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HCO_3^--Dependent Impact of $Na^+,K^+,2Cl^-$ Cotransport in Vascular Smooth Muscle Excitation-Contraction Coupling

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Key Words

Na^+ • K^+ • $2Cl^-$ • Cotransport • Vascular smooth muscle cells • Contraction • Bicarbonate

Abstract

In smooth muscles, inhibition of $Na^+,K^+,2Cl^-$ cotransport (NKCC) by bumetanide decreased intracellular Cl^- content ($[Cl^-]_i$) and suppressed the contractions triggered by diverse stimuli. This study examines whether or not bicarbonate, a regulator of several Cl^- transporters, affects the impact of NKCC in excitation-contraction coupling. Addition of 25 mM $NaHCO_3$ attenuated the inhibitory action of bumetanide on mesenteric artery contractions evoked by 30 mM KCl and phenylephrine (PE) by 5 and 3-fold, respectively. In cultured vascular smooth muscle cells, $NaHCO_3$ almost completely abolished inhibitory actions of bumetanide on transient depolarization and $[Ca^{2+}]_i$ elevation triggered by PE. In bicarbonate-free medium, bumetanide decreased $[Cl^-]_i$ by ~ 15%; this effect was almost totally abrogated by $NaHCO_3$. The addition of $NaHCO_3$ resulted in 2-fold inhibition of NKCC activity and 3-fold attenuation of $[Cl^-]_i$. These data strongly suggest that extracellular HCO_3^- diminishes the NKCC-sensitive component of excitation-contraction coupling via suppression of this carrier.

Introduction

$Na^+,K^+,2Cl^-$ cotransporters (NKCCs) belong to the superfamily of Cl^--coupled carriers that provide electroneutral symport of Na^+, K^+ and Cl^-. Two NKCC isoforms have been cloned from vertebrate cDNA libraries. NKCC1 was found in all cell types studied so far, including vascular smooth muscle cells (VSMC), whereas renal-specific NKCC2 is expressed exclusively in the apical membranes of the thick ascending limb and macula densa [1]. Under baseline conditions ($[Na^+]_o >> [Na^+]_i$ and $[Cl^-]_o^2 >> [Cl^-]_i^2$), NKCC provides inwardly-directed net ion flux and maintenance of intracellular chloride ($[Cl^-]_i$) above values predicted by Nernst equilibrium potential. Importantly, unlike the dominant contribution of K^+ permeability (P_K) to resting membrane potential (E_m) in skeletal and cardiac muscles, P_K and P_{Cl} values in VSMC are similar [2], indicating that NKCC-mediated modulation of $[Cl^-]_i$ in these cells affects the E_m-dependent component of excitation-contraction coupling. Indeed, high-ceiling

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diuretics (HCD), such as furosemide and bumetanide, known to be potent NKCC inhibitors, decreased $[Cl^-]_i$ [3-5], hyperpolarized VSMC [4], attenuated Ca^{2+} influx via voltage-gated L-type channels [5] and reduced baseline tone [6-8] as well as contractions of vascular and non-vascular smooth muscles triggered by diverse stimuli, including K^+_o-induced depolarization [5], phenylephrine (PE) [5, 9-11], histamine [12], angiotensin II [13], thromboxane A_2 [14, 15] and oxytocin [16, 17]. These findings and decreased blood pressure in *NKCC1*$^{-/-}$ knock-out mice [10, 18, 19] led some researchers to propose that augmented NKCC1 activity, revealed in erythrocytes and in VSMC from spontaneously hypertensive rats [20] and rats with DOCA-induced hypertension [21], contributes to the maintenance of high blood pressure via elevation of vascular tone and peripheral resistance [10, 22]. It should be mentioned, however, that the absolute values of HCD-induced decrements of VSMC contraction, documented by different research teams, were drastically different. Thus, we observed that bumetanide almost completely blocked the contractions of rat aortic rings triggered by PE [5], whereas Akar and co-workers reported that it attenuated these responses by only 5-10% [9]. Two hypotheses might explain this controversy. First, at high concentrations, HCD inhibit VSMC contractions by interacting with targets distinct from NKCC. This assumption is consistent with data showing the direct effect of these compounds on thromboxane A_2 receptors [14] and cAMP phosphodiesterase [16, 17]. Second, the discrepancy may be caused by methodological features of the experimental procedures employed in these studies. Thus, for example, HCO_3^--free *vs* HCO_3^--buffered medium was used in our investigation and in experiments performed by Akar and co-workers, respectively. Considering this, we compared the actions of HCO_3^- on NKCC activity, $[Cl^-]_i$ and the bumetanide-sensitive components of contractile responses. Data obtained here disclose that extracellular bicarbonate inhibited NKCC activity in cultured VSMC and sharply attenuated the actions of bumetanide on contractions of mouse mesenteric arteries evoked by modest depolarization and PE.

