Superficial buffer barrier and preferentially directed release of Ca\(^{2+}\) in canine airway smooth muscle

LUKE J. JANSSSEN, PIERRE A. BETTI, STUART J. NETHERTON, AND DENISE K. WALTERS

Asthma Research Group and Smooth Muscle Research Group, Department of Medicine, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Superficial buffer barrier and preferentially directed release of Ca\(^{2+}\) in canine airway smooth muscle. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L744–L753, 1999.—We examined cytosolic concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]) in canine airway smooth muscle using fura 2 fluorimetry (global changes in [Ca\(^{2+}\)]), membrane currents (subsarcolemmal [Ca\(^{2+}\)]), and contractions (deep cytosolic [Ca\(^{2+}\)]). Acetylcholine (10^{-4} M) elicited fluorimetric, electrophysiological, and mechanical responses. Caffeine (5 mM), ryanodine (0.1–3 mM), and 4-chloro-3-ethylphenol (0.1–0.3 mM), all of which trigger Ca\(^{2+}\)-induced Ca\(^{2+}\) release, evoked Ca\(^{2+}\) transients and membrane currents but not contractions. The sarcoplasmic reticulum (SR) Ca\(^{2+}\)-pump inhibitor cyclopiazonic acid (CPA; 10 µM) evoked Ca\(^{2+}\) transients and contractions but not membrane currents. Caffeine occluded the response to CPA, whereas CPA occluded the response to acetylcholine. Finally, KCl contractions were augmented by CPA, ryanodine, or saturation of the SR and reduced when SR filling state was decreased before exposure to KCl. We conclude that 1) the SR forms a superficial buffer barrier dividing the cytosol into functionally distinct compartments in which [Ca\(^{2+}\)] is regulated independently; 2) Ca\(^{2+}\)-induced Ca\(^{2+}\) release is preferentially directed toward the sarcolemma; and 3) there is no evidence for multiple, pharmacologically distinct Ca\(^{2+}\) pools.

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CA\(^{2+}\) is sequestered within the sarcoplasmic reticulum (SR) by sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) (1); this uptake is selectively inhibited by agents such as thapsigargin or cyclopiazonic acid (CPA) (17, 20). Agonists can release this stored Ca\(^{2+}\) by stimulating phospholipase C to generate inositol 1,4,5-trisphosphate [Ins(1,4,5)P\(_3\)]_, which, in turn, activates Ca\(^{2+}\)-permeable channels on the SR (1). Ca\(^{2+}\) can also be released through channels on the SR membrane that are gated by an elevation in the cytosolic concentration of Ca\(^{2+}\) ([Ca\(^{2+}\); Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR)) (2). Ryanodine, a plant-derived muscle-paralyzing alkaloid, binds to a high-affinity site on this channel and induces channel opening; thus these channels are also referred to as ryanodine receptors. At higher concentrations, ryanodine also binds to low-affinity sites on the channel and induces channel closure (2), thereby complicating the interpretation of data obtained with this ligand. Recently, 4-chloro-3-ethylphenol (CEP) has been shown to mimic the ability of ryanodine to open these channels but without the inhibitory effects on channel function, although it may have nonspecific inhibitory effects on the contractile apparatus (15). Caffeine at millimolar concentrations increases the Ca\(^{2+}\) sensitivity of CICR channels such that basal levels of [Ca\(^{2+}\)] are sufficient to induce channel opening; however, like other methylxanthines, caffeine also inhibits phosphodiesterases (leading to accumulation of cAMP) and blocks adenosine receptors. Thus there are a wide variety of agents available to examine the uptake and release of Ca\(^{2+}\) from the SR, although care must be taken to consider their possible nonspecific actions.

According to the superficial buffer barrier (SBB) hypothesis, the peripheral SR separates the cytosol into a subsarcolemmal compartment and a deep cytosolic compartment and “buffers” elevations in [Ca\(^{2+}\)] in the latter space due to Ca\(^{2+}\) influx (21). It is also proposed that Ca\(^{2+}\) from the SR is vectorially “leaked” into the subsarcolemmal space and then extruded from the cell by Na\(^{+}^-\)/Ca\(^{2+}\) exchange and/or the sarcolemmal pump. Although this hypothesis is supported by data from studies of vascular smooth muscle (SM) (3, 21), it has not been examined in airway SM. Ca\(^{2+}\)-sensitive dyes such as fura 2 provide a global estimate of [Ca\(^{2+}\)] throughout the entire cell. Patch-clamp recordings of Ca\(^{2+}\)-dependent membrane currents and contractile responses, on the other hand, can serve as indirect indexes of Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]i) within the subsarcolemmal space and the deeper cytosol, respectively (3, 9, 13, 14, 16); it should be noted, though, that these responses do not precisely mirror changes in [Ca\(^{2+}\)]i under all conditions so these data must be interpreted with caution. For example, contractions can occur without any corresponding change in [Ca\(^{2+}\)]i (19). Similarly, although activation of Ca\(^{2+}\)-dependent Cl\(^{-}\) channels appears to be solely Ca\(^{2+}\) dependent, this is soon followed by phosphorylation and consequent inactivation of the channels (23).

