Na\(^+\) entry via store-operated channels modulates Ca\(^{2+}\) signaling in arterial myocytes

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Arnon, Assaf, John M. Hamlyn, and Mordecai P. Blaustein. Na\(^+\) entry via store-operated channels modulates Ca\(^{2+}\) signaling in arterial myocytes. Am. J. Physiol. Cell Physiol. 278: C163–C173, 2000.—In many nonexcitable cells, hormones and neurotransmitters activate Na\(^+\) influx and mobilize Ca\(^{2+}\) from intracellular stores. The stores are replenished by Ca\(^{2+}\) influx via “store-operated” Ca\(^{2+}\) channels (SOC). The main routes of Na\(^+\) entry in these cells are unresolved, and no role for Na\(^+\) in signaling has been recognized. We demonstrate that the SOC are a major Na\(^+\) entry route in arterial myocytes. Unloading of the Ca\(^{2+}\) stores with cyclopiazonic acid (a sarcoplasmic reticulum Ca\(^{2+}\) pump inhibitor) and caffeine induces a large external Na\(^+\)-dependent rise in the cytosolic Na\(^+\) concentration. One component of this rise in cytosolic Na\(^+\) concentration is likely due to Na\(^+\)/Ca\(^{2+}\) exchange; it depends on elevation of cytosolic Ca\(^{2+}\) and is insensitive to 10 mM Mg\(^{2+}\) and 10 µM La\(^{3+}\). Another component is inhibited by Mg\(^{2+}\) and La\(^{3+}\), blockers of SOC; this component persists in cells preloaded with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid to buffer Ca\(^{2+}\) transients and prevent Na\(^+\)/Ca\(^{2+}\) exchange-mediated Na\(^+\) entry. This Na\(^+\) entry apparently is mediated by SOC. The Na\(^+\) entry influences Na\(^+\)/pump activity and Na\(^+\)/Ca\(^{2+}\) exchange and has unexpectedly large effects on cell-wide Ca\(^{2+}\) signaling. The SOC pathway may be a general mechanism by which Na\(^+\) participates in signaling in many types of cells.

ACTIVATION OF MOST ANIMAL cells by hormones or other agents usually elevates the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)), which, in turn, triggers a variety of biological responses (3, 8). Some of this “signal Ca\(^{2+}\)” enters cells from the extracellular space, and some is released from intracellular Ca\(^{2+}\) stores in the sarcoplasmic (in muscle) or endoplasmic reticulum (S/ER) (3, 8). Emptying of the Ca\(^{2+}\) stores initiates a mechanism to refill the stores, termed “capacitative Ca\(^{2+}\) entry” (CCE) (36), that is mediated by “store-operated” channels (SOC) located in the plasmalemma (PL) (50). This widely distributed mechanism (10) has been reported to occur in vascular smooth muscle cells (31, 47), where it may play an important role in the regulation of tonic vascular tension (“tone”) (11). Several types of SOC have been proposed as mediators of S/ER Ca\(^{2+}\) store depletion-dependent Ca\(^{2+}\) entry. These include the Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channel (17, 23, 24) and various other channels (16, 27, 29), some of which are homologous to the transient receptor potential (TRP) and TRP-like (TRPL) channels of Drosophila photoreceptors (25, 35, 50, 51).

In mammals, recent observations suggest that most SOC are permeable to Na\(^+\) as well as Ca\(^{2+}\) (23, 35, 50), with permeability ratios (P\(_{Ca}/P_{Na}\)) usually on the order of 10:1 under normal physiological conditions (35, 50), although relatively nonelective cation channels have also been described (25, 26, 51). Thus, with extracellular Ca\(^{2+}\) and Na\(^+\) concentrations ([Ca\(^{2+}\)]\(_{o}\) and [Na\(^+\)]\(_{o}\)) on the order of 1 and 150 mM, respectively, large amounts of Na\(^+\) may enter the cells through SOC. The physiological significance of this potentially important Na\(^+\) entry pathway has not attracted attention. Although many hormones and neurotransmitters mobilize sarcoplasmic reticulum (SR) Ca\(^{2+}\) and activate vascular smooth muscle cells, they also promote Na\(^+\) entry (6, 7) via unknown pathways. Vascular smooth muscle cells have few, if any, “classical” voltage-gated Na\(^+\) channels (44, 48) and do not exhibit Na\(^+\)-dependent action potentials. This raises the possibility that the Na\(^+\) entry evoked by hormones and neurotransmitters in these cells may be mediated, in part, by SOC.

Here we use digital imaging methods to investigate SOC in mesenteric artery myocytes and to determine whether these channels influence Na\(^+\), as well as Ca\(^{2+}\), homeostasis. We show that SOC mediate the entry of Na\(^+\) and Ca\(^{2+}\) measured as changes in cytosolic Na\(^+\) concentration ([Na\(^+\)]\(_{cyt}\)) and [Ca\(^{2+}\)]\(_{cyt}\) with Na\(^+\)-binding benzofuran isophthalate (SBFI) and fura 2, respectively.1 Furthermore, Na\(^+\) entry through the SOC apparently augments whole cell Ca\(^{2+}\) signaling by reducing the extrusion of Ca\(^{2+}\) in these cells; this effect is especially pronounced when the activity of a subset of Na\(^+\) pumps is suppressed. SOC also appear to be an important route of Na\(^+\) entry in arterial myocytes at rest.

MATERIALS AND METHODS

Primary cell culture. Mesenteric artery myocytes were obtained from female Sprague-Dawley rats (150–200 g) and

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were grown in primary culture for 4–6 days, as described previously (40).

