Muscarinic excitation-contraction coupling mechanisms in tracheal and bronchial smooth muscles

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J Appl Physiol 91: 1142–1151, 2001.—We investigated the mechanisms underlying muscarinic excitation-contraction coupling in canine airway smooth muscle using organ bath, fura 2 fluorimetric, and patch-clamp techniques. Cyclopiazonic acid (CPA) augmented the responses to submaximal muscarinic stimulation in both tracheal (TSM) and bronchial smooth muscles (BSM), consistent with disruption of the barrier function of the sarcoplasmic reticulum. During maximal stimulation, however, CPA evoked substantial relaxation in TSM but not BSM. CPA reversal of carbachol tone persisted in the presence of tetraethylammonium or high KCl, suggesting that hyperpolarization is not involved; CPA relaxations were absent in tissues preconstricted with KCl alone or by permeabilization with β-escin, ruling out a non-specific effect on the contractile apparatus. Peak contractions were sensitive to inhibitors of tyrosine kinase (genistein) or Rho kinase (Y-27632). Sustained responses were dependent on Ca\(^{2+}\) influx in TSM but not BSM; this influx was sensitive to Ni\(^{2+}\) but not La\(^{3+}\). In conclusion, there are several mechanisms underlying excitation-contraction coupling in canine smooth muscle, the relative importance of which varies depending on tissue and degree of stimulation.

airway smooth muscle contraction; airway hyperresponsiveness; Rho kinase; tyrosine kinase; phosphatidylinositol 3-kinase; calcium ion-adenosinetriphosphatase; genistein; calcium ion-dependent chloride ion current

AIRWAY HYPERRESPONSIVENESS and variable airflow obstruction are key features of airway-related diseases such as asthma; indeed, one might say that these are the most clinically relevant features of such airway disorders. For this reason, it is essential to have a good understanding of the mechanisms underlying excitation-contraction coupling in canine smooth muscle (ASM).

Excitation-contraction coupling in cardiac and skeletal muscles, as well as certain types of smooth muscle (vascular and gastrointestinal), involves membrane depolarization, resulting in Ca\(^{2+}\) entry via voltage-dependent (“L-type”) Ca\(^{2+}\) channels. Thus L-type Ca\(^{2+}\) channel blockers are very effective in controlling vascular and cardiac contractions in hypertension. In ASM, excitation is also accompanied by membrane depolarization via activation of large inward Cl\(^{-}\) and nonselective cation currents (14, 36). This depolarization activates voltage-dependent Ca\(^{2+}\) current (8, 18), which is sufficient for contraction, as attested to by the robust responses evoked by high millimolar concentrations of KCl (11, 16). Unfortunately, however, Ca\(^{2+}\) channel blockers are ineffective in treating bronchoconstriction. Ca\(^{2+}\) entry in ASM may also involve nonselective cation channels (7, 36) and/or “receptor-operated” Ca\(^{2+}\) channels (22).

The next advance in our understanding of ASM excitation-contraction coupling came with a series of studies showing that spasmogens act by activation of phospholipase C and generation of inositol 1,4,5-trisphosphate (IP\(_3\)), which, in turn, triggers release of Ca\(^{2+}\) stored within the sarcoplasmic reticulum (31, 38). It is now known that the sarcoplasmic reticulum forms sheets around the periphery of the cytosol, thereby dividing the cytosol into two pools, the peripheral space immediately underneath the plasmalemma where ion channels are found (many of them being regulated by Ca\(^{2+}\) and the deep cytosolic space where the contractile apparatus is found, and that intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in these two spaces can be regulated very differently (11). There have been numerous studies of the mechanisms underlying Ca\(^{2+}\) handling in ASM (2, 11, 15, 16, 29, 38). Surprisingly, substantial ASM contraction can still be evoked under conditions in which internally sequestered Ca\(^{2+}\) is not available for contraction. For example, repeated stimulation of Ca\(^{2+}\) release in the presence of agents that inhibit the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) leads to complete abrogation of Ca\(^{2+}\)-dependent Cl\(^{-}\) currents, suggesting that the store has been functionally depleted, and yet contractions in those same cells are completely unaffected (15)! Others have used contractile responses as an index of [Ca\(^{2+}\)]\(_i\) in ASM (albeit a much less reliable one) and have come to
similar conclusions (2). Instead, the voltage-dependent Ca\(^{2+}\) channels may play an important role in refilling the sarcoplasmic reticulum (1, 2, 15, 25). Clearly then, other excitation-contraction coupling mechanisms remain to be examined.