There may also be regional heterogeneity or specialization with respect to the SR itself. For example, in some cell types, it seems that there are multiple, pharmacologically distinguishable Ca\(^{2+}\) pools: subsets of SR are sensitive to caffeine (i.e., express CICR sites), whereas the remainder are sensitive to agonists (i.e., express Ins(1,4,5)P\(_3\);gated release sites) (1, 5). Furthermore, there is evidence that the Ins(1,4,5)P\(_3\)-sensitive Ca\(^{2+}\) pools, but not the caffeine-sensitive Ca\(^{2+}\) pools, are sensitive to CPA (5). Alternatively, Ca\(^{2+}\) release sites may be concentrated on one side of the SR (e.g., that which faces the deep cytosol or the subsarcolemmal space) and mediate a preferential or vectorial release in a certain direction (21).

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In this study, we sought to examine Ca\(^{2+}\) handling in canine airway SM. Using fura 2 fluorimetry, patch-clamp recordings, and contractions to monitor changes in [Ca\(^{2+}\)], we provide evidence that the SR does, in fact, divide the cytosol into two functionally distinct compartments, that CICR is preferentially directed toward the sarcolemma, and that there is no evidence for pharmacologically distinct Ca\(^{2+}\) pools. Some of these data have been presented in abstract form (7).

**METHODS**

Preparation of tissues and cell dissociation. Adult mongrel dogs were euthanized with pentobarbital sodium (100 mg/kg). Tracheae were excised and kept in a physiological solution. The trachea was isolated by removing connective tissue, vasculature, and epithelium, then cut into strips parallel to the muscle fibers (=1 mm wide). For single-cell studies, tracheal SM (TSM) strips (0.5–1.0 g wet weight) were transferred to dissociation buffer (composition given in Solutions and chemicals) containing collagenase (type IV, 2.7 U/ml), elastase (type IV, 12.5 U/ml), and BSA (1 mg/ml), then were either used immediately or stored at 4°C for up to 48 h later. Janssen and Sims (8) have previously found that used cells immediately and those used after 48 h of refrigeration exhibit similar functional responses (i.e., contraction and activation of Ca\(^{2+}\)-dependent ion conductances). To liberate single TSM cells, tissues in an enzyme-containing solution were incubated at 37°C for 60–120 min, then gently trituated.

Fura 2 fluorimetry. Single cells were studied with a DeltaScan system (Photon Technology International, South Brunswick, NJ). After settling onto a glass coverslip mounted onto a Nikon inverted microscope, the cells were loaded with fura 2 (fura 2-AM; 2 µM for 30 min at 37°C), then superfused continuously with Ringer buffer at 37°C (2–3 ml/min). The cells were illuminated alternately (0.5 Hz) at the excitation wavelengths, and the emitted fluorescent emissions (measured at 510 nm) were converted to [Ca\(^{2+}\)] using the equation

\[
\frac{F_{340} - F_{380}}{F_{340}} = \frac{[Ca^{2+}]_i}{[Ca^{2+}]_o}
\]

where [Ca\(^{2+}\)]\(_o\) is the free Ca\(^{2+}\) concentration in the extracellular solution (assumed to be 100 nM). These experiments were performed within 1 h before the specific experiments were begun. At the conclusion of the experiments, cells were fixed and counterstained with fura 2 (DMSO) and ryanodine (95% ethanol).

Solutions and chemicals. The dissociation buffer contained (in mM) 125 NaCl, 5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, pH 7.4, 0.1 EDTA, 10 d-glucose, and 1 L-taurine, pH 7.0. Single cells were studied in Ringer buffer containing (in mM) 130 NaCl, 5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 20 HEPES, and 10 d-glucose, pH 7.4. Intact tissues were studied with Krebs-Ringer buffer containing (in mM) 116 NaCl, 4.2 KCl, 2.5 CaCl\(_2\), 1.6 NaH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 22 NaHCO\(_3\), and 10 d-glucose, bubbled to maintain pH at 7.4. Chemicals were obtained from Sigma with the exception of fura 2-AM (Calbiochem, La Jolla, CA). All agents were prepared as aqueous solutions except for CPA (DMSO), fura 2 (DMSO), and ryanodine (95% ethanol).