Measurement of \([Ca^{2+}]_{cyt}\) and \([Na^+]_{cyt}\). The cells, on 25-mm coverslips, were loaded with fura 2 or SBI-F by incubation with 3.3μM fura 2-AM for 40 min or 10μM SBI-F-AM (TEFLABS, Austin, TX) for 1 h at 22–25°C. \([Ca^{2+}]_{cyt}\) and \([Na^+]_{cyt}\) were then studied with digital imaging methods (6, 13). The coverslips were mounted in a 1-ml chamber on the stage of an inverted fluorescence microscope. The cells were superfused at a rate of 5 ml/min (chamber washout half time ~30 s) with a physiological salt solution (PSS). Experiments were conducted at 32°C. Before the experimental protocols were started, cells were superfused for 40 min with PSS to wash away extracellular dye and permit the intracellular esterases to cleave the fura 2-AM or SBI-F-AM.

Fura 2 was calibrated as described previously (13). SBI-F, which can detect 0.5 mM changes in \([Na^+]_{cyt}\) (6), was calibrated after each experiment (32). Digital images of fields containing five to eight cells were acquired, background subtracted, and transformed to \(Ca^{2+}\) (or \(Na^+\)) concentration images with a MetaFluor Imaging System (Universal Imaging, West Chester, PA). Fluorescence ratio data for each cell were obtained from a 5 × 5 pixel (1.5 × 1.5 μm) nonnuclear region (1/cell). To enhance the signal-to-noise ratio, 32 consecutive frames were averaged at video frame rate, except when agonist and/or ouabain were added; then only 4 frames were averaged to enhance temporal resolution. Images were acquired at a rate of two per minute, except at times of agonist application, when the rate was increased to two per second.

Solutions and reagents. The normal PSS contained (in mM) 140 NaCl, 5.0 KCl, 1.2 NaH2PO4, 5 NaHCO3, 1.4 MgCl2, 1.8 CaCl2, 11.5 glucose, and 10 mM HEPES (titrated to pH 7.4 with NaOH). CaCl2 was omitted from the \(Ca^{2+}\)-free PSS, and 0.2 mM EGTA was added. The Ca2+-free, 5 mM Na+ PSS (0 Ca2+-5 Na+ PSS) contained 5 mM NaCl and 140 mM N-methylglucamine. NaH2PO4 and NaHCO3 were omitted from PSS containing LaCl3. The osmolarity of all solutions was adjusted to 320 mosM with sucrose. In some experiments (see results), cells were preloaded with 1,2-bis(2-aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid (BAPTA) by incubation with 20 μM BAPTA-AM for 30 min at 22–25°C.

ANG II was purchased from Peninsula Laboratories (Belmont, CA). All other compounds were reagent grade or the highest grade available and were purchased from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Data analysis and statistics. Data (ratios and calibrated values) were analyzed and plotted with Sigma Plot software (Jandel, San Rafael, CA). Significance of differences between means of different groups (means ± SE) was calculated by Student’s paired t-test.

RESULTS

Characteristics of SOC in arterial myocytes: studies with fura 2. Some properties of SOC in rat mesenteric artery myocytes, as indicated by changes in \([Ca^{2+}]_{cyt}\), are illustrated in Figs. 1 and 2. The SOC are activated by unloading \(Ca^{2+}\) stores with 10μM cyclopiazonic acid (CPA), which inhibits S/ER \(Ca^{2+}\)-ATPase (SERCA) (18), and 10 μM caffeine (Caff), which induces \(Ca^{2+}\) release through ryanodine-sensitive S/ER channels (30) (Fig. 1, A and B). SR \(Ca^{2+}\) also is mobilized by neurotransmitters and hormones such as serotonin, vasopressin, phenylephrine (PE), and ANG II (31, 47; see below); this, too, opens SOC in vascular smooth muscle cells (31, 47; see below).

In \(Ca^{2+}\)-free (0 μM \(Ca^{2+}\)) medium, emptying the stores with CPA + Caff caused \([Ca^{2+}]_{cyt}\) to rise rapidly and then decline below the original resting level (Fig. 1A). When extracellular \(Ca^{2+}\) was restored in the continued presence of CPA + Caff, to prevent store refilling (and with 10 μM nifedipine to block L-type \(Ca^{2+}\)-channels), a second large \(Ca^{2+}\) transient, the “hallmark” of SOC activation and CCE (36), was observed. This transient depended on store \(Ca^{2+}\) depletion and on external \(Ca^{2+}\). The \([Ca^{2+}]_{cyt}\) needed to evoke half-maximal \(Ca^{2+}\) transients was 1.1 mM (Fig. 1D), consistent with reported EC50 values for \(Ca^{2+}\) entry through SOC (~0.8–3.3 mM) (9, 17). These \([Ca^{2+}]_{cyt}\)-dependent \(Ca^{2+}\) transients were blocked reversibly by 10 mM Mg2+ (Fig. 1, A and C) and 10 μM La3+ (Fig. 1, B and C), known blockers of SOC (12, 35, 46, 47). Neither Mg2+ nor La3+ inhibits agonist-evoked \(Ca^{2+}\) transients in normal medium (2, 38, 46; see below). Moreover, the CPA- and agonist-evoked \(Ca^{2+}\) transients were prevented by pretreatment with the \(Ca^{2+}\) chelator BAPTA (see below), which buffers intracellular \(Ca^{2+}\) (17, 28, 29).

To test for inactivation of the SOC (23, 24, 46), external \(Ca^{2+}\) was restored for 10–15 min in the presence of CPA + Caff. \([Ca^{2+}]_{cyt}\) rapidly rose to a peak and then declined (by ~13%) to a plateau that was maintained for ~10–15 min (Fig. 2A). This decline might reflect accelerated \(Ca^{2+}\) sequestration (e.g., in mitochondria) and/or extrusion (26), as well as some (limited) inactivation. There was, however, no long-term inactivation: the peak amplitudes of the \(Ca^{2+}\) transients remained constant when the cells were repetitively exposed (for ~2 min) to \(Ca^{2+}\)-containing medium at ~30-min intervals (Fig. 2B).