Recently, it has come to be appreciated that spasmodens can constrict smooth muscle both by increasing [Ca\(^{2+}\)], and by increasing the sensitivity of the contractile apparatus to Ca\(^{2+}\), such that even basal ("resting") levels of [Ca\(^{2+}\)], are sufficient to evoke contraction (or that a given level of excitation leads to a greater level of contraction). Although some suggest that this involves the diacylglycerol-protein kinase C pathway (which is coincident with the IP\(_3\)-Ca\(^{2+}\)-release pathway), direct evidence for this, at least in ASM, is lacking. Instead, many studies point to the role of Rho and Rho kinase, the latter of which phosphorylates myosin light chain phosphatase, leading to accumulation of phosphorylated myosin light chain and thus contraction (3, 4, 26, 32, 39).

A growing body of evidence attests to differences in structure and function as one progresses down the airways, including differential expression of ion conductances, receptors, and functional responses, to name only a few (10, 13, 30). This fact may call into question the value of many studies of ASM physiology, because these have used tracheal tissues and not smaller airways.

During the course of our laboratory’s earlier studies of Ca\(^{2+}\) handling in ASM (13, 16), we found cyclopiazonic acid (CPA; inhibitor of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase) to have mixed effects on tone in carbachol (CCh)-stimulated ASM, evoking either a contraction or a relaxation, depending on several factors, including the degree of preconstriction and the type of tissue (tracheal vs. bronchial). In this study, we sought to examine the mechanism(s) underlying 1) the CPA-induced enhancement of tone during modest muscarinic stimulation; 2) the CPA-induced relaxation seen during aggressive muscarinic stimulation; and 3) the tone that persists in the presence of CPA, particularly with a view to understanding the differences between tracheal (TSM) and bronchial smooth muscle (BSM) tissues.

METHODS

Preparation of isolated tissues and single cells. Whole lobes of lung and tracheae were obtained from dogs that had been euthanized using pentobarbital sodium (100 mg/kg). TSM was isolated by removing connective tissue, vasculature, and epithelium, and then was cut into strips parallel to the muscle fibers (~1 mm wide). Lobes of lung were pinned out, the overlying parenchyma and pulmonary vasculature were removed, and ring segments (~4–5 mm long) of fifth- to sixth-order bronchi (outer diameter, 2–6 mm) were excised. For single-cell studies, TSM strips (0.5–1.0 g wet weight) were transferred to dissociation buffer (composition given below) containing collagenase (type IV; 2.7 U/ml), elastase (type IV; 12.5 U/ml), and bovine serum albumin (1 mg/ml) and then were either dissociated immediately or stored at 4°C for dissociation at a later time (<48 h later). We have previously found that cells used 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 dissociate into single TSM cells, tissues in enzyme-containing solution were incubated at 37°C for 60–120 min and then gently triturated. Tissues were either used immediately or stored at 4°C for use the next day; we found no functional differences in tissues that were studied immediately compared with those used after 24-h refrigeration.

Muscle bath technique. Ring segments were mounted into 3-ml muscle baths using stainless steel hooks inserted into the lumen. One hook was fastened to a Grass FT-03 force transducer using silk thread (Ethicon 4-0); the other was attached to a Plexiglas rod, which served as an anchor. Tissues were bathed in Krebs-Ringer buffer (see below for composition) containing indomethacin (10 μM), bubbled with 95% O\(_2\)-5% CO\(_2\), and maintained at 37°C; tissues were passively stretched to impose a preload tension of ~1 g (determined to allow maximal responses). Isometric changes in tension were amplified and plotted using a chart recorder or were digitized (2 samples/s) and recorded on-line (Digimed System Integrator, MicroMed, Louisville, KY) for plotting on the computer. Tissues were equilibrated for 2 h before the experiments were commenced, during which time the tissues were challenged with 60 mM KCl at least once to assess the functional state of each tissue.

