Na\(^+-\)Ca\(^{2+}\) Exchange, Myoplasmic Ca\(^{2+}\) Concentration, and Contraction of Arterial Smooth Muscle

Christopher M. Rembold, Howard Richard, and Xiao-Liang Chen

Na\(^+-\)Ca\(^{2+}\) exchange is proposed to be an important regulator of myoplasmic intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and contraction in vascular smooth muscle. We investigated the role of Na\(^+-\)Ca\(^{2+}\) exchange in regulating [Ca\(^{2+}\)]\(_i\) in swine carotid arterial tissues that were loaded with aequorin to allow simultaneous measurement of [Ca\(^{2+}\)]\(_i\) and force. Reversal of Na\(^+-\)Ca\(^{2+}\) exchange, by reduction of extracellular Na\(^+\) concentration ([Na\(^+\)]\(_o\)) to 1.2 mM, induced a large increase in aequorin-estimated [Ca\(^{2+}\)]\(_i\), and a low [Ca\(^{2+}\)] sensitivity. The contraction induced by 1.2 mM [Na\(^+\)]\(_o\) was partially caused by depolarization and opening of L-type Ca\(^{2+}\) channels because 10 \(\mu\text{M}\) diltiazem partially attenuated the 1.2 mM [Na\(^+\)]\(_o\)-induced increases in [Ca\(^{2+}\)]\(_i\). High dose ouabain (10 \(\mu\text{M}\)), a putative endogenous Na\(^+,K^+\)-ATPase inhibitor, increased both [Ca\(^{2+}\)]\(_i\) and force. However, the increases in [Ca\(^{2+}\)]\(_i\) and force were mostly blocked by 10 \(\mu\text{M}\) phentolamine, suggesting the predominant effect of ouabain was to increase norepinephrine release from nerve terminals. In the presence of 10 \(\mu\text{M}\) phentolamine, 10 \(\mu\text{M}\) ouabain slightly accentuated 1 \(\mu\text{M}\) histamine-induced increases in [Ca\(^{2+}\)]\(_i\) and force. The ouabain dose necessary to induce contraction in the absence of phentolamine was significantly less than the ouabain dose necessary to accentuate histamine-induced contractions in the presence of phentolamine. These results suggest that Na\(^+-\)Ca\(^{2+}\) exchange exists in swine arterial smooth muscle. These data also suggest that ouabain (which should increase [Na\(^+\)]\(_i\)) and inhibit Na\(^+-\)Ca\(^{2+}\) exchange) primarily enhances contractile function in the swan carotid artery by releasing catecholamines from nerve terminals; direct action of Na\(^+,K^+\)-ATPase inhibitors on smooth muscle appears to occur only with very high doses. (Hypertension 1992;19:308–313)

KEY WORDS • calcium • digitalis-like factors • norepinephrine • ouabain • phentolamine • vascular smooth muscle

The Na\(^+-\)Ca\(^{2+}\) exchange is proposed to be an important mechanism responsible for physiologically regulating smooth muscle myoplasmic intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). The Na\(^+-\)Ca\(^{2+}\) exchanger moves one Ca\(^{2+}\) ion across the plasma membrane in exchange for three or four Na\(^+\) ions moving in the opposite direction. Its function is passive and depends on the relative extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)), [Na\(^+\)]\(_o\), [Ca\(^{2+}\)]\(_i\), and possibly the membrane potential. Since [Na\(^+\)]\(_o\) is approximately 140 mM and [Na\(^+\)]\(_i\) is approximately 10–20 mM in smooth muscle, the Na\(^+-\)Ca\(^{2+}\) exchanger normally functions to transport Ca\(^{2+}\) from the intracellular to the extracellular space. [Na\(^+\)]\(_i\) is predominantly regulated by the activity of the plasma membrane Na\(^+,K^+\)-ATPase. Recently, an endogenous regulator of the Na\(^+,K^+\)-ATPase has been identified as ouabain. Other vasoactive compounds may also regulate the Na\(^+,K^+\)-ATPase. Theoretically, inhibition of the Na\(^+,K^+\)-ATPase would increase [Na\(^+\)]\(_i\), thereby inhibiting Na\(^+-\)Ca\(^{2+}\) exchange, increasing [Ca\(^{2+}\)]\(_i\), and enhancing the contractility of vascular smooth muscle. This effect could contribute to the pathophysiology of hypertension because elevations in endogenous Na\(^+,K^+\)-ATPase inhibitors have been observed in human hypertension and abnormalities of the Na\(^+-\)Ca\(^{2+}\) exchanger per se have been inferred in experimental hypertension.

