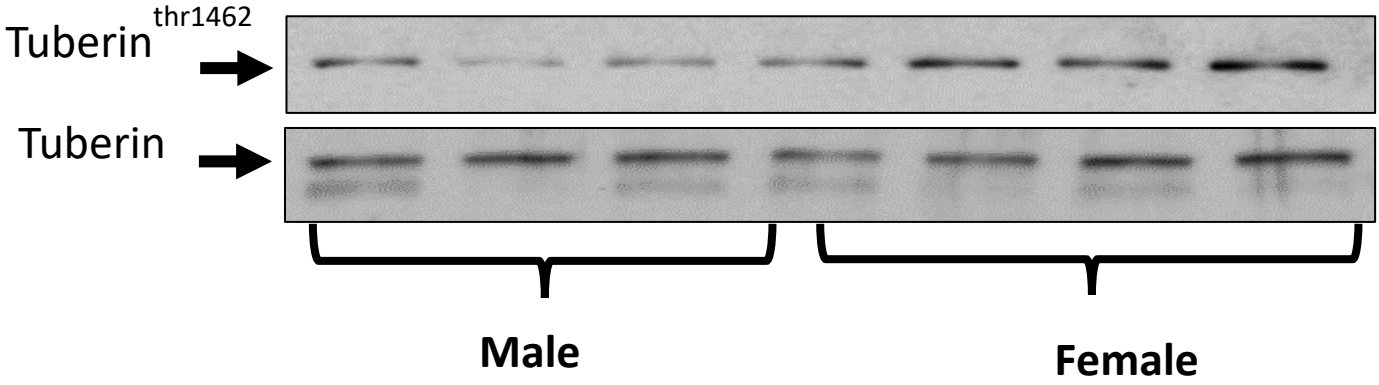

Raptor/GAPDH
0.64
0.48
0.32
0.16
*
Male
Female

Figure 3C



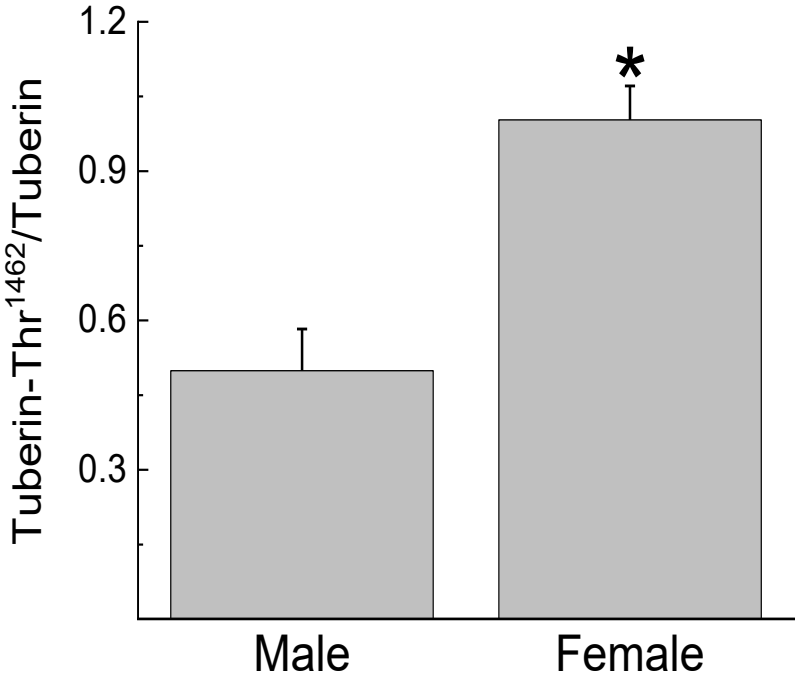


Figure 3

Figure 4

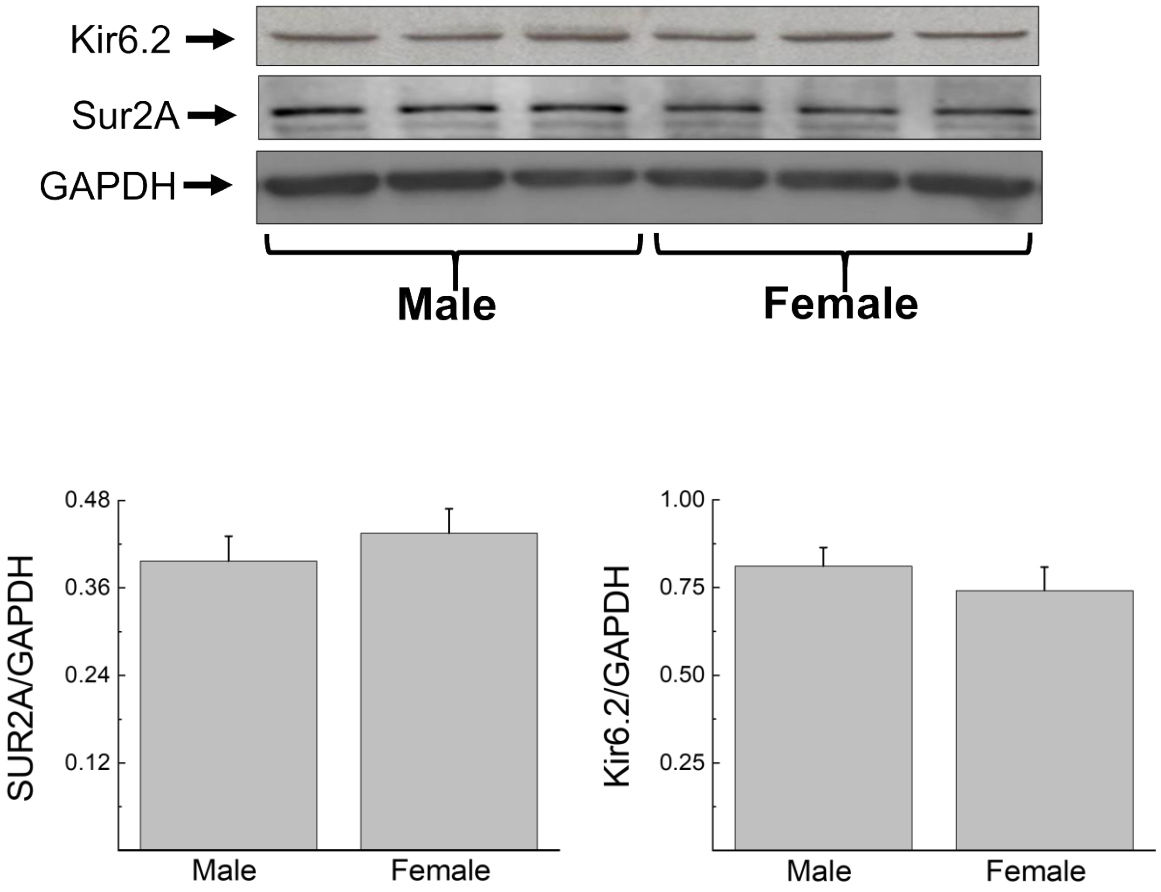


Figure 4

Figure 5 A, B

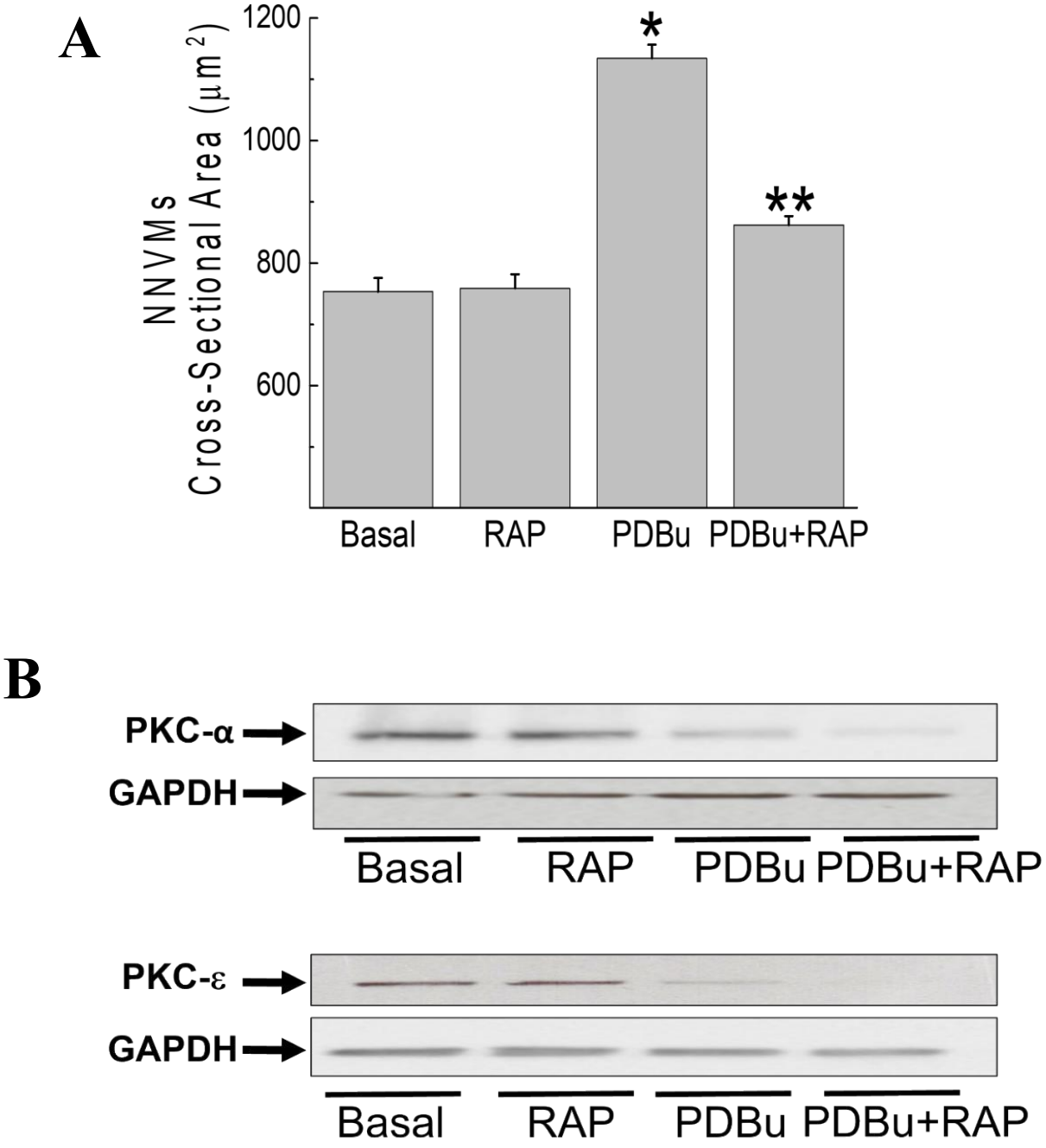


Figure 5

Figure 6A

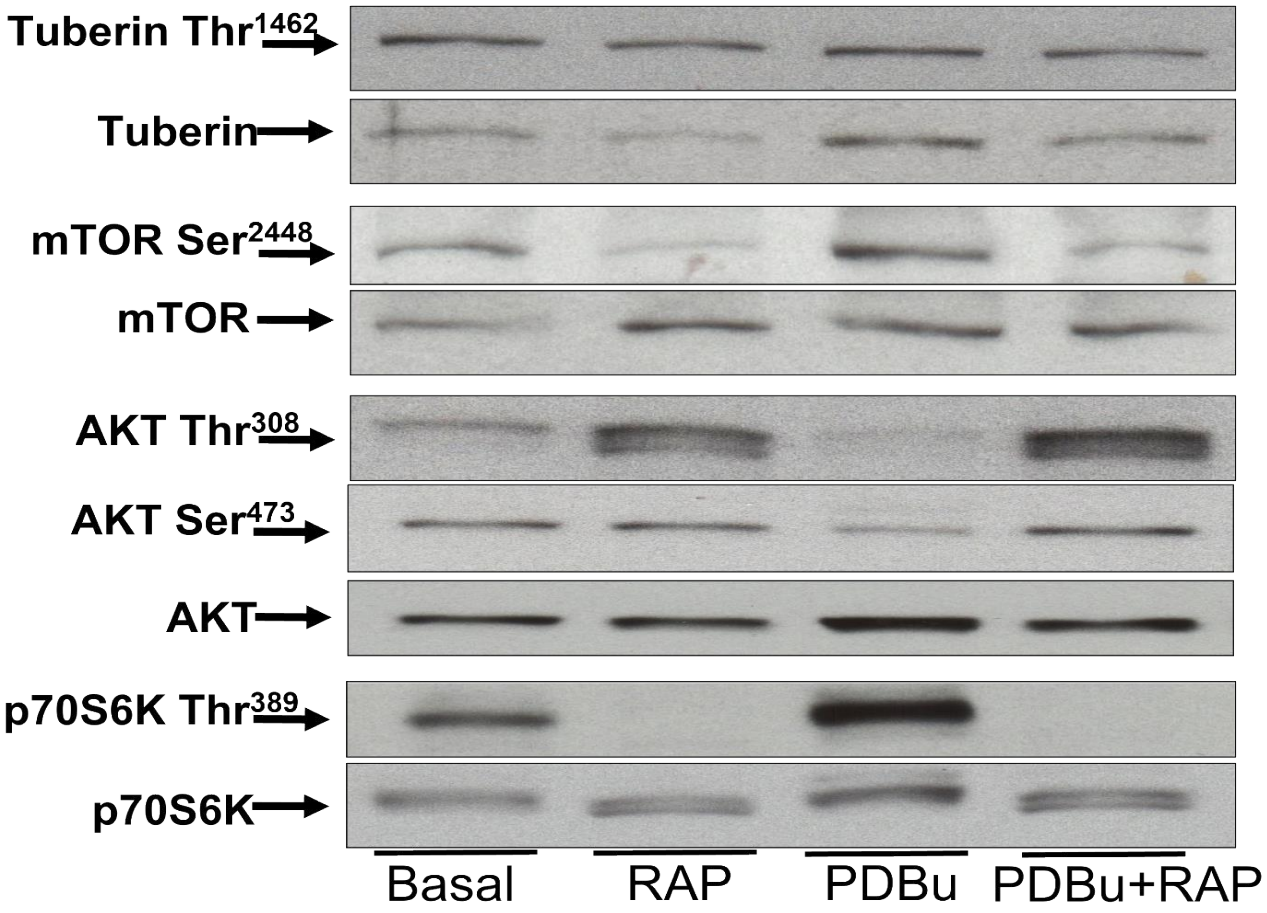

Tuberin Thr1462
Tuberin
mTOR Ser2448
mTOR
AKT Thr308
AKT Ser473
AKT
p70S6K Thr389
p70S6K
Basal
RAP
PDBu
PDBu+RAP