Data analysis. Responses are reported as means ± SE and were compared with two-tailed Student's t-test (paired or unpaired as appropriate), with P values < 0.05 being considered significant.

**RESULTS**

*ACh*-induced Ca\(^{2+}\) release. We first investigated ACh-induced Ca\(^{2+}\) release. In single cells studied at 37°C, ACh (10\(^{-4}\) M) induced a rapid spikelike elevation in [Ca\(^{2+}\)] that reached a peak within 5–10 s after onset of the application, then decayed toward basal levels (Fig. 1A, Table 1); the initial spikelike elevation and the subsequent plateau have previously been shown to represent release of internal Ca\(^{2+}\) and influx of external Ca\(^{2+}\), respectively (13, 18, 26). In cells held under voltage clamp at −60 mV and studied with the perforated-patch configuration so that intracellular signaling pathways would remain intact, ACh (10\(^{-4}\) M) evoked a large transient inward current that peaked back and changes in cell length were quantified with a line drawn through the central axis of the cell (SigmaScan, Jandel, Corte Madera, CA) as Janssen and Sims have previously described (9).

Microelectrode studies. Intact tissues were carefully pinned out in a chamber having a bath volume of ~10 ml; Krebs-Ringer buffer (composition given in Solutions and chemicals) was bubbled with 95% O\(_2\)-5% CO\(_2\), heated to 37°C, and superfused over the tissues at a rate of 3 ml/min. Microelectrodes (tip resistance of 30–80 MΩ when filled with 3 M KCl) were pulled from borosilicate capillary tubes and used to impale single SM cells. Membrane potential changes were observed on a dual-beam oscilloscope (Tektronix D13, 5A22N differential amplifier, and 5B12 dual-time base) and recorded on 0.25-inch magnetic tape with a Hewlett-Packard instrumentation recorder. Portions of these data were played back, digitized (Digidata 1200), and sampled with pCLAMP 6 software (Axon Instruments), then fitted with pCLAMP 6 and/or exported to SigmaPlot (Jandel) for graphic presentation.

Organ bath studies. TSM strips were mounted vertically in 3-ml organ baths with bath (Ethicon 4-0) tied to either end of the strip. One of which was fastened to a Grass FT.03 force transducer while the other was anchored. Isometric changes in tension were digitized and recorded with an on-line program (DigiMed System Integrator, MicroMed, Louisville, KY). Tissues were bathed in Krebs-Ringer buffer (see Solutions and chemicals for composition) containing indomethacin (10 µM), bubbled with 95% O\(_2\)-5% CO\(_2\), and maintained at 37°C. Preload tension was ~1.25 g (determined previously to allow maximal responses). Tissues were first equilibrated for 1 h before the specific experiments were begun. At the conclusion of the experiments, tissue dry weight was obtained and used to standardize the contractile responses.
within 5 s after onset of the application, then reversed completely to basal levels before the application of ACh had ended (Fig. 1B, Table 1). This membrane current response has been shown previously (8) to represent activation of Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channels in response to the release of internally sequestered Ca\textsuperscript{2+}. Cells that responded to ACh in this way also shortened to 28 ± 2% of their initial length (data not shown, but see Ref. 9).

Isometric contractile responses were studied in intact tissues with the standard organ bath technique; under these experimental conditions, ACh evoked powerful and sustained contractions (Fig. 1C, Table 1).

These data indicate that ACh elevates [Ca\textsuperscript{2+}], in the deep cytosol (leading to contraction) as well as within the subsarcolemmal region (causing activation of Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channels).

Ca\textsuperscript{2+} release induced by antagonism of SR Ca\textsuperscript{2+} pump. CPA selectively blocks SERCA activity (17), which normally compensates for a continuous leakage of Ca\textsuperscript{2+} from the SR (1, 19). As a result, Ca\textsuperscript{2+}-pump inhibition leads to a net release of internally sequestered Ca\textsuperscript{2+}; these responses have been described in detail elsewhere (9, 13, 18).