The mechanism whereby increases in myoplasmic [Ca\(^{2+}\)] regulate contractile force is complex. With a few exceptions, increases in myosin light chain phosphorylation appear to be the primary regulator of contractile force in arterial smooth muscle. However, the amount of myoplasmic [Ca\(^{2+}\)] necessary to increase the level of myosin light chain phosphorylation (i.e., the [Ca\(^{2+}\)] sensitivity) is dependent on the form of stimulation. [Ca\(^{2+}\)] sensitivity is high (i.e., high phosphorylation at lower [Ca\(^{2+}\)]) when smooth muscle is stimulated with contractile agonists (e.g., histamine or phenylephrine) that activate receptors linked to inositol-1,4,5-triphosphate and induce release of the intracellular store. Observed [Ca\(^{2+}\)] sensitivity is lower when stimuli only induce Ca\(^{2+}\) influx (e.g., KCl depolarization or Bay K 8644).
This study will address the following questions: 1) Does the Na+·Ca2+ exchanger exist in swine arterial smooth muscle? 2) Does the Na+-Ca2+ exchanger reduce elevations in [Ca2+]? 3) If Ca2+ entry occurs by reversal of the Na+-Ca2+ exchanger, is the [Ca2+] sensitivity low (i.e., similar to that induced by Ca2+ entry from KCl depolarization)? 4) Do inhibitors of the Na+/K+-ATPase increase [Ca2+], and contractility? Unlike prior studies, we measured [Ca2+], and contractile force simultaneously in an intact smooth muscle tissue.

Methods

Swine common carotid arteries were obtained from a slaughterhouse and transported at 2°C in physiological salt solution (PSS). Dissection of medial strips and mounting were performed as illustrated by Driska et al. The intimal surface was mechanically rubbed to remove the endothelium.

PSS contained (mM): NaCl 140, KCl 5, 3-morpholinopropanesulfonic acid (MOPS) 2, CaCl2 1.6, MgCl2 1.2, Na2HPO4 1.2, d-glucose 5.6 (pH adjusted to 7.4 at 37°C). Agonist stimulation was performed by injecting an appropriate volume of 10 mM stock histamine into the tissue bath. Histamine stock solutions were prepared daily.

Aequorin (batch 2, purchased from Dr. J. Blasco, Friday Harbor Laboratories, Friday Harbor, Wash.) was loaded intracellularly by isoosmotic hyperpermeabilization. Simultaneous light and force measurements were made in a light-tight enclosure. Force was measured and muscle length controlled with a 300H servo (Cambridge Technology, Cambridge, Mass.) in the isometric mode. A Metabyte DASH 16 AD board (Taunton, Mass.) in an AT&T personal computer was used to store force measurements. Stress was calculated as force per cross-sectional area, which was estimated from measured length, weight, and a density of 1.050 g/cm3. Aequorin light signals were collected digitally, counted by the Metabyte DASH 16 AD board, and were presented in the form log L/Lμm change units, where L is the photon count (in counts per second) and Lμm is an estimate of the peak light intensity that would be recorded if all of the aequorin in the tissue were instantaneously exposed to 5 mM CaCl2. Aequorin light emission was calibrated in a series of Ca2+/ethylene glycol bis(β-aminoethyl ether) N,N,N'N'-tetraacetic acid (EGTA) buffers with 0.5 mM Mg2+ at 37°C. Estimated basal [Ca2+], (i.e., at 0 log L/Lμm change units) was 75±7 nM in the 24 preparations shown in Figures 1–4. Based on this calibration, 0.3 log L/Lμm change units was estimated as 135 nM, 0.6 log L/Lμm change units was estimated as 205 nM, and 1.2 log L/Lμm change units was estimated as 375 nM.