Figure 6B

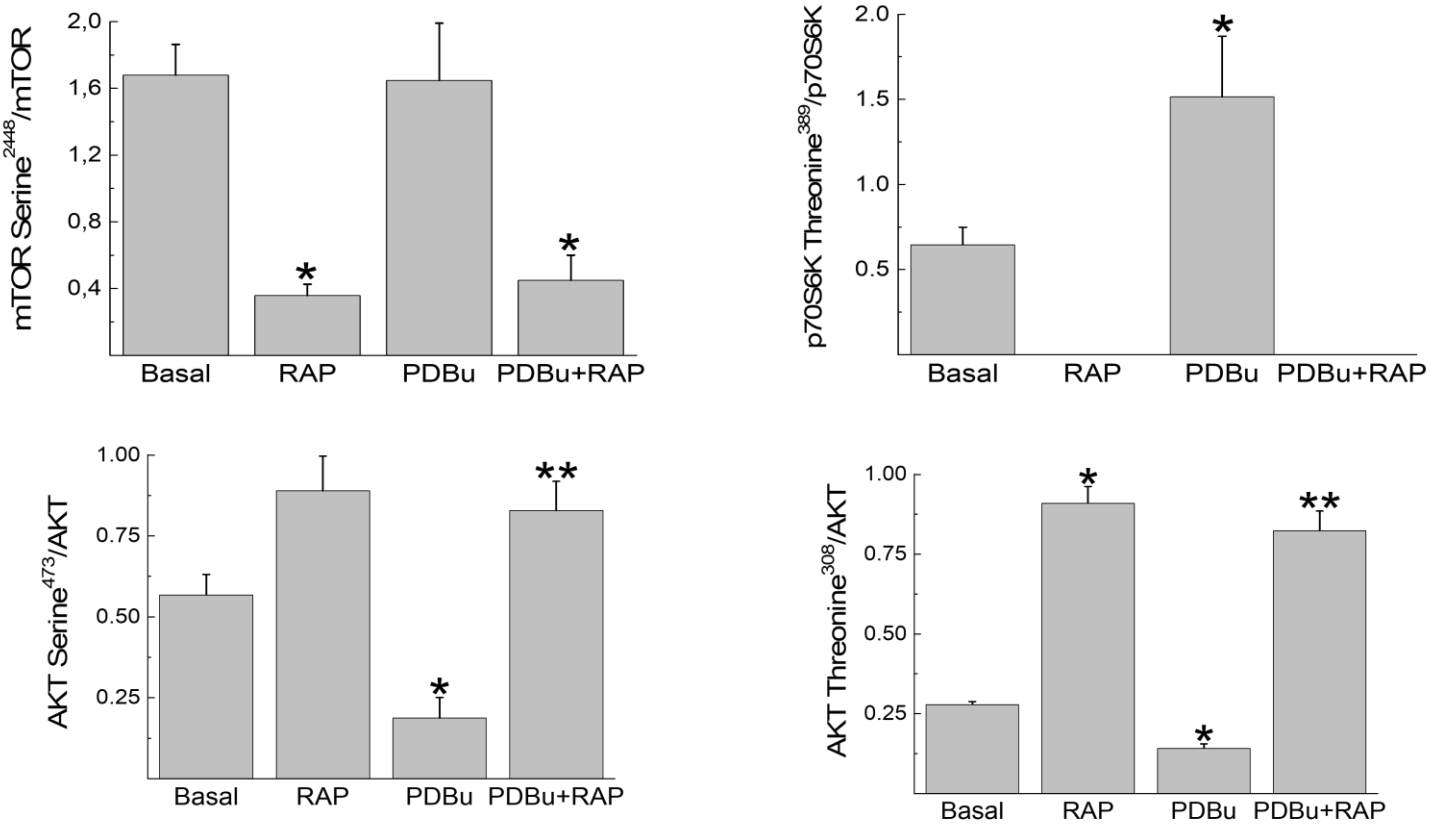


Figure 6

Figure 7

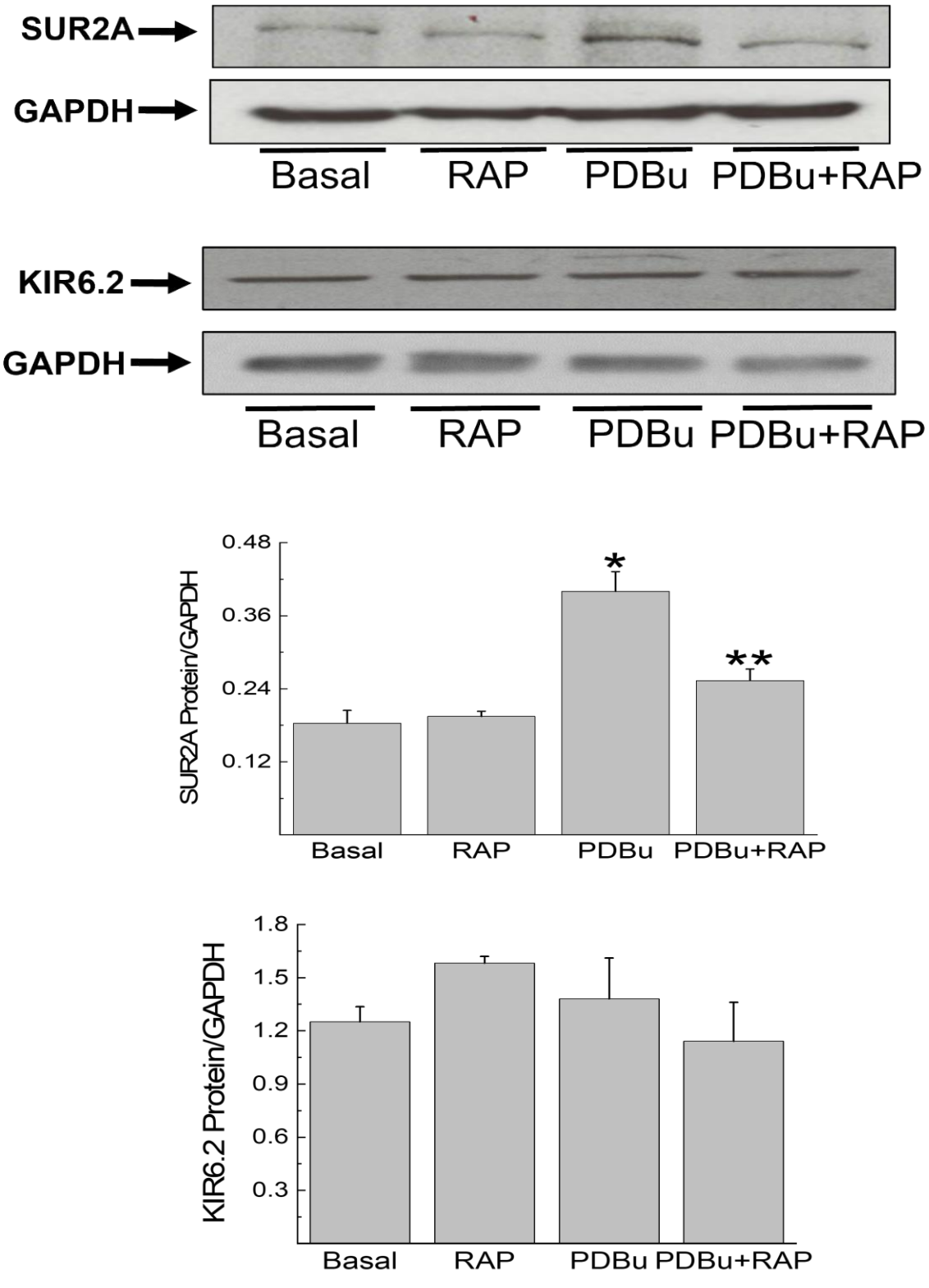
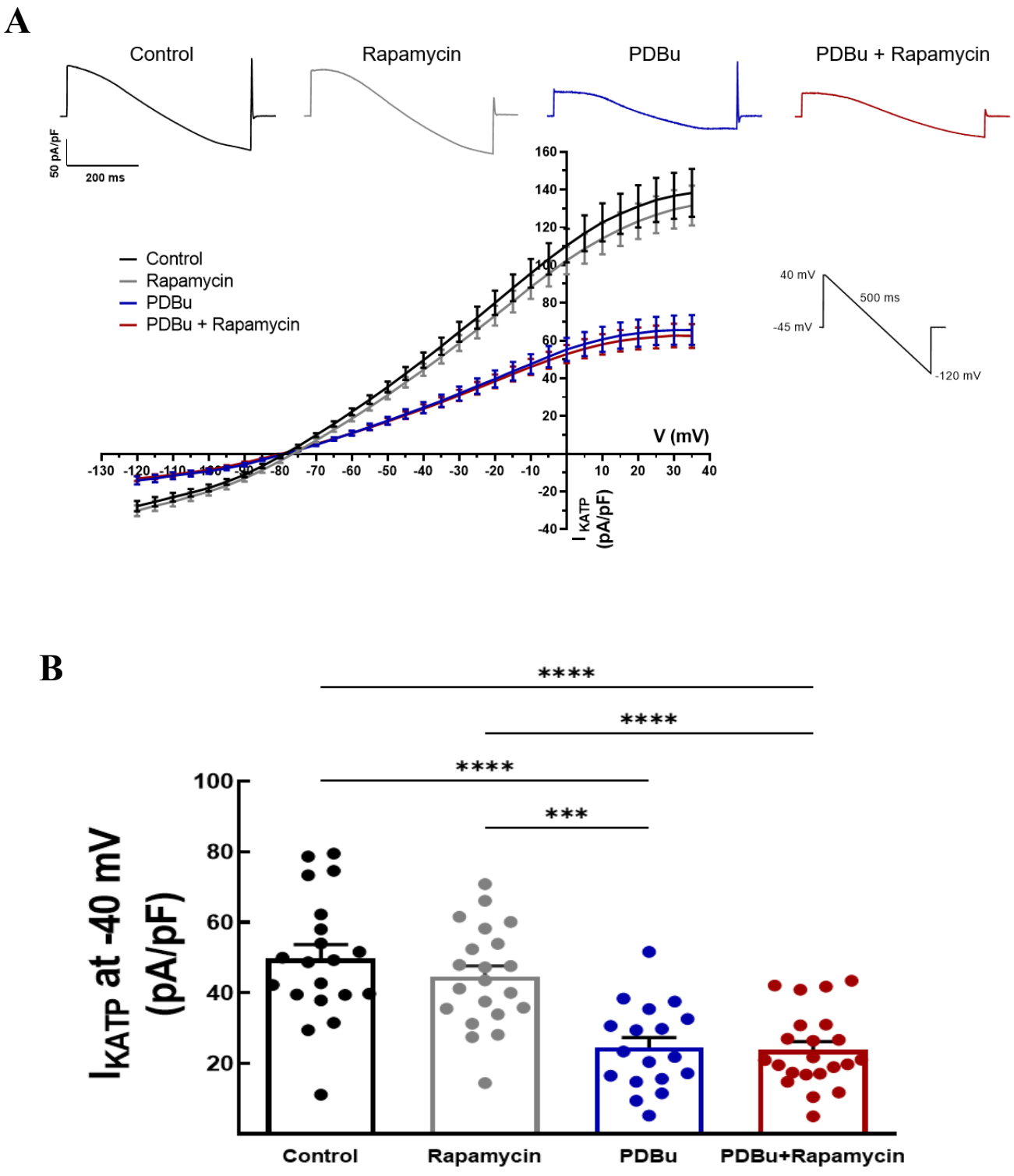


Figure 7

Figure 8


A
Control
Rapamycin
PDBu
PDBu + Rapamycin
50 pA/pF
200 ms
Control
Rapamycin
PDBu
PDBu + Rapamycin
40 mV
500 ms
-45 mV
-120 mV
V (mV)
I KATP (pA/pF)
B
****
****
****
***
I KATP at -40 mV (pA/pF)
Control
Rapamycin
PDBu
PDBu+Rapamycin

C

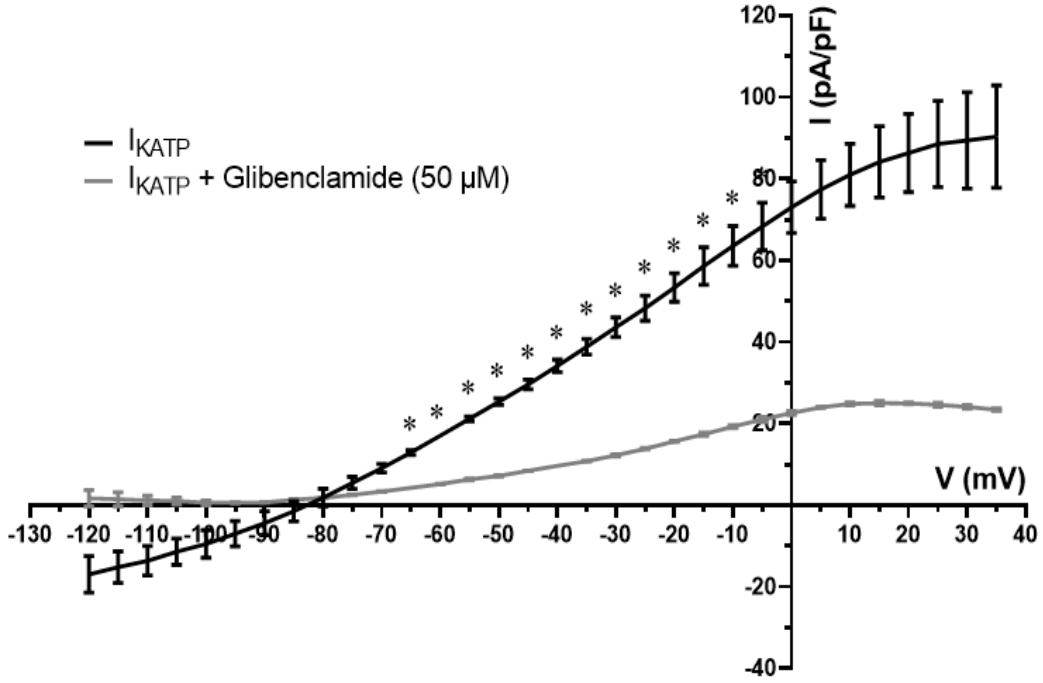

I (pA/pF)
V (mV)
I_{KATP}
I_{KATP} + Glibenclamide (50 µM)
120
100
80
60
40
20
-20
-40
-130 -120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20 -10 10 20 30 40
* * * * * * * * * * *

Figure 8

Chapter 5: Discussion

5.1. The Role of Ion Channels in Cardiac Remodeling Following Ischemic Damage

Following ischemic damage to the myocardium, the loss of viable cardiomyocytes leads to the formation of a permanent collagen-containing scar preserving the structural integrity of the compromised heart. Moreover, the loss of ventricular tissue following an ischemic insult further promotes a concomitant hypertrophic response of individual cardiomyocytes residing in the non-infarcted left ventricle providing contractile support to the compromised heart. Thus, scar formation and hypertrophy of ventricular cardiomyocytes residing in the non-infarcted region represent essential biological events that prevent cardiac rupture and provide inotropic support, respectively to the ischemically damaged heart. In addition to their electrophysiological role in cardiac contractility, Ca^{2+} channels and K_{ATP} channels play a seminal role in remodeling post-myocardial infarction. The accumulation of intracellular Ca^{2+} following activation of voltage-dependent calcium channels contributes in part to the recruitment of Ca^{2+}-dependent signaling events implicated in cardiomyocyte hypertrophy. With regard to the K_{ATP} channel, several studies have reported that the channel provides a sex-specific cardioprotective mechanism limiting scar damage of the female rodent heart following an ischemic insult. Interestingly, K_{ATP} channels were also identified in the male rodent heart; albeit the channel fails to provide cardioprotection in the ischemically damaged male heart. In this regard, the selective protection provided by K_{ATP} channels to the ischemically damaged female rodent heart may be dependent upon their more rapid recruitment following an ischemic challenge. Thus, a major AIM of the thesis was to examine in greater detail the regulation of Ca^{2+} channel subunits during the hypertrophic response of neonatal rat ventricular cardiomyocytes in response to sympathetic stimulation. The second major AIM employed an *in vivo* model of myocardial infarction and neonatal rat ventricular cardiomyocytes to highlight a sex-specific effect on scar formation and further identify signaling events that may rapidly recruit the K_{ATP} channel selectively in female rats. Collectively, the data obtained from these studies will further highlight the seminal role of ion channels in specific events of cardiac remodeling following myocardial infarction of the adult heart.

5.2. $Ca_V\alpha2\delta1$ subunit of the $Ca_V1.2$ channels is upregulated following sympathetic stimulation of hypertrophied NRVMs

Several *in vitro* and *in vivo* studies have identified the role of calcium-dependent signaling cascades in hypertrophic response. Increased cytosolic Ca^{2+} concentration forms the Ca^{2+}-calmodulin complex and serine/threonine protein phosphatase calcineurin. Ca^{2+}-calmodulin complex activates the protein kinase Cα (PKCα) and the calcium/calmodulin-dependent protein kinase type II (CaMKII), which acts *via* myocyte enhancer factor (MEF2) to stimulate the expression of pro-hypertrophic genes. Besides CaMKII, calcineurin is a serine/threonine phosphatase that dephosphorylates and activates its downstream target, nuclear factor of activated T cells (NFAT) [198,199]. NFAT binds to and activates GATA4 to induce the transcription of hypertrophic genes. Despite the latter unveiled Ca^{2+}-dependent mechanisms, the tie-in between increased intracellular Ca^{2+} and the expression of the voltage-gated L-type Ca^{2+} channels following hypertrophic stimulus remains unresolved. The study's main aim in Chapter 3 was to test the hypothesis that sympathetic-induced hypertrophy of neonatal rat ventricular cardiomyocytes (NRVMs) results in the elevation of Ca^{2+} influx secondary to an increase in the protein expression of the $Ca_V\alpha2\delta1$ subunit. A former study from our lab demonstrated that the co-expression of $Ca_V\alpha2\delta1$ and $Ca_V\alpha1C$ subunits increased $Ca_V1.2$ peak current density 5-10-fold and fostered the opening of the $Ca_V1.2$ channels at physiological voltages in recombinant cells[370]. To examine the hypothesis, the protein expression of $Ca_V1.2$ subunits was investigated in NRVMs treated with Norepinephrine (NE).

NE is a neurotransmitter released by the sympathetic system following a chronic sustained increase of preload or afterload to the adult heart. It was first established by Simpson et *al.* that 1 μM NE induced cardiac hypertrophy in cultured NRVMs, which was assessed by the increased surface area of the cardiomyocytes and enhanced protein synthesis. In the present study, we show a significant increase in the surface area of NRVMs following exposure to NE for 24 hours, confirming the pro-hypertrophic role of NE [130,601,602]. Moreover, and as expected, $Ca_V1.2$ peak current density was significantly elevated in NE-induced hypertrophied NRVMs. In parallel, the total protein expression of $Ca_V\alpha2\delta1$ and $Ca_V\beta3$ was upregulated in NRVMs secondary to exposure to NE for 24h, whereas $Ca_V\alpha1C$ and $Ca_V\beta2$ subunits remain unaltered.

The modification of $Ca_V1.2$ currents in response to β-adrenergic stimulation [403,410] and animal models of cardiac hypertrophy have been extensively studied [424,603,604]. As early as in the 1960s, it was established that activation of β-adrenergic signaling leads to elevation of $Ca_V1.2$ currents[605]. Exposure of adult ventricular cardiomyocytes to isoproterenol increased $Ca_V1.2$ peak current density that was reversed following treatment with β1-adrenergic antagonist[606]. Similarly, I_{CaL} was increased in response to isoproterenol in guinea pig ventricular myocytes[412]. These data were in conformity with *in vivo* studies demonstrating that mice treated with isoproterenol exhibited augmented I_{CaL}, resulting in an enhanced force of cardiac contractions[607]. The data in this dissertation further confirmed that sympathetic stimulation increased $Ca_V1.2$ peak current density in NRVMs 24h following exposure to NE. Nevertheless, activation kinetics of $Ca_V1.2$ Ca^{2+} currents weren't modified in response to NE treatment.