CPA (10 µM) induced an elevation in [Ca\textsuperscript{2+}], that was significantly smaller than the cholinergic response (Table 1); this elevation peaked ≈1–2 min after the addition of CPA (Fig. 2A) and subsided to baseline after 10–15 min. CPA also evoked a contraction that was significantly smaller (Table 1) and developed more slowly than the ACh-evoked response (Fig. 2C); after 10–15 min, CPA-induced tone spontaneously decayed to baseline levels. However, CPA did not significantly increase membrane current in any of 14 cells held within 5 s after onset of the application, then reversed completely to basal levels before the application of ACh had ended (Fig. 1B, Table 1). This membrane current response has been shown previously (8) to represent activation of Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channels in response to the release of internally sequestered Ca\textsuperscript{2+}. Cells that responded to ACh in this way also shortened to 28 ± 2% of their initial length (data not shown, but see Ref. 9). Isometric contractile responses were studied in intact tissues with the standard organ bath technique; under these experimental conditions, ACh evoked powerful and sustained contractions (Fig. 1C, Table 1).

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Table 1. Peak effects of Ca\textsuperscript{2+}-releasing agents on [Ca\textsuperscript{2+}], membrane current, and tone

<table>
<thead>
<tr>
<th>Agent</th>
<th>Ca\textsuperscript{2+} Transient, nM</th>
<th>Membrane Current, pA</th>
<th>Contraction, g/mg dry wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh (100 µM)</td>
<td>318 ± 44 (15)</td>
<td>782 ± 98 (16)</td>
<td>10.6 ± 1.3 (21)</td>
</tr>
<tr>
<td>CPA (10 µM)</td>
<td>118 ± 14 (6)</td>
<td>10 ± 16* (14)</td>
<td>4.5 ± 0.8 (9)</td>
</tr>
<tr>
<td>Caffeine (5 mM)</td>
<td>485 ± 134 (6)</td>
<td>537 ± 130 (12)</td>
<td>−0.3 ± 0.2* (5)</td>
</tr>
<tr>
<td>Ryanodine (30 µM)</td>
<td>190 ± 120 (7)</td>
<td>Variable (see text)</td>
<td>0.6 ± 1.1* (7)</td>
</tr>
<tr>
<td>CEP (0.3 mM)</td>
<td>692 ± 101 (9)</td>
<td>ND</td>
<td>0.3 ± 0.2* (6)</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses, no. of cells or tissues. [Ca\textsuperscript{2+}], cytosolic Ca\textsuperscript{2+} concentration; CPA, cyclopiazonic acid; CEP, 4-chloro-3-ethylphenol; ND, not done. *Not significantly different from zero, P > 0.05.
under voltage clamp at −60 mV (Fig. 2B, Table 1), although subsequent exposure to ACh evoked no response (Fig. 2B; n = 6 cells), indicating that internally sequestered Ca\(^{2+}\) had been released. Consistent with this, CPA had no significant effect on membrane potential in intact tissues studied with the intracellular microelectrode electrophysiological technique (Fig. 2D); the mean membrane potential was −61 ± 2 mV before CPA and −64 ± 3 mV after exposure to CPA (net change of 3 ± 4 mV; n = 8 cells).

These observations suggest that, on application of CPA, there is an elevation in [Ca\(^{2+}\)]i within the deep cytosol but not in the region immediately beneath the sarcolemma.

CICR. Caffeine (5 mM) elicited a transient elevation in [Ca\(^{2+}\)]i (Fig. 3A), the magnitude of which was not significantly different from that of the response to ACh (Table 1). In cells studied under voltage-clamp conditions, caffeine also activated membrane currents, with similar magnitudes and time courses as those for the response to ACh (Fig. 3B, Table 1). Intact tracheal tissues exhibited little or no contractile response on exposure to caffeine (5 mM; Fig. 3C, Table 1), although the CPA-evoked contraction was essentially abolished in all tissues treated with caffeine (Fig. 3C); the mean CPA response was −0.2 ± 0.1 g/mg in caffeine-treated tissues compared with 3.8 ± 2.0 g/mg in paired control tissues (n = 5). In contrast, pulmonary venous tissues studied under identical experimental conditions exhibited substantial contractions on exposure to caffeine (n = 11; Fig. 3D), suggesting that the lack of response in airway tissues is not due merely to an inability of caffeine to diffuse quickly through an intact tissue.