At specific times after stimulation, we calculated a stress/[Ca2+] ratio as a quantitative index of [Ca2+], sensitivity. Stress was normalized such that maximal force was 1.0 and unstimulated force was 0.0. [Ca2+], was calculated from the aequorin light signal. Assuming that the phosphorylation/stress relation does not change, high stress/[Ca2+] ratios would indicate high [Ca2+], sensitivity and low stress/[Ca2+] ratios would indicate low [Ca2+], sensitivity.

Phosphorylation was estimated in a second set of tissues that underwent identical protocols except the tissues were not loaded with aequorin. Aequorin loading has been previously shown not to change the time course of phosphorylation or stress development in the swine carotid. Tissues were frozen by immersion in a dry ice/acetone slurry (20 g/20 ml) at −78°C. Phosphorylation of the smooth muscle-specific 20 kd myosin light chain was determined by the method of Driska et al with precautions noted by Aksoy et al. Phosphorylation is reported as moles P/moles total smooth muscle-specific light chain. Force was measured isometrically with force transducers (model FT.03, Grass Instruments Co., Quincy, Mass.) in the tissues that were assayed for phosphorylation.

Results

Substantial reductions of extracellular Na+ will reverse the plasma membrane Na+ gradient and should induce Ca2+ entry into the cell by the Na+-Ca2+ exchanger. We expected that contractions induced by reversal of the Na+-Ca2+ exchanger would exhibit a low [Ca2+], sensitivity because other stimuli that induced only Ca2+ entry also induced a low [Ca2+], sensitivity. Reduction of [Na+], to 1.2 mM by equimolar substitution with choline+ increased aequorin-estimated myoplasmic [Ca2+], myosin light chain phosphorylation, and stress values (Figure 1, left panel). Subsequent addition of 100 μM histamine induced a transient increase in [Ca2+], and a further increase in phosphorylation and stress. Addition of histamine also significantly increased the stress/[Ca2+] ratio from 2.88 to 3.91 (measured 10 minutes after addition of histamine) indicating an increase in [Ca2+], sensitivity (Table 1). Restoration of 140 mM [Na+], and removal of choline+ decreased [Ca2+], phosphorylation, and stress. The stress/[Ca2+] ratio increased from 3.91 to 4.74, but the change was not significant (Table 1). These data show that substitution of all but 1.2 mM extracellular Na+ with choline+ was associated with a lower [Ca2+], sensitivity and that histamine stimulation was capable of increasing [Ca2+], sensitivity despite the presence of low [Na+].

We evaluated whether low [Na+], increasing [Ca2+], partially by depolarization and opening of L-type Ca2+ channels, In the presence of 10 μM diltiazem, the 1.2 mM [Na+],-induced [Ca2+], increase was only partially attenuated but the contraction was more significantly decreased (Figure 1, right panel). The stress/[Ca2+] ratio was 0.78, indicating a lower [Ca2+], sensitivity in the presence of diltiazem than in its absence (Table 1). These data suggest that the [Ca2+], sensitivity is lowest when [Ca2+], was derived mostly from Na+-Ca2+ exchange, intermediate when [Ca2+], was derived from Ca2+ entry through L-type Ca2+ channels, and highest in the presence of histamine. Subsequent histamine stimulation transiently increased [Ca2+], and induced a maximal contraction with a high stress/[Ca2+] ratio of 5.30 (indicating a high [Ca2+], sensitivity). On restoration of 140 mM [Na+], in the continued presence of histamine and diltiazem, both [Ca2+], and force declined to lower values. These results suggest that under these conditions, Na+-Ca2+ exchange is at least partially responsible for decreasing histamine-induced increases in [Ca2+].