Although previous studies revealed contradictory data concerning the mechanism underlying LTCC modulation, it is inevitable that the $Ca_V1.2$ auxiliary subunits serve as an indispensable mediator of channel regulation by sympathetic activation. The molecular, biophysical, and functional properties of LTCC are greatly bolstered by the association of the auxiliary calcium channel subunits: $Ca_V\beta$ and $Ca_V\alpha2\delta1$[311,373,414]. Through a high-affinity interaction with $Ca_V\alpha1C$, $Ca_V\beta$ serves as a chaperone protein whereby it contributes to the proper maturation of the $Ca_V1.2$ channel and enhances its trafficking to the plasma membrane [347,608,609, p. 3]. A host of studies employing transgenic mice revealed that disruption of $Ca_V\alpha1$-$Ca_V\beta$ interaction resulted in reduced LTCC currents and was associated with multiple physiological abnormalities[330,610,611]. Moreover, during β1-adrenergic stimulation, $Ca_V\beta$ serves as a mediator of LTCC regulation through either direct phosphorylation by protein kinases[612] or association with RGK proteins (mainly Rad) [421].

In addition to the $Ca_V\beta$ subunit, the extracellular subunit $Ca_V\alpha2\delta1$ has emerged as a stalwart contributor to LTCC fine-tuning. A former study from our lab demonstrated that the co-expression of $Ca_V\alpha2\delta1$ and $Ca_V\alpha1C$ subunits increased $Ca_V1.2$ peak current density 5-10-fold and fostered the opening of the $Ca_V1.2$ channels at physiological voltages[370]. Moreover, the functional importance of $Ca_V\alpha2\delta1$ was also delineated *in vivo* in mice with homozygous knockout of the CACNA2D1 gene[359]. These mice exhibited reduced I_{CaL} associated with impaired cardiac contractility[359], further confirming the regulatory role of $Ca_V\alpha2\delta1$ on LTCC.

To date, a modicum of data suggests an inter-related alliance between $Ca_V\alpha2\delta1$, $Ca_V1.2$ activity and cardiac function. Hence, in this study, it is tempting to postulate that the elevated current density observed in NE-induced hypertrophied NRVMs was secondary to the upregulation of $Ca_V\alpha2\delta1$ protein expression. The data in this study revealed that sympathetic stimulation upregulated the total protein expression of $Ca_V\alpha2\delta1$ and $Ca_V\beta3$ in hypertrophied NRVMs. Nevertheless, $Ca_V\alpha1C$ and $Ca_V\beta2$ subunits remained unchanged. These results are partly in agreement with data highlighting that expression of $Ca_V\alpha1C$ subunit in cardiomyopathic hamsters was unaltered when compared to control [613]. By contrast, $Ca_V\alpha1C$ protein expression was increased in the hypertrophied septum of patients suffering from hypertrophic obstructive cardiomyopathy compared to control patients [431]. The discrepancies in these data might be attributed to the stage of the cardiac hypertrophy, wherein it was shown that the protein expression of $Ca_V\alpha1C$ increased in the early stages of cardiomyopathy but decreased in a more advanced stage of heart failure, indicating that the change in the protein expression of $Ca_V\alpha1C$ fluctuates with the progression of the disease [614]. A previous study from our lab demonstrated that co-expressing the pore-forming subunit with $Ca_V\beta$ alone was insufficient to enhance the $Ca_V1.2$ peak current density[370]. In this regard, the latter data excludes the possibility that the upregulation of $Ca_V\beta3$ protein expression is responsible for the increased I_{CaL} currents following NE treatment. Hence, these results reveal that the increase in I_{CaL} observed in NE-induced hypertrophied NRVMs was secondary to the upregulation of $Ca_V\alpha2\delta1$ protein expression.

Depending on the cell type, $Ca_V\alpha2\delta1$ enhances the $Ca_V1.2$ peak current density by several proposed mechanisms, including facilitating channel activation at more physiological voltages [357,373,414,615], promoting the channel's expression at the plasma membrane[616], and stabilizing the channel complex at the plasma membrane. In this study, patch-clamp results showed that gating kinetics of $Ca_V1.2$ were not changed, and the I-V curve did not show any negative shift in NE-induced hypertrophied NRVMs. Moreover, the possibility that the $Ca_V\alpha2\delta1$ would increase the cell surface expression of the channel can also partially be ruled out because, despite being qualitative, the confocal images displayed that the expression of $Ca_V\alpha2\delta1$ and $Ca_V\alpha1C$ subunits at the plasma membrane was not altered. More quantitative assays such as flow cytometry are warranted further to corroborate our confocal imaging data[617]. Therefore, the potential possible explanation is that the upregulation of $Ca_V\alpha2\delta1$ protein expression might stabilize the $Ca_V1.2$

channel complex at the plasma membrane. Although not being tackled in this dissertation, assessing $Ca_V1.2$ turnover and stabilization in response to sympathetic stimulation can decipher the mechanism that accounts for the impact of $Ca_V\alpha2\delta1$'s upregulation on the biophysical properties of $Ca_V1.2$.

5.3. Upregulation of the $Ca_V\alpha2\delta1$ subunit protein expression is mediated by the β1-adrenergic receptor-induced phosphorylation of ERK 1/2 pathway

An explored mechanism is the signaling pathway coupled to the upregulation of $Ca_V\alpha2\delta1$ and $Ca_V\beta3$ in hypertrophied NRVMs following exposure to NE. NE is a non-selective catecholamine that acts *via* alpha and beta-adrenergic receptors, activating Gq/11 and Gs family of G proteins, respectively. β-adrenergic receptors account for 90% of the total cardiac adrenergic receptors, with β1-adrenergic receptor being the major predominant type[119,120], while $\boldsymbol{\alpha}_1$-adrenergic receptors account for approximately 10% [121].

During the fight-or-flight response, the prompt release of norepinephrine enhances cardiac contractility and relaxation through the β1-adrenergic receptor-mediated activation of cyclic adenosine monophosphate/PKA (cAMP/PKA) signaling cascade[122–124]. By contrast, the activation of α1-adrenergic receptor results in elevated cytosolic Ca^{2+} levels through the stimulation of phospholipase C and subsequent generation of inositol trisphosphate (IP3) and diacylglycerol (DAG). In turn, IP3 and DAG trigger Ca^{2+} release of the SR and activate PKC, respectively. It has been long known that the $Ca_V1.2$ channel is a target of PKA secondary to β1-adrenergic stimulation, which results in increased $Ca_V1.2$ peak current density. Based on the latter premise and the β1-adrenergic receptor's role in cardiac contractility, it was tempting to speculate that the increased protein expression of the $Ca_V\alpha2\delta1$ was mediated through the β1-adrenergic receptor-mediated signaling.

A pharmacological approach revealed that NE-mediated upregulation of $Ca_V\alpha2\delta1$ subunit protein levels occurred through the β1-adrenergic receptor subtype. Specifically, metoprolol antagonism of the β1-adrenergic receptor inhibited NE-induced upregulation of $Ca_V\alpha2\delta1$ whereas $Ca_V\beta3$ protein levels were not altered. These data are consistent in part with previous findings of an earlier work by Tsien, Greengard, and Reuter[618–620] that revealed that the cardiac LTCC

was a target of protein kinase A (PKA) secondary to β1-adrenergic receptor stimulation. Moreover, data obtained in recombinant cells and isolated cardiac myocytes showed that the cardiac α_{1C} and β_{2a} subunits of the $Ca_V1.2$ channel are head-on targets of β1-adrenergic stimulation [421,621–623]. Herein, we show that $Ca_V\alpha2\delta1$ is the novel target of β1-adrenergic signaling influencing LTCC expression and activity (Figure 1).

Despite the inhibition of $Ca_V\alpha2\delta1$ subunit upregulation, metoprolol failed to attenuate NE-induced hypertrophy. These date are consistent with previous published papers highlighting the prominent role of the α1-adrenergic receptor in NE-induced hypertrophy of ventricular cardiomyocytes [624,625]. These data indicate that the upregulation of $Ca_V\alpha2\delta1$ is attributed to the β1-adrenergic stimulation by norepinephrine and is independent of cardiac hypertrophy. By contrast, the elevation of $Ca_V\beta3$ protein expression levels may be contingent on the hypertrophic response of NRVMs following sympathetic stimulation(Figure 1).

A seminal signaling event coupled to the β1-adrenergic receptor in ventricular cardiomyocytes is the ERK 1/2 pathway [626]. The role of ERK 1/2 following sympathetic stimulation and during the progression of cardiac hypertrophy has been extensively studied [131,627–631]. However, little is known regarding its implication in LTCC regulation[632,633]. P-ERK 1/2 was activated in guinea pigs subjected to thoracic aortic banding-induced-pressure overload cardiac hypertrophy[627], as well as in neonatal rat cardiomyocytes treated with norepinephrine, endothelin-1, or phenylephrine [625,628,634]. In the present study, NE treatment of NRVMs increased ERK 1/2 phosphorylation in a biphasic pattern, and the maximal response observed at 24h coincided with cardiac hypertrophy. Thus, additional experiments were performed to assess whether ERK 1/2 was involved in the upregulation of $Ca_V\alpha2\delta1$ protein expression in response to NE-mediated hypertrophy. Pharmacological inhibition of ERK 1/2 signaling with U0126 attenuated NE-stimulated upregulation of $Ca_V\alpha2\delta1$ but did not alter the proteins levels of $Ca_V\beta3$. Moreover, despite its inhibitory action on $Ca_V\alpha2\delta1$, U0126 failed to prevent the hypertrophic response of NRVMs to NE. Therefore, the β1-adrenergic stimulation of ERK 1/2 signaling plays a prominent role in the modulation of $Ca_V1.2$ calcium currents *via* the upregulation of $Ca_V\alpha2\delta1$ subunit protein levels (Figure 1).

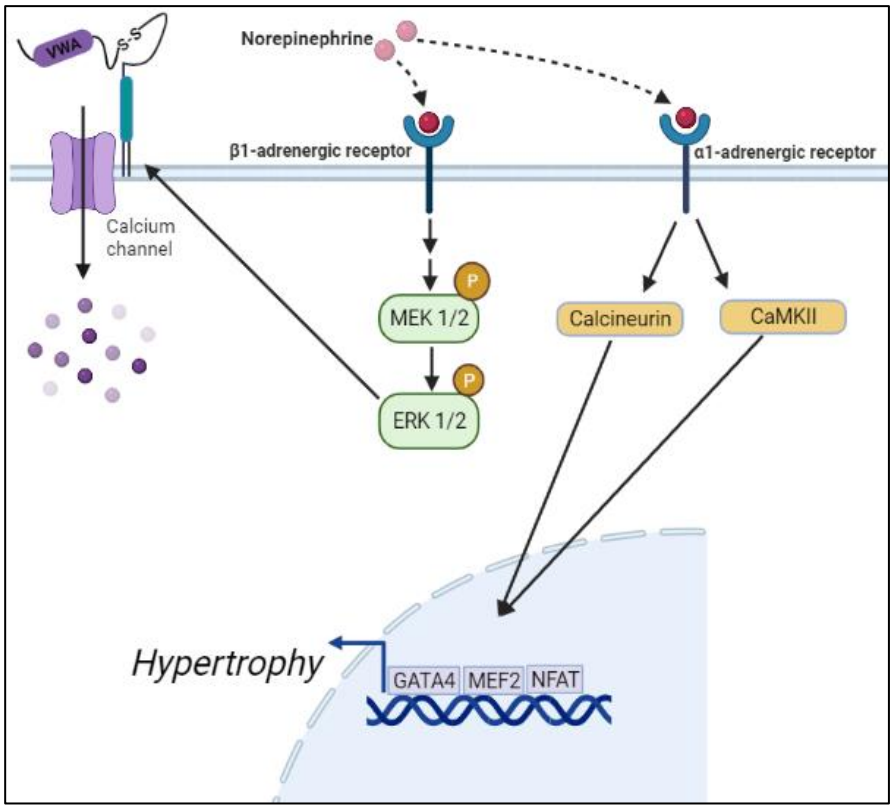


Figure 1: Scheme depicting the signaling pathways partaking in cardiac hypertrophy and $Ca_V1.2$ modulation in neonatal rat cardiomyocytes (Recapitulating findings of project -1)

5.4. The relationship between sex and myocardial infarction; role of K_{ATP} channels

As previously indicated, myocardial infarction represents a leading cause of death in Western society. Despite these data, epidemiological studies have reported that the incidence and prevalence of ischemia are significantly higher in men than in age-matched women and onset of the disease in women occurs later in life [635,636]. Several clinical investigations have demonstrated that the incidence of acute MI is two-fold higher in males than females[551,637,638]. The observed sex disparity reported in clinical settings were recapitulated in experimental studies employing animal models of myocardial infarction. Female rodent hearts were shown to exhibit increased resistance to ischemia and ischemia-reperfusion injury, which was manifested by reduced infarct size and improved contractile performance[21,639–641]. Thus, ongoing studies have attempted to elucidate the underlying mechanism attributed to the apparent selective cardioprotection mechanism in females. The observed sex difference secondary to ischemic injury has led to the observation that ovarian hormones estrogen and progesterone

provide sex-dependent cardioprotection. Indeed, several studies have suggested that sex hormones partially confer the protection since women with hormonal disturbances, such as polycystic ovarian syndrome, encounter atherosclerosis and MI earlier than healthy women[642,643]. By contrast, an experimental study revealed that cardiac contractility of female mice subjected to ovariectomy did not exacerbate cardiac function as compared to non-ovariectomized rats [644,645]. Furthermore, the treatment of ovariectomized rats with 17-β-estradiol did not attenuate cardiac dysfunction or cause further dilatation post-MI, suggesting that estrogen deficiency does not influence cardiac dysfunction post-MI [644,645]. Likewise, previous work from our lab and others have reported that the loss of circulating ovarian sex hormones estrogen and progesterone did not influence scar formation after complete coronary artery ligation of the adult female rat and mouse heart [644–646]. Moreover, hormonal replacement therapy to postmenopausal women did not provide a cardioprotective effect. Randomized clinical trials involving postmenopausal women revealed that treatment with estrogen did not attenuate myocardial infarction[552,647]. Moreover, women receiving estrogen treatment were associated with a higher risk of developing coronary heart disease (e.g., thromboembolic complications) as compared to placebo group[552,647]. Collectively, these data suggest that the absence of a significant beneficial effect of female sex hormones on cardiac function or scar formation following myocardial infarction has provided the impetus to search out alternative mechanisms.

Several studies have revealed that female rodents were associated with more pronounced activation of the sarcolemma cardiac K_{ATP} channels, suggesting that activation and/or expression of the channel might contribute to the sex disparities in response to ischemia[21]. As electrical channels, K_{ATP} channels act as metabolic sensors that couple the cellular metabolic state to the electrical activity. In response to ATP depletion following ischemia, K_{ATP} channels are activated, favoring K^+ efflux and hyperpolarization, providing cardioprotection by limiting Ca^{2+} influx. The prominence of K_{ATP} channels in cardioprotection against ischemia has been well established following pharmacological blockade or K_{ATP} knockout animal models[511,513,648,649]. Moreover, it would appear that cardioprotective effect of K_{ATP} channels was sex-dependent. Johnson et *al.* revealed that sarcolemma K_{ATP} channels governed the sex-selective protection during ischemia as pharmacological blockade led to scar expansion selectively in infarcted female rat hearts[21]. The selective sex-dependent effect of K_{ATP} channels was not attributed to a disparate level of expression as work presented in the thesis revealed that Kir6.2 and SUR2A protein levels

in the left ventricle of normal adult male and female rats were equivalent. These data were consistent in-part with a previous study demonstrating that mRNA and protein levels of Kir6.2 subunits were similar in both male and female adult guinea pig hearts[650]. However, the latter study did reveal that protein levels of SUR2A and Kir6.2 on the plasma membrane were higher in females as compared to male hearts, suggesting that female hearts might have more functional K_{ATP} channels [650]. By contrast, a previous study reported that protein expression of Kir6.2 and SUR1/2 subunits were lower in normal adult male hearts compared to female hearts [641]. This difference could be attributed to age-dependent factors as the latter study employed older rats of 5-8 months compared to 2-2.5 months old rats used in our study [641]. Collectively, these data suggest that the selective sex-protective effect of sarcolemma K_{ATP} channels in female versus male rodent hearts following ischemic injury may not be necessarily related to expression levels of the channel but rather attributed in part to their selective recruitment in response to ischemia.