Ryanodine (3 × 10\(^{-5}\) M) evoked a Ca\(^{2+}\) transient in only three of seven cells, an example of which is given in Fig. 4A; the response in this cell had a time course similar to that evoked by caffeine, with peak activation occurring within 5 s, followed by a decay to a superbasal plateau level. There was little or no change in [Ca\(^{2+}\)]i in the remaining four cells challenged with 3 × 10\(^{-5}\) M ryanodine nor in any of six cells challenged with 10\(^{-6}\) M ryanodine (Fig. 4C). The effects of ryanodine (10\(^{-5}\) to 10\(^{-4}\) M) on membrane currents were tested in five cells and found to vary considerably between a large inward current (>200 pA) shortly after onset of exposure (n = 2 cells), a small inward current (35 pA) after a 30-s delay (n = 1 cell), or no inward current whatsoever (n = 2 cells). Similarly, ryanodine had mixed effects on mechanical tone. There was little or no change in baseline tone in 16 of 20 tissues exposed to 3 × 10\(^{-5}\) M ryanodine (mean change of 5.3 ± 4.3%; n = 7; Fig. 4D; see Fig. 7A for an example) nor in any of 22 tissues exposed to 10\(^{-6}\) M ryanodine (mean change of −7 ± 4%; n = 7; Fig. 4D). In the remaining four tissues exposed to 3 × 10\(^{-5}\) M ryanodine, however, the baseline was increased >50%, albeit after a delay of up to 10 min (Fig. 4, B and D).

Like caffeine, CEP (0.1 and 0.3 mM) produced a large elevation in [Ca\(^{2+}\)]i (Fig. 5A, Table 1); however, these responses were sustained in contrast to the transient responses evoked by caffeine or ryanodine. CEP alone did not trigger contractions (Fig. 5B, Table 1), even though the tissues were still able to respond to ACh (Fig. 5B), indicating that the contractile apparatus was still functional.

These data suggest that agents that trigger CICR elevate [Ca\(^{2+}\)]i in the subsarcolemmal space to a greater extent than that in the deep cytosol.

Barrier function of the SR. It has been proposed that, in vascular SM, the superficial SR forms a barrier to Ca\(^{2+}\) entry, allowing the SR to modulate the elevation in [Ca\(^{2+}\)], that accompanies agonist stimulation (21). We investigated this possibility by examining contractions evoked by KCl (which triggers voltage-dependent Ca\(^{2+}\) influx) under conditions in which SR buffering capacity was altered.
KCl elicited contractions in a dose-dependent fashion, with a maximal response of $5.8 \pm 0.8$ g/mg dry weight (Fig. 6, A and C). After a 30-min exposure to CPA (10 µM; which had no discernable effect of its own on tone in the tissue represented in Fig. 6A), the responses to 15 and 30 mM KCl were significantly enhanced, whereas those to higher concentrations of KCl were not (Fig. 6, B and D). Likewise, ryanodine ($3 \times 10^{-5}$ M) also induced a leftward shift in the KCl dose-response curve (Fig. 7, A–C) and accelerated the rate of rise of KCl-evoked contractions (Fig. 7, B and D) without directly inducing a mechanical response of its own (Fig. 7A). The lower concentration of ryanodine ($10^{-6}$ M) had no effect on mechanical activity and little or no affect on KCl responses ($n = 7$ tissues; data not shown).

In the experiments summarized in Figs. 6 and 7, we often found that, in a given tissue exposed to vehicle, the response to a second challenge with KCl 30 min later was somewhat enhanced compared with the first or control response, although this augmentation was much less than that in tissues exposed to CPA or ryanodine (as indicated in Figs. 6D and 7D). It may be that during the first exposure to KCl (S1), the SR became more fully loaded and was therefore less able to buffer the second response (S2). To test this directly, SR unloading was facilitated by bathing some tissues in nominally Ca$^{2+}$-free medium during the 30-min period separating S1 and S2 (external Ca$^{2+}$ was reintroduced 1–2 min before S2); under these conditions, S2 developed more slowly and was reduced in height compared with S1 (Fig. 8). Overall, the rate of rise and magnitude of S2 were decreased when S2 was preceded by 30 min in Ca$^{2+}$-free medium and increased when external Ca$^{2+}$ was present for the full 30 min before S2 (Fig. 8, B and C); one-tailed paired t-test analysis showed these trends to be significant.
**DISCUSSION**