Methods and Materials

Contraction of mesenteric arteries

Experiments were performed on 2- to 4-month-old male C57B1/6 mice obtained from Charles River Canada (Montreal, PQ). The animals' intestines were removed surgically with the mesenteric beds attached and placed in ice-cold physiologically-balanced salt solution (PSS) containing 120 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose, 50 mM mannitol and 20 mM HEPES-tris (pH 7.4; 37°C). Dissected segments of second- and third-order arteries, 1-2 mm in length and with an intraluminar diameter in the range of 150 to 200 μm, were isolated from the intestinal tract and fat tissues under a dissection microscope (Motic, Richmond, BC). Then, the arteries were transferred to a 15-ml arteriographic chamber cannulated at both ends with micropipettes and mounted in a Motic inverted microscope with a video-monitored perfusion system (Living System Instrumentation Inc., Burlington, VT). The signal derived from this system was processed by a video dimension analyzer for continuous measurement of intraluminal diameter. After 30-min perfusion with PSS under 30 mmHg, the arteries were subjected to modest and full-scale depolarization, caused by equimolar substitution of NaCl with 30 or 80 mM KCl, respectively, or treatment with 10 μM PE. To enrich the medium with HCO_3^- in the range from 5 to 25 mM, 50 mM mannitol was isosmotically substituted with $NaHCO_3$ and pH adjusted at 7.4. For more details, see figure and table legends.

VSMC

The precise measurement of ion fluxes in blood vessels is complicated by a relatively large extracellular space and cellular heterogeneity. On the other hand, cultured VSMC rapidly downregulate the expression of several specific genes that define their contractile phenotype in vivo. Keeping this in mind, we adopted a low-density seeding strategy for VSMC derived from the aortae of Wistar-Kyoto (WKY) rats to select a cell line possessing the highest expression of smooth muscle-specific α-actin, SM22 protein, myosin light chain kinase as well as angiotensin II- and endothelin-1-induced ERK1/2 phosphorylation [23]. In the present study, WKY-7 cells were grown for 48-72 hr in Dulbecco's modified Eagle Medium supplemented with fetal and newborn calf serum (10% of each), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). To establish quiescence, VSMC were incubated before the experiments for 48 hr in the presence of 0.2% calf serum.

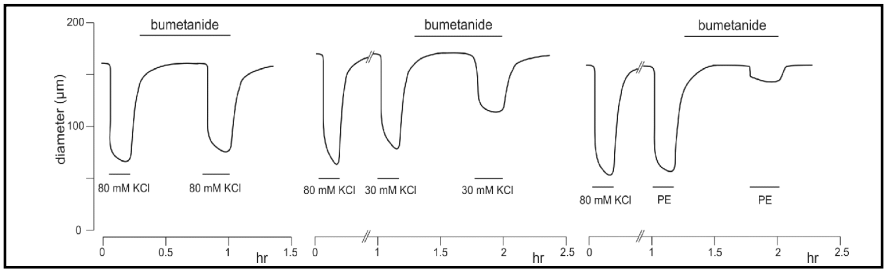
A) NKCC activity was measured as the bumetanide-sensitive component of the ^{86}Rb influx rate. Cells seeded in 24-well plates were washed twice with 2-ml aliquots of PSS and preincubated in the same medium for 1 hr at 37°C. Then, the medium was aspirated, and 0.5 ml of PSS with 1 μCi/ml ^{86}Rb, 5 mM ouabain ± $NaHCO_3$ and 100 μM bumetanide was added. After 15-min incubation at 37°C, isotope uptake was terminated by the addition of 2 ml of ice-cold medium W containing 100 mM $MgCl_2$ and 10 mM HEPES-tris buffer (pH 7.4). The cells were washed 3 times with 2 ml of medium W, and radioactivity of the incubation medium and cell lysate was measured with a liquid scintillation analyzer. The rate of ^{86}Rb influx (V, nmol per mg of protein per 5 min) was calculated as $V = A/am$, where A was the radioactivity of the samples (cpm), a was the specific radioactivity of K^+ (^{86}Rb) in the medium (cpm/nmol), and m was protein content measured with the modified Lowry method. For more details, see [24]. *$[Cl^-]_i$ content* was measured as the steady-state distribution of ^{36}Cl after 1-hr incubation with radioisotope, as described previously in detail [25].