5.5. Rapamycin treatment unmasks a cardioprotective mechanism selectively in the ischemically damaged female rat heart

Rapamycin (sirolimus) is a macrocyclic antibiotic used as an immunosuppressive agent to suppress the rejection of transplanted organs and coronary restenosis following stent implantation [651]. Moreover, several studies have examined the effect of rapamycin on cardiac function and remodeling following ischemic injury. Several studies reported that rapamycin increased infarct size and escalated cardiomyocyte death induced by oxidative stress post-ischemia reperfusion[652–654]. By contrast, Buss et *al.* revealed that rapamycin administration to a chronic MI animal model reduced infarct size[598]. The latter paradox may be explained by the concentration and the time by which rapamycin is administered during MI. Previous work from Dr. Calderone's lab reported that administration of rapamycin to the ischemically damaged female Sprague-Dawley rat heart led to a two-fold increase in scar weight and scar surface area[655]. To examine whether the latter observation was sex-dependent, rapamycin was administered to adult male Sprague-Dawley rats 24 hours after complete coronary artery ligation of the heart and continued for an additional 5 days. These data revealed that rapamycin administration to the infarcted male heart did not alter scar size or scar surface area compared to untreated myocardial

infarcted adult male rats whereas left ventricular contractility of infarcted male rat hearts was improved. Our data were in part consistent with echocardiographic analysis revealing that rapamycin administration to a chronic male rat MI model ameliorated cardiac function and reduced cardiomyocyte apoptosis[656]. Furthermore, the apparent sex-dependent effect of rapamycin was not limited to scar formation following myocardial infarction as numerous studies have revealed that rapamycin preferentially extended the lifespan of female rodents versus males [657,658]. Besides, Apelo et *al*. have reported an anti-aging potential of rapamycin with minimal concomitant negative effects following intermittent administration of the macrolide rapamycin[659].

A potential mechanism attributed to rapamycin-induced sex-dependent scar expansion may be associated with a disparate effect of the drug on the serine/threonine kinase mammalian target of rapamycin (mTOR) signaling. mTORC1 is the main target for inhibition by rapamycin and is shown to be activated in the remote region of the myocardium due to elevated load during ventricular remodeling following MI[598,599]. Indeed, in the non-infarcted left ventricle of the post-MI female and male rat heart, mTOR protein levels were significantly increased as compared to untreated rat heart. Rapamycin administration abolished phosphorylation of the serine^{2448} residue of mTOR in sham and post-MI female rats but failed to reduce phosphorylation of mTOR in sham and post-MI male rat heart, indicating that rapamycin exhibited sex-selective effect on mTOR phosphorylation.

Rapamycin inhibits mTORC1 by binding to FKBP12 and destabilizing the mTOR-Raptor complex, ultimately promoting the dissociation of raptor from mTOR. In this study, raptor protein levels were lower in the normal female rat heart as compared to male rat heart. Accordingly, pharmacological effects of rapamycin may be more pronounced and sustained in female rats secondary to a lower expression of the target raptor. Collectively, these data have identified a sex-specific effect of rapamycin on scar formation and mTOR phosphorylation in the infarcted adult rat heart and has provided the impetus to examine the underlying mechanism attributed to the latter paradigm.

5.6. Sex-dependent regulation of mTOR signaling and relationship with K_{ATP} channels

The mammalian target of rapamycin (mTOR) is the primary target of rapamycin and implicated in cardiac function and remodeling in various models of cardiovascular disease[574,660]. Moreover, the cardioprotective effect observed exclusively in the infarcted heart of female rat hearts recapitulated the analogous phenotype observed after pharmacological inhibition of K_{ATP} channels providing a potential relationship between mTOR and K_{ATP} channels. Indeed, several studies have reported mTOR-dependent modulation of K_{ATP} channel subunit expression and activity [661,662,663]. Thus, based on the observation that K_{ATP} channel subunit expression was equivalent in the normal male and female rat heart, experiments were performed to assess whether greater mTOR signaling in the female rat heart may translate to a more rapid recruitment of K_{ATP} channels. First, phosphorylation of the serine^{2448} residue of mTOR was similar in the normal adult female rat heart as compared to the normal adult male heart. By contrast, a greater inactivation of the upstream inhibitor tuberin was observed in the female heart characterized by the higher phosphorylated state of the threonine^{1468} residue. These data suggest that the greater inactivated state of the upstream inhibitor tuberin may translate to a more rapid recruitment of mTOR following ischemic injury. Moreover, sex-dependent regulation of tuberin was identified predominantly in young women in a rare multisystem disease referred to as lymphangioleiomyomatosis (LAM) [664,665]. LAM is a low grade, destructive, metastasizing neoplasm and the incipient cause is an acquired mutational inactivation of tuberin leading to a hyperactivated state of mTORC1 and uncontrolled cell proliferation. Collectively, these data support a significant relationship between sex and tuberin activation. Moreover, multiple signals including stress, energy, and hypoxia influence tuberin regulation *via* ERK signaling and the AMPK cascade[666,667]. In the study presented in the thesis, the input and mechanism accounting for the greater phosphorylated inactivated state of tuberin in female hearts was not investigated.

Based on the selective protective role of K_{ATP} channels in the ischemically damaged female rodent, we hypothesized that the greater phosphorylated state of mTOR in the adult heart of female rats might positively regulate sarcolemma K_{ATP} channels leading to the upregulation of $K_{ir}6.2$/SUR2A protein expression during the acute phase of an ischemic insult thereby suppressing cardiomyocyte apoptosis translating to a smaller scar. To address this hypothesis, the impact of mTOR recruitment in response to PKC stimulation on K_{ATP} channel subunit expression was

examined in NRVMs. Considerable evidence has revealed the mTOR represented a downstream target of PKC signaling[668]. In adult cardiomyocytes, PKCε and PKCδ mediated ET-1-triggered mTORC1 activation[669]. Furthermore, previous studies have reported that PKC-ε represents a protective mechanism in the female heart against ischemic damage. Inhibition of the PKC-ε prior to ischemia resulted in an expansion of infarct size in female, but not male hearts[640]. Moreover, cardiac K_{ATP} channels were identified as a target of PKC and implicated in regulating cardiac function and excitability[670,671]. The paper presented in the thesis revealed that PDBu treatment of NRVMs increased phosphorylation of the threonine^{389} residue of 70S6K and induced hypertrophy and both responses were inhibited with rapamycin pretreatment thereby supporting a direct role of the mTORC1 complex. By contrast, changes in mTOR and tuberin phosphorylation were not observed after a 24-hour treatment with PDBu suggesting that phosphorylation of either protein must have occurred prior to the development of a hypertrophic response. These results were in part consistent with the data by Rolfe et *al.* in adult rat cardiomyocytes as tuberin phosphorylation increased at 10 minutes and was maintained for 60 minutes, but decreased at 90 minutes following treatment with phenylephrine, an α1 adrenergic agonist coupled to hypertrophy[672]. This implies that tuberin is phosphorylated and inactivated shortly after treatment, which thereafter leads to the activation of the downstream target p70S6K translating to gene expression and protein synthesis.

A second mTOR complex denoted mTORC2 was identified as mTOR binds selectively to Rictor and plays a prominent role in the activation of AKT *via* promoting the phosphorylation of the serine^{473} residue [673]. Phosphorylated serine^{473} of AKT then facilitates the phosphorylation of the threonine^{308} residue of AKT by phosphoinositide-dependent protein kinase-1 (PDK1) translating to a fully activated serine/threoine kinase. Chu et *al*. postulated that serine^{473} phosphorylation activates AKT by impeding the autoinhibition conformation[674]. Moreover, AKT is implicated in cardioprotection during myocardial infarction through its anti-apoptotic/pro-survival function[675]. However, mTORC1 was reported to inhibit mTORC2 activity and thus AKT phosphorylation[574,660]. Interestingly, phosphorylation of the serine and threonine residues of the serine/threonine kinase AKT was significantly reduced following PDBu treatment of NNVMs and the response was abolished by rapamycin pretreatment. These data support the negative feedback loops between mTORC1 and mTORC2 as mTORC1 suppressed mTORC2-mediated phosphorylation of the serine^{473} residue of AKT[676]. However, the concomitant

inhibition of threonine^{308} phosphorylation of AKT by PDBu and complete reversal following rapamycin pretreatment represents a novel effect of the mTORC1 complex. Despite the latter novel observation, the underlying mechanism attributed to mTORC1 complex regulation of threonine^{308} phosphorylation of the serine/threonine kinase AKT remains unknown finding.

Establishing recruitment of the mTORC1 complex in NNRVMs following PDBu treatment provided the impetus to assess its role in K_{ATP} channel subunit expression. Indeed, PDBu treatment significantly increased SUR2A protein levels in NRVMs and rapamycin pretreatment attenuated the response. By contrast, Kir6.2 subunit protein levels were unaffected by PDBu treatment of NRVMs, and rapamycin pretreatment likewise failed to alter expression. These data suggest that increased SUR2A expression may significantly enhance K_{ATP} activity as previous studies reported that SUR2 subunit was responsible for the trafficking and localization of K_{ATP} channels to the plasma membrane[466–468]. Moreover, a previous study revealed that mice subjected to SUR2A gene knockout exhibited non-functional K_{ATP} channels and ventricular cardiomyocyte-restricted re-expression of SUR2A to the SUR2A-null mice restored K_{ATP} channel activity [677]. Thus, our study has identified a novel signaling pathway in ventricular cardiomyocytes as mTORC1 activation was associated with the upregulation of the SUR2A subunit that may in turn translate to increased presence of functional of K_{ATP} channels at the plasma membrane (Figure 2). However, a limiting factor in our study is whether increased SUR2A protein levels in PDBu-treated NNVMs may be in part dependent on the hypertrophic response as rapamycin inhibition of hypertrophy coincided with attenuation of subunit expression. Lastly, additional studies are required to assess whether phorbol ester-mediated recruitment of the mTORC1 complex in ventricular cardiomyocytes is mediated by PKC-α or PKC-ε and/or requires a collectively effort of both isoforms.

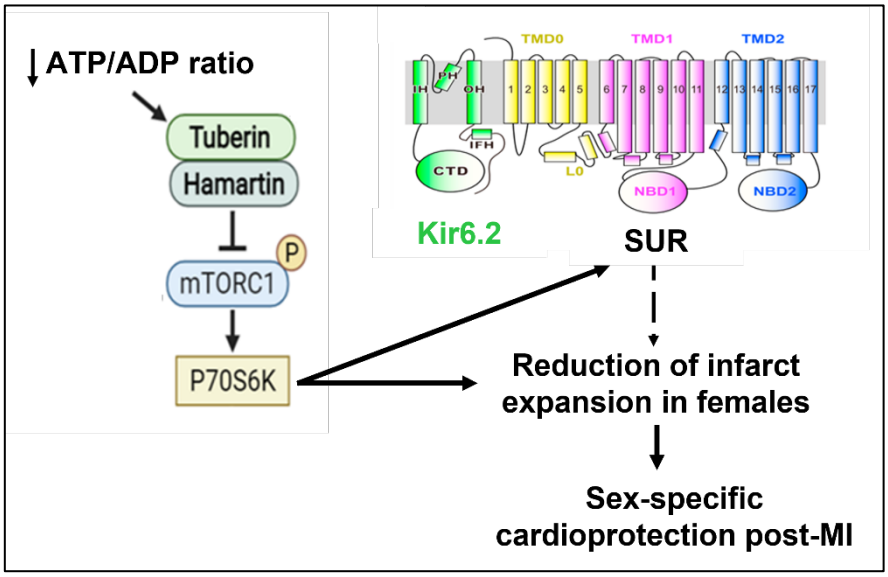


Figure 2: Scheme depicting the signaling pathways partaking in cardioprotection post-MI. (Recapitulating findings of project -2)

Chapter 6: Conclusion, limitations, and future directions

The findings in Chapter 3 of this dissertation demonstrates a mechanism whereby norepinephrine, through the activation of the β1-adrenergic receptor/ MEK1/2 / ERK1/2 signaling pathway, upregulates the total protein expression of $Ca_V\alpha2\delta1$ auxiliary subunit in neonatal rat ventricular cardiomyocytes. The increase of $Ca_V\alpha2\delta1$ protein expression was sufficient to enhance the $Ca_V1.2$ peak current density, which might prompt Ca^{2+}-dependent signaling caused by sustained sympathetic overactivation observed during cardiac hypertrophy. However, the β1-adrenergic receptor-mediated elevation of the $Ca_V\alpha2\delta1$ subunit protein expression was independent of the hypertrophic response. However, further experiments are required to evaluate whether the plasma membrane expression of $Ca_V\alpha2\delta1$ and $Ca_V1.2$ is also enhanced following treatment with norepinephrine. An important factor that is unexplored in this study is the effect of the P-ERK 1/2 blocker, U0126, on $Ca_V1.2$ peak current density. Although Duran et *al.* have demonstrated that blocking P-ERK 1/2 reduced $Ca_V1.2$ peak current density in GH3 cells[632], measuring $Ca_V1.2$ peak current density in the presence of U0126 in NRVMs secondary to NE treatment has to be performed to further confirm the contribution of P-ERK 1/2 cascade in regulating $Ca_V1.2$ calcium

currents. To better understand the mechanism of $Ca_V1.2$ channel modulation, the next steps would be to examine whether the transcriptional upregulation of $Ca_V\alpha2\delta1$ by ERK 1/2 is established *via* an intermediate such as a transcription factor.

Moreover, this dissertation further outlined that mTORC1 provides a correlation between ischemic cardioprotection and K_{ATP} channels (Chapter 4). This correlation was achieved by the upregulation of SUR2A subunit protein expression by mTORC1/P70S6K cascade, which might increase the Kir6.2 channel activity and account for the sex-specific cardioprotection during MI. However, further experiments are warranted to examine whether the increased SUR2A protein expression is associated with upregulation of K_{ATP} channel currents. Furthermore, the data identified a sex-specific effect of rapamycin on scar formation and mTOR phosphorylation in the infarcted adult rat heart. These findings serve as a stepping stone to understand the mechanism underlying the mTOR/K_{ATP}-dependent cardioprotective effects in the female ischemic heart. Therefore, Rapamycin stands-out as a potential candidate for large scale research to fine-tune its cardiac effects through the mTOR/P70S6K/K_{ATP} channel cascade and potentially harbor them in clinical research.

Lastly, it is noteworthy that the studies investigating signaling cascades were performed in neonatal rat ventricular cardiomyocytes in order to avoid any additional factors that might intervene in the molecular mechanism if performed *in vivo*.

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Annex

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Title

L-type calcium channels in health and disease: The case of heart failure

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Abstract

Ca^{2+} entry through L-type Ca^{2+} channels (LTCCs) is crucial for cardiac excitability and contraction. During the fight-or-flight response, β-adrenergic stimulation of LTCC currents contributes to the increase of the cardiac contractility. Nevertheless, long-term stimulation alters Ca^{2+} cycling, leading to heart failure. To date, the contribution of LTCC to heart failure remains speculative. Modifying auxiliary subunits in transgenic mouse models altered LTCC currents and are associated with heart failure. This review recapitulates a vast and evolving literature on the protein interaction within the LTCC complex, its impact on LTCC-mediated-Ca^{2+} signaling, and its trend in cardiac hypertrophy and heart failure.

Calcium ions are ubiquitous second messengers

Calcium controls cardiac function

Calcium is a central second messenger that regulates diverse physiological processes. Normal Ca^{2+} intracellular homeostasis is essential for excitability, gene expression, and foremost cardiac

contractility (Fearnley et *al*. 2011; Carafoli, 2002). In cardiac muscle cells, also referred to as cardiomyocytes, Ca^{2+}-dependent signaling is highly regulated and determines the force of cardiac muscle contraction. In particular, the contractile function is controlled by a rapid ten-fold increase in the intracellular Ca^{2+} concentration leading to contraction (systole) and a subsequent recapture resulting in muscle relaxation (diastole) (Gorski et *al*. 2015; Donohoe et *al*. 2000; Wang et al. 2001; Bers and Perez-reyes, 1999). During the systole, depolarization triggers the opening of the voltage-gated L-type calcium channels (LTCC) (Fearnley et *al*., 2011) clustered in the T-tubules (Carl et *al*. 1995; Kawai et *al*. 1999; Scriven et al. 2000; Musa et *al*. 2002) (Figure 1). Ca^{2+} influx through LTCC induces Ca^{2+} release from ryanodine receptor 2 proteins located in the sarcoplasmic reticulum, resulting in cardiac muscle contraction (Scriven et *al*. 2000; Hong et *al*. 2010; Shaw and Colecraft, 2013; Rougier and Abriel, 2016). Disturbances in Ca^{2+} cycling lead to cardiac diseases (Kehat and Molkentin, 2010), highlighting the importance of Ca^{2+} regulation in cardiomyocytes. This current review focusses on a family of proteins, the voltage-gated Ca^{2+} channel expressed at the cell membrane, also referred to as the sarcolemma in cardiomyocytes, and specifically some of the recent knowledge in the field.