Multiple Ca\(^{2+}\) pools? Some tissues possess multiple, functionally distinct Ca\(^{2+}\) pools expressing Ins(1,4,5)P\(_3\)-gated and/or Ca\(^{2+}\)-gated release sites (1, 5); in some cases, the agonist-sensitive pool is CPA sensitive (i.e., is refilled by SERCA), whereas the caffeine-sensitive pool is not (5). We found that caffeine completely occluded the contractile response to CPA (Fig. 3C), suggesting that the caffeine-sensitive and CPA-sensitive Ca\(^{2+}\) pools overlap completely. ACh and caffeine liberate the same intracellular pool of Ca\(^{2+}\) in this tissue (8, 18), and CPA can completely deplete the ACh-sensitive pool (9, 13, 18). Likewise, ryanodine reduced the total cellular Ca\(^{2+}\) content in canine TSM to the same extent as carbachol, and carbachol had no additional effect on tissue Ca\(^{2+}\) content after pretreatment with ryanodine (4). CPA completely depletes the caffeine-sensitive Ca\(^{2+}\) pool in equine TSM (23). Thus airway SM cells do not appear to possess multiple, heterogeneous Ca\(^{2+}\) pools as may be the case in other preparations (1, 5). In porcine TSM, the ACh-sensitive and caffeine-sensitive Ca\(^{2+}\) pools appear to be linked (allowing ACh response to be occluded by caffeine and vice versa) but seem to be refilled by different mechanisms (14).

The fact that the CEP-triggered Ca\(^{2+}\) response is much larger and more prolonged than that of ACh or caffeine does not contradict the claim that all three agents are acting on the same Ca\(^{2+}\) pool; Ins(1,4,5)P\(_3\) and caffeine-triggered Ca\(^{2+}\) release are both subject to feedback regulatory mechanisms (e.g., suppression when [Ca\(^{2+}\)]\(_i\) reaches micromolar levels) (1, 2), whereas CEP is reported to lack such inhibitory effects on SR Ca\(^{2+}\)-channel function (15).

Multiple cytosolic regions? Although we found no evidence for multiple functionally distinct Ca\(^{2+}\) pools in canine airway SM (see Multiple Ca\(^{2+}\) pools?), this does not rule out the possibility of heterogeneity within the cytosol. In fact, we found that ACh, CPA, caffeine, ryanodine, and CEP all elevated [Ca\(^{2+}\)]\(_i\) (indicated directly with fura 2 fluorimetry) but that this elevation did not seem to be uniform throughout the cell.

For example, CPA evoked contraction but did not activate Ca\(^{2+}\)-dependent Cl\(^{-}\) current in cells studied under voltage-clamp conditions (Fig. 2B) nor alter membrane potential in intact tissues (Fig. 2D); likewise, in equine TSM, CPA did not increase the activity of Ca\(^{2+}\)-dependent K\(^{+}\) current (22), which may be somewhat more sensitive to changes in [Ca\(^{2+}\)]\(_i\) than the Cl\(^{-}\) current (12). These observations suggest that on blockade of SERCA activity in airway SM, there is a net increase in [Ca\(^{2+}\)]\(_i\) in the deep cytosolic space but not in the region around the ion channels (Fig. 9). This does not necessarily imply that the spontaneous leak of Ca\(^{2+}\) from the SR is preferentially directed toward the deep cytosol; instead, it may be that this leak from the SR is uniform in all directions but that some Ca\(^{2+}\) extrusion pathway prevents subsarcolemmal [Ca\(^{2+}\)]\(_i\) from reaching levels that would increase membrane channel

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Fig. 6. CPA enhances KCl contractions. A: representative tracing showing protocol used to examine effects of CPA (+CPA; 30 µM) on contractions evoked by KCl added in cumulative fashion (15–75 mM). B: expanded traces of contractions (i and ii) evoked by 15 mM KCl in A, highlighting increased magnitude and rate of rise in response after exposure to CPA. C: mean KCl dose ([KCl])-response relationship from tissues exposed to CPA or its vehicle (n = 5). *P < 0.05. D: mean rate of rise of contractile responses evoked by 15 mM KCl in tissues exposed to CPA or its vehicle (n = 5) standardized as a percent change from 1st or control response to KCl. *P < 0.05.
activity (Fig. 9). Ca\(^{2+}\) that was released toward the deep cytosol, on the other hand, would mediate contraction until it diffused to the periphery, whereupon it would be rapidly ejected.

In marked contrast, those agents that cause CICR (i.e., caffeine, ryanodine, and CEP) are relatively ineffective in triggering contraction in airway SM (although vascular SM exhibited substantial responses; Fig. 3D).