B) Free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in VSMC was quantified in accordance with a previously-described method [26]. Briefly, WKY-7 cells grown on glass cover-slips were incubated for 30-40 min in PSS containing 5-10 μM fura 2-

Table 1. Effect of bumetanide and bicarbonate on contraction of mesenteric arteries triggered by K^+_o-induced depolarization and PE. Means ± S.E. obtained in n independent experiments are shown. The decrease of mesenteric artery diameter triggered by 80 mM KCl in the absence of bumetanide was taken as 100%. NS - non-significant.

Bumetanide, μM	$NaHCO_3$, mM	Decrement of mesenteric artery diameter, %		
		$[K^+]_o$ = 30 mM	$[K^+]_o$ = 80 mM	PE
1. None	None	76.6±5.6 (n=7)	100.0±8.3 (n=4)	99.3±8.2 (n=4)
2. 100	None	41.6±5.6 (n=7)	96.1±4.7 (n=4)	21.4±7.5 (n=4)
3. None	25	88.4±2.0 (n=5)	104.3±6.6 (n=4)	103.6±9.9 (n=4)
4. 100	25	81.5±3.6 (n=5)	102.4±9.0 (n=4)	70.9±15.9 (n=4)
$P_{1,3}$		NS	NS	NS
$P_{1,2}$		<0.001	NS	<0.001
$P_{3,4}$		NS	NS	NS

Fig. 1. Typical recording showing the effect of extracellular K^+ (80 and 30 mM), PE (10 μM) and bumetanide (100 μM) on the internal diameter of a mouse resistance mesenteric artery incubated in bicarbonate-free PSS.



AM. Then, the cells were washed, mounted in a diagonal position in a 1x1-cm perfused cuvette, and fluorescence was measured under permanent stirring at 37°C (λ_{ex} = 340 and 380 nm, slit 4 nm; λ_{em} = 510 nm, slit 12 nm), using a SPEX FluoroMax spectrofluorimeter (Edison, NJ). Free $[Ca^{2+}]_i$ was quantified as $[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)$, where K_d is the dissociation constant of the Ca^{2+}-fura 2 complex (224 nM at 37°C), and $R = F_{340}/F_{380}$ is the ratio of fluorescence at λ_{ex} = 340 and 380 nm. To evaluate F_{max}, the cells were treated with 0.5 μM ionomycin in the presence of 1 mM $CaCl_2$. To determine F_{min}, $MnCl_2$ was added at a final concentration of 2 mM.

C)Plasma membrane electrical potential (E_m) was measured by the whole-cell patch clamp nystatin technique [27, 28]. WKY-7 cells grown on plastic cover-slips were placed in the chamber perfused with PSS at 37°C and mounted on the stage of the inverted microscope. After a giga-ohm seal formed between the cell membrane and the glass pipette filled with "intracellular" high-K^+ solution (110 mM K-gluconate, 30 mM KCl, 10 mM NaCl, 0.7 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM EGTA, 5 mM glucose, 20 mM HEPES-tris, pH 7.1, input resistance of 5 mΩ), 100 ng/ml nystatin was added. A flowing KCl electrode served as reference. Previously, it was shown that this procedure triggered non-selective cation conductance [29]. The resulting E_m was recorded in current-clamp mode of the amplifier (Puschino, Russia) with current I set at 0.

Chemicals

Ouabain, bumetanide, phenylephrine, nystatin, nicardipine - Sigma (St. Louis, MO); $^{86}RbCl$, $H^{36}Cl$ - Dupont (Boston, MA) and Isotope (Russia); salts, D-glucose and buffers - Sigma and Anachemia (Montreal, PQ).