Fura 2 fluorometry. Freshly dissociated cells were studied by using a filter-based photometric system (DeltaScan; Photon Technology International, South Brunswick, NJ). After settling onto a glass coverslip mounted onto a Nikon TMD inverted microscope, cells were loaded with the membrane-permeant form of fura 2 (fura 2-acetoxymethyl ester, 2 μM for 30 min at 37°C) and then superfused continuously with Ringer buffer (2–3 ml/min) at 37°C. Cells were illuminated alternately (0.5 Hz) at the excitation wavelengths and the emitted fluorescence (measured at 510 nm) induced by 340-nm excitation (F\(_{340}\)) and that induced by 380-nm excitation (F\(_{380}\)) was measured using a photomultiplier tube assembly. Agonists were applied by pressure ejection from a puffer pipette (Picospritzer II, General Valve, Fairfield, NJ).

Patch-clamp electrophysiology. Single TSM cells were allowed to settle and adhere to the bottom of a recording chamber (1-ml bath volume perfused at 2–3 ml/min) and were studied within 6 h after dissociation. Membrane currents were recorded using the nystatin perforated-patch method, which our laboratory has described in detail previously (10, 11, 16). Briefly, cells were held under voltage clamp at ~60 mV using an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA). Electrodes had a tip resistance of 1–3 MΩ and were filled with an electrode solution containing the following (in mM): 140 KCl, 0.4 CaCl\(_2\), 1 MgCl\(_2\), 1 EGTA, 20 HEPES, pH 7.2, and nystatin (final concentration of 200 μg/ml). Access resistance ranged from 10 to 40 MΩ, and 60–80% series resistance compensation was employed. Data were filtered at 1 kHz, sampled at 2 kHz using pCLAMP6 software (Axon Instruments), and stored on the computer hard drive for later analysis using pCLAMP6 and SigmaPlot software. Corrections were not made for liquid junction potentials [previously found to be only ~2 mV (14)]. Agonists were applied by pressure ejection from a puffer pipette (Picospritzer II; General Valve). The apparatus is designed to hold two different puffer pipettes, and we are sometimes able to replace these with a new one during the course of a voltage-clamp experiment (only when the cell can be lifted up off the coverslip while maintaining a gigohm seal on the electrode); thus at least two different concentrations of ACh (Acetylcholine) could be applied to a given cell.

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For data analysis, we included only cells that were able to respond to \(10^{-8}\) M ACh.

**Solutions and chemicals.** Dissociation buffer contained the following (in mM): 125 NaCl, 5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 0.25 EDTA, 10 D-glucose, and 10 L-taurine, pH 7.0.

Single cells were studied in Ringer buffer containing the following (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 using Krebs-Ringer buffer containing the following (in mM): 116 NaCl, 4.2 KCl, 1.25 CaCl\(_2\), 1.8 NaH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 22 NaHCO\(_3\), and 11 D-glucose, bubbled to maintain pH at 7.4. Indomethacin (10 \(\mu\)M) was also added to the latter to prevent generation of cyclooxygenase metabolites of arachidonic acid. Nominally Ca\(^{2+}\)-free medium was prepared by omitting CaCl\(_2\) and adding EGTA (either 0.1 or 1.0 mM, as indicated).

Chemicals were obtained from Sigma Chemical with the exception of Y-27632 (kindly provided by A. Yoshimura of the Welfide, Osaka, Japan). Stock solutions (10 mM) were prepared in aqueous media (CCh, LaCl\(_3\), NiCl\(_2\), tetraethylammonium (TEA)), DMSO (CPA, genistein, Y-27632, LY-294002), or 95% EtOH (niflumic acid). The final bath concentration of DMSO and EtOH did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity. The effects of LaCl\(_3\) were tested while some tissues were bathed in Krebs and others in Ringer buffer [because La\(^{3+}\) may precipitate out as La(CO\(_3\))\(_3\) salts]; we found no qualitative difference between these two approaches.

**Data analysis.** At the end of the experiments, tissues were dried and weighed; dry weight was used to standardize the qualitative difference between these two approaches.

**RESULTS**

**Mixed effects of CPA on muscarinic responses.** CPA \((6 \times 10^{-5}\) M\) had mixed effects on tone evoked by CCh in canine TSM: it evoked substantial contraction in tissues pretreated with relatively low concentrations of CCh [i.e., \(10^{-8}\) M, which elicits ~10% of the maximal contractile response (12)] but dramatic relaxations in tissues exposed to half-maximally effective concentrations of CCh or greater (Fig. 1). Approximately 10–20 min after the addition of CPA, many tracheal tissues exhibited phasic activity, “oscillations” in tone, similar in time course to those that our laboratory has described previously (13). Figure 1B shows the mean peak magnitude of mechanical activity after stimulation with each concentration of CCh, as well as tone existing after the addition of CPA. The CPA-induced increase in tone during low levels of muscarinic stimulation and the CPA-induced decrease in tone during more aggressive muscarinic stimulation were both statistically significant. Interestingly, phasic activity was seen in the majority (20 of 33) of TSM tissues preconstricted with \(10^{-8}\)–\(10^{-6}\) M CCh but not in any of 32 tissues constricted with \(10^{-6}\)–\(10^{-4}\) M CCh.

