Role of sarcoplasmic reticulum in regulation of tonic contraction of rabbit basilar artery

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Received 2 February 2001; accepted in final form 9 May 2001

Szado, Tania, Megan McLarnon, Xiaodong Wang, and Casey van Breemen. Role of sarcoplasmic reticulum in regulation of tonic contraction of rabbit basilar artery. Am J Physiol Heart Circ Physiol 281: H1481–H1489, 2001.—Superficial sarcoplasmic reticulum (SR) regulates smooth muscle force development directly by Ca2+ release and removal to and from the cytoplasm (Somlyo and Somlyo, J Cardiovasc Pharmacol 8, Suppl 8: S42–S47, 1986) by buffering Ca2+ influx and contributing to Ca2+ extrusion (Mueller and van Breemen, Nature 281: 682–683, 1979) and indirectly by releasing Ca2+ near Ca2+-activated K+ channels (KCa) to hyperpolarize the plasma membrane (Bolton and Imaizumi, Cell Calcium 20: 141–152, 1996 and Nelson et al. Science 270: 633–637, 1995). In the rabbit basilar artery, relative contributions of direct effects and those mediated through activation of KCa were evaluated by measuring force and tetraethylammonium ion (TEA). A large contraction was observed in response to KCa blockade with either 3 mM TEA or 100 nM IbTX and also after addition of 10 μM ryanodine or 2 μM thapsigargin. When KCa was blocked first with TEA or IbTX, subsequent addition of thapsigargin or ryanodine also increased force. Measurements of fura 2 fluorescence showed parallel increases in [Ca2+]i in response to sequential blockade of sarco(endo)plasmic reticulum Ca2+-ATPase and KCa regardless of the order of application. It appears that a significant fraction of KCa remains activated in the absence of SR function and that SR contributes to relaxation after blockade of KCa. We found that depletion of SR before stimulating Ca2+ influx through voltage-gated Ca2+ channels markedly reduced force development rate and that thapsigargin abolished this effect. We conclude that the SR of rabbit cerebral arteries modulates constriction by direct and indirect mechanisms.

cerebral artery; spectrofluorometry; calcium; buffering

Since Devine et al’s first description (10) of sarcoplasmic reticulum morphology in vascular smooth muscle, many functions have been attributed to this organelle. The sarcoplasmic reticulum is specialized in Ca2+ storage, functioning both to relax smooth muscle by removing Ca2+ via sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA) from the cytoplasm and activation by release through d-myo-inositol 1,4,5-trisphosphate and ryanodine receptors. Subsequently, more refined aspects of these general functions were discovered. Iino et al. (18) and Saida and van Breemen (37) showed there was calcium-induced calcium release from the Ca2+ entry signal is amplified by opening of the Ca2+-sensitive ryanodine channels in the superficial sarcoplasmic reticulum. This was later confirmed by Ganitkevich and Isenberg (11) by showing that sarcoplasmic reticulum depletion decreases Ca2+ signals accompanying opening of voltage-gated Ca2+ channels (VGCC). Another indirect mechanism potentially regulating vascular constriction is activation of Ca2+ entry through sarcoplasmic reticulum depletion (5, 8, 36, 42).

On the other hand, van Breemen (44) showed that the superficial sarcoplasmic reticulum could also attenuate the VGCC-mediated Ca2+ signal by sequestering Ca2+ from a restricted subsarcolemmal space. This was made all the more plausible because of Somlyo and Franzin-Armstrong’s (39) distinction between the superficial and deep sarcoplasmic reticulum. In fact, Somlyo and colleagues (10, 38, 41) showed specialized functional areas where the two membranes are separated by only 10–15 nm.