We evaluated whether these results were specific to the use of choline+. Reduction of [Na+], to 1.2 mM by equimolar substitution with Li+ induced a large increase
in [Ca\(^{2+}\)], a very slight contraction, and a very low stress/[Ca\(^{2+}\)] ratio (0.09), indicating a very low [Ca\(^{2+}\)] sensitivity (Figure 2). Addition of histamine increased stress and the stress/[Ca\(^{2+}\)] ratio despite little sustained increases in [Ca\(^{2+}\)]. This result suggests that low [Na\(^{+}\)] induced a low [Ca\(^{2+}\)] sensitivity regardless of the substituting cation. However, the degree of the decrease in [Ca\(^{2+}\)] sensitivity was dependent on the cation replacing Na\(^{+}\): Li\(^{+}\) induced a lower [Ca\(^{2+}\)] sensitivity than choline\(^{+}\).

Reduction of [Na\(^{+}\)] to 1.2 mM demonstrated the characteristics of Na\(^{+}\)-Ca\(^{2+}\) exchange in the swine carotid; however, such substantial changes in [Na\(^{+}\)] are nonphysiological. We studied a more physiological system by evaluating the effects of ouabain, a putative endogenous inhibitor of the Na\(^{+}\),K\(^{-}\)-ATPase. High-dose ouabain (10 \(\mu\)M) induced a gradual increase in aequorin-estimated myoplasmic [Ca\(^{2+}\)] and stress (Figure 3, left panel). The stress/[Ca\(^{2+}\)] ratio was 3.28±0.63, apparently suggesting that ouabain induced a high [Ca\(^{2+}\)] sensitivity. However, incubation in 10 \(\mu\)M phentolamine (an \(\alpha\)-adrenergic blocker) significantly attenuated the ouabain-induced increase in [Ca\(^{2+}\)], and force (\(p<0.01\) by Neuman-Keuls test) and the stress/[Ca\(^{2+}\)] ratio was low (0.33±0.07; Figure 3, right panel). This result suggests that the high-dose ouabain-induced contraction was predominantly induced by activation of nerve terminals and endogenous norepinephrine release (confirming earlier reports\(^{28}\)). Endogenous release of norepinephrine would be expected to induce a high [Ca\(^{2+}\)] sensitivity because we previously showed that phenylephrine (an \(\alpha\)-adrenergic agonist) induced a high [Ca\(^{2+}\)] sensitivity.\(^{11}\)

It has been suggested that ouabain can also directly alter smooth muscle contractility.\(^{1-3}\) We evaluated whether ouabain can enhance histamine-induced contractions in the swine carotid artery. Tissues were incubated in 10 \(\mu\)M phentolamine to inhibit the effects of norepinephrine release. The tissues were sequentially stimulated three times with 1 \(\mu\)M histamine (a concentration that induced a submaximal contraction). The second contraction was performed in the presence of 10 \(\mu\)M ouabain (Figure 4). The increase in [Ca\(^{2+}\)] and stress observed during the second histamine-induced contraction (in the presence of 10 \(\mu\)M ouabain) was slightly larger than the [Ca\(^{2+}\)], and stress observed during the first and third histamine contractions (with no ouabain). The increase in [Ca\(^{2+}\)] and stress observed during the second contraction (with ouabain) was significantly larger than the mean of the [Ca\(^{2+}\)], and stress observed during the first and third histamine contractions (when compared with paired \(t\) tests, \(p<0.05\) for both). However, the standard errors of these measurements were large, and when the data were compared with the Neuman-Keuls test, these changes were not significant. These results suggest that ouabain may also directly increase contractility of the smooth muscle by increasing [Ca\(^{2+}\)], in addition to its effects on enhancing norepinephrine release from nerve terminals. However, these effects were small and of marginal significance.