L-type Ca^{2+} channels are activated by positive membrane potential

LTCCs are activated at stronger depolarization (threshold ≈ -40 mV) than the voltage-gated Na^{+} channels and are thus considered to be high-voltage activated channels (Cohen and Lederer, 1987). Under physiological Ca^{2+} concentrations, LTCCs are maximally activated at ≈ +5 mV (Cohen and Lederer, 1987; Isenberg and Klöckner, 1982) with a peak current density varying between -4 to -12 pA.pF-1 (Yang et *al.* 1996; Xiao et *al.* 1997; Xin et *al.* 2012; Zhang et *al.* 2012; Yue et al. 2015). The absolute peak current densities increase steeply with recording temperatures (with Q10 factors ranging from 1.8 to 3.5) (Donohoe et *al.* 2000). LTCCs activate with slower activation and inactivation kinetics than Na_V currents (Cohen and Lederer, 1987). Depolarization longer than 50 ms is required to fully inactivate LTCCs (Cohen and Lederer, 1987), a process that is largely driven by a local increase in intracellular Ca^{2+} (Ababou et *al.* 2017). Ca^{2+} influx through LTCCs during phase 2 of the action potential promotes faster inactivation, thus contributing to the cardiomyocyte repolarization (Grant, 2009). Conversely, slower inactivation kinetics increases total Ca^{2+} influx, which in turn, prolongs the duration of the action potential (Giudicessi and Ackerman, 2016). LTCCs have been historically designated L-type, as they have slower inactivation kinetics than

other voltage-dependent Ca^{2+} channels when measured in the presence of Ba^{2+} ions. Among all voltage-activated Ca^{2+}channels, LTCCs are uniquely inhibited by so-called "Ca^{2+} channel blockers". Three major classes of pharmacological compounds: dihydropyridines (DHP), benzothiazepines, and phenylalkylamines inhibit Ca^{2+} channel activity in the nanomolar to the micromolar range (Fleckenstein, 1983). DHP compounds have been widely used for the treatment of hypertension and angina pectoris (Ferrari et *al.* 2018; Campbell, 2007; Chobanian et *al.* 2003).

L-type Ca^{2+} channels are upregulated by neurohumoral factors

The determinants of cardiac contractility, Ca^{2+} cycling amplitude and kinetics, are regulated by β-adrenergic-mediated cascades (Donohoe et *al.* 2000). During the fight or flight response, catecholamines (such as norepinephrine and epinephrine) activate β1-adrenergic receptors that induce adenylate cyclase and trigger cAMP-dependent protein kinase A (PKA) (Fearnley et *al*., 2011; Fuller et *al*. 2010). β-adrenergic stimulation increases the firing of the sinoatrial node (chronotropic effect), thus increasing the heart rate (Carlsson et *al.* 1977). Downstream effectors of PKA modulate multiple target proteins in ventricular cardiomyocytes that collectively increase intracellular Ca^{2+} levels and increase the force of ventricular contraction (positive inotropic response) (Van Der Heyden et *al.* 2005). As early as in the 1960s, the β-adrenoceptor stimulation was found to increase cardiac LTCC currents (Katchman et *al*. 2017; Mcdonald et *al.* 1994; Kamp and Hell, 2000; Weiss et *al*. 2013) in animal models (rats (Brown et *al.* 1975), guinea pig (Reuter, 1965), and bovine cardiomyocytes (Isenberg and Klöckner, 1982) (Reuter, 1967)). In particular, β-adrenergic stimulation could double the LTCC peak current density (Gomez et *al*. 1996) mainly through an increase in the channel open probability (Po) (55%) coupled with a shorter mean close time (60%) (Schroder et *al.* 1999). As we will describe in section 5, excessive catecholamine activation in chronic hypertension results in intracellular Ca^{2+} overload, leading to cardiac hypertrophy to normalize wall stress and maintain tissue perfusion (Fiedler and Wollert, 2004; Calderone, 2014). The link between sympathetic overdrive, Ca^{2+} overload, and LTCC has long eluded the cardiac physiologists and has only been recently elucidated. The underpinnings of this discovery require to understand the molecular structure of LTCCs.

The L-type Ca^{2+} channel is an oligomeric complex

LTCCs are expressed in endocrine cells (Safayhi et *al*. 1997), neurons (Zhang et *al.* 2020), cardiac (Mcdonald et *al*. 1994), smooth (Ghosh et *al*. 2017; Triggle et *al*. 1989), and skeletal muscle (Rosenberg, 2009). Initial biochemical studies on LTCC purified from skeletal muscle T-tubules found that LTCCs are oligomeric protein complexes comprised of a pore-forming $Ca_V\alpha1$ subunit (250 kDa) associated with auxiliary $Ca_V\beta$ (60 kDa) and $Ca_V\alpha2\delta$ (175 kDa) subunits (Curtis and Catterall, 1986). Cryogenic electron microscopy studies carried out with purified skeletal muscle T-tubules confirmed its oligomeric structure (Figure 2) (Wu et *al*. 2015; Wu et *al*. 2016)

$Ca_V\alpha1$ subunits of L-type Ca^{2+} channels

$Ca_V\alpha1$ forms the pore of L-type Ca^{2+} channels

The pore-forming $Ca_V\alpha1$ subunit accounts for Ca^{2+} influx in all these cell types including cardiomyocytes and confers defining features of LTCC, namely the ion selectivity, pharmacological sensitivity to DHP compounds and other Ca^{2+} channel blockers, activation gating, and inactivation kinetics (Buraei and Yang, 2010). $Ca_V\alpha1C$ is formed by 24 transmembrane segments (4 x S1-S6) folded into 4 homologous domains and flanked by intracellular NH2 and COOH termini. The pore domain is lined by residues contributed from the folding of the S6 transmembrane regions (Mikala et *al*. 1993; Bodi et *al*. 2005; Tianhua et *al*. 2018) and enables the inward flow of partially hydrated Ca^{2+} into the central cavity (Tang et *al*. 2014). The inwardly directed loop linking S5-S6 shapes the selectivity filter where four conserved glutamate residues (4x Glu) account for the micromolar Ca^{2+} selectivity (Parent and Gopalakrishnan, 1995; Bodi et *al*. 2005; Neely and Hidalgo, 2014; Klockner et *al*. 1996). Four different genes encode $Ca_V\alpha1C$ subunit of LTCC, giving rise to Ca_V1 ($Ca_V1.1$-$Ca_V1.4$). $Ca_V1.2$ ($Ca_V\alpha1C$) and $Ca_V1.3$ ($Ca_V\alpha1D$) are LTCC members identified in the heart. CaV1.3 activates at slightly more negative potentials (≈ -50 mV) than $Ca_V1.2$ and contributes to the cardiac pacemaker activity in sinoatrial and in atrioventricular nodes (Kodama et *al*. 1997). $Ca_V\alpha1C$ is subjected to alternative splicing: $Ca_V1.2a$ isoform predominates in the cardiac muscle with exons 1a/8a/-9*/32/33 (Atsushi et *al*. 1989; Liao et *al*. 2007; Liao and Soong, 2010), while the $Ca_V1.2b$ isoform, mostly found in smooth muscle, contains exons 1b/8/9*/32/33 (Biel et *al*. 1990).

$Ca_V\alpha1C$ dictates the unique functional properties of LTCC

Inactivation kinetics, which limits Ca^{2+}overload, is driven by Ca^{2+}-Calmodulin (CaM). CaM is constitutively bound to the C-terminus of $Ca_V\alpha1C$ in either Ca^{2+}-deplete (apoCaM) or Ca^{2+}-replete forms (Yamniuk et *al*. 2007). Upon $Ca_V1.2$ activation and subsequent local Ca^{2+} elevation, Ca^{2+} binds to CaM and triggers its conformational change. This, in turn, results in a conformational change of $Ca_V1.2$ that accelerates inactivation kinetics (Ababou et *al*. 2017). Significant decreases in the inactivation kinetics slow down cardiomyocyte repolarization and predispose to life-threatening arrhythmias, as seen in the Timothy syndrome (Splawski et *al*. 2004). Genetic mutations associated with prolonged repolarization interval (for instance, G402S (Splawski et *al*. 2005), G406R (Splawski et *al*. 2004; Splawski et *al*. 2005), A1473G (Gillis et *al*. 2012), and G1911R (Hennessey et *al*. 2014)) are located near the intracellular face of the S6 of $Ca_V\alpha1C$ (Splawski et *al*. 2004; Rosen, 2002; Raybaud et *al*. 2006), indicating that inactivation kinetics are somehow coupled with the intracellular activation gate.

Downregulation of $Ca_V\alpha1C$ in animal models: mixed results

Initial clinical investigations supported the cardioprotective effect of LTCC blockers in patients with essential hypertension (Onose et *al*. 2001; Takami and Shigematsu, 2003). As a specific target of LTCC blockers, $Ca_V\alpha1C$'s role in heart failure was closely studied in animal models with a host of controversial conclusions (Winslow et *al*. 1999). To seemingly simplify the number of variables, protein expression of $Ca_V\alpha1C$ was directly manipulated in transgenic mice. The first strategy aimed to downregulate the cardiac-specific expression of $Ca_V\alpha1C$. Given that homozygous genetic deletion of $Ca_V\alpha1C$ leads to embryonic lethality (Seisenberger et *al*. 2000), Goonasekera et *al*. used a transgenic mice model with cardiac-specific conditional heterozygous knockdown of $Ca_V\alpha1C$ in adult mice (Goonasekera et *al*. 2012). The 40% reduction in the $Ca_V\alpha1C$ protein resulted in a smaller 25% decrease in whole-cell currents. Lower protein expression of $Ca_V\alpha1C$ did not protect the mice from developing cardiac dysfunction, even as early as at 2 months of age (Goonasekera et *al*. 2012). Subjecting the transgenic mice (10 weeks) to pathological stimuli further decreased cardiac performance, indicating that hypertrophy is not attenuated by a reduction in $Ca_V\alpha1C$ protein expression (Goonasekera et *al*. 2012). These data are in line with clinical studies showing that the negative impact of long-term blockage of LTCC outweighs its beneficial effects such that their negative inotropic effects may exacerbate heart failure (De Vries et *al*. 2000; Packer et *al*. 2013; Patel et *al*. 2014).

Overexpression of Cavα1C in isolated tissues and animal models

The second approach aimed to investigate the longitudinal impact of overexpressing Cavα1C in cardiomyocytes (Muth et *al*. 2001; Song et *al*. 2002; Wang et *al*. 2009). Again, the genetic manipulation of Cavα1C led to small changes in the LTCC currents peak density (↑30%). Cardiac remodeling was modest at 4 months of age (Muth et *al*. 2001; Song et *al*. 2002; Wang et *al*. 2009), and only a fraction of the 8- to 11-month-old mice developed heart failure (Muth et *al*. 2001; Song et *al*. 2002; Wang et *al*. 2009). More interestingly, the mice showing the functional and morphological impairment associated with heart failure exhibited an increase in the LTCC slow inactivation kinetics (Muth et *al*. 2001; Song et *al*. 2002; Wang et *al*. 2009). These results suggest that the transition from hypertrophy to heart failure could involve an increase in total Ca^{2+} influx without any significant change in the protein expression of Cavα1C or a change in the LTCC peak density.

Genetic Manipulation of Cavα1C: an ongoing journey

The role of LTCC in hypertrophy and transition to heart failure remains contentious. Animal models of heart failure set forth controversial data with either increased (Winslow et *al*. 1999), decreased (Mukherjee et *al*. 1995; Mukherjee et al. 1998; Briston et *al*., 2011), or unaltered LTCC currents (Gómez et *al*. 2001; Ahmmed et *al*. 2000; Pogwizd et *al*. 1999; He et *al*. 2001). Similarly, LTCC currents were shown to either decrease (Chen et *al*. 2002) or remain unchanged (Schröder et *al*. 1998; Beuckelmann et *al*. 1991) in patients with heart failure (Chen et *al*. 2002). These findings could result from the discrepancies in the role of the Cavα1C protein in heart failure. It is also notable that the overexpression or downregulation of Cavα1C resulted in slight changes in LTCC currents. In support of these observations, Rosati et al. reported little change in LTCC peak current density in a heterozygous conditional knockout performed in 7-week-old mice (Rosati et *al*. 2011). Altogether, these data indicate that manipulating the protein expression of Cavα1C is not a limiting determinant of LTCC function and kinetics, which may be significantly influenced

by the complex interaction between auxiliary subunits (Goonasekera et *al*. 2012; Rosati et *al*. 2011).

Ca$_V$α2δ1is an extracellular auxiliary subunit

Ca$_V$α2δ1is widely distributed in muscle tissues

Ca$_V$α2δ1 subunits have been traditionally overlooked in many voltage-gated Ca^{2+} channels. First cloned at the beginning of 1990s after purification of the multisubunit complex, Ca$_V$α2δ is a glycosylphosphatidylinositol (GPI)-anchored protein with a large extracellular domain formed by Ca$_V$α2 held to the smaller Ca$_V$δ subunit by a series of disulfide bonds (Takahashi et al. 1987; Ellis et *al*., 1988; De Jongh et *al*. 1990; Jay et *al*., 1991). Of the four different CACNA2D genes coding for the four isoforms of Ca$_V$α2δ, Ca$_V$α2δ1 is dominant in striated, smooth, and cardiac muscle (Ellis et *al*. 1988; Gong et *al*. 2001). Ca$_V$α2δ2 and Ca$_V$α2δ3 are more abundant in the central nervous system (Barclay and Rees, 2000; Hobom et *al*. 2000), often associated with GABA-mediated neurons (Cole et *al*. 2005). Ca$_V$α2δ4 is expressed in the retina (Wycisk et *al*. 2006) and endocrine tissues (Qin et *al*. 2002). Ca$_V$α2δ1 variants (Kim et *al*. 1992; Angelotti and Hofmann, 1996) are associated with LTCC in the brain (Cole et *al*. 2005), endocrine cells (Mastrolia et *al*. 2017), skeletal (Angelotti and Hofmann, 1996), smooth (Bannister et *al*. 2012; Zhang et *al*. 2018) and cardiac muscle (Hatano et *al*. 2006; Tuluc et *al*., 2007), but not with T-type Ca^{2+} channels (Zhao et *al*. 2019). Ca$_V$α2δ2 and Ca$_V$α2δ3 form oligomeric protein complexes with P/Q-type (Nimmrich and Gross, 2012), N-type (Sutton et *al*. 2002), and LTCC in the brain (Striessnig et *al*. 2014). Both Ca$_V$α2δ1 and Ca$_V$α2δ2 promote larger currents of Ca$_V$1.2 (Fuller-Bicer et *al*. 2009; Bourdin et *al*. 2010; Singer et *al*. 1991; Felix et *al*. 1997; Parent et *al*. 1997), but Ca$_V$α2δ1 is the predominant isoform in cardiomyocytes (Gong et *al*. 2001; Fuller-Bicer et *al*. 2009) with more abundant mRNA expression in atrium than ventricle (Hatano et *al*. 2006).

N-linked glycosylation of Ca$_V$α2δ1 is essential for its expression

All Ca$_V$α2δ isoforms share a similar folding. Primary sequence homology is higher between Ca$_V$α2δ1 and Ca$_V$α2δ2 (69%) and Ca$_V$α2δ3 and Ca$_V$α2δ4 (78%) (Whittaker and Hynes, 2002) with less than 50% homology between two subgroups with predicted molecular weights varying from 125 to 128 kDa. The N-terminal of the 150-kDa Ca$_V$α2δ subunit is extracellular in

agreement with the presence of the signal sequence (Wolf et al. 2003). $Ca_V\alpha2$ is hovering over the pore-forming $Ca_V\alpha1$ subunit. This was shown in the low- (27Å) (Canti et *al*. 2005) and high- (3.6Å) (Wu et al. 2016) cryo-electron microscopy structure of the homologous skeletal muscle LTCC $Ca_V1.1$ complex. The mature extracellular native $Ca_V\alpha2\delta$ protein is highly glycosylated, adding up to 50 kDa of complex branched sugars from 16 predicted Asparagine residues (Jay et *al*. 1991; Marais et *al*. 2001; Tétreault et *al*. 2016). The substitution of each of the Asparagine by Alanine residues progressively reduced the cell surface expression of $Ca_V\alpha2$, thus indirectly impairing the upregulation of LTCC (Tétreault et *al*. 2016). Cycloheximide chase assays demonstrated that preventing glycosylation promotes faster degradation and decreased protein stability. Glycosylation of Asparagine-663 (rat numbering) is most critical for protein integrity (Tétreault et *al*. 2016). Glycosylation at this position may bridge two structural CACHE domains within $Ca_V\alpha2$ (Tétreault et *al*. 2016; Bourdin et *al*. 2017; Briot et *al*. 2018).

$Ca_V\alpha2\delta1$ is loosely tethered at the membrane

$Ca_V\alpha2\delta1$ incubated in harsh reducing conditions (such as 100 mM DTT) produced a migration pattern with two bands of ≈ 150-kDa (Jay et *al*. 1991; Kadurin et *al*. 2012; Andrade et *al*. 2007; Riveraa et *al*. 2012) and ≈ 25-kDa (Jay et *al*. 1991; Kadurin et *al*. 2012) in SDS-PAGE gels. From these early observations, it was suggested that $Ca_V\alpha2\delta1$ was inserted in the membrane as a multi-disulfide linked complex formed by a large extracellular ($Ca_V\alpha2$) and a smaller single transmembrane $Ca_V\delta$ protein (Andrade et *al*. 2007; Riveraa et *al*. 2012). With more than 20 cysteine residues, the precise location of the cleavage site between $Ca_V\alpha2$ and $Ca_V\delta2$ has yet to be identified (Riveraa et *al*. 2012; Zhao et *al*. 2019). $Ca_V\alpha2\delta1$ is also proteolytical cleaved at a glycosylphosphatidylinositol (GPI)-anchoring ω-site (Eisenhaber et *al*. 1998; Segura et *al*. 2017), which is found at positions Gly-1060 and Gly-1061 (rat isoform) (Gly-1075 and Gly-1076 in rabbit). Cleavage at the di-glycine site produces a small 4-kDa fragment that is compatible with $Ca_V\delta$ losing its hydrophobic domain (Segura et *al*. 2017). Substitution of these two glycine residues prevents the release of the transmembrane domain, decreases the cell surface expression of the extracellular $Ca_V\alpha2$ fragment, loosens protein interaction with the $Ca_V\alpha1$ subunit, and impairs LTCC function in HEK-293 cells and rat cardiomyocytes (Segura et *al*. 2017). As other GPI-anchored proteins, $Ca_V\alpha2\delta1$ can partition into lipid rafts (Davies et *al*. 2010). The increased

mobility of Cavα2δ1 was reported with live single-molecule tracking, hence supporting assembly and disassembly of Cavα2δ1 with the Cavα1/CaVβ complex and within the plasma membrane.