In the 1980s, Bulbring and Tomita (4) suggested that localized release of Ca2+ could have a relaxing action on intestinal smooth muscle as seen during α-adrenergic stimulation. This mechanism was established on a firm basis by Benham and Bolton (1), who showed localized activation of clusters of K+ channels by spontaneous Ca2+ release from the sarcoplasmic reticulum, also termed spontaneous transient outward currents (STOCs). More recently, Nelson et al. (30) provided direct evidence for such local Ca2+ release by imaging Ca2+ sparks. It is of particular interest that sparks more frequently occur peripherally and that there seem to be specific sites that function as hot spots (19) or frequent discharge sites (1, 35). This work led to the suggestion that, at least in small cerebral arteries, the main function of the sarcoplasmic reticulum is to relax the blood vessels as a consequence of sparks and or STOCs. This hypothesis fits well with the earlier evidence that resistance arteries are mainly activated by...
Ca\textsuperscript{2+} influx rather than by intracellular Ca\textsuperscript{2+} release (7, 30). In addition, this concept may have clinical relevance in that the activity and expression of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (K\textsubscript{Ca}) are enhanced in genetically hypertensive rats. The enhanced Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} current through BK\textsubscript{Ca} blockers may function as a compensatory mechanism to balance the enhanced activity of VGCC observed in vascular smooth muscle cells of another hypertensive rat model (24).

Considering all of these possible mechanisms of sarcoplasmic reticulum-mediated regulation, we were interested in determining the relative contributions of each of these in a conduit, cerebral blood vessel, the basilar artery. We approached this problem by pharmacologically manipulating Ca\textsuperscript{2+} pumps and channels in the sarcoplasmic reticulum and plasmalemma and by measuring force and intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in rabbit basilar arteries. We found that both activation of K channels by sarcoplasmic reticulum Ca\textsuperscript{2+} release and buffering of Ca\textsuperscript{2+} influx are important mechanisms in maintaining the basilar artery in a relaxed state. We found no evidence for store-operated channels (SOCs).

### MATERIALS AND METHODS

**Tissue preparation.** Adult New Zealand White rabbits weighing 2.0–2.5 kg were killed by CO\textsubscript{2} asphyxiation followed by exsanguination from the carotid arteries. The brain was removed, immediately placed in cold physiological saline (PSS), and placed in the refrigerater. The basilar artery was dissected from the brain in cold PSS continually and was removed, immediately placed in cold physiological saline, followed by exsanguination from the carotid arteries. The brain vessel on filter paper. The tissue was placed in cold HEPES in a specially designed chamber mounted on two stainless steel wires (Multi Myograph 610M, Danish Myo Technology) with two 30-μm stainless steel wires ~2.5 cm in length. The tissues were constantly kept at 37°C and continually bubbled with 95% O\textsubscript{2}-5% CO\textsubscript{2} cut into approximately four 2-mm segments, and mounted on a wire myograph. The stock solutions of Indo, thapsigargin, dil-tiazem, nifedipine, ibotenic acid (IbTX), apamin, charybdotoxin (CtX), Mn\textsuperscript{2+}, tetraethylammonium ion (TEA), ryanodine, and DMSO were obtained from Sigma (St. Louis, MO). Fura 2-AM was obtained from Molecular Probes (Eugene, OR). The stock solutions of Indo, nifedipine, ryanodine, and thapsigargin were dissolved in DMDO. TEA was dissolved in PSS or HEPES-PSS; all others were dissolved in distilled water. Serial dilutions of all chemicals were made in buffered PSS. The maximal concentration of any vehicle to which preparations were exposed was 0.1%, which had no effect on contraction or fura 2 signals.

**Statistics.** Results are shown as means ± SE and are analyzed for significance by paired or unpaired t-tests where noted.