We compared the dose response of the ouabain-dependent increase in neural transmission and the dose response of ouabain's direct action on smooth muscle contractility. In the absence of phentolamine, 0.3 \(\mu\)M ouabain induced a half-maximal contraction (Figure 5A). In the presence of 10 \(\mu\)M phentolamine, the ouabain-induced contraction was significantly reduced at all tested doses (\(p<0.01\) by Neuman-Keuls). In the presence of 10 \(\mu\)M phentolamine, only the 10-\(\mu\)M ouabain dose enhanced histamine-induced contractions (Figure 5B). However, the 10-\(\mu\)M ouabain-induced increase in stress was only significant when evaluated with a paired \(t\) test; analysis by Neuman-Keuls revealed no significant difference. These data suggest that release of endogenous norepinephrine is much more sensitive to ouabain than the direct effects of ouabain on smooth muscle.

**Discussion**

The present study demonstrated that Na\(^{+}\)-Ca\(^{2+}\) exchange exists in swine arterial smooth muscle and that it
TABLE 1. Stress/Ca²⁺ Concentration Ratios Observed With Varying Extracellular Na⁺ Concentration

<table>
<thead>
<tr>
<th>Figure</th>
<th>Time (min)</th>
<th>[Na⁺]o (mM)</th>
<th>Cation</th>
<th>Diltiazem (µM)</th>
<th>Histamine (µM)</th>
<th>Stress/[Ca²⁺] ratio (µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>1.2</td>
<td>Choline</td>
<td>...</td>
<td>...</td>
<td>2.88±0.26</td>
</tr>
<tr>
<td>1</td>
<td>130</td>
<td>1.2</td>
<td>Choline</td>
<td>10</td>
<td>...</td>
<td>0.78±0.17</td>
</tr>
<tr>
<td>1</td>
<td>140</td>
<td>1.2</td>
<td>Choline</td>
<td>10</td>
<td>100</td>
<td>3.91±0.24</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>140</td>
<td>Choline</td>
<td>...</td>
<td>100</td>
<td>5.30±0.62</td>
</tr>
<tr>
<td>1</td>
<td>150</td>
<td>140</td>
<td>Lithium</td>
<td>...</td>
<td>100</td>
<td>4.65±0.26</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>1.2</td>
<td>Lithium</td>
<td>...</td>
<td>100</td>
<td>4.74±0.33</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>1.2</td>
<td>Lithium</td>
<td>...</td>
<td>100</td>
<td>5.30±0.43</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>140</td>
<td>Lithium</td>
<td>...</td>
<td>100</td>
<td>6.03±0.20</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>140</td>
<td>Lithium</td>
<td>...</td>
<td>100</td>
<td>6.33±0.20</td>
</tr>
</tbody>
</table>

At specific time points (always before the succeeding solution change and at least 9 minutes after the prior solution change), normalized stress and estimated intracellular Ca²⁺ concentration ([Ca²⁺]) (in µM) were calculated from the data shown in Figures 1 and 2. The stress/[Ca²⁺] ratio was then calculated and changes in stress/[Ca²⁺] ratios were compared by the Neuman-Keuls test. [Na⁺], extracellular sodium concentration.

*p<0.01 significant difference among the three groups. Within each of the above three groups the values were not significantly different.

is a potential mechanism for decreasing stimulus-induced elevations in [Ca²⁺], (Figure 1; see the decrease in [Ca²⁺], and force after restoration of extracellular Na⁺ in the presence of diltiazem and histamine). High doses of a putative endogenous inhibitor of the Na⁺,K⁺-ATPase (ouabain) slightly accentuated agonist-induced increases in [Ca²⁺], and stress (Figure 4). This ouabain effect was of marginal significance. These data suggest that inhibitors of the Na⁺,K⁺-ATPase could enhance smooth muscle contractility by directly acting on smooth muscle. However, the contractile effects of ouabain in the absence of α-blockade were seen at much lower ouabain doses than the marginally significant enhancement of histamine-induced contraction (Figure 5). These data suggest that the primary action of ouabain in the swine carotid was to enhance release of endogenous norepinephrine.