\(Na^+\) entry mediated by the \(Na^+/Ca^{2+}\) exchanger: studies with SBI-F. Figures 3–5 present evidence that \(Ca^{2+}\) store unloading also activates \(Na^+\) entry. Figure 3 shows data from an experiment analogous to that shown in Fig. 1A, except \(Na^+\) entry was estimated by measuring changes in the cytosolic \(Na^+\) concentration (\([Na^+]_{cyt}\)) with the \(Na^+\)-sensitive dye SBI-F (6). Here, SOC were blocked with 10 mM Mg2+ (Fig. 1, A and C) or 10 μM La3+ (Fig. 1, B and C) in the presence of the \(Ca^{2+}\) and \(Na^+\) channel blockers nifedipine (10 μM) and tetrodotoxin (50 μM). When the \(Ca^{2+}\) stores were unloaded with CPA + Caff in 0 \(Ca^{2+}\)-PSS, there was an initial transient rise in \([Na^+]_{cyt}\) as well as \([Ca^{2+}]_{cyt}\) (not observed here, but see Fig. 1, A and B). This first evoked \(Na^+\) transient appeared to be the result of \(Na^+/Ca^{2+}\) exchange, because it depended on external \(Na^+\) (Figs. 4A and 5B) and on the rise in \([Ca^{2+}]_{cyt}\). This \(Na^+\) transient was insensitive to 10 mM Mg2+ (Figs. 3 and 5B) or 10 μM La3+ (Fig. 5B), but it was eliminated when the \(Ca^{2+}\) released from the stores was buffered with BAPTA (Figs. 4, B and C, and 5B). The top \([Ca^{2+}]_{cyt}\) record in Fig. 4C confirms that intracellular BAPTA abolished the evoked \(Ca^{2+}\) transient. In other words, this \(Na^+\) transient did not depend directly on
the unloading of the SR Ca\(^{2+}\) stores but, rather, on the rise in [Ca\(^{2+}\)\(_{\text{cyt}}\)].

In most experiments, 0 Ca\(^{2+}\) PSS was employed (Figs. 3A and 5B) to rule out the participation of an external Ca\(^{2+}\)-dependent mechanism, but a similar 10 mM Mg\(^{2+}\)-insensitive Na\(^{+}\) transient was observed in PSS with 1.8 mM Ca\(^{2+}\) (not shown). Inhibition of Na\(^{+}\)/Ca\(^{2+}\) exchange by removal of external Na\(^{+}\) or antisense knockdown of the exchanger greatly augments and prolongs the Ca\(^{2+}\) transients evoked by store unloading (38, 39), implying that much of the decline of [Ca\(^{2+}\)\(_{\text{cyt}}\)] is mediated by the Na\(^{+}\)/Ca\(^{2+}\) exchanger. Neither 10 mM Mg\(^{2+}\) (unpublished data) nor 10 µM La\(^{3+}\) (38) interferes with Ca\(^{2+}\) extrusion via the Na\(^{+}\)/Ca\(^{2+}\) exchanger. Therefore, the initial rise in [Na\(^{+}\)\(_{\text{cyt}}\)] after store depletion (Fig. 3) apparently results from Na\(^{+}\)/Ca\(^{2+}\) exchange-mediated Na\(^{+}\) entry (NCXNE) that is promoted by the elevation of [Ca\(^{2+}\)\(_{\text{cyt}}\)].
Na⁺ entry mediated by SOC: studies with SBFI. When the SOC were subsequently unblocked by reducing Mg²⁺ to 1.4 mM (Figs. 3 and 4, B–E) or removing La³⁺ (Fig. 5A), a second large transient rise in [Na⁺]_{cyt} was induced. This Na⁺ transient also was abolished reversibly when [Na⁺]_{o} was reduced to 5 mM (Figs. 4D and 5A), but, unlike the first Na⁺ transient in Fig. 3 (also see Fig. 5B), the second Na⁺ transient was blocked by 10 mM Mg²⁺ and by 10 µM La³⁺ (Fig. 5A). Furthermore, this second Na⁺ transient (Fig. 3) was

![Fig. 3](http://ajpcell.physiology.org/)

Fig. 3. Effect of unloading sarcoplasmic reticulum (SR) Ca²⁺ stores with CPA (10 µM) + Caff (10 mM) on cytosolic Na⁺ concentration ([Na⁺]_{cyt}) in a cell bathed in 0 Ca²⁺ PSS containing 10 or 1.4 mM external Mg²⁺ ([Mg²⁺]_{o}; horizontal bar below record in A). Solution changes are indicated by horizontal bars below records. All solutions contained 10 µM nifedipine to block voltage-gated, L-type Ca²⁺ channels and 50 µM tetrodotoxin (TTX) to block voltage-gated Na⁺ channels. A: time course for data from B. B–E: “Na⁺ images” for times indicated by a–e in A. Unlabeled panel (top left) is an original fluorescent image (340-nm excitation) of 2 cells; horizontal bar, 5 µm. Small white boxes surround 5 × 5 pixel areas used for analysis (see A and Fig. 5). SFBI, Na⁺-binding benzofuran isophthalate.