### RESULTS

To examine the contribution of the sarcoplasmic reticulum to Ca\textsuperscript{2+} activation of K\textsubscript{Ca}, it was important to determine the maximal concentrations of thapsigargin required for depletion of the store. Therefore, a concentration-response curve for thapsigargin was determined by measuring depletion of the sarcoplasmic reticulum Ca\textsuperscript{2+} content as reflected by inhibition of a caffeine response (Fig. 1). In Fig. 1A, we show reproducible contractile responses to 10 mM caffeine that are initiated by Ca\textsuperscript{2+} release from the sarcoplasmic reticulum and are used in subsequent experiments as a control measure of sarcoplasmic reticulum Ca\textsuperscript{2+} content. The extent of sarcoplasmic reticulum depletion is measured by the ratio of the caffeine response after a 10-min exposure to thapsigargin (Fig. 1B) divided by excessive external fura 2-AM. The chamber, maintained at 37°C, was placed on the stage of an inverted microscope (Nikon Diaphot). The bottom of the basilar artery was exposed to alternating 340- and 380-nm (bandwidth 10 nm) ultraviolet light (1/s) that was passed through a 510-nm (bandwidth 40 nm) cut-off filter before acquisition by an intensified charge-coupled device camera (model 4093G, 4810 series). Fluorescence signals were recorded as digital image data using the software program Northern Eclipse by Empix Imaging Systems housed in a Pentium processor personal computer.

The single line trace in each figure is the average of the simultaneously measured fluorescence ratio (F\textsubscript{340}/F\textsubscript{380}) of individual or groups of smooth muscle cells in the chosen field of the basilar artery preparation. Traces shown in Figs. 1–7 were each representative of similar responses obtained from at least three experiments. Each trace was obtained from the same basilar artery preparation where noted. Drugs were applied as indicated by the horizontal bars or arrows in each figure.

**Intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) measurement.** Briefly, the rabbit basilar artery was inverted with forceps to expose the endothelial cell side, and the endothelium was removed by gently rubbing the inverted vessel on filter paper. The tissue was placed in cold HEPES in a specially designed chamber mounted on two stainless steel rods and stretched slightly. Loading solution was added to the chamber with the following composition: HEPES with 5 μM membrane-permeant fura 2-acetoxymethyl ester (fura 2-AM; 1 mM stock in DMSO) and 5 μM pluronic acid to facilitate loading for 2 h at the dark at room temperature. After incubation with fura 2, the tissue was washed two to three times with HEPES and left for ~20 min to remove excessive external fura 2-AM. The chamber, maintained at 37°C, was placed on the stage of an inverted microscope (Nikon Diaphot). The bottom of the basilar artery was exposed to alternating 340- and 380-nm (bandwidth 10 nm) ultraviolet light (1/s) that was passed through a 510-nm (bandwidth 40 nm) cut-off filter before acquisition by an intensified charge-coupled device camera (model 4093G, 4810 series). Fluorescence signals were recorded as digital image data using the software program Northern Eclipse by Empix Imaging Systems housed in a Pentium processor personal computer.

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**Solutions and drugs.** PSS contained (in mM) 119 NaCl, 4.7 KCl, 2.5 CaCl\textsubscript{2}, 1.17 MgSO\textsubscript{4}, 1.18 KH\textsubscript{2}PO\textsubscript{4}, 24.9 NaHCO\textsubscript{3}, and 11.1 glucose bubbled continuously with 5% O\textsubscript{2}-5% CO\textsubscript{2}, pH 7.4 at 37°C. For high extracellular K\textsuperscript{+} solution (80 K-PSS), 75.3 mM NaCl was replaced with equimolar KCl to make the final concentration equal to 80 mM. HEPES-PSS contained (in mM) 140 NaCl, 5 KCl, 1.5 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose, and 5 HEPES (pH 7.4 at 37°C). For high extracellular K\textsuperscript{+} solution (HEPES-80 K), 75 mM NaCl was replaced with equimolar KCl to make the final concentration equal to 80 mM. Experimental solutions used for the experiments illustrated in Fig. 7 consisted of Krebs solution with only 0.5 mM CaCl\textsubscript{2} and, in cases where 0 Ca\textsuperscript{2+} was used, no Ca\textsuperscript{2+} was added and 0.1 mM EGTA was included in the solution.