Given the important role played by \([Ca^{2+}]_i\); in contraction of smooth muscle, we also examined responses in single canine tracheal myocytes loaded with fura 2. Although this approach allows one to measure \([Ca^{2+}]_i\) directly, it is important to point out that the measurements represent the average \([Ca^{2+}]_i\) across the entire cell. CCh \((10^{-8}\) M\) evoked no discernible or statistically significant change in the ratio of F\(_{340}/F_{380}\) (Fig. 2, B and D; \(n = 5\)); however, subsequent addition of CPA \((10^{-5}\) M\) triggered a sudden elevation in this ratio (Fig. 2, B and D), which was faster in onset and larger in amplitude than the response to CPA alone (Fig. 2A), which our laboratory has described previously (11, 16). CCh \((10^{-4}\) M\), on the other hand, elicited a large spikelike elevation in F\(_{340}/F_{380}\) followed by a sustained “plateau,” as described previously (11, 16, 29), after which CPA caused a further modest elevation in F\(_{340}/F_{380}\) (Fig. 2, C and D; \(n = 8\)).

We also used ACh-evoked membrane currents as an indirect assay of \([Ca^{2+}]_i\), because these currents are predominantly Ca\(^{2+}\)-dependent Cl\(^{-}\) currents triggered by release of internally sequestered Ca\(^{2+}\) (14); although this approach is indirect, it specifically indicates \([Ca^{2+}]_i\) in the subplasmalemmal space rather than averaging across the entire cell (11). Single canine tracheal myocytes were studied under voltage-clamp conditions (holding potential of ~60 mV) and challenged with at least two different [ACh] \((10^{-10}\)–\(10^{-4}\) M in the application pipette); only those that responded to \(10^{-5}\) M ACh were included in the accompanying statistical analysis. All cells challenged with \(10^{-6}\) M ACh exhibited substantial membrane current; however, the fraction of responsive cells decreased with decreasing [ACh]. Although less than one-half of the cells tested were able to respond to \(10^{-10}\) M ACh, many of those that did respond still exhibited substantial membrane currents (Fig. 3); in other words, the magnitude of the responses was somewhat “all or none.”

In canine BSM, CPA also evoked a statistically significant increase in mechanical tone during submaximal stimulation (i.e., \(10^{-8}\) and \(10^{-7}\) M CCh). The relaxant effect of CPA, however, was not as profound in BSM tissues as it was in TSM: there was essentially no change in tissues constricted with \(10^{-6}\) M CCh and only a small (albeit significant) reduction during more aggressive muscarinic stimulation (Fig. 1C). Also, phasic activity was seen in only 1 of 41 BSM tissues tested (that one had been constricted with \(10^{-6}\) M CCh).

**Mechanism underlying salutary effect of CPA on CCh-evoked contraction.** Nelson et al. (17, 23) have shown that CPA (and other agents that release internally sequestered Ca\(^{2+}\)) increases vascular tone by decreasing Ca\(^{2+}\) spark frequency, thereby decreasing Ca\(^{2+}\)-dependent K\(^{+}\)-channel activity, leading to membrane depolarization and voltage-dependent Ca\(^{2+}\) influx. This mechanism may also account in part for the CPA-evoked enhancement of tone in our tissues. On the other hand, by allowing more Ca\(^{2+}\) to accumulate in the subplasmalemmal space, CPA may increase tone by increased activation of Ca\(^{2+}\)-dependent Cl\(^{-}\) channels and thus voltage-dependent Ca\(^{2+}\) influx. We tested these two possibilities using TEA to block Ca\(^{2+}\)-dependent K\(^{+}\) channels and niflumic acid to block...
Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channels (Fig. 4). Whereas niflu-
mic acid (10\textsuperscript{-4} M) suppressed the contraction evoked
by 10\textsuperscript{-8} M CCh, subsequent addition of CPA evoked a
further eightfold increase in tone (to 802 ± 205% of
that evoked by CCh). TEA at 5 mM had no effect on
baseline tone or muscarinic tone, and subsequent ad-
dition of CPA also still evoked a statistically significant
increase (to 178 ± 24%) of tone. At 30 mM, however,
TEA markedly increased baseline tone, thereby occlud-
ing the subsequent responses to 10\textsuperscript{-8} M CCh and to
CPA (not shown).