![Fig. 4](http://ajpcell.physiology.org/)

Fig. 4. A: effect of extracellular Na⁺ concentration ([Na⁺]_{o}) on rise of [Na⁺]_{cyt} evoked by unloading Ca²⁺ stores in 0 Ca²⁺ PSS containing 10 mM Mg²⁺. B: effect of preincubation with 20 µM 1,2-bis(2-aminophenoxy)ethane-N,N',N",N"-tetraacetic acid (BAPTA) on rise of [Na⁺]_{cyt} evoked by unloading Ca²⁺ stores in 0 Ca²⁺ PSS at 10 and 1.4 mM Mg²⁺. C: effects of BAPTA preincubation on increases of [Na⁺]_{cyt} (bottom record) and [Ca²⁺]_{cyt} (top record, parallel experiment) evoked by unloading Ca²⁺ stores in PSS at 10 and 1.4 mM Mg²⁺. D: effect of [Na⁺]_{o} on rise of [Na⁺]_{cyt} evoked by unloading Ca²⁺ stores in 0 Ca²⁺ PSS containing 10 or 1.4 mM Mg²⁺ in BAPTA-pretreated cells. E: effects of 15 min of incubation in 0 Ca²⁺ PSS on increases of [Na⁺]_{cyt} (bottom record) and [Ca²⁺]_{cyt} (top record, parallel experiment) evoked by lowering [Mg²⁺]_{o} to 1.4 mM and (for [Ca²⁺]_{cyt} measurement only) adding back Ca²⁺ (= PSS). F: data from experiments similar to E, except cells were incubated in 0 Ca²⁺ for only 1 min before [Mg²⁺]_{o} was lowered to 1.4 mM. Time course curves are data from single representative cells; summary data are presented in Fig. 5. All solutions contained 10 µM nifedipine to block voltage-gated, L-type Ca²⁺ channels and 50 µM TTX to block voltage-gated Na⁺ channels. Solution changes are indicated by horizontal bars below records.
observed when cytosolic Ca\(^{2+}\) was buffered with BAPTA (Figs. 4, B–D, and 5A; see top record in Fig. 4C); thus it depended on Ca\(^{2+}\) store depletion and not on the rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\). Also it did not require the reintroduction of external Ca\(^{2+}\) after the stores were unloaded (Figs. 4, B and D, and 5A). These results are consistent with the view that the second Na\(^{+}\) transient in Fig. 3 is the result of Na\(^{+}\) entry through SOC (i.e., SOC-mediated Na\(^{+}\) entry (SOCNE)).

SOCNE also was stimulated when the Ca\(^{2+}\) stores were depleted by nonpharmacological means. Incubation in 0 Ca\(^{2+}\) PSS (with 10 mM Mg\(^{2+}\), to block SOC) for 15 min to deplete the Ca\(^{2+}\) stores by slow “leakage” and extrusion of Ca\(^{2+}\) led to a slow decline in [Na\(^{+}\)]\(_{\text{cyt}}\) (Fig. 4E, bottom record). The subsequent lowering of extracellular Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]\(_{\text{o}}\)) from 10 to 1.4 mM evoked a rise in [Na\(^{+}\)]\(_{\text{cyt}}\) (Figs. 4E, bottom record, and 5A). In parallel experiments on cells from the same cultures, the simultaneous reintroduction of external Ca\(^{2+}\) induced a Ca\(^{2+}\) transient, indicating that the SOCs were activated (Fig. 4E; top record; Ca\(^{2+}\) was not restored for the [Na\(^{+}\)]\(_{\text{cyt}}\) measurement to prevent NCXNE). In contrast, when Mg\(^{2+}\) was lowered from 10 to 1.4 mM after only 1 min of incubation in 0 Ca\(^{2+}\) PSS, reintroduction of external Ca\(^{2+}\) had little effect on [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Fig. 4F, top record) or [Na\(^{+}\)]\(_{\text{cyt}}\) (Fig. 4F, bottom record), indicating that the stores had not yet unloaded and most SOC were still closed, so that SOCNE was not stimulated. With continued incubation in 0 Ca\(^{2+}\) PSS, however, [Na\(^{+}\)]\(_{\text{cyt}}\) rose slowly (Fig. 4F, bottom record), reflecting progressive depletion of the Ca\(^{2+}\) stores and some stimulation of SOCNE and a consequent rise in [Na\(^{+}\)]\(_{\text{cyt}}\).

Some Na\(^{+}\) enters unstimulated myocytes through SOC. In resting cells incubated in PSS, the mean [Na\(^{+}\)]\(_{\text{cyt}}\) (8.7 ± 0.1 mM, n = 385 cells; Fig. 5A) was constant. Elevation of [Mg\(^{2+}\)]\(_{\text{o}}\) to 10 mM in standard PSS with Ca\(^{2+}\) (Figs. 4C and 6, A and B) or in 0 Ca\(^{2+}\) PSS (Fig. 4E) caused [Na\(^{+}\)]\(_{\text{cyt}}\) in the unstimulated cells to decline slowly to a new steady level. Over the course of 40–60 min, [Na\(^{+}\)]\(_{\text{cyt}}\) reversibly declined by ~50%, to 5.1 ± 0.4 mM when [Mg\(^{2+}\)]\(_{\text{o}}\) was elevated from 1.4 to 10 mM Mg\(^{2+}\) in the PSS (Fig. 4, A and B). The final steady [Na\(^{+}\)]\(_{\text{cyt}}\) was inversely dependent on [Mg\(^{2+}\)]\(_{\text{o}}\), with apparent half-maximal decline of [Na\(^{+}\)]\(_{\text{cyt}}\) observed at [Mg\(^{2+}\)]\(_{\text{o}}\) of 3.0 mM (Fig. 6C). Comparable results were obtained when the SOC were inhibited with 10 µM La\(^{3+}\) (Fig. 6B). Indeed, when 10 µM La\(^{3+}\) was added to PSS that already contained 10 mM Mg\(^{2+}\), no further effect on [Na\(^{+}\)]\(_{\text{cyt}}\) was observed (Fig. 6B), which implies that the effects of these two cations on SOCNE are not additive. These results suggest that some SOC are open at rest and that SOCNE contributes to the resting [Na\(^{+}\)]\(_{\text{cyt}}\) in mesenteric artery myocytes under normal physiological conditions. We should, however, ignore other possible routes of Na\(^{+}\) entry that might also be inhibited by La\(^{3+}\) and high [Mg\(^{2+}\)]\(_{\text{o}}\), such as nonselective cation channels and Na\(^{+}\)/Mg\(^{2+}\) exchange. Also, we have not ruled out the possibility that these polyvalent cations might affect the membrane potential and, thus, the driving force for Na\(^{+}\) entry. There is no evidence, however, that the myocytes possess nonselective cation channels (see Relative permeabilities of SOC to Ca\(^{2+}\) and Na\(^{+}\)) or that La\(^{3+}\) inhibits Na\(^{+}\)/Mg\(^{2+}\) exchange, and it seems unlikely that 10 mM Mg\(^{2+}\) and 10 µM La\(^{3+}\) will have comparable effects on the membrane potential (indeed, neither cation affected the evoked Ca\(^{2+}\) transients; see Possible role of SOC in the augmentation of Ca\(^{2+}\) transients by low-dose ouabain).