Results

Mesenteric artery contraction

Figure 1 displays the kinetics of modulation of mesenteric artery inner diameter in HCO_3^--free PSS by

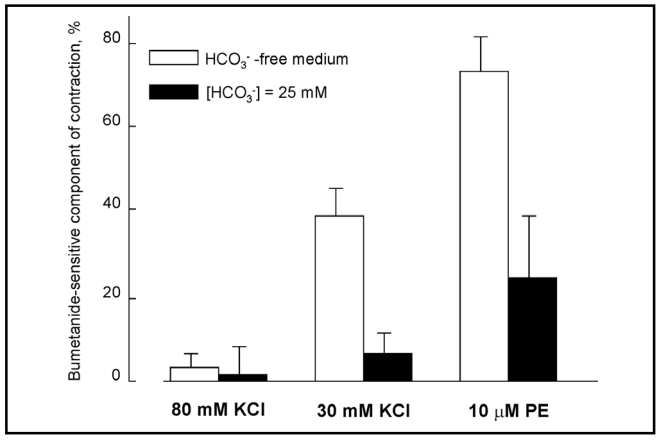


Fig. 2. Bumetanide-sensitive components of mesenteric artery contraction triggered by 80 mM KCl, 30 mM KCl and 10 mM PE in bicarbonate-free PSS and in the presence of 25 mM $NaHCO_3$. Contractions triggered by these stimuli in the absence were taken as 100%. Means ± S.E. for the bumetanide-sensitive components of contraction were calculated from data shown in Table 1.

K^+_o-induced depolarization and activation of α-adrenoceptors with PE. We observed that 30-min preincubation with 100 μM bumetanide did not affect contractions evoked by full-scale depolarization (80 mM KCl), attenuated contractions in vessels subjected to modest depolarization with 30 mM KCl, and almost completely abolished the contractions triggered by activation of α-adrenoceptors with PE. These results are consistent with previous data on the inhibition, by bumetanide, of isometric contractions of vascular rings from the rat aorta [5].

Isosmotic substitution of 50 mM mannitol with 25 mM $NaHCO_3$ did not significantly affect contractions

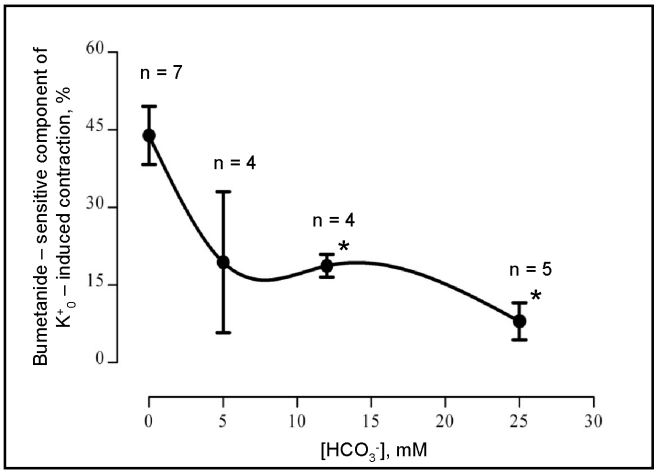
Fig. 3. Dose-dependent action of bicarbonate on the bumetanide-sensitive component of mouse resistance mesenteric artery contraction evoked by 30 mM KCl. Bicarbonate concentration in PSS was increased up to 25 mM by isosmotic substitution of mannitol with $NaHCO_3$. Means ± S.E. for n experiments with arteries from different mice are shown. * - $p<0.001$ compared to bicarbonate-free medium.

evoked by depolarization and PE (Table 1) but attenuated the inhibitory action of bumetanide on contractions of mesenteric arteries treated with 30 mM KCl and PE by 5- and 3-fold, respectively (Fig. 2). Thus, in HCO_3^--free medium, bumetanide suppressed K^+_o- and PE-induced contractions by 46 and 78%, whereas in the presence of 25 mM HCO_3^-, these values decreased up to 8 and 31%, respectively (Table 1). Importantly, the addition of 10 mM $NaHCO_3$, was sufficient to reduce bumetanide-sensitive contractions triggered by modest K^+_o-induced depolarization from 44±6 to 17±2% ($p<0.001$); further elevation of $[NaHCO_3]_o$ up to 25 mM depressed the inhibitory action of bumetanide by only ~5% (Fig. 3).