Mechanism underlying CPA-evoked relaxations.
CPA may trigger relaxations by allowing more Ca\textsuperscript{2+}
to accumulate in the subplasmalemmal space, leading to
increased activation of Ca\textsuperscript{2+}-dependent K\textsuperscript{+}
channels and membrane hyperpolarization (17, 23). To test this
hypothesis, we pretreated tissues with TEA (30 mM;
n = 6) to block Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels or with
KCl (120 mM; n = 6) to prevent membrane hyperpo-
larization even if the K\textsuperscript{+} channels were to open, before
assessing CPA-evoked relaxations. CPA could still re-
verse CCh tone under these conditions (Fig. 5), and the
magnitudes of those relaxations were not statistically
significantly different from the control responses (65 ±
6 vs. 59 ± 4%). However, CPA did not evoke a relax-
ation in tissues precontracted with 120 mM KCl alone
(Fig. 5; n = 6) or in tissues permeabilized using β-escin
and precontracted directly by Ca\textsuperscript{2+} in the bathing
medium (Fig. 5; n = 5). In fact, CPA elevated tone
under those circumstances by 5.0 ± 2.5 and 20.2 ± 80%,
respectively. The absence of relaxations in the pres-
ence of KCl alone or of β-escin indicates that CPA
relaxation is not due to some nonspecific effect on the
contractile apparatus. We, therefore, conclude that the
CPA-evoked relaxation reflects some kind of “Ca\textsuperscript{2+}-
recycling” phenomenon: that is, sustained CCh-in-

fig. 1. Mixed effects of cyclopiazonic acid (CPA)
on muscarinic tone. A: representative tracings
showing the contractile or relaxant responses
evoked by CPA (6 × 10\textsuperscript{-5} M) in canine tracheal
smooth muscle (TSM) tissues preconstricted to
varying degrees using carbachol (CCh; 10\textsuperscript{-8}–
10\textsuperscript{-4} M). Mean (±SE) magnitudes (g tension/mg
tissue) of responses in TSM (B) and bronchial
smooth muscle (BSM) tissues (C) studied in this
fashion are given. n = 5 for all. Open bars, tone
existing before addition of CPA; solid bars, tone
in those same tissues after addition of CPA.
*Statistically significant change in muscarinic
tone on addition of CPA, P < 0.05.
duced release of internally sequestered Ca\textsuperscript{2+} requires ongoing refilling of the sarcoplasmic reticulum to maintain a certain level of tone.

Ca\textsuperscript{2+}-influx pathways. The Ca\textsuperscript{2+}-recycling mechanism proposed above would require a certain degree of ongoing Ca\textsuperscript{2+} influx across the membrane to maintain the filling state of the sarcoplasmic reticulum (see DISCUSSION). Others have investigated the extent to which this Ca\textsuperscript{2+} enters via voltage-dependent Ca\textsuperscript{2+} channels (2, 25); we used a number of strategies to examine whether voltage-independent pathways are involved.

Tissues were stimulated with 10\textsuperscript{-4} M CCh, and the magnitude of the peak response, as well as tone remaining after 20 min, was recorded (Fig. 6A). Under control conditions, these two measurements were not significantly different in both TSM and BSM (compare open and solid bars in Fig. 6). When Ca\textsuperscript{2+} was omitted from the bathing medium and 0.1 mM EGTA was added to chelate trace Ca\textsuperscript{2+}, the initial peak response in TSM was significantly decreased, and tone continued to decline over the course of the 20-min stimulation. When 1 mM EGTA was used, the initial response was smaller yet, and tone declined essentially to zero over the course of 20 min. In BSM, however, neither the initial peak response nor the sustained response was significantly different from control under either experimental condition. Thus Ca\textsuperscript{2+} entry is clearly important for the sustained response to maximal muscarinic stimulation in TSM but apparently not in BSM.