Relative permeabilities of SOC to Ca\(^{2+}\) and Na\(^{+}\). The data in Figs. 3–5 demonstrate that depletion of SR Ca\(^{2+}\) stores induces a rise in [Na\(^{+}\)]\(_{\text{cyt}}\) by a process with the same properties as those of SOC. Therefore, the rising phases of the Ca\(^{2+}\) and Na\(^{+}\) transients evoked by Ca\(^{2+}\) store depletion were used to estimate P\(_{\text{Ca}}\)/P\(_{\text{Na}}\). The

### Table 1. Effects of Mg\(^{2+}\) on Na\(^{+}\) Entry

<table>
<thead>
<tr>
<th>Condition</th>
<th>Na(^{+}) Entry</th>
<th>P(<em>{\text{Ca}})/P(</em>{\text{Na}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.0 ± 0.1 mM</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Mg(^{2+}) 0.1 mM</td>
<td>10.0 ± 0.1 mM</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Mg(^{2+}) 1.4 mM</td>
<td>9.0 ± 0.1 mM</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Mg(^{2+}) 10.0 mM</td>
<td>8.0 ± 0.1 mM</td>
<td>0.8 ± 0.2</td>
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### Figure 5. Summary of effects of Na\(^{+}\), Mg\(^{2+}\), La\(^{3+}\), and BAPTA on resting and transient peak [Na\(^{+}\)]\(_{\text{cyt}}\) during SOC-mediated Na\(^{+}\) entry (SOCNE) and Na\(^{+}\)/Ca\(^{2+}\) exchange (NCXNE)-mediated Na\(^{+}\) entry (NCXNE) (see Figs. 3 and 4 for representative original experiments). Left-hand bar and thin horizontal line correspond to resting ([Na\(^{+}\)]\(_{\text{cyt}}\) 8.7 ± 0.1 mM, n = 385 cells). A: effects of 5 mM Na\(^{+}\), 10 mM Mg\(^{2+}\), 10 µM La\(^{3+}\), and pretreatment with 20 µM BAPTA on SOC-mediated Na\(^{+}\) transients (SOCNE) induced by 10 µM CPA + 10 mM Caff. Effects of Ca\(^{2+}\) depletion by 1 and 15 min of incubation in 0 Ca\(^{2+}\) PSS before reducing Mg\(^{2+}\) from 10 to 1.4 mM are also shown (2 right-hand bars). B: effects of 5 mM Na\(^{+}\), 10 mM Mg\(^{2+}\), 10 µM La\(^{3+}\), and pretreatment with 20 µM BAPTA on NCXNE induced by 10 µM CPA + 10 mM Caff. In all experiments, all solutions contained 10 µM nifedipine to block voltage-gated, L-type Ca\(^{2+}\) channels and 50 µM TTX to block voltage-gated Na\(^{+}\) channels. Solution changes are indicated by horizontal bars below records. Error bars, SE; numbers of cells observed for each condition are in parentheses. *Significantly different from control (P < 0.01). Bars labeled “control” in A and B represent control [Na\(^{+}\)]\(_{\text{cyt}}\) transients.
volume ratios were assumed to be constant. The permeabilities (P) were calculated as follows: $P = -J/C$ (cm/s), where $J$ is molar flux (mol·cm$^{-2}$·s$^{-1}$) and $C$ is the difference between extracellular and cytosolic ion concentration (15).

The calculated $P_{Ca}/P_{Na}$ of 8.2 ± 1.0 in PSS containing 1.8 mM Ca$^{2+}$ is comparable to values obtained in electrophysiological studies on TRP/TRPL channels, ~8–10:1 (35, 46). Moreover, measurements of the maximum rate of rise of $[Na]_{cyt}$ in BAPTA-loaded cells (i.e., in the absence of NCXNE; Fig. 8) as a function of $[Ca^{2+}]_{o}$ indicated that $P_{Na}$ was reduced nearly sixfold as $[Ca^{2+}]_{o}$ was increased from 0 to 10 mM. This is consistent with the low monovalent cation permeability of CRAC channels measured in high-Ca$^{2+}$ medium (17).