Cavα2δ1: a staunch contributor of LTCC function

Cavα2δ1 enhances the function of LTCC by improving channel activation at physiological voltages and increasing by 5 to 10-fold its peak current density (Bourdin et al. 2017). The Cavα2δ1-induced increase in peak current density results from the relative combination of multiple mechanisms: 1) channel activation at more physiological potentials (Tétreault et *al*. 2016; Segura et *al*. 2017; Mastrolia et *al*. 2017; Yasuda et *al*. 2004); 2) increased cell surface expression (Canti et *al*. 2005; Rosa et *al*. 2018); and 3) stronger stability of the LTCC protein complex (Shakeri et *al*. 2012). We surmise that the relative importance of each mechanism is family- and isoform-dependent (Hobom et *al*. 2000; Shakeri et *al*. 2012). Point in case, Cavα2δ1 possesses a tighter interaction with Cav1.2 than with Cav2.2 and Cav2.1 (Voigt et *al.* 2016) furthering the notion that Cavα2δ1 modulation could be family-dependent.

The structural complexity of Cavα2δ1: a cryo-EM analysis

Upregulation of Cav1.2 channel activity requires direct protein interaction between the extracellular Cavα2 and the pore-forming Cavα1C subunit (Segura et *al*. 2017; Bourdin et *al*. 2017; Briot et *al*. 2018). The high-resolution cryo-EM structures (3.6 Å) of the skeletal muscle Cav1.1 channel complex (Wu et *al*. 2016) provided the best structural investigation of the LTCC complex, and by extension, Cavα2δ1. The Von Willebrand factor domain (VWA from 251-443) (Canti et *al*. 2005) sits directly above the Cavα1 protein. The VWA domain contains a metal-ion adhesion (MIDAS) motif (Asp-259, Ser-261, Ser-263, Thr-331, and Asp-363) that is folded by the coordination of a divalent cation, Ca^{2+} or Mg^{2+}, and is known to promote protein interaction in bacterial systems (Whittaker and Hynes, 2002). The VWA primary sequence homology between human Cavα2δ subunits are respectively 87%, 61%, and 60% for Cavα2δ2, Cavα2δ3, and Cavα2δ4 compared with Cavα2δ1. Four other structural domains Cache1 to Cache4 are suitably located to anchor an extracellular networking hub for LTCC (Briot et *al*. 2016). The lack of electronic density below Cys-1047 in CaVδ is compatible with our data about the GPI-anchoring of the cleaved C terminus at position Gly-1060 in Cav1.2 (Segura et *al*. 2017). Rather than the single disulfide bond postulated between Cys-404 and Cys-1047, multiple intra- and inter- disulfide bonds were

identified through LC-MS/MS analysis (Wu et *al*. 2016), which could explain the variability in the reported molecular mass for $Ca_V\delta$.

Identifying the interaction site of $Ca_V\alpha2\delta1$ with $Ca_V\alpha1C$

The cryo-EM structure proposes multiple contact areas between the Cache1, VWA, and Cache2 domains of $Ca_V\alpha2\delta1$ and the extracellular loops of repeat I and III of $Ca_V\alpha1$ (Wu et *al*. 2016; Wu et *al*. 2015; Briot et *al*. 2017). We developed a multi-pronged approach combining fluorescent detection of proteins at the cell surface using flow cytometry assays, co-immunoprecipitation assays of full-length proteins, functional patch-clamp measurements and molecular dynamics simulations of 3-D computer models of the channel complex to investigate this network of molecular interactions (Bourdin et *al*. 2017). The insertion of residues in the first extracellular loop (IS1S2) of $Ca_V1.2$ prevented the co-immunoprecipitation of $Ca_V1.2$ with $Ca_V\alpha2\delta1$. Pull-down assays identified Asp-181 in the extracellular loop of $Ca_V1.2$ as the anchor between $Ca_V\alpha1C$ and $Ca_V\alpha2\delta1$. The interaction of $Ca_V\alpha2\delta1$with the voltage-sensor domain of $Ca_V\alpha1C$, where IS1S2 is located, was already predicted to play an important role in the upregulation of LTCC by $Ca_V\alpha2\delta1$ (Savalli et *al*. 2016). It remains to be seen, nonetheless, whether this molecular mechanism can be transposed to other voltage-gated calcium channels, although we know that the "Pro-Glu-Asp-Asp" locus is conserved in the extracellular IS1-S2 loop of $Ca_V\alpha1$ subunits from the Ca_V2 family channel.

Defining the structural and interaction domains of $Ca_V\alpha2\delta1$

The interaction site of $Ca_V\alpha1C$ onto $Ca_V\alpha2\delta1$ was investigated using the same approach. Although substitutions of Asp-259 of $Ca_V\alpha2\delta1$ prevented channel modulation, this site is predicted by the three-dimensional structure to be inaccessible for extracellular residues of $Ca_V\alpha1C$. Nonetheless, manipulations of this site proved that substitutions at position Asp-259 negatively affected protein stability, and flow cytometry experiments showed a sharp decrease in cell surface expression. Using molecular dynamics simulations, we concluded that Asp-259 plays a crucial role in coordinating the binding of a single Ca^{2+} ion within MIDAS. Folding of MIDAS around a divalent cation has been documented in many systems and probably occurs in the endoplasmic reticulum (Briot et *al*. 2018). It plays a crucial role in providing protein stability. In addition, the virtual simulations concur that the side-chain hydrogen atoms of $Ca_V\alpha2\delta1$ Ser-261 and Ser-263 interact

with the oxygen atoms of Cav1.2 Asp-181. Altogether, Asp-259 of Cavα2δ1 initiates the protein folding around the Ca^{2+} ion, which stabilizes the conformation of VWA domain that secures Cavα2δ1 Ser-261 and Ser-263 within atomic distance with Cavα1C Asp-181 (Briot et *al*. 2018). Furthermore, this three-way inter-molecular network is responsible for the functional modulation of Cav1.2 by the auxiliary subunit Cavα2δ1 (Briot et *al*. 2018). It has been reported elsewhere that mutations within the MIDAS motif abolish Cavα2δ1 and Cavα2δ2 modulation of Cav1 and Cav2 channels (Canti et *al*. 2005; Hoppa et *al*. 2012), but we believe that this effect is caused by preventing proper protein folding of Cavα2δ1 (Bourdin et *al*. 2015).

Manipulating Cavα2δ1 expression in animal models

Cell-surface localization of Cavα2δ1 is required for the upregulation of LTCC in myocytes. Homozygous knockout of the CACNA2D1 gene in mice is not embryonically lethal (Fuller-Bicer et *al*. 2009). Although the hearts appear morphologically normal, myocardial contractility was significantly impaired. These mice showed no obvious sign of cardiac hypertrophy, indicating that sustained smaller Ca^{2+} influx through LTCC does not provoke cardiac remodeling. The inactivation of the gene caused a -10-mV depolarizing shift in the activation potential and a ≈ 45% decrease in the LTCC peak current density (Fuller-Bicer et al. 2009).

Cell surface expression of Cavα2δ1 is a strong determinant of peak current density (Bourdin et *al*. 2015). This feature endows Cavα2δ1 with the unique property of fine-tuning LTCC function in physiological and pathological settings. The significant contribution of Cavα2δ1 to cardiac contractility (Fuller-Bicer et *al*. 2009) prompts interest as to whether changes in Cavα2δ1 expression trigger cardiac hypertrophy and heart failure, given its large impact on LTCC currents (Fuller-Bicer et *al*. 2009; Bourdin et *al*. 2010; Singer et *al*. 1991). In fact, Cavα2δ1 was shown to be upregulated upon activation of TGF-β/Smad-NFkB cascade in neuroblastoma cells (Martínez-Hernández et al. 2013), a signaling pathway that is also induced during pressure-overload cardiac hypertrophy (Schluter et *al*. 1995; Azakie et *al*. 2006; Wang et *al*. 2018). Thus, it is possible that Cavα2δ1 protein expression might be responsible for changes in LTCC peak current density in models of cardiac hypertrophy.

Cavβ is a small cytoplasmic auxiliary subunit

Cavβ2 is the major isoform in the adult heart

Four distinct CACNB genes encode Cavβ subunits, giving rise to Cavβ1 - Cavβ4 isoforms (Buraei and Yang, 2010; Link et *al*. 2009), some of which are subjected to alternatively splicing (Birnbaumer et *al*. 1998; Foell et *al*. 2004). Cavβ1 and Cavβ3 were reported in human and canine ventricles (Foell et *al*. 2004; Hullin et *al*. 2003), and we have detected protein expression of Cavβ3 in neonatal rat ventricular cardiomyocytes (unpublished data). Cavβ2 was the first isoform identified in rat and rabbit hearts (Perez-reyes et *al*. 1992; Hullin et al. 1992) and is considered the typical Cavβ found in cardiomyocytes. Alternative splicing of human Cavβ2 generates five variants Cavβ2a - Cavβ2e (Buraei and Yang, 2010; Link et *al*. 2009), whose expression varies with cardiac developmental stages. Cavβ2a shares a high level of sequence homology (63.9%) with Cavβ2b (Zhou et *al*. 2008). Cavβ2a differs from Cavβ2b by alternative splicing at exon 5 encoding two N-terminal cysteines accounting for the palmitoylation site, as well as at exon 7 that encodes for the central variable region of Cavβ2 (Foell et *al*. 2004). Cavβ2a and Cavβ2b predominate in adult hearts (Colecraft et *al*. 2002), whereas Cavβ2c-e are more prominent at the earlier stage of development (Chen et *al*. 2002). In vivo, complete disruption of the Cavβ2 gene elicited cardiac impairment manifested by pericardial effusion and abnormal heart tubes, resulting in embryonic lethality (Weissgerber et *al*. 2006; Striessnig and Koschak 2008) that can be rescued by cardiac-specific expression of Cavβ2 (Ball et *al*. 2002).

Cavβ is a MAGUK protein

Cavβ subunit belongs to the family of scaffold proteins known as membrane-associated guanylate kinase (MAGUK) family (Buraei and Yang 2010; Fang and Colecraft, 2011; Oliva et *al*. 2011). MAGUK are characterized by the arrangement of Src homology 3 domain (SH3) and guanylate kinase (GK) homologous domains, which are generally folded in the following fashion in mammalian Cavβ subunits: N-terminus-SH3-HOOK-GK-C-terminus (Colecraft et *al*. 2002; Opatowsky et *al*. 2003) with heterogeneity in the C-terminus, the N-terminus, and the HOOK region (Opatowsky et al. 2003). The GK-like domain is known to be catalytically inactive as the P-loop, which binds ATP, is absent. MAGUK proteins are ubiquitous and promote protein-protein interactions with cytoskeleton proteins, microtubule/actin-based machinery, and molecules involved in signal transduction. The SH3 domain binds non-calcium channel proteins and could play a role in the non-canonical function of Cavβ (Van Petegem et *al*. 2004). The GK-like domain

confers the interaction with Cavα1 (Buraei and Yang, 2010; Chen et *al*. 2004; Gonzalez-Gutierrez et *al*. 2007; Sparks et *al*. 1996; Berrou et *al*. 2002; Berrou et *al*. 2005). High-affinity interaction between Cavβ and Cavα1C (De Waard et *al*. 1995; Pragnell et *al*. 1994; Derrick et *al*. 1995) ranges between Kd = 2-54 nM (Buraei and Yang, 2010; Hidalgo et *al*. 2006). Three high-resolution crystal structures revealed that Cavβ binds to Cavα1C-interacting domain (AID domain), a highly conserved 18 amino acid sequence in the I-II cytoplasmic loop of Cavα1C, via a hydrophobic cleft residing in its GK-like domain (Shakeri et *al*. 2012; Buraei and Yang, 2010; Van Petegem et *al*. 2004; De Waard et *al*. 1995; Chen et *al*. 2004; Opatowsky et *al*. 2004). We have shown that the MMQKAL motif of Cavβ is required for the high-affinity interaction to AID (Bourdin et *al*. 2010). Trp-440 and Ile-441 in the AID of the Cavα1C of Cav1.2 account for the Cavβ -Cavα1 high-affinity interaction (Van Petegem et *al*. 2008). Opposing surfaces of the GK-like domain remain available to interact with signaling proteins, such as the Rad/Rem/Rem2/Gem/Kir (RGK) family of Ras-like GTPases (Finlin et *al*. 2006; Fan et *al*. 2010). Rad and Rem are the most abundant RGK proteins in the heart (Wang et *al*. 2010; Reynet and Kahn, 1993). Although being a family member of small GTPases, most studies concur that RGK proteins per se do not possess GTPase activity (Sasson et *al*. 2011; Yang and Colecraft, 2013). RGK proteins have however emerged as potent inhibitors of LTCC via direct interaction with Cavβ (Béguin et *al*. 2001). Co-expressing either Rem, Gem, or Rad with CaVα1C and Cavβ2a in recombinant cells suppressed LTCC currents, thus identifying RGKs as negative regulators of LTCC channel function (Finlin et *al*. 2003; Finlin et *al*. 2005).

Cavβ promotes the expression of Cavα1C in recombinant cells

The cytoplasmic Cavβ auxiliary subunit is canonically known to regulate the cell surface trafficking and biophysical gating properties of Cavα1C (Yeon et al. 2018; Dalton et al. 2005; Buraei and Yang, 2010). In recombinant mammalian cells, the expression of Cavα1C alone produces perinuclear staining of Cav1.2, whereas its co-expression with Cavβ2 promotes the cell surface localization of Cavα1C (Gao et *al*. 1999). Such a large increase in Cav1.2 trafficking could not be reproduced by expressing or over-expressing calmodulin or CaV□2δ1 (Bourdin et al. 2010). Cavβ requirement was further supported by adenovirus-based studies showing two and three-fold increase of LTCC currents density along with increased voltage-dependent inactivation kinetics in cultured young adult ventricular myocytes overexpressing recombinant Cavβ2a or Cavβ3 (Wei et

al. 2000). In adult cardiomyocytes, overexpression of exogenous $Ca_V\beta$ enhanced LTCC currents two to five-fold, depending on the $Ca_V\beta$ isoform (Colecraft et *al*. 2002; Miriyala et *al*. 2008), by increasing single channel-open probability as well as the number of $Ca_V1.2$ channels at the plasma membrane (Colecraft et *al*. 2002).

Modest upregulation of LTCC by $Ca_V\beta$ in animal models

In contrast to the findings in recombinant systems, several studies exploiting transgenic mice have challenged $Ca_V\beta$'s quintessential role in channel trafficking. $Ca_V\beta2$ null mice demonstrated a mere 33% reduction of LTCC currents (Weissgerber et *al*. 2006), and lentiviral-based shRNA knockdown of cardiac-specific $Ca_V\beta2$ gene strongly reduced (57%) but did not abolish LTCC currents in adult mice (Cingolani et *al*. 2007). Tamoxifen-induced cardiomyocyte-specific disruption of the CACNB2 gene in adult mice showed only 29% reduction in LTCC current density without any change of voltage-dependent activation and inactivation kinetics (Meissner et *al*. 2011) while excluding the compensatory upregulation of other $Ca_V\beta$ isoforms (Meissner et *al*. 2011). The most convincing series of observations were reported recently in transgenic mice with cardiac-specific inducible expression of AID-impaired $Ca_V\alpha1C$ subunit. The $Ca_V\alpha1C$ construct substituted the highly conserved tryptophan residue critical for binding $Ca_V\beta$ (Yang et al. 2019) and was engineered to eliminate the sensitivity to the inhibition by dihydropyridine (DHP) compounds. Although biochemical assays verified that AID-impaired $Ca_V\alpha1C$ is not capable of binding $Ca_V\beta$ in transgenic mice, confocal imaging revealed that $Ca_V\alpha1C$ was localized at the plasma membrane of adult cardiomyocytes isolated from transgenic mice. Additionally, LTCC peak current density as well as activation and inactivation kinetics of the AID-impaired DHP-insensitive $Ca_V\alpha1C$ construct were similar to endogenous LTCC (Yang et *al*. 2019), indicating that AID-impaired $Ca_V1.2$ channels were still capable of trafficking to the plasma membrane and maintaining their function in vivo (Yang et al. 2019). While it is possible that the slower turnover of LTCC in adult cardiomyocytes accounts in part for these observations, these data are shaking up the long-held dogmatic role of $Ca_V\beta$. It turns out that the binding of $Ca_V\beta$ to AID is critical for β-adrenergic-mediated regulation of $Ca_V1.2$ currents (Yang et *al*. 2019). AID-impaired $Ca_V\alpha1C$ constructs in mice are insensitive to isoproterenol stimulation (Yang et *al*. 2019), indicating that $Ca_V\beta$ mediates β-adrenergic upregulation of LTCC currents in ventricular cardiomyocytes (Gerhardstein et *al*. 1999). Marx and colleagues have further addressed the underlying mechanism

(Liu et *al*., 2020). Cavβ2 overexpression alone failed to promote the PKA-upregulation of LTCC and was found to require co-expression of Rad to carry out the process in a recombinant system (Liu et *al*. 2020). The association of unphosphorylated Rad to Cavβ maintains LTCC at a low functioning basal state. Upon β-adrenergic stimulation, PKA-mediated phosphorylation of Rad induces its dissociation from Cavβ, thus promoting LTCC to a higher functioning level (Liu et *al*. 2020). This indicates that Cavβ can serve as a hub of interacting proteins, hence contributing to cardiac function modulation in many different and complementary roles.