E_m and $[Ca^{2+}]_i$

Keeping in mind the strongest inhibitory action of bumetanide on PE-induced contractions of mesenteric arteries that was sharply diminished in the presence of bicarbonate (Fig. 1, Table 1), we compared the effects of bumetanide and $HCO_{3\,o}^-$ on modulation of E_m and $[Ca^{2+}]_i$ by PE in cultured VSMC. In accordance with previous data [27, 28], 10 min of cell treatment with nystatin was sufficient to measure baseline E_m values ($E_{m/base}$) (Fig. 4). In the absence of bicarbonate, these values varied in the range of -41±3 mV (Table 2). PE transiently depolarized VSMC whereas bumetanide led to sustained and reversible hyperpolarization ($E_{m/base}$ = -58±4 mV, Table 2). Five-min treatment of VSMC with 100 μM bumetanide resulted in 3-fold attenuation of maximal values of PE-induced depolarization compared to control cells (ΔE = 5±2 and 15±3 mV, respectively, Table 2). The addition of 25 mM $NaHCO_3$ did not significantly affect $E_{m/base}$ values and the depolarizating action of PE but almost completely abolished the modulation of these parameters by bumetanide (Table 2).

Side-by-side with depolarization, PE triggered bimodal $[Ca^{2+}]_i$ elevation in cultured VSMC that was completely abolished in 5 min of stimulation (Fig. 5). We characterized transient and sustained phases of $[Ca^{2+}]_i$ responses as the maximal increment of $[Ca^{2+}]_i$ (Δ_1) and its value in 2 min of PE addition (Δ_2) (Fig. 5). In HCO_3^--free medium, bumetanide did not significantly alter



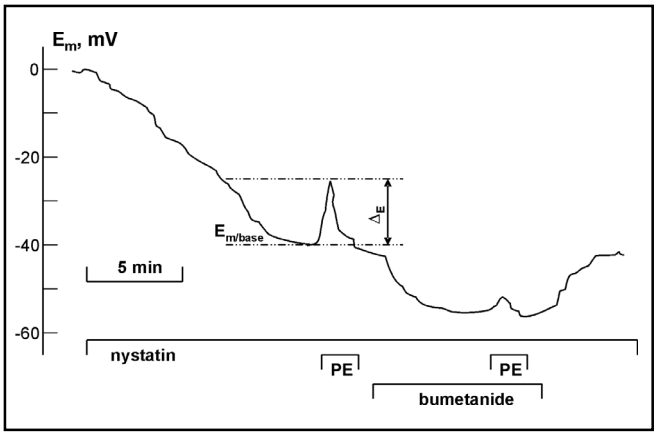


Fig. 4. Typical recording showing the effects of 10 μM PE and 100 μM bumetanide on plasma membrane electrical potential (E_m) in WKY-7 VSMC after rupture of a giga-ohm seal between the plasma membrane and the glass pipette with 100 ng/ml nystatin.

Bumetanide, μM	$NaHCO_3$, mM	$E_{m/base}$, mV	ΔE, mV
1. None	None	-41±3	15±3
2. 100	None	-58±4	5±2
3. None	25	-44±5	18±4
4. 100	25	-49±6	13±3
$P_{1,3}$		NS	NS
$P_{1,2}$		<0.01	<0.02
$P_{3,4}$		NS	NS

Table 2. Effect of bumetanide and bicarbonate on baseline electrical membrane potential ($E_{m/base}$) and maximal values of PE-induced depolarization (ΔE) in WKY-7 cells. Cells were treated with 10 μM PE, as shown in Fig. 4. Means ± S.E. obtained in 5 independent experiments are reported. NS - non-significant.

baseline $[Ca^{2+}]_i$ and its maximal increase evoked by PE (Δ_1) but almost completely abrogated sustained phase of $[Ca^{2+}]_i$ signaling (Δ_2 = 106±23 and 4±16 nM in control and bumetanide-treated cells, respectively, Table 3).

Table 3. Effect of bumetanide and bicarbonate on baseline intracellular Ca^{2+} concentration ($[Ca^{2+}]_{i\text{-base}}$) and on rapid ($\Delta_1$) and sustained ($\Delta_2$) increments of $[Ca^{2+}]_i$ in WKY-7 cells triggered by PE. Cells were treated with 10 μM PE. $[Ca^{2+}]_{i\text{-base}}$, Δ_1 and Δ_2 parameters were measured as shown in Fig. 5. Means ± S.E. obtained in 4 independent experiments are reported. NS - non-significant.