Rise in $[Na]_{cyt}$ mediated by SOCNE stimulates the Na$^+$ pump. The transiency of the SOCNE-mediated rise in $[Na]_{cyt}$ (Fig. 3) was unexpected, because SOC exhibits little inactivation (Fig. 2, A and B). To determine whether the decline in $[Na]_{cyt}$ during SOCNE (Figs. 3 and 4, A and C–E) might reflect accelerated rates of rise of $[Ca^{2+}]_{o}$, (Fig. 1A) and $[Na]_{cyt}$ (Fig. 7B) in PSS, during the 2nd min after $[Mg^{2+}]_{o}$ was lowered from 10 to 1.4 mM, were used to estimate $P_{Ca}$ (6.48 ± 0.80 × 10$^{-6}$ cm/s, n = 43 cells) and $P_{Na}$ (0.79 ± 0.23 × 10$^{-6}$ cm/s, n = 37 cells). The $[Na]_{cyt}$ data were obtained in cells preloaded with BAPTA (to eliminate NCXNE) with 1 mM ouabain present (to avoid underestimating SOCNE because of Na$^+$ pump-mediated Na$^+$ extrusion); we did not, however, compensate for possible Ca$^{2+}$ efflux via the PL Ca$^{2+}$ pump, which may have caused us to underestimate $P_{Ca}$. Inhibition of virtually all the Na$^+$ pumps with 1 mM ouabain (4) in the absence of CPA and Caff induced comparable elevation of $[Na]_{cyt}$ (not shown, but see Ref. 6.) Cell surface-to-
Na⁺ extrusion by PL Na⁺ pumps, we used 1 mM ouabain to inhibit all Na⁺ pumps (4). With NCXNE blocked by BAPTA, activation of SOCNE by reducing [Mg²⁺]₀ from 10 to 1.4 mM caused [Na⁺]₀ to rise at a rate of 0.25 mM/min, whereas in the presence of 1 mM ouabain, this rate doubled to 0.51 mM/min (Fig. 7, A and C); the difference between these two rates (0.26 mM/min) also reflects ouabain-sensitive Na⁺ pump activity (Fig. 7C). Thus the Na⁺ pump rate increased almost threefold (from 0.26 to 0.74 mM/min) during the course of the SOCNE transient. This may reflect modulation of Na⁺ pump activity (4).

Possible role of SOC in the augmentation of Ca²⁺ transients by low-dose ouabain. The Ca²⁺ transients evoked by a 30-s exposure to 10 nM ANG II (Fig. 9, A and D) and 100 nM PE (Fig. 9D) were significantly augmented by the simultaneous application (i.e., for 30 s) of 100 nM ouabain. There was, however, no measur-
The increase in whole cell \([\text{Na}^+]_{\text{cyt}}\) during these brief exposures to the low concentrations of agonists and ouabain (not shown). Nevertheless, the augmentation of the \(\text{Ca}^{2+}\) transients by ouabain depended on external \(\text{Na}^+\), but not \(\text{Ca}^{2+}\) (Fig. 9, B and C). This augmentation may be mediated by SOC, because it was abolished by 10 mM Mg\(^{2+}\) and 10 \(\mu\text{M} \text{La}^{3+}\). In contrast, the response to ANG II or PE, alone (i.e., the “controls”), were unaffected by these cations (Fig. 9, A and D).

**DISCUSSION**

SOC and \(\text{Ca}^{2+}\) entry in arterial myocytes. SOC, which apparently serve as a mechanism for replenishing depleted S/ER \(\text{Ca}^{2+}\) stores, have been studied in many types of cells (50), including vascular smooth muscle cells (31, 47). We examined the properties of SOC in primary cultured rat mesenteric artery myocytes by measuring changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) with fura 2. When SR \(\text{Ca}^{2+}\) was unloaded with CPA + Caff in cells bathed in \(\text{Ca}^{2+}\)-free media, a brief rise in \([\text{Ca}^{2+}]_{\text{cyt}}\), a rapid recovery, and then a decline below the original resting level occurred. The restoration of extracellular \(\text{Ca}^{2+}\) evoked a larger rise in \([\text{Ca}^{2+}]_{\text{cyt}}\), indicative of \(\text{Ca}^{2+}\) entry through SOC (36). This external \(\text{Ca}^{2+}\)-dependent rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) was half-maximally activated by \([\text{Ca}^{2+}]_{o}\) of 1.1 mM and was prevented by pretreating the cells with BAPTA to buffer the cytosolic \(\text{Ca}^{2+}\). Furthermore, the rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) was reversibly blocked by 10 mM Mg\(^{2+}\) or 10 \(\mu\text{M} \text{La}^{3+}\), known blockers of SOC (12, 35, 46). Thus the general properties of the SOC in the arterial myocytes appear comparable to those in many other types of cells (10).

The molecular nature of the channels responsible for SOC activity is not known. Several candidate channels have, however, been identified and characterized by molecular genetics and/or electrophysiological methods. These candidate channels include the CRAC channels of mast cells, leukocytes, and leukemia cells (17, 23, 24) and a variety of TRP and TRPL channels (25, 35, 46, 50, 51). Particularly important for the present investigation is the recent observation that many of these channels are permeable to \(\text{Na}^+\) as well as \(\text{Ca}^{2+}\) (23, 35, 46). Indeed, CRAC and TRP/TRPL channels may have sometimes been characterized by measuring the \(\text{Na}^+\) currents that they conduct, simply because these currents are larger than the respective \(\text{Ca}^{2+}\) currents under comparable conditions (23, 24, 46).

Two modes of \(\text{Na}^+\) entry in arterial myocytes. Many studies indicate that vascular smooth muscle cells express few, if any, classical tetrodotoxin-sensitive, voltage-gated \(\text{Na}^+\) channels. Indeed, rat mesenteric artery myocytes do not exhibit a depolarization-activated inward current (48). Moreover, in the present study we were unable to detect any effects of the \(\text{Na}^+\) channel opener veratridine (44) or the blocker tetrodotoxin on \([\text{Na}^+]_{\text{cyt}}\). Through what pathway(s), then, do \(\text{Na}^+\) enter these cells? Our results reveal two mechanistically distinct pathways through which \(\text{Na}^+\) can enter the myocytes after cell activation and show how these pathways can influence cell function.