![Fig. 7. Ryanodine enhances KCl contractions. A: same protocol illustrated in Fig. 6A was used to examine effects of ryanodine on KCl-evoked contractions. B: expanded traces of contractions (i and ii) evoked by 15 mM KCl before and after exposure to ryanodine. C: mean KCl dose-response curves from tissues exposed to ryanodine or its vehicle (n = 5). *P < 0.05. D: mean rate of rise of contractions evoked by 15 mM KCl in tissues exposed to ryanodine or its vehicle (n = 5) standardized as a percent change from 1st or control response to KCl. *P < 0.05.](image)

![Fig. 8. Ca\(^{2+}\) influx saturates sarcoplasmic reticulum (SR) and compromises buffering capacity of SR. A: contractile response to KCl (15 mM) was assayed before (S1) and 30 min after (S2) bath in nominally Ca\(^{2+}\)-free medium; magnitude and rate of rise in S2 were decreased compared with those of S1. B and C: absolute rate of rise and peak magnitude, respectively, of paired responses to 15 mM KCl obtained before (i.e., S1) and after (i.e., S2) 30 min in Ca\(^{2+}\)-free (n = 7) or Ca\(^{2+}\)-containing (n = 7) medium. P values were obtained by paired t-test analysis.](image)
but nonetheless activate membrane current (Figs. 3–5). We do not feel that the relative inability of these three agents to trigger contraction is due to their nonspecific pharmacological actions because each has a unique profile of nonspecific effects. For example, although caffeine might cause a slowly developing accumulation of cAMP and thereby antagonize mechanical activity, ryanodine and CEP do not alter phosphodiesterase activity. Similarly, although caffeine and ryanodine may have paradoxical effects on CICR due to adaptation and/or induction of a subconductance state of the ryanodine receptor, CEP does not (15) (Fig. 9). Finally, although high concentrations of CEP can directly antagonize the contractile apparatus, this is not seen with lower concentrations of CEP (15) nor with caffeine or ryanodine. Although it might be argued that the inability of these agents to evoke contraction is due to their inability to increase Ca\(^{2+}\) sensitivity (as is the case for ACh), we would point out that CPA also does not sensitize the contractile apparatus but did trigger contractions with only a modest increase in [Ca\(^{2+}\)] (much less than that evoked by either ACh or caffeine). The property that these diverse chemicals have in common, however, is the ability to trigger CICR and elevate [Ca\(^{2+}\)] but to do so in a manner (or a cytosolic region) that does not result in a significant mechanical response.

ACh, however, evokes membrane current as well as contraction (Fig. 1); the same is true for histamine (10) and substance P (11). Thus physiological agonists that trigger Ins(1,4,5)P\(_3\)-gated Ca\(^{2+}\) channels seem to release Ca\(^{2+}\) toward both the sarcolemma and the deeper cytosol (Fig. 9).

**SBB function of the SR.** This study also provides strong evidence that the SR serves as an SBB in airway SM (Fig. 9) as was first proposed for vascular SM (21). First, CPA induced a marked and significant leftward shift in the KCl dose-response curve and accelerated the rate of rise in KCl-induced contractions (Fig. 6). We do not feel that CPA did this by elevating [Ca\(^{2+}\)] and thereby displacing the muscle into a steeper region of the [Ca\(^{2+}\)]-tension relationship because we tested the KCl responses 30 min after the addition of CPA (Fig. 6), long after the transient elevation in [Ca\(^{2+}\)] caused by CPA would have subsided (12). Similarly, saturating the SR by preexposing the tissues to a high concentration of KCl 30 min before examining the responses to a second challenge with KCl mimicked the effects of CPA (i.e., enhanced rate of rise and magnitude of second response; Fig. 8); the opposite changes were seen when SR unloading was facilitated by bathing the tissue in Ca\(^{2+}\)-free medium during the intervening 30-min period (Fig. 8). We interpret these findings to indicate that voltage-dependent Ca\(^{2+}\) influx during the first exposure to KCl increased uptake and saturated the SR, thereby compromising the ability of the SR to buffer the response to the second KCl exposure. Furthermore, the data indicate that the SR can discharge some of its Ca\(^{2+}\) load while at rest (see Vectorial Ca\(^{2+}\) release and SR unloading) to be able to serve as a Ca\(^{2+}\) buffer.

**Vectorial Ca\(^{2+}\) release and SR unloading.** Generally, there is an ongoing spontaneous release of Ca\(^{2+}\) from the SR that may account, in part, for the spontaneous transient outward currents often recorded from SM preparations (8). SERCA compensates for this sponta-
neous leak; as such, agents such as CPA unmask this spontaneous release (Fig. 2A). The nature of this leak pathway is uncertain but may involve the stochastic flickering of the Ca^{2+}-permeable channels on the SR.