Clinical perspectives

Dysfunction in calcium handling causes cardiac diseases

Alteration in β-adrenergic regulation is a major hallmark of heart failure. Excessive catecholamine activation in chronic hypertension results in intracellular Ca^{2+} overload, leading to cardiac hypertrophy to normalize wall stress and maintain tissue perfusion (Fiedler and Wollert, 2004; Calderone, 2014). Over time, the imposed chronic hemodynamic stress induces pathological cardiac hypertrophy (Calderone, 2014). When left untreated, pathological hypertrophy results in a decreased β-adrenergic response that ultimately impairs sarcoplasmic reticulum-mediated Ca^{2+} uptake and release, compromising excitation-contraction coupling (Gwathmey et *al*. 1987; Hajjar et *al*. 1998; Port and Bristow, 2001; Chen et *al*. 2002). The consequent systolic and diastolic contractile dysfunctions set the stage for heart failure with a maladaptive ability to improve cardiac pumping function and maintain blood flow (Oka and Komuro, 2008). β1 agonists are sometimes used to treat acute decompensated heart failure in an effort to maintain hemodynamic stability; however, they are rarely used as a long-term treatment as they may also provoke ventricular arrhythmias or promote the development of hypokalemia (Colucci et *al*. 1986; Tariq and Aronow, 2015). Whereas treatment with β-receptor antagonists is known to improve left ventricular systolic performance and reduce mortality in chronic heart failure (Filippo, 2007), long-term use may lead to worsening heart failure by interfering with the positive inotropic effects of endogenous catecholamines (Filippo, 2007). This led cardiac physiologists to explore the role of the auxiliary subunits of LTCCs as molecular targets in heart failure.

Cavβ contributes to cardiac hypertrophy in animal models

Failing human myocardium displayed a small 20% upregulation of $Ca_V\beta2$ protein expression without an associated change in the protein expression of $Ca_V\alpha1C$ or $Ca_V\alpha2\delta1$ subunits (Hullin et al. 2007), but it is unclear if this upregulation altered LTCC function (Hullin et *al.* 2007). The upregulation of $Ca_V\beta$ in heart failure was also observed in studies exploiting transgenic animal models. For example, protein expression of $Ca_V\beta2$ and $Ca_V\beta3$ was upregulated four to five-fold in a transgenic mouse model overexpressing $Ca_V\alpha1C$ but only when the transgenic mice entered the heart failure stage at 9 months (Hullin et *al.* 2007). Moreover, Nakayama et al. investigated the longitudinal effects of cardiac-specific overexpression of $Ca_V\beta2$. Results showed that the mice overexpressing $Ca_V\beta2$ developed cardiac hypertrophy as early as 4 months of age and heart failure by 6-12 months of age (Nakayama et *al.* 2007). These hypertrophied cardiomyocytes exhibited a 70% increase in LTCC currents that were reversed upon treatment with verapamil, a calcium channel blocker, implying that $Ca_V\beta$ is crucial for mediating cardiac hypertrophy (Nakayama et *al.* 2007). In a different study using a similar mouse model, the cAMP analog, 8-Br-cAMP, failed to enhance LTCC currents in transgenic mice with cardiac-specific $Ca_V\beta2$ overexpression, indicating that LTCC are maximally activated in these mice (Beetz et *al.* 2009). Whereas overexpression promotes hypertrophy, downregulation of $Ca_V\beta$ by lentiviral-based shRNA attenuates the development of cardiac hypertrophy in an aortic banding model (Cingolani et *al.* 2007). $Ca_V\beta2$ knockdown also reduced cardiomyocyte size and protein synthesis in a phenylephrine-induced neonatal rat cardiomyocytes model (Cingolani et *al.* 2007). However, only a partial reduction of LTCC currents was observed in these $Ca_V\beta2$-deficient mice (Cingolani et *al.* 2007), indicating that $Ca_V\beta$ might regulate cardiac function without altering Ca^{2+} influx. Such a non-canonical function of $Ca_V\beta$ was deciphered in a study showing that $Ca_V\beta2b$, but not $Ca_V\alpha2\delta1$, mediates $Ca_V1.2$-induced nuclear signaling and gene transcription independently of Ca^{2+} in recombinant cells (Servili et *al.* 2018). Altogether these data endorse $Ca_V\beta$ as a go-between for regulating cardiac function via canonical and non-canonical paradigms.

$Ca_V\beta$ is essential for β-adrenergic mediated regulation of LTCC

β-adrenergic regulation of LTCC and the subsequent acute modifications of the Ca^{2+} cycling confers the rapid changes in cardiac output during fight or flight response. Thanks to the comprehensive work by the group of Steven Marx (Liu et *al*. 2020), we currently know that acute β-adrenergic stimulation relieves the constitutive inhibition of LTCC by monomeric G Rad

proteins (Liu et *al.* 2020). However, long-term hemodynamic stress results in Ca^{2+} handling alterations that elicit cardiac hypertrophy and heart failure. Both increased as well as decreased LTCC activity predisposes for heart failure (Mukherjee et *al.* 1995; Mukherjee et al. 1998; Briston et *al.* 2011; Chen et *al.* 2002; Cingolani et *al.* 2007; Hullin et *al.* 2007; Nakayama et *al.* 2007). LTCC currents result from the concerted contribution of the auxiliary subunits that fine-tune LTCC's activity and expression. Transgenic animal models overexpressing or downregulating the auxiliary subunits permitted scrutinizing their pathophysiological roles on LTCC current and their prominence on cardiac function. The most steadfast contributor for LTCC currents is the $Ca_V\alpha2\delta1$-subunit, but few studies have carefully investigated the changes of $Ca_V\alpha2\delta1$ in cardiac hypertrophy and heart failure. Time-dependent remodeling of $Ca_V\alpha2\delta1$ could account for the changes in LTCC peak current density without significant alteration in the protein expression of its pore-forming subunit during transitioning to heart failure.

Could $Ca_V\beta$ be a clinical target for heart failure?

$Ca_V\beta$ appears to be critical for β-adrenergic mediated regulation of LTCC currents. The identification of Rad as an essential link between adrenergic stimulation and LTCC peak current density sparks interest as to whether $Ca_V\beta$-RGK interaction can stand out as a clinically promising target for heart failure (Colecraft, 2020). β-adrenergic receptors antagonists are currently used to improve survival in patients with chronic heart failure by reducing heart rate, contractility, and wall tension, ultimately preserving cardiac output and myocardial performance (Ruffolo et *al.* 1997; Lombardi and Gilbert, 2001). *In vivo*, norepinephrine and epinephrine stimulate both β1 and β2-adrenergic mediated pathways (Lymperopoulos et *al*. 2013; Cros and Brette, 2013), with the former resulting in the upregulation of LTCC current density through the recently demonstrated phosphorylation of Rad (Liu et *al.* 2020; Roybal et al. 2020). However, sustained activation, often observed in hypertension-driven cardiac remodeling, provokes desensitization of β1 adrenoreceptors (with little change in the density of β2 receptors) through the biased activation of the β-arrestin and PI3K/AKT pathways (Patel et *al*. 2008; Perino et al. 2011). Moreover, the PI3K/AKT signaling pathway could directly inhibit LTCC currents, thus offsetting the PKA-mediated positive inotropic effect (Leblais et *al*. 2004). Clinically, the third generation β-antagonist, carvedilol, was shown to exert its cardioprotective effects via a biased β-arrestin pathway (Walker et *al*. 2011; Violin et *al*. 2013; Woo et *al*. 2015; Wang et *al.* 2018; Carr et *al.*

2016). Therefore, biased β-adrenergic signaling could account for the confounding observations on the role and time-dependent changes in LTCC function reported in different stages of heart failure.

Conclusion

Further research is warranted to investigate the combinatorial changes of Cavα1C and its auxiliary subunits in cardiac hypertrophy and heart failure. Despite the clinical efforts in dealing with LTCC as a therapeutic target for cardiac diseases, treating cardiac hypertrophy by specifically targeting the modulation of the cardiac LTCC auxiliary subunits could open up novel perspectives, given their major impact on LTCC function and kinetics. Elucidating the auxiliary subunits will not only help us understand the functional changes of LTCC but also provide new targets to set out a novel generation of targeted therapies combatting cardiac hypertrophy and heart failure.

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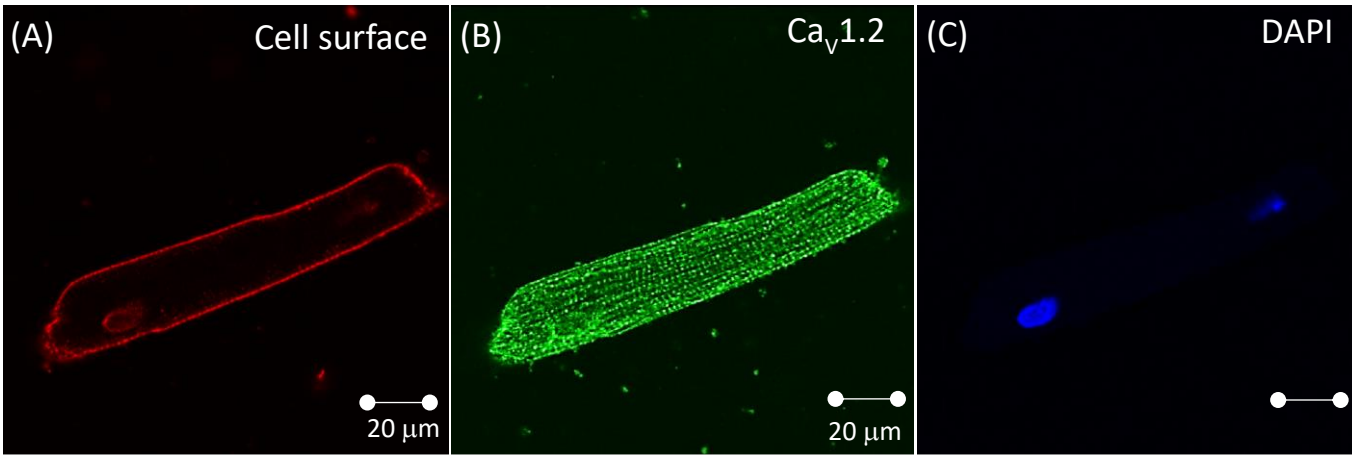

Figure 1: Expression of $Ca_V1.2$ in adult rat cardiomyocytes**.** Cardiac muscle consists of interlacing bundles of cardiomyocytes (cardiac muscle cells). Cardiomyocytes are striated due to the arrangement of actin and myosin filaments that extend from end to end of each cardiomyocyte. Cardiomyocytes are rectangular being about 100 μm long and 25 μm in diameter. Cardiomyocytes are often branched and contain many mitochondria, which provide the energy required for contraction. Cardiomyocytes isolated from adult rat heart were labeled with fluorescent antibodies to identify the cell surface membrane (in red) and the nucleus (in blue). $Ca_V1.2$ is abundant in T-tubules, which are invaginations of the sarcolemma, extending into the interior of the muscle fiber as the sarcotubular system. (A) Wheat germ agglutinin (WGA)-Alexa 647 in red for plasma membrane staining, (B) $Ca_V1.2$ is shown in green, and (C) DAPI for nucleus staining. Images were captured with a confocal microscope with 63X objective. Scale bar corresponds to 20 μm.

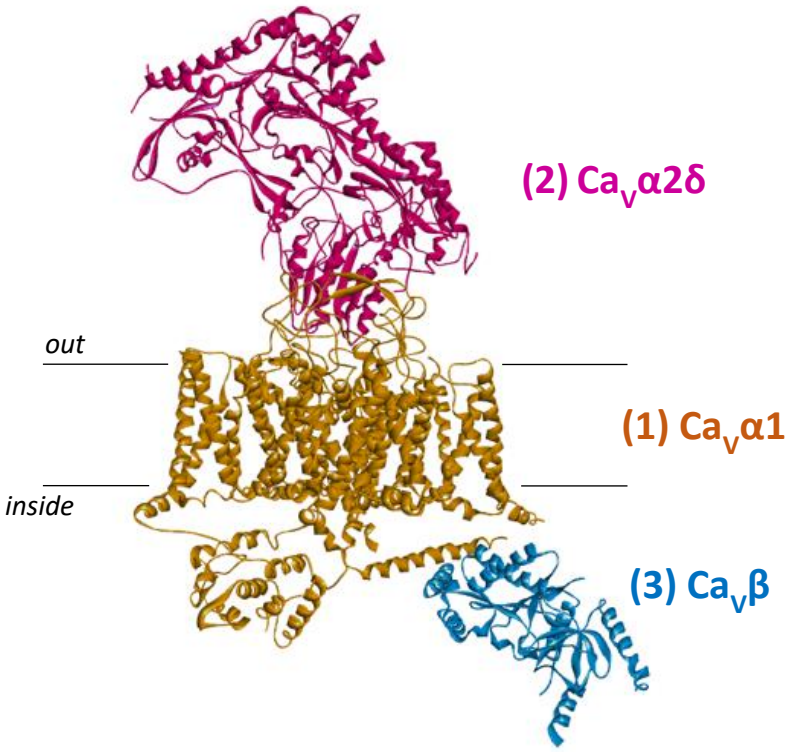

Figure 2: Architecture and membrane topology of the $Ca_V1.1$. The figure shows a cartoon model illustrating the Cryo-EM 3D structure of $Ca_V1.1$ oligomeric complex at 3.6 Å (PDB: 5GJV). The cryo-EM reconstruction is constituted by individual rapidly frozen macromolecules which are consistent in conformation, making the positions of atoms superimposable within a few angstroms from copy to copy. This consistency permits information to be merged from images of these particles, ultimately providing the final density map. $Ca_V1.1$ is a member of the LTCCs, mainly expressed in the skeletal muscle. LTCCs share similar structure, being composed of the (1) pore-forming subunit, $Ca_V\alpha 1$, (2) an extracellular auxiliary subunit, $Ca_V\alpha 2\delta 1$, and (3) $Ca_V\beta$, an intracellular subunit bound to $Ca_V\alpha 1$ through the intracellular helix linking repeats I and II of $Ca_V\alpha 1$. In addition, the transmembrane $Ca_V\gamma 1$ subunit (absent in $Ca_V1.2$) has been omitted. The $Ca_V\alpha 1S$ subunit is composed of four homologous domains, each domain comprises six transmembrane domains and the N- and C-termini of $Ca_V1.1$ are facing the intracellular domains. The cryo-EM structure is colored according to different subunits. $Ca_V\alpha 1S$, $Ca_V\beta$, and $Ca_V\alpha 2\delta 1$ subunits are shown in yellow, blue, and pink respectively. $Ca_V\alpha 1C$ and $Ca_V\alpha 1S$ share 81% homology in their primary protein sequence. Transmembrane $Ca_V\alpha 1S$ protein is characterized by the presence of α-helices (shown as yellow ribbons) spanning the membrane. The α-helix structure is dominant in the cytoplasmic $Ca_V\beta$ whereas anti-parallel β-sheets characterize the protein organization of $Ca_V\alpha 2\delta 1$. Image was produced by Discovery Studio 2020 (BIOVIA Pipeline Pilot 2020).