One \(\text{Na}^+\) entry pathway depends on the rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) and is abolished when cytosolic \(\text{Ca}^{2+}\) is buffered by BAPTA. This \(\text{Na}^+\) entry mechanism is unaffected by 10 mM Mg\(^{2+}\) or 10 \(\mu\text{M} \text{La}^{3+}\). \(\text{Ca}^{2+}\) transients induced by unloading the SR \(\text{Ca}^{2+}\) stores in these cells are greatly prolonged when external \(\text{Na}^+\) is removed and \(\text{Ca}^{2+}\) extrusion via \(\text{Na}^+/\text{Ca}^{2+}\) exchange is prevented (38, 39). Thus it appears that one \(\text{Na}^+\) entry pathway (which can account for the first \(\text{Na}^+\) transient in Fig. 3A) is via the \(\text{Na}^+/\text{Ca}^{2+}\) exchanger operating in the \(\text{Na}^+\) entry/\(\text{Ca}^{2+}\) exit mode (i.e., NCXNE).

A second \(\text{Na}^+\) entry pathway depends on the unloading of the SR \(\text{Ca}^{2+}\) stores, but not on the rise in \([\text{Ca}^{2+}]_{\text{cyt}}\), as much as it is not inhibited by buffering the cytosolic \(\text{Ca}^{2+}\) with BAPTA. This pathway is inhibited by 10 mM Mg\(^{2+}\) and 10 \(\mu\text{M} \text{La}^{3+}\). These properties are consistent with the idea that the SOC are responsible for this \(\text{Na}^+\) entry. Our data also indicate that elevating external \(\text{Ca}^{2+}\) inhibits \(\text{Na}^+\) entry and that these channels have a \(P_{\text{Ca}}/P_{\text{Na}}\) of ~8, which is in the range of published values for some SOC channels (35, 46). Because many SOC are permeable to \(\text{Na}^+\), \([\text{Na}^+]_{o} \gg [\text{Ca}^{2+}]_{o}\), and arterial myocytes possess these channels, we might expect the SOC to be a significant \(\text{Na}^+\) entry pathway in these cells. Indeed, vasoconstrictors such as serotonin and vasopressin, which mobilize SR \(\text{Ca}^{2+}\) and evoke \(\text{Ca}^{2+}\) transients, also induce \(\text{Na}^+\) entry and elevate \([\text{Na}^+]_{\text{cyt}}\) (6, 7). Therefore, it seems surprising that the role of SOC in \(\text{Na}^+\) entry and \(\text{Na}^+\) homeostasis has not been appreciated.

\(\text{Na}^+\) entry through SOC and resting \([\text{Na}^+]_{\text{cyt}}\). SOC are normally activated by unloading the SR \(\text{Ca}^{2+}\) stores; nevertheless, some of these channels are likely to be spontaneously active under normal resting conditions when the \(\text{Ca}^{2+}\) stores are replete. In resting cells, we observed that \([\text{Na}^+]_{\text{cyt}}\) was markedly influenced by \([\text{Mg}^{2+}]_{o}\): lowering \([\text{Mg}^{2+}]_{o}\) from 1.4 to 0.33 mM elevated \([\text{Na}^+]_{\text{cyt}}\) from 8.7 to 9.4 mM, whereas raising \([\text{Mg}^{2+}]_{o}\), to 10 or 20 mM (or adding 10 \(\mu\text{M} \text{La}^{3+}\)) for long periods caused \([\text{Na}^+]_{\text{cyt}}\) to decline to 5.1 mM. Mg\(^{2+}\) and \(\text{La}^{3+}\) may not be selective inhibitors of SOC channels, so we cannot be certain that the resting \(\text{Na}^+\) entry inhibited by Mg\(^{2+}\) is mediated solely by SOC. Nevertheless, the 10 mM Mg\(^{2+}\)-induced decline in \([\text{Na}^+]_{\text{cyt}}\) raises the possibility that SOC may contribute as much as 40–50% of the entering \(\text{Na}^+\) (i.e., the Mg\(^{2+}\)-inhibitable component) in resting mesenteric artery myocytes. These results reveal that external Mg\(^{2+}\) is an important regulator of \(P_{\text{Na}}\) in these cells.

A prolonged rise in \([\text{Na}^+]_{\text{cyt}}\) stimulates \(\text{Na}^+\) pump activity. Another unanticipated finding was that prolonged elevation of \([\text{Na}^+]_{\text{cyt}}\) increases the rate of extrusion of \(\text{Na}^+\) via the ouabain-sensitive \(\text{Na}^+\) pump (Fig. 7). This increase in \(\text{Na}^+\) pump activity occurred with a delay of 5–10 min. It seems most unlikely that this delay was a direct result of an increase in pump “substrate” (i.e., internal \(\text{Na}^+\)), because the rise in \([\text{Na}^+]_{\text{cyt}}\) preceded the increase in \(\text{Na}^+\) pump activity. The delay suggests that a secondary event, such as a phosphorylation of the \(\text{Na}^+\) pump (4), may be respon-
sible. This may serve as a “physiological safety valve,” so that adequate Na\(^+\) can be extruded under conditions of chronic stimulation in which [Na\(^+\)]\text{cyt} may rise above normal.