It should be noted that endogenous levels of the Na⁺-K⁺ inhibitor appear to be much lower (less than 1 nM) than the ouabain doses (0.3–10 µM) used in this current study. Low and potentially physiological doses of ouabain (0.1 nM) have been shown to inhibit endothelial-dependent relaxation in human resistance arteries. The effects of ouabain on norepinephrine release or directly on smooth muscle may occur only at supra...
FIGURE 4. Line graphs show change in log L/L_{max} (myoplasmic [Ca^{2+}]) and active stress observed on three successive 10-minute submaximal stimulations with 1 μM histamine (Hist.) at 10, 40, and 70 minutes. Ouabain (10 μM) was added at 25 minutes and washed out at 50 minutes. Phentolamine (10 μM) was present throughout the experiment to inhibit the effects of norepinephrine release. Data is presented as mean (solid lines) ±1 SEM (dotted lines) averaged at 30-second intervals to decrease noise with n=4 for each set of tissues. Changes in log L/L_{max} and stress was compared by analysis of variance and revealed no significant differences between the three contractions. However, changes in log L/L_{max} and stress were significantly larger (p<0.05) when the second contraction (with ouabain) was compared with the average of the first and third with Student's paired t test.

This study also demonstrated that the [Ca^{2+}]_{i} sensitivity induced by reversal of the Na^{+}-Ca^{2+} exchanger was low. There are two mechanisms proposed that could alter [Ca^{2+}]_{i} sensitivity: 1) Stull et al.\(^{28}\) have proposed that large increases in [Ca^{2+}]_{i} can activate Ca^{2+}-calmodulin-dependent protein kinase II, which is known to phosphorylate and thereby decrease the Ca^{2+}-sensitivity of myosin light chain kinase. Stull et al.\(^{28}\) have proposed that large increases in [Ca^{2+}]_{i} can activate Ca^{2+}-calmodulin-dependent protein kinase II, which is known to phosphorylate and thereby decrease the Ca^{2+}-sensitivity of myosin light chain kinase. We have found that the Ca^{2+}-sensitivity of myosin light chain kinase extracted from depolarized smooth muscle tissues is lower than the Ca^{2+}-sensitivity of myosin light chain kinase extracted from unstimulated or histamine-stimulated tissues.\(^{32}\) It is likely that reversal of the Na^{+}-Ca^{2+} exchanger by removal of extracellular Na^{+} induces a lower [Ca^{2+}]_{i} sensitivity (Figure 1) in the same manner as KCl depolarization: the large increases in [Ca^{2+}]_{i} induce myosin light chain kinase phosphorylation and thereby decrease the Ca^{2+}-sensitivity of myosin light chain kinase.

2) Activation of G proteins by contractile agonists, GTP analogues, or AlF_{4}^{-} can increase [Ca^{2+}]_{i} sensitivity.\(^{19,23-35}\) Several groups have suggested that G protein activation could decrease myosin light chain phosphatase activity, resulting in increased myosin light chain phosphorylation despite similar changes in [Ca^{2+}]_{i}; however, data on regulated phosphatases have yet to be presented except in abstract form.\(^{36}\) This mechanism could explain the increase in [Ca^{2+}]_{i} sensitivity observed when histamine was added to the 1.2 mM Na^{+}-induced contraction (Table 1).

In conclusion, Na^{+}-Ca^{2+} exchange appears to exist in swine arterial smooth muscle. Despite large increases in [Ca^{2+}]_{i}, derived from reversal of Na^{+}-Ca^{2+} exchange, these increases in [Ca^{2+}]_{i} are associated with a low [Ca^{2+}]_{i} sensitivity. Inhibition of the Na^{+},K^{+}-ATPase with ouabain (which increases [Na^{+}]_{i}) and therefore should inhibit Na^{+}-Ca^{2+} exchange appears to primarily enhance contractile function in the swine carotid artery by enhancing norepinephrine release from nerve terminals rather than a direct action on smooth muscle per se, although both actions require high and potentially supraphysiological concentrations of ouabain.

Acknowledgment

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