Both voltage-dependent Ca\textsuperscript{2+} channels (8, 10, 18) and nonselective cation channels (14, 35, 36) have been described in this tissue, and both are sensitive to inorganic ions such as Ni\textsuperscript{2+} or La\textsuperscript{3+}. We found that neither Ni\textsuperscript{2+} (0.1 or 1 mM) nor La\textsuperscript{3+} (1 mM) had any significant effect on contractions (peak or sustained) in BSM tis-
sues nor did Ni\textsuperscript{2+} in TSM tissues (Fig. 6); however, La\textsuperscript{3+} did significantly decrease sustained tone in TSM tissues.

"Pharmacomechanical" coupling in TSM and BSM. Several studies have shown that, whereas electromechanical coupling is important during submaximal stimulation (5, 6), pharmacomechanical coupling mechanism(s) predominates at the other extreme of the cholinergic dose-response relationship (31). The nature of the latter mechanism(s) is still unclear; however, tyrosine kinases and Rho kinase are emerging as important candidate effectors (3, 4, 26, 32, 39). Also, phosphatidylinositol 3-kinase is coupled to muscarinic receptors in ASM (35) and increases Ca\textsuperscript{2+} sensitivity in

Fig. 3. ACh-evoked membrane currents. A: in 1 cell held under voltage clamp at -60 mV, ACh (10\textsuperscript{-10} M) evoked large inward current; a subsequent application of 10\textsuperscript{-4} M ACh 3 min later evoked a response of comparable magnitude. Fraction (%) of cells responding (B) and absolute magnitude of the currents evoked (C) in 23 cells (n = 5) studied in this way are given.

Fig. 4. CPA-evoked enhancement of muscarinic tone. Representative tracings show CPA-induced enhancement of tone evoked by 10\textsuperscript{-8} M CCh in tracheal tissues pretreated with niflumic acid (10\textsuperscript{-4} M; A) or tetraethylammonium (TEA 5 mM; B).

Fig. 5. CPA-evoked reversal of muscarinic tone. Representative tracings illustrate ability of CPA (6 × 10\textsuperscript{-5} M) to reverse tone evoked by 10\textsuperscript{-4} M CCh, even during exposure to TEA (25 mM; D) or high KCl (120 mM; C) but its inability to reverse tone evoked by KCl alone (120 mM; A) or during permeabilization of the membrane by β-escin (10\textsuperscript{-4} M; B).
colonic smooth muscle (37). We, therefore, compared the effects of the nonspecific tyrosine kinase inhibitor genistein (10^{-2}4 M), CPA (10^{-5}5 M), the Rho kinase inhibitor Y-27632 (10^{-5}5 M), and the phosphatidylinositol 3-kinase inhibitor LY-294002 (10^{-2}5 M) on the CCh dose-response relationship in TSM and BSM tissues.

Genistein alone had very little effect on CCh contractions in TSM but significantly reduced those in BSM (Fig. 7A). When genistein was added together with CPA, however, contractions were further reduced in both tissue types (Fig. 7A). Y-27632 significantly reduced the responses to all concentrations of CCh in both tissue types (Fig. 7B). It should be pointed out, though, that substantial contractions could still be evoked by 10^{-6}–10^{-4} CCh under all of the experimental conditions described above (Fig. 7). LY-294002 did not significantly reduce CCh responses in either TSM nor BSM (Fig. 7C).

**DISCUSSION**

Many have investigated electromechanical coupling in ASM and found it to be important to muscarinic excitation-contraction coupling only during submaximal levels of stimulation. That is, the upper one-half of the concentration-response relationship shows no correlation with membrane depolarization (5) and is essentially unaffected when voltage-dependent Ca^{2+} influx is prevented using L-type Ca^{2+}-channel blockers (6) or K^{+}-channel openers (2, 28) or directly using voltage-clamp techniques (15). The responses to other spasmogenic autacoids, such as histamine or leukotrienes, may exhibit a different dependence on electromechanical coupling.