Additions, μM	$NaHCO_3$, mM	$[Ca^{2+}]_{i\text{-base}}$, μM	Δ_1, μM	Δ_2, μM
1. None	None	84±7	801±90	106±23
2. Bumetanide, 100	None	79±6	707±99	4±16
3. Nicardipine, 0.1	None	82±8	781±102	7±18
4. None	25	106±12	789±104	221±22
5. 100	25	97±6	777±83	101±21
6. Nicardipine, 0.1	25	104±13	723±88	16±23
$P_{1,4}$		NS	NS	<0.02
$P_{1,2}$		NS	NS	<0.01
$P_{1,3}$		NS	NS	<0.02
$P_{4,5}$		NS	NS	<0.01
$P_{4,6}$		NS	NS	<0.001

The addition of 25 mM $NaHCO_3$ did not change Δ_1 but increased the sustained phase of PE-induced signaling (Δ_2 = 221±22 vs 106±23 in HCO_3^--free PSS). In the presence of bicarbonate, bumetanide suppressed the sustained phase of PE-induced $[Ca^{2+}]_i$ signaling by only 2-fold that was in contrast to the more than 25-fold inhibition by bumetanide seen in HCO_3^--free PSS (Table 3). Unlike bumetanide, the potent and selective inhibitor of L-type Ca^{2+} channels, nicardipine, almost completely abolished the sustained phase of PE-induced signaling independently of bicarbonate addition.

NKCC activity and $[Cl^-]_i$

In cultured VSMC, we observed that the addition of 5 mM $NaHCO_3$ resulted in 2-fold inhibition of NKCC activity, measured as the bumetanide-inhibited component of ^{86}Rb influx; further elevation of bicarbonate concentration up to 25 mM did not significantly increase its inhibitory action on this carrier (Fig. 6A).

Figure 6B shows that the addition of 5 mM $NaHCO_3$ decreased $[Cl^-]_i$ by ~30%. Importantly, in contrast to its action on NKCC, elevation of $[NaHCO_3]$ from 5 to 25 mM led to 2-fold attenuation of $[Cl^-]_i$ (Fig. 6B). We also noted that in bicarbonate-free medium, bumetanide decreased $[Cl^-]_i$ by ~15%; this effect was almost completely abolished by the addition of 5 mM $NaHCO_3$ (Fig. 6C).

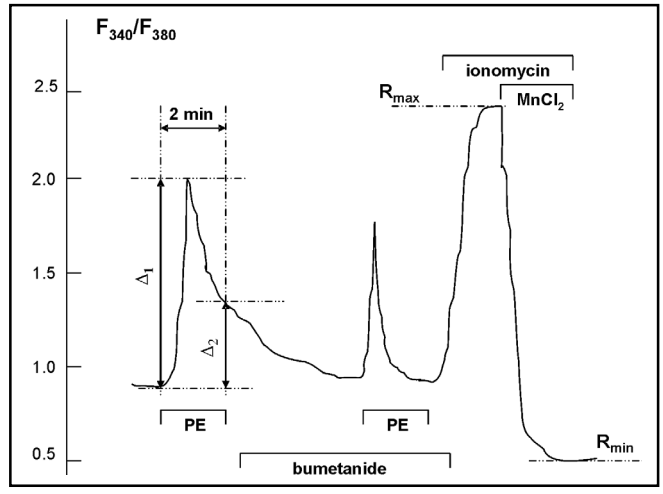


Fig. 5. Typical recording showing the effects of 10 μM PE, 100 μM bumetanide, 0.5 μM ionomycin and 2 mM $MnCl_2$ on the F_{340}/F_{380} ratio in fura-2-loaded WKY-7 VSMC.

Discussion

The data obtained in this study show that similarly to endothelium-denuded rat aortic rings [5], preincubation with bumetanide decreased the contraction of mouse resistant mesenteric arteries subjected to modest depolarization ($[K^+]_o$ = 30 mM) as well as the activation of α-adrenergic receptors with PE (Fig. 1). Importantly, we observed that the inhibitory action of bumetanide on K^+_o- and PE-induced contractions was sharply diminished in bicarbonate- containing medium (Fig. 2). At least 2 hypotheses might explain this finding (Fig. 7).