Analytical grade reagents for PSS, HEPES-PSS, N-nitro-L-arginine methyl ester (L-NAME), histamine, bradykinin, acetylcholine (ACh), indomethacin (Indo), thapsigargin, dil-tiazem, nifedipine, ibotenic acid (IbTX), apamin, charybdotoxin (CtX), Mn\textsuperscript{2+}, tetraethylammonium ion (TEA), ryanodine, and thapsigargin were dissolved in DMDO. TEA was dissolved in PSS or HEPES-PSS; all others were dissolved in double-distilled water. Serial dilutions of all chemicals were made in buffered PSS. The maximal concentration of any vehicle to which preparations were exposed was 0.1%, which had no effect on contraction or fura 2 signals.

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AJP-Heart Circ Physiol • VOL 281 • OCTOBER 2001 • www.ajpheart.org
that if the only functional consequence of abolishing the basilar artery to 10 mM thapsigargin were used because they eliminated relaxing responses to all solutions. These concentrations or incorporated into the initial bathing solution during the experimental time period. These concentrations were used because they eliminated relaxing responses of the basilar artery to 10 mM ACh and 10 mM bradykinin (data not shown). Other studies have shown that all endothelium-dependent relaxations in rabbit basilar artery can be blocked with NO synthase and prostacyclin inhibitors and that there is no indication for the action of an endothelium-derived hyperpolarizing factor other than NO or a prostanoid in this artery (25). Mechanical removal of the endothelium also inhibited vasodilatory responses to ACh and bradykinin, but these tissues were not used in these experiments because of compromised smooth muscle function.

After establishing a procedure for the complete depletion of the sarcoplasmic reticulum, we compared the effects of this procedure with direct blockade of large BKCa with IbTX. The rationale for this experiment is that if the only functional consequence of abolishing sarcoplasmic reticulum function is inactivation of BKCa, then the two procedures should be indistinguishable and nonadditive (3). Blockade of the BKCa channel with 100 nM IbTX added to the bath solution produced a sustained contraction presumably due to depolarization (Fig. 2A, left). Subsequent additive application of 2 mM thapsigargin caused further contraction indicating an action at sites other than BKCa (Fig. 2A, right). Figure 2B examines the Ca2+ signals measured in parallel with the above contraction experiments. IbTX alone caused an increase in [Ca2+]i (Fig. 2B, left) and additional subsequent blockade of SERCA with thapsigargin increased [Ca2+]i further (Fig. 2B, right). These results suggest that the sarcoplasmic reticulum regulation of vasoconstriction is more complex than exclusively regulating KCa activity.

In the reverse situation where SERCA was blocked initially followed by blockade of BKCa, we again would expect that the two procedures would be nonadditive if the sarcoplasmic reticulum is solely responsible for releasing Ca2+ toward the BKCa. Abolishing the sarcoplasmic reticulum function first with 2 mM thapsigargin resulted in an increase in tension (Fig. 3A) paralleled by an increase in [Ca2+]i (Fig. 3B). When BKCa was then blocked in the absence of sarcoplasmic reticulum function, there were additional large increases in tension (Fig. 3A) and [Ca2+]i (Fig. 3B). These additional increases induced by IbTX application reached values of significance in the case of force development and [Ca2+]i (Figs. 3, A, right, and B, right). These results suggest that even though the sarcoplasmic reticulum is empty and SERCA is blocked, BKCa remain.

Fig. 1. Thapsigargin (TG) concentration-response curve. A: two consecutive applications of 0 Ca2+/0.1 mM EGTA/10 mM caffeine (arrows). The second concentration of caffeine was applied after removing it from the solution by a 10-min washout in physiological saline solution (PSS) indicated by the slashed bars in the time scale. B: same as A but 1 mM TG was applied to all solutions. C: TG concentration-response curve constructed from the ratio of second caffeine responses in B divided by control caffeine responses over time in A. N-nitro-L-arginine methyl ester (L-NAME, 200 μM) and indomethacin (Indo, 10 μM) were added 30 min before the experiment. Note that 1–2 mM TG was found to completely deplete sarcoplasmic reticulum (SR) stores. SERCA, sarcoplasmic reticulum Ca2+ ATPase.
active. Therefore, BKCa must also be activated by Ca\(^{2+}\) from sources other than the sarcoplasmic reticulum.