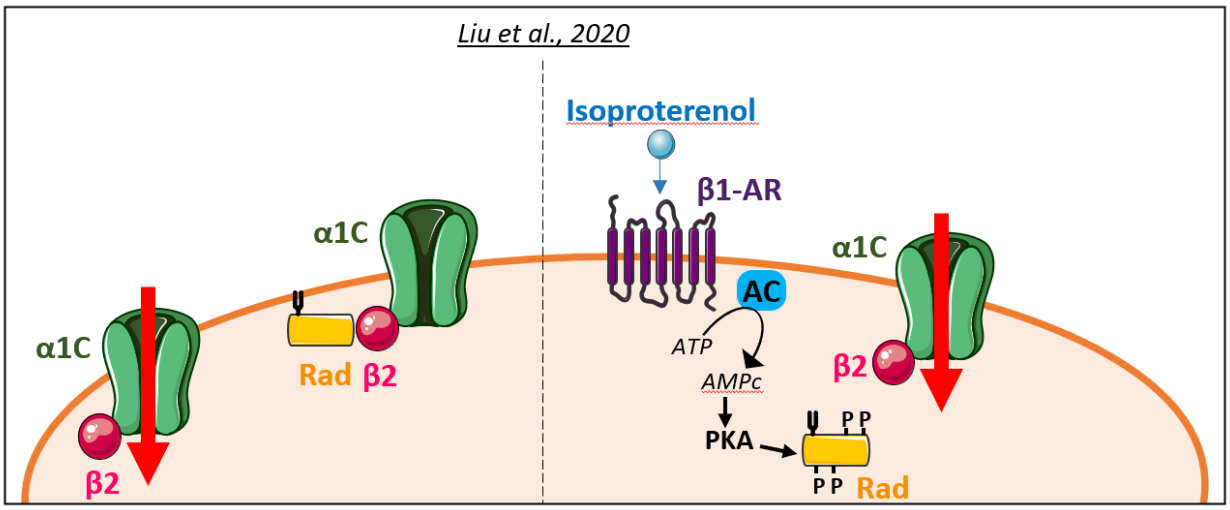

Figure 3: Proposed mechanism of Cav1.2 activation by PKA in cardiomyocytes. **A.** In the basal state (without stimulation), Rad is bound to Cavβ subunit thus limiting calcium influx through the pore-forming subunit Cavα1C of the Cav1.2 channel. **B.** The activation of the β1-adrenergic receptor signaling cascade by ISO (isoproterenol) promotes the dissociation of Rad from the Cavβ subunit. The β1-AR is a G-protein coupled receptor, a family of integral membrane proteins, coupled to the trimeric G-protein identified by Gαs. Upon stimulation by the agonist, Gαs dissociates from the Gβγ subunits to activate the adenylate cyclase enzyme (AC) that promotes the conversion of intracellular ATP to cyclic AMP (cAMP). Protein kinase A (PKA) is activated by the binding of cyclic AMP (cAMP). PKA, in turn, phosphorylates Rad causing its dissociation from Cavβ subunit, ultimately releasing the Rad-induced low activity mode of Cav1.2.

β1-AR: β1-adrenergic receptor; AC: Adenylate cyclase; ATP: Adenosine triphosphate; cAMP: Cyclic adenosine monophosphate; PKA: Protein kinase A; Rad: Ras-related protein associated with diabetes.

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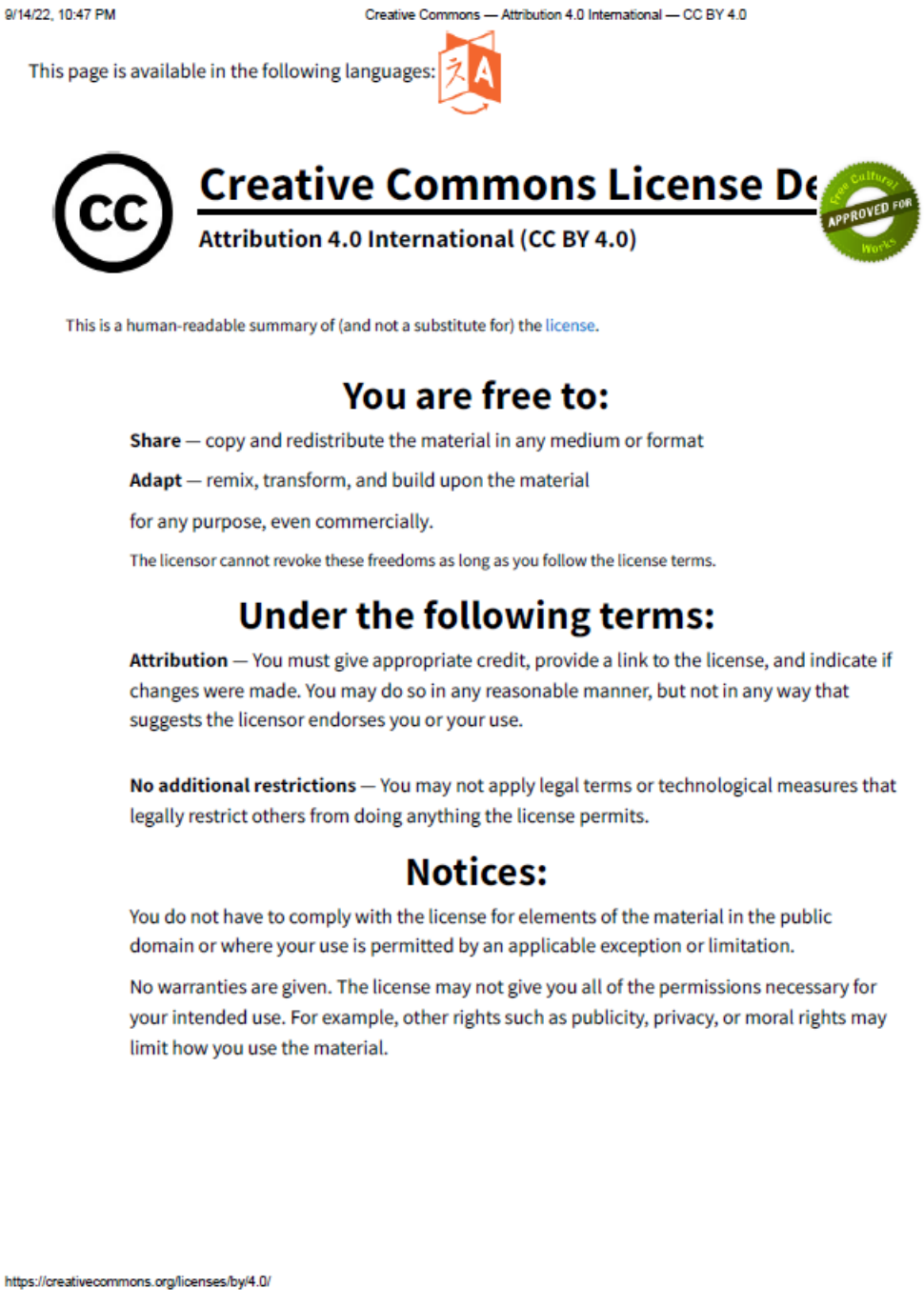

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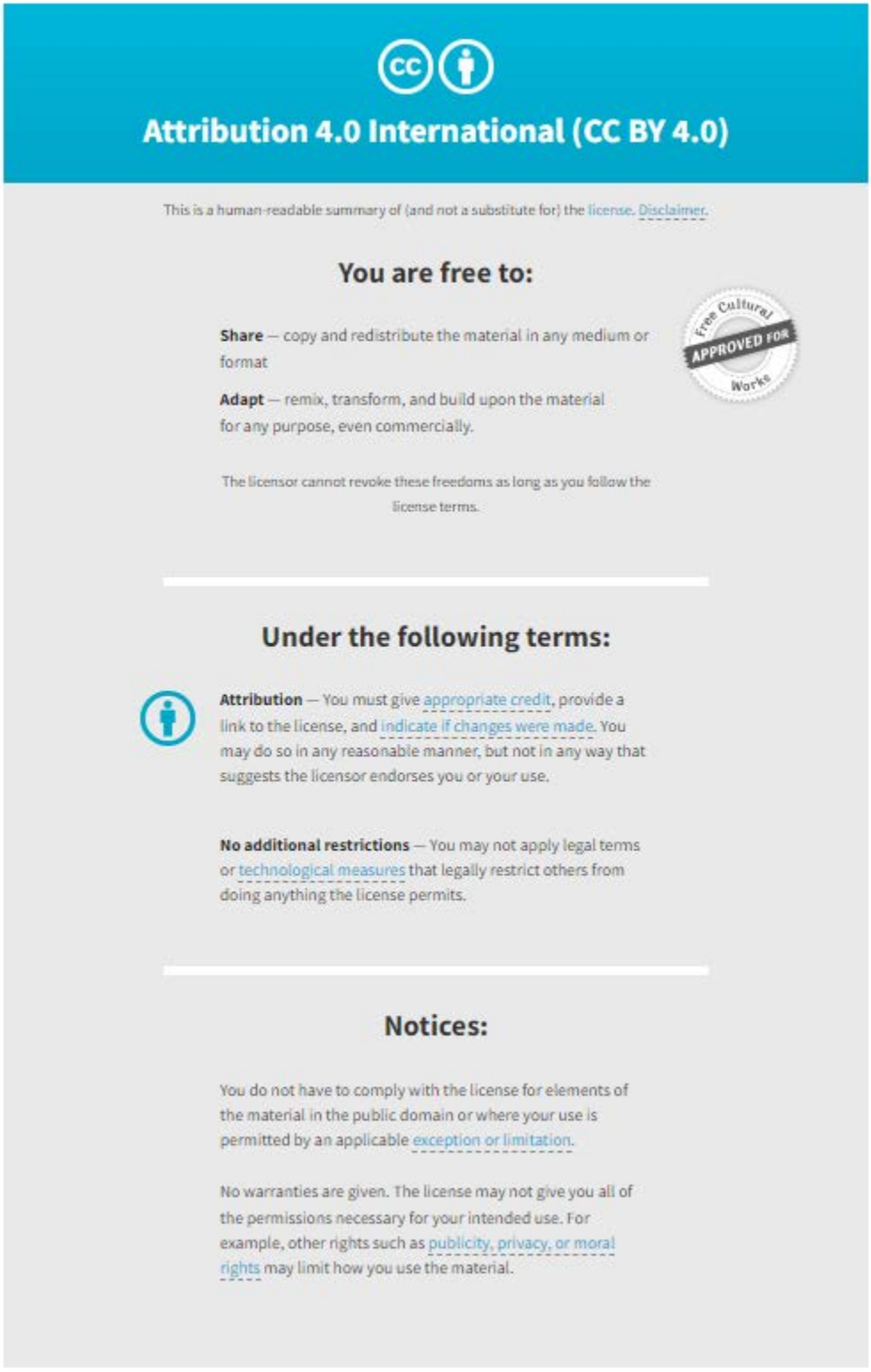

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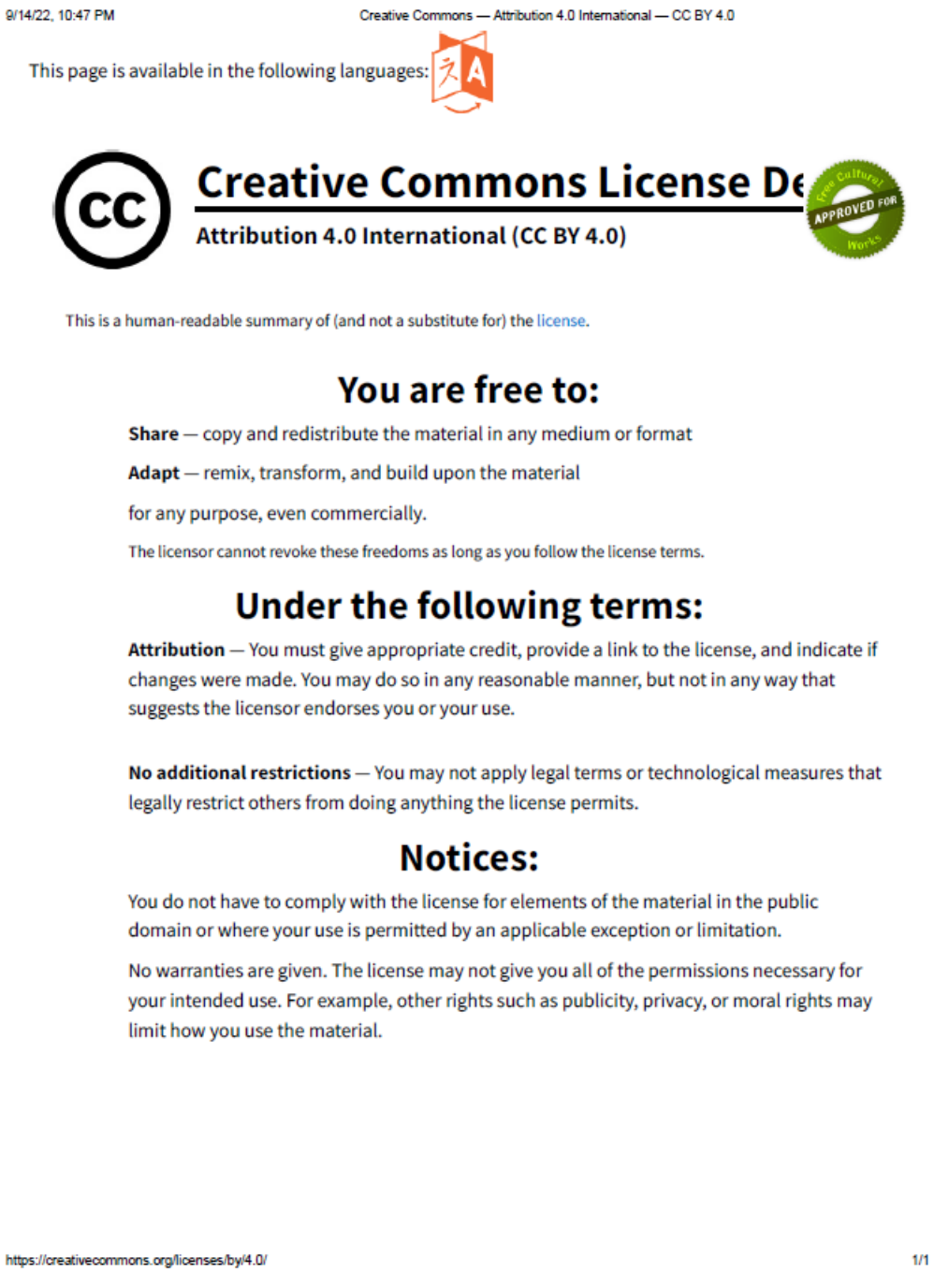

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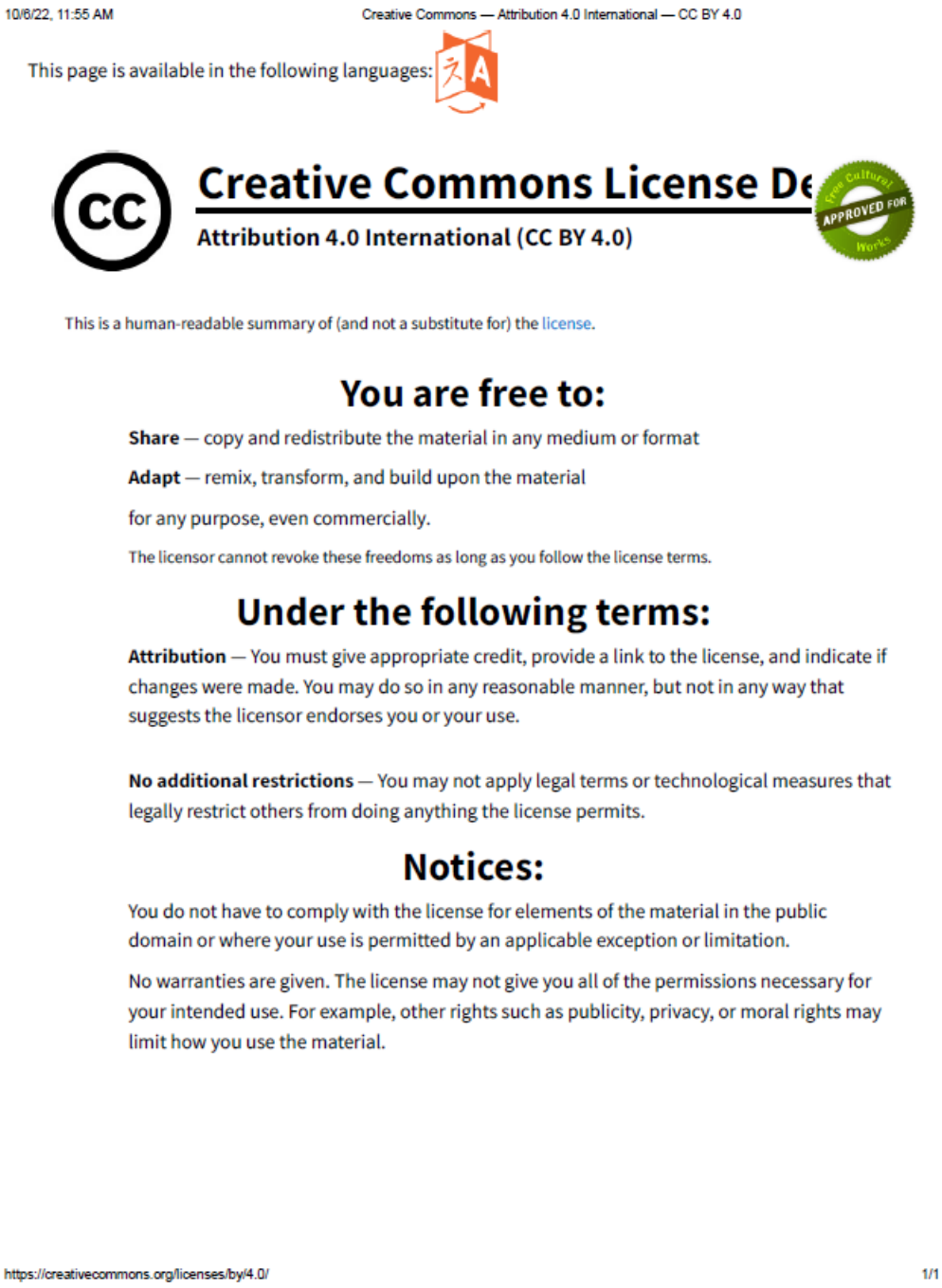

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