First, HCO_3^- inhibits NKCC1 activity by competing with Cl^- for anion-binding sites of the carrier. Indeed, we noted that addition of $NaHCO_3$ decreased NKCC in cultured VSMC by 2-to 3-fold (Fig. 6A, curve 3). We also noted that, similarly to its action on NKCC, the addition of 5 mM $NaHCO_3$ was sufficient to inhibit the bumetanide-sensitive component of mesenteric artery contractions by more than 5-fold (Fig. 3). This hypothesis predicts that HCO_3^- suppresses the NKCC-dependent component of E_m and $[Ca^{2+}]_i$ modulations by vasoconstrictors. Indeed, bicarbonate sharply suppressed VSMC hyperpolarization evoked by bumetanide as well as the inhibitory action of the HCD on PE-induced depolarization (Table 2). Neither baseline $[Ca^{2+}]_i$ nor its maximal increment evoked by PE (Δ_1) and mediated by phospholipase C activation, inositol-1,4,5-triphosphate production and Ca^{2+} release from endoplasmic reticulum [30] was affected by bumetanide. However, bumetanide

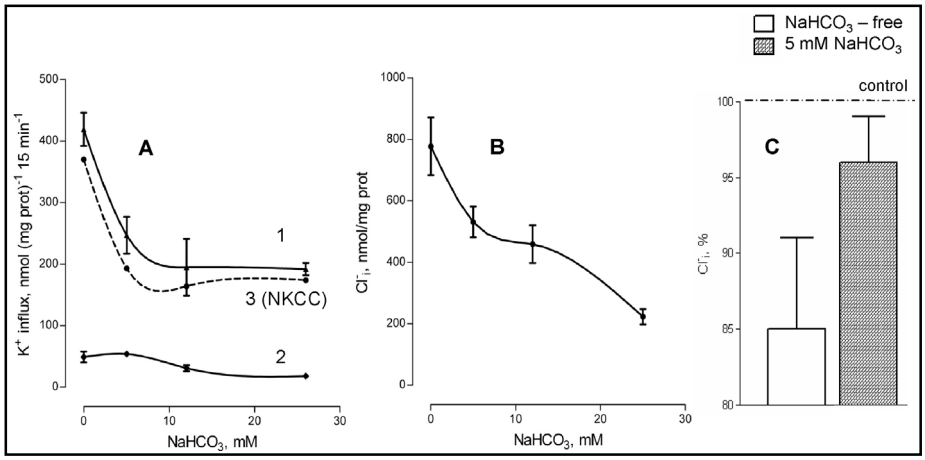


Fig. 6. Effect of bicarbonate on K^+ (^{86}Rb) influx and intracellular Cl^- content (Cl^-_i) in VSMC from the rat aorta. A. Dose-dependent action of $NaHCO_3$ on ^{86}Rb influx in the presence of 5 mM ouabain (1) and ouabain + 100 μM bumetanide (2). NKCC activity (3) was calculated as the bumetanide-sensitive component of ^{86}Rb influx. Bicarbonate concentration in PSS was increased up to 25 mM by isosmotic substitution of mannitol with $NaHCO_3$. B. Dose-dependent action of $NaHCO_3$ on $[Cl^-]_i$ in the absence of ouabain and bumetanide. C. Effect of bumetanide on $[Cl^-]_i$ in HCO_3^--free PSS and in PSS containing 5 mM $NaHCO_3$. Control values measured in the absence of bumetanide were taken as 100%. Means ± S.E. for experiments performed in quadruplicates are shown. * $p<0.05$ compared to Cl^-_i content in the absence of bumetanide (control).

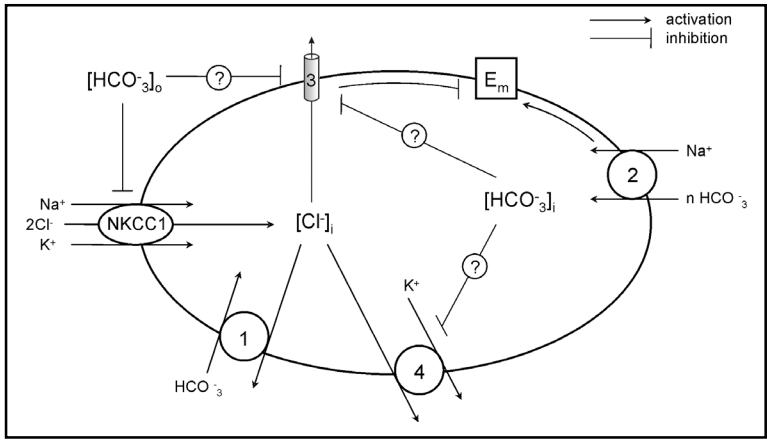


Fig. 7. Possible mechanism of the involvement of bicarbonate anions in the regulation of $[Cl^-]_i$ and electrical membrane potential (E_m) in VSMC. 1 - anion exchanger; 2 - Na^+,HCO_3^- cotransport; 3 - Cl^- channels; 4 - K^+,Cl^- cotransport. For other abbreviations, see text.