A mechanism has been proposed whereby the SR can unload sequestered Ca^{2+} by preferentially releasing it into the subsarcolemmal space, followed by ejection of that Ca^{2+} out of the cell via Na^{+}/Ca^{2+} exchange and/or the sarcolemmal Ca^{2+}-ATPase (21). The data obtained in this study indicate that this vectorial release involves CICR because caffeine and CEP have a much greater effect on subsarcolemmal [Ca^{2+}] than that in the deep cytosol (Figs. 3–5). Previously, Janssen and Sims (8, 9) have shown that caffeine can trigger an instantaneous and massive dumping of the SR into the subsarcolemmal space (for example, cyclooxygenases, phospholipases, and nitric oxide synthases) and ion channels can be activated without necessarily evoking a change in tension. In fact, others (24) have shown that β-agonists elevate [Ca^{2+}] in the periphery of bovine TSM cells while simultaneously decreasing [Ca^{2+}], in the deeper cytosolic regions and mediate relaxation. This relaxant response may involve the mechanism proposed by Nelson et al. (16), in which localized elevations in subsarcolemmal [Ca^{2+}], referred to as Ca^{2+} sparks, activate Ca^{2+}-dependent K⁺ channels, leading to membrane hyperpolarization, deactivation of Ca^{2+} channels, and relaxation. In other words, relaxants may act by causing a localized increase in [Ca^{2+}], which, in turn, triggers a more globalized decrease in [Ca^{2+}]. Spasmogens, on the other hand, release internally sequestered Ca^{2+} and activate Ca^{2+}-dependent Cl⁻ and nonselective cation channels, which, in turn, depolarize the membrane and thereby open voltage-dependent Ca^{2+} channels, leading to contraction (8, 9, 11, 13, 18). Clearly then, agonist-mediated responses involve a complicated interaction between the SR and the sarclemma. The mechanism(s) by which an elevation in [Ca^{2+}] in the subsarcolemmal space leads to activation of Cl⁻ channels in the presence of a spasmogen (8, 9, 11) but to activation of K⁺ channels in the presence of a relaxant (24) needs to be examined.

Experimental implications. These findings have important ramifications for studies of agonist-induced responses. First, physiologically important information is lost when global changes in [Ca^{2+}] are monitored by photometry of whole cells or intact tissues in which [Ca^{2+}] in the subsarcolemmal and deep cytosolic spaces...
becomes averaged. In addition, care must be taken when comparing data obtained with an indicator dye that tends to partition in membranes (e.g., aequorin) with data obtained with dyes that partition more uniformly throughout the cell. These data also underscore the need for caution when using contractions and membrane currents as indexes of global \([\text{Ca}^{2+}]\). The differential regulation of \([\text{Ca}^{2+}]\) in the subsarcolemmal and deep cytosolic spaces may also account, in part, for the frequently reported discrepancies between myosin light chain phosphorylation and changes in \([\text{Ca}^{2+}]\) in SM (19). Finally, this confirmation of the SBB hypothesis in airway SM is of utmost importance with respect to the physiological and pathophysiological changes that take place at or near the membrane, including those that involve second messenger signaling pathways that are \(\text{Ca}^{2+}\) dependent, such as phospholipases \(A_2\) and \(C\), protein kinase \(C\), cylooxygenases, nitric oxide synthases, and caveolae.

Summary and conclusion. We found that agents that induce CICR directly (e.g., caffeine, ryanodine, and CEPI) increase subsarcolemmal \([\text{Ca}^{2+}]\) and membrane current activity but are much less effective in elevating \([\text{Ca}^{2+}]\) in the deeper cytosol and tone, suggesting that CICR is preferentially directed toward the sarcolemma (Fig. 9). CPA, on the other hand, has the opposite effects: transient elevation of \([\text{Ca}^{2+}]\) in the deep cytosol, contraction, and augmentation of KCl-evoked responses but not of membrane currents (Fig. 9). Cholinergic stimulation elevates \([\text{Ca}^{2+}]\) in both cytoplasmic regions and thereby triggers membrane currents as well as contraction (Fig. 9). Thus the SR in canine TSM forms an SBB and allows for a complex regulation of \([\text{Ca}^{2+}]\) (Fig. 9).

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Address for reprint requests and other correspondence: L. J. Jansen, Dept. of Medicine, HSC-3U1, McMaster Univ., 1200 Main St. West, Hamilton, Ontario, Canada L8N 325 (E-mail: janssenl@hs. csu.mcmaster.ca).

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