When the previous experiments were repeated with 10 mM ryanodine in place of thapsigargin, the same trends were observed. Ryanodine alone increased force and subsequent application of 3 mM TEA additionally increased tension (Fig. 4A). [Ca\(^{2+}\)]\(_i\) measurements confirmed that the increases in tension were due to increases in [Ca\(^{2+}\)]. Subsequent addition of 3 mM TEA, after the sarcoplasmic reticulum was no longer functional, further increased [Ca\(^{2+}\)]\(_i\) (Fig. 4B). These results were repeated with 100 nM IbTX, and the same
trends were observed (data not shown). Therefore, regardless of the mode of inactivation of the sarcoplasmic reticulum, i.e., either with thapsigargin (via SERCA blockade) or ryanodine (via depletion due to opening of ryanodine receptors), there still seemed to be activation of BKCa independent of the sarcoplasmic reticulum.

Because TEA is a nonspecific blocker of all KCa, it was important to determine the relative contribution of each KCa channel when blocked with this agent. Therefore, the contribution of small conductance KCa (SKCa) was tested with 100 nM apamin, a selective inhibitor of SKCa (16). When added subsequently to treatment with ryanodine or thapsigargin and IbTX (blocks BKCa specifically), apamin had no additional effect (Fig. 5), suggesting that in the absence of a functional sarcoplasmic reticulum, SKCa do not play a significant role. Intermediate conductance KCa (IKCa) were also examined by the addition of 100 nM CTx after IbTX and apamin were present in the bathing solution. Although CTx blocks all KCa subtypes (16), its addition after blockade of SKCa with apamin and BKCa with IbTX would test specifically for the involvement of IKCa. Addition of CTx did appear to have a small effect (Fig. 5), and, therefore, IKCa may also contribute to the regulation of membrane potential (Em) under these conditions. In addition, experiments performed with 3 mM TEA tended to show slightly larger effects than in the presence of 100 nM IbTX, which may be explained by a nonspecific action on IKCa (data not shown). Thus, although TEA is not entirely specific, we were confident that the major portions of the responses observed after depletion of the sarcoplasmic reticulum using ryanodine were due to activation of BKCa. This idea was supported by use of the specific BKCa blocker, IbTX, in experiments using thapsigargin.

The increases in [Ca2+]i and force observed with sarcoplasmic reticulum-depleting agents or BKCa blockers were completely abolished by application of 1 μM nifedipine or 10 μM diltiazem. The effects of all sequences of SERCA and KCa blockers were completely reversed with VGCC blockers (data not shown). In addition, preincubation with 1 μM nifedipine for 20 min almost completely prevented a high K+ response (data not shown). These data support the notion that

Fig. 4. Ryanodine (Ry, 10 μM) produces trends similar to those observed with 2 μM TG. A, left: isometric tension measurements in rabbit basilar arteries show an increase in force with 10 μM Ry and an additional increase when 3 mM tetraethylammonium ion (TEA) was subsequently added. Results represent at least 5 tissues from at least 5 different rabbits. L-NAME (200 μM) and Indo (10 μM) were included in the bathing solution. Right, summary of results compared with 80 mM K+ contractions. *P < 0.05 resulting from an unpaired t-test. B: fura 2-AM spectrofluorometry in inside-out rabbit basilar arteries denuded of endothelium. Left, 10 μM Ry increases [Ca2+]i, and additional blockade of BKCa further increases [Ca2+]i. Right, summary of [Ca2+]i data comparing increases from baseline and analyzed with a paired t-test. The more-specific blocker of BKCa, IbTX, has the same effect as TEA, indicating that BKCa is active without a functional SR.