Is SOC the route of Na\(^+\) entry for modulating Ca\(^{2+}\) signaling by ouabain? As illustrated in Fig. 9, brief (30-s) exposure of the arterial myocytes to 100 nM ouabain augmented the Ca\(^{2+}\) transients evoked by vasoconstrictors (e.g., ANG II and PE) that mobilize SR Ca\(^{2+}\); this augmentation depended on external Na\(^+\). Inasmuch as the exposure to this low concentration of ouabain was brief, it is unlikely to have raised “bulk”[Na\(^+\)]\text{cyt}, because the most prevalent Na\(^+\) pump catalytic (α) subunit in these cells is the α\(_{1}\)-subtype (21), which has a low affinity for ouabain (IC\(_{50}\) ∼ 100 μM) (4). Indeed, our unpublished results reveal that a 10-min exposure to 100 nM ouabain does not raise [Na\(^+\)]\text{cyt} in these myocytes. Thus the augmentation by low-dose ouabain must be mediated by the ouabain-sensitive (IC\(_{50}\) < 50 nM) α\(_{1}\)-isoform of the Na\(^+\) pump (4), which is restricted to PL microdomains that overlie the myocyte “functional” (subplasmalemmal) SR (jSR) (21). Interestingly, SOC (19, 25) and the Na\(^+\)/Ca\(^{2+}\) exchangers (20, 33) are likely to be clustered with the high ouabain affinity Na\(^+\) pumps in these same PL microdomains. Therefore, we propose the following hypothesis to account for the results in Fig. 9: Normally, at rest, the Na\(^+\) that enters via SOCNE (Fig. 6) is very rapidly extruded by the nearby α\(_{1}\)-type Na\(^+\) pumps (21). When these pumps are inhibited by 100 nM ouabain, applied along with the vasoconstrictors, the local [Na\(^+\)]\text{cyt} within the tiny diffusion-restricted volume of cytosol (estimated to be 10^\text{−19}−10^{−18} \text{liters}) between the PL and adjacent jSR (separated by 10−15 nm) (39, 41) rises within seconds, i.e., before the peak of the agonist-evoked Ca\(^{2+}\) transient. At this low concentration of ouabain, the uniformly distributed, “housekeeping” α\(_{1}\)-type Na\(^+\) pumps are not affected, so that the bulk [Na\(^+\)]\text{cyt} does not change. However, the reduced Na\(^+\) gradient across the PL microdomains impairs Ca\(^{2+}\) extrusion locally, because the Na\(^+\)/Ca\(^{2+}\) exchangers are located here (20, 33). The rise in local Ca\(^{2+}\) concentration should enhance Ca\(^{2+}\)-induced Ca\(^{2+}\) release from more central regions of the SR and, thereby, rapidly amplify the “global” Ca\(^{2+}\) transients. Additional factors that may contribute to the augmented signaling include increased release of “trigger Ca\(^{2+}\)” from the jSR because of sensitization of the inositol trisphosphate receptors and/or increased Ca\(^{2+}\) storage in the jSR and, possibly, increased Ca\(^{2+}\) entry across the PL; these possibilities are, however, too speculative to warrant further discussion here.

The aforementioned sequence of events not only may explain the vasomotor actions of low-dose cardiotonic steroids (42) but also may reveal a more general mechanism. Via this mechanism, isomorph-specific changes in Na\(^+\) pump activity, mediated by protein kinases (4) or endogenous inhibitors (14), may regulate Ca\(^{2+}\) signaling and cell responsiveness in many other types of cells.

Implications of Na\(^+\) entry through SOC for the modulation of vascular tone. The mechanism described in 1soc the route of Na\(^+\) entry for modulating Ca\(^{2+}\) signaling by ouabain? may also provide a role for SOCNE in the tonic modulation of vascular contractility. Others have suggested that Ca\(^{2+}\) entry via SOC may help regulate smooth muscle tone (11). The present results now suggest that interplay between the rate of SOCNE and the rate of Na\(^+\) pump-mediated Na\(^+\) extrusion, which may be altered by neurotransmitters such as dopamine (5) and inhibitors such as ouabain, may affect arterial tone by altering the rate of Ca\(^{2+}\) extrusion via Na\(^+\)/Ca\(^{2+}\) exchange.

Our findings may also help elucidate the “calcium paradox” phenomenon in vascular smooth muscle (49) and the antihypertensive action of Mg\(^{2+}\) (45). Withdrawal of extracellular Ca\(^{2+}\) and Mg\(^{2+}\) promotes an Na\(^+\) influx in arterial myocytes that is unaffected by L-type Ca\(^{2+}\) channel blockers, whereas restoration of Ca\(^{2+}\) then induces contraction [the calcium paradox (49)]. Under these conditions, we suggest that Na\(^+\) entry may occur through SOC, inasmuch as the entry is inhibited by divalent cations such as Mg\(^{2+}\), Mn\(^{2+}\), and Ni\(^{2+}\) (49), all of which block SOC (17, 46, 47, 52). When Ca\(^{2+}\) is restored, the elevated [Na\(^+\)]\text{cyt} may induce a contraction by promoting Ca\(^{2+}\) entry via Na\(^+\)/Ca\(^{2+}\) exchange, although Ca\(^{2+}\) entry through SOC and through L-type Ca\(^{2+}\) channels (activated by depolarization) and receptor-operated channels may also contribute (2, 11).

The influence of Mg\(^{2+}\) on blood pressure has been the subject of numerous studies (1, 45). Epidemiological data reveal an inverse relationship between dietary Mg\(^{2+}\) and blood pressure (45). Although clinical trials of dietary Mg\(^{2+}\) supplementation in patients with essential hypertension have yielded inconsistent results (22), intravenous Mg\(^{2+}\) (as MgSO\(_{4}\)) has long been used to lower blood pressure in the toxemias of pregnancy (37). Our results now suggest that these effects may be due, at least in part, to the ability of Mg\(^{2+}\) to block SOC and thereby diminish Na\(^+\) and Ca\(^{2+}\) entry and inhibit vasoconstriction.

In summary, our results demonstrate that Ca\(^{2+}\) signaling in arterial myocytes is critically influenced by the coentry of Na\(^+\) and Ca\(^{2+}\) through the SOC pathway. Under resting conditions, some Na\(^+\) enters cells via the SOC, and mobilization of stored Ca\(^{2+}\) opens these channels and greatly increases Na\(^+\) entry. In turn, the entering Na\(^+\) influences Ca\(^{2+}\) storage and release and, thereby, plays a key role in modulating cell signaling. The magnitude of this effect is determined by the amount of Na\(^+\) that enters and by the activities of the Na\(^+\) pumps and the Na\(^+\)/Ca\(^{2+}\) exchangers colocalized with SOC in junctional microdomains of the PL. This role of dual-ion permeation in Ca\(^{2+}\) signaling may be applicable to numerous types of cells in which SOC have been reported (10).
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