abolished the sustained nicardipine-sensitive phase of $[Ca^{2+}]_i$ elevation (Δ_2, Table 3) triggered by activation of Ca^{2+}_i-sensitive anion channels and membrane depolarization [31]. Previously, we reported that bumetanide diminished the nicardipine-sensitive component of ^{45}Ca influx triggered by modest K^+_o-induced depolarization [5]. Viewed collectively, these data show that bumetanide decreases mesenteric artery contractions via NKCC inhibition, $[Cl^-]_i$ attenuation, membrane hyperpolarization and reduced Ca^{2+}-influx via nicardipine-sensitive E_m-gated L-type Ca^{2+} channels. These actions of bumetanide are diminished by $HCO_{3\,o}^-$ due to partial NKCC inhibition.

Second, we noted that $[NaHCO_3]$ elevation in the range from 5 to 25 mM resulted in more than a 2-fold decrease of $[Cl^-]_i$ (Fig. 6B). Keeping in mind that bicarbonate did not significantly affect NKCC activity in this concentration range (Fig. 6A), it may be assumed that $[Cl^-]_i$ attenuation is caused by activation of anion exchanger-mediated $Cl^-_i/HCO_{3\,o}^-$ countertransport that raises $[HCO_3^-]_i$ (Fig. 7). Importantly, side-by-side with electroneutral anion exchanger, $[HCO_3^-]_i$ elevation might be mediated by electrogenic Na^+,HCO_3^- cotransporters and members of the anion channel family that also contribute to the decline of $[Cl^-]_i$ via inhibition of outwardly-directed Na^+-independent K^+,Cl^- cotransport (KCC). All these transporters have been detected in smooth muscle cells [32-35]. Indeed, KCC has benn shown first time by Lauf to be inhibited by increasing concentrations of $NaHCO_3$ with apparent affinity of 38 mM and hence lower than reported in our work on the NKCC, however, with a Hill coeficient of 1.9 suggesting more than one site of bicarbonate action [36]. However, the absence of selective inhibitors complicates the estimation of their relative impact on the $HCO_{3\,o}^-$-dependent component of $[Cl^-]_i$ regulation by NKCC1. Thus, for example, SITS, DIDS and other 2,2'-stilbenedisulfonic acid derivatives, known to be potent inhibitors of anion exchanger, also suppress anion channels [37]. In human erythrocytes, KCC is inhibited by alcanoic acid derivatives such as DIOA

[38-40]. However, in VSMC, this compound also blocked Na^+,K^+-ATPase and NKCC [41]. More recently, Lauf and co-workers reported that hyposmotic activation of intermediate K^+ conductance channels in human lens epithelial cells can be also completely inhibited by DIOA [42].

Under normal conditions, plasma [HCO_3^-] is about 24 mM with minor differences (0.52 mM) between venous and arterial blood [43]. It has also been shown that the maximal changes of plasma [HCO_3^-] detected in patients with severe metabolic alkalosis and acidosis do not exceed 55 and 6 mM, respectively [44-46]. We observed almost complete suppression of NKCC and the NKCC-dependent component of mesenteric artery contractions at $[HCO_3^-]_o$ = 5 mM. Considering this issue, it is important to underline that the relative impact of NKCC1 and other anion transporters on VSMC contractions seems to be different in blood vessels of distinct origins. Indeed, in contrast to very modest suppression of mesenteric artery contractions by bumetanide in HCO_3^--containing PSS (Table 1), the diuretic completely abolished the contraction of renal afferent arterioles evoked by angiotensin II in the presence of 30 mM $NaHCO_3$ [15]. In addition, the range of local $[HCO_3^-]_o$ modulation in normal and pathophysiological conditions might be essentially higher than in plasma. Thus, additional studies should be performed to examine the role of acid-base homeostasis in the regulation of NKCC activity, VSMC contractions and other NKCC-dependent cellular functions.

In conclusion, our results show that extracellular HCO_3^- diminishes the NKCC-sensitive component of excitation-contraction coupling. They also strongly suggest that this action of bicarbonate is caused by its inhibitory effect on the carrier's activity. Mechanisms of this phenomenon as well as the possible involvement of anion exchanger and other HCO_3^--sensitive transporters should be verified in additional experiments.

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