Fig. 5. Contributions of small conductance KCa (SKCa), intermediate conductance KCa (IKCa), and BKCa blockades to additional contraction after SERCA blockade. Blockade with TG increased force and additional blockade with IbTX additionally increased force. Apamin had no additional effects, whereas charybdotoxin (CTx) slightly increased force. Rabbit basilar arteries were pretreated with 200 μM L-NAME and 10 μM Indo. Results represent 8 tissues from 3 different rabbits.
cerebral vessels rely solely on the VGCC as a source of Ca$^{2+}$ as has been shown in smaller, resistance-sized cerebral vessels (7, 14).

To test for a possible contribution by SOC, 5 μM Mn$^{2+}$ was added to a nominally Ca$^{2+}$-free PSS and fura 2 fluorescence recorded at the isobestic wavelength of 360 nm. There was no change in slope when either 10 μM ryanodine and/or 2–5 μM thapsigargin were added to the superfusate, even though subsequent high K$^+$ depolarization caused a fourfold increase in the rate of Mn$^{2+}$ entry (Fig. 6). These data are consistent with the presence of VGCC and absence of SOC in the basilar artery of the rabbit.

Finally, it was demonstrated in these cerebral arteries that the sarcoplasmic reticulum is capable of actively buffering Ca$^{2+}$ influx. All solutions for this experiment contained low CaCl$_2$ (refer to MATERIALS AND METHODS) to slow down the rate of force development. Figure 7 shows rate of force development when Ca$^{2+}$ is restored and the K$^+$ concentration is elevated in the superfusate of basilar arteries with a depleted sarcoplasmic reticulum. Before the increase in extracellular [K$^+$] the sarcoplasmic reticulum was depleted by caffeine in 0 Ca$^{2+}$/PSS and the caffeine then washed out for 10 min. Under these conditions, addition of thapsigargin to the high K$^+$ solution markedly enhances the rate of force development (Fig. 7B), presumably because it prevents Ca$^{2+}$ uptake into the sarcoplasmic reticulum as it enters the smooth muscle cells through VGCC.

**DISCUSSION**

Major findings in this communication are 1) effects of thapsigargin or ryanodine and blockade of KCa on force development and [Ca$^{2+}$]; are additive; 2) depletion of sarcoplasmic reticulum does not enhance Mn$^{2+}$ influx; and 3) inhibition of SERCA enhances force developed during high K$^+$-induced depolarization. After SERCA is blocked with thapsigargin, KCa blockers additionally increased [Ca$^{2+}$]; and force, suggesting that K$_{Ca}$ is at
least partially activated in the absence of a functional sarcoplasmic reticulum. In addition, when $K_{Ca}$ is blocked first, subsequent SERCA blockade still increases $[Ca^{2+}]_i$ and force. These data indicate that in the rabbit basilar artery 1) $BK_{Ca}$ activity is not totally dependent on functional sarcoplasmic reticulum; and 2) SERCA lowers $[Ca^{2+}]_i$ at least in part by mechanisms other than regulation of $E_m$, most likely by buffering $Ca^{2+}$ entry. It has been reported that the $Ca^{2+}$ sensitivity of $K_{Ca}$ is too low to respond to global $[Ca^{2+}]_i$ (12, 43) and, therefore, requires local $[Ca^{2+}]_i$ elevation by virtue of release of quanta of $Ca^{2+}$ from the sarcoplasmic reticulum in the form of sparks (2, 30). Yet, after complete blockade of SERCA, which has been shown to eliminate sparks and STOCs (35, 45), $K_{Ca}$ of the basilar artery is still activated. We postulate that, under control conditions, SERCA removes $Ca^{2+}$ from a restricted subplasmalemmal space and that its blockade leads to accumulation of $Ca^{2+}$ in this space (see Fig. 8). The portion of $K_{Ca}$ that faces these restricted cytoplasmic regions would then be activated on SERCA blockade.

These observations were made in the rabbit basilar artery. Although this artery is not strictly speaking a resistance artery, it is an important conduit cerebral artery, which has many characteristics in common with cerebral resistance arteries. For example, vasoconstriction is completely dependent on $Ca^{2+}$ influx through VGCC and the smooth muscle displays spontaneous activity. Smooth muscle cells isolated from the basilar artery of the rat have been used in the study of sarcoplasmic reticulum function in small, cerebral arteries (35). In addition, Omote and Mizusawa (32) have studied fluctuations in basilar artery myogenic responses generated by variable activity of VGCC. They proposed that this $Ca^{2+}$ entry activated $BK_{Ca}$, leading to hyperpolarization of the membrane and, thus, providing negative feedback on the opening of $Ca^{2+}$ channels. Similar mechanisms have been suggested in cerebral arteries from spontaneously hypertensive rats (33) and in rabbit mesenteric arteries (31). In addition, this latter study suggested that the endothelium does not contribute to myogenic activity because its removal did not affect the oscillations. This may further implicate a role for $BK_{Ca}$ because there is some recent evidence suggesting that $BK_{Ca}$ is not active in the endothelium (22).

We chose the rabbit basilar artery as an appropriate model for the investigation of the roles the sarcoplasmic reticulum plays in a cerebral artery. The main question to be resolved is: What is the function of the sarcoplasmic reticulum in a blood vessel in which force development is totally dependent on $Ca^{2+}$ entry through VGCC? It appears unlikely that even agonist-induced contractions have a significant component of sarcoplasmic reticulum $Ca^{2+}$ release because histamine contractions are almost completely blocked by nifedipine. In peripheral resistance arteries, norepinephrine-induced contractions are readily abolished by removal of extracellular $Ca^{2+}$ (6). $Ca^{2+}$ sequestration by the sarcoplasmic reticulum without interaction between the plasma membrane and sarcoplasmic reticulum would also not be able to explain the steady-state results obtained in this study because such a putative function would cease on saturation of the sarcoplasmic reticulum and, therefore, be transient. In 1995, Nelson et al. (30) proposed that in cerebral resistance arteries, the sole function of the sarcoplasmic reticulum is to hyperpolarize the plasma membrane by releasing $Ca^{2+}$ in the vicinity of $K_{Ca}$. The authors based their conclusion on the observation that the effects of sarcoplasmic reticulum depletion and $K_{Ca}$ blockade on diameter, $[Ca^{2+}]_i$, and $E_m$ were identical and nonadditive. Because they subsequently reported on spark activation of $K_{Ca}$ in cerebral resistance vessels of other species, including humans, their hypothesis appeared to have universal validity. There is, indeed, an abundance of recent evidence suggesting the importance of $Ca^{2+}$ sparks in regulating $E_m$ and vascular tone specifically in resistance-sized vessels (21, 27, 28, 30) (for a review see Ref. 20). However, in the rabbit basilar artery, it appears that activation through $Ca^{2+}$ sparks may not be the only way the $E_m$ is regulated in these larger cerebral vessels.

The idea that localized $Ca^{2+}$ release could cause relaxation though activation of $K_{Ca}$ was first proposed by Bulbring and Tomita (4) to explain $\alpha$-adrenergic relaxation of intestinal smooth muscle (13). The existence of this mechanism was established by the studies of Benham and Bolton (1) showing that STOCs arose from transient $Ca^{2+}$ releases by the sarcoplasmic reticulum. Nelson et al.’s elegant and extensive studies (30) of sparks and related events in cerebral resistance arteries have established their contribution as a feedback regulatory mechanism. However, evidence for other sarcoplasmic reticulum-mediated mechanisms have been reported for vascular smooth muscle, namely buffering of $Ca^{2+}$ influx by the superficial sarcoplasmic reticulum and the activation of SOC by sar-
coplasmic reticulum depletion. Clearly, the significant thapsigargin-induced increase in \([Ca^{2+}]\text{i}\), blockade of BKCa by IbTX or TEA supports the involvement of either of these two latter mechanisms. In this instance, \(Ca^{2+}\) entry through VGCC would be enhanced by the depolarization caused by blockade of KCa.

To test for the activity of SOC in the rabbit basilar artery, we measured the effect of thapsigargin on Mn\(^{2+}\) quenching of intracellular fura 2-AM. The negative outcome of this experiment makes the SOC possibility unlikely, although it cannot be completely ruled out at this point. In some cells other than smooth muscle, \(Ca^{2+}\) release-activated channels have been described, which were highly selective for \(Ca^{2+}\) (17, 34). However, in our experiments, the effects of thapsigargin were completely blocked by nifedipine (at 1 or 10 \(\mu M\)), which is not known to inhibit SOC. In contrast, Mn\(^{2+}\) entry through VGCC was clearly observed. Additionally, it has been shown previously that either cadmium or diltiazem (both \(Ca^{2+}\) channel blockers) inhibited Mn\(^{2+}\) influx in aortic smooth muscle cells isolated from rats (24) and in venous smooth muscle of the rabbit (9), respectively.

Having thus exhausted all the other possible mechanisms, we conclude that the most likely candidate for explaining the actions of thapsigargin and ryanodine after blockade of KCa is inhibition of the \(Ca^{2+}\)-buffering action of the superficial sarcoplasmic reticulum. This is supported by our observation that thapsigargin enhanced the rate of force development in response to \(Ca^{2+}\) entry through VGCC. In the experiment reported herein, an effect on \(E_m\) was ruled out by the large depolarization induced by the 80 mM K\(^+\) solution. This result would also not be explained by inhibition of calcium-induced calcium release, because this would have the opposite effect.

In addition, our results support the idea of Guia et al. (15) that in rabbit coronary artery smooth muscle cells, there may be coupling of VGCC and BKCa, which has been shown previously in hippocampal neurons (26). \(Ca^{2+}\) influx through VGCC may directly cause accumulation of \(Ca^{2+}\) in the junctional space and directly activate BKCa, as demonstrated by whole cell patch-clamp and simultaneous [\(Ca^{2+}\)]i measurements in vascular smooth muscle cells (15). Guia et al. (15) showed that abolishing the sarcoplasmic reticulum function with CPA or ryanodine had no effect on the transient opening of BKCa. In fact, the transient stimulation of KCa they observed appeared to be independent of sarcoplasmic reticulum function. Our data are consistent with the notion that local \(Ca^{2+}\) entry may directly activate at least a fraction of BKCa.

This leads to the concept of the existence of cytoplasmic microdomains within the smooth muscle cell. In some areas of vascular smooth muscle, it has been observed that the relative distance of the sarcoplasmic reticulum from the plasma membrane is on the order of 20 nm (20). Therefore, colocalization and functional interactions of various channels and ion pumps within this small, restricted space seems probable. Although our data are consistent with those of Guia et al. (15), suggesting a direct functional interaction between VGCC and KCa, we cannot rule out the possibility that blockade of \(Ca^{2+}\) removal from a restricted space may lead to accumulation of \(Ca^{2+}\), and activation of KCa in the absence of \(Ca^{2+}\) sparks. Thus, if SERCA (located in the superficial sarcoplasmic reticulum) removes \(Ca^{2+}\) from the restricted cytoplasmic space (Fig. 8), it would not only decrease the flow of \(Ca^{2+}\) into the deeper cytoplasmic space, but it would also lower \([Ca^{2+}]i\) in the vicinity of KCa. Blocking SERCA would then not only abolish sparks and their activation of KCa but, in addition, \(Ca^{2+}\) removal from a subplasmalemmal space, would explain all the data reported herein.

In conclusion, our results demonstrate for the first time that in an intact cerebral small artery, BKCa remains active in the absence of \(Ca^{2+}\) release from the sarcoplasmic reticulum and that the superficial sarcoplasmic reticulum buffers \(Ca^{2+}\) entry through VGCC. Additionally, our data suggest that SOC do not significantly contribute to elevation of \([Ca^{2+}]i\) in the basilar artery.

We gratefully acknowledge Christian Caritey for technical assistance. This work was supported by the Heart and Stroke Foundation of Canada.

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