Generally speaking then, voltage-independent mechanisms seem to be most important for contraction in ASM. The first such mechanism to be described was the phosphoinositide cascade, resulting in IP3-induced release of internal Ca^{2+} (38). This Ca^{2+} release is barely discernible in cells challenged with 10^{-8} M ACh [a concentration that is sufficient to increase tone (19)] using photometric techniques that measure the average change in [Ca^{2+}]_{i} throughout the entire cell (Fig. 2; Refs. 27, 29, 38). However, we could demonstrate substantial responses even down to 10^{-10} M ACh using patch-clamp techniques to monitor [Ca^{2+}]_{i} in the subplasmalemmal space (Fig. 3). Interestingly, another group could visualize Ca^{2+} transients in response to 10^{-10} M ACh using whole cell photometry in serum-deprived canine TSM cells but not cells grown in serum-containing media (21). They also showed that the fraction of cells responding to ACh decreased with decreasing [ACh], consistent with our own data (Fig. 3). Ongoing SERCA activity serves to refill this intracellular Ca^{2+} pool and moderates changes in [Ca^{2+}]_{i}, resulting from Ca^{2+} influx across the membrane (11) (see below). Previously, our laboratory showed that CPA causes a slow and modest elevation of [Ca^{2+}]_{i} (≈100 nM) in ASM cells at rest (16). When added to cells stimulated with “threshold” concentrations of agonist, however, CPA elevates [Ca^{2+}]_{i}, several hundred nanomolar (Fig. 2). Thus the CPA-induced enhancement of tone seen in tissues during mild stimu-

![Fig. 6. Ca^{2+} influx and sustained muscarinic contraction. Contractions were evoked by CCh (10^{-4} M) in TSM (A) and BSM (B) tissues, and the mean peak tone (open bars) and tone existing 20 min after addition of CCh (solid bars) were quantified. This was done under control conditions, as well as in nominally Ca^{2+}-free media with either 0.1 or 1.0 mM EGTA to chelate trace Ca^{2+}, or in the presence of Ni^{2+} (0.1 or 1.0 mM) or La^{3+} (1.0 mM) as indicated. Values are means ± SE; n = 5 for all. *Significant change in tone over the course of the 20-min stimulation, P < 0.05.](http://jap.physiology.org/doi/10.220.33.1 on August 27, 2017)
lation is due to disruption of a “superficial buffer barrier,” resulting in a net greater elevation of \([\text{Ca}^{2+}]\) in the vicinity of the contractile apparatus. During more aggressive muscarinic stimulation, however, \(\text{Ca}^{2+}\) release likely surpasses \(\text{Ca}^{2+}\) uptake, and a significant amount of \(\text{Ca}^{2+}\) would be discharged via the plasmalemmal \(\text{Ca}^{2+}\)-pump. This “loss” of \(\text{Ca}^{2+}\) would need to be compensated for in some way by \(\text{Ca}^{2+}\) influx to prevent a progressive depletion of the sarcoplasmic reticulum; such \(\text{Ca}^{2+}\) recycling would account for the collapse of the muscarinic responses in TSM tissues bathed in the absence of external \(\text{Ca}^{2+}\) (Fig. 6). Thus the CPA-evoked relaxation might reflect a greater uncompensated ejection of \(\text{Ca}^{2+}\) from the cell by plasmalemmal \(\text{Ca}^{2+}\)-ATPase activity. Presently, there are no selective blockers for plasmalemmal \(\text{Ca}^{2+}\)-ATPase that would allow us to test this hypothesis.

Thus there is somewhat of a paradox: muscarinic contractions are mediated largely by release of internal \(\text{Ca}^{2+}\) and yet are dependent to some degree on \(\text{Ca}^{2+}\) influx. Liu and Farley (19) have also shown the same to be true for maintained \(\text{Cl}^{-}\)-current oscillations during sustained cholinergic stimulation in TSM. In fact, there is accumulating evidence that this may represent an important function of the voltage-dependent \(\text{Ca}^{2+}\) channels in these tissues (1, 2, 15); when the intracellular \(\text{Ca}^{2+}\) pool is eliminated by agents such as CPA, the relative importance of voltage-dependent \(\text{Ca}^{2+}\) influx to excitation-contraction coupling is dramatically increased (33). Other studies suggest that \(\text{Ca}^{2+}\) may also enter via nonspecific cation channels (7, 36), which are activated by cholinergic stimulation (14). \(\text{Ni}^{2+}\) at millimolar concentrations is expected to be sufficient to block both of these pathways and yet does not mimic the effect of removing external \(\text{Ca}^{2+}\) (Fig. 6); others have demonstrated refilling of the sarcoplasmic reticulum in ASM via a \(\text{Ni}^{2+}\)-insensitive \(\text{Ca}^{2+}\)-influx pathway (20). \(\text{La}^{3+}\) did inhibit muscarinic responses (Fig. 6) but may have done so by a number of other nonspecific effects.
Interestingly, contractions in BSM are much less dependent on Ca\(^{2+}\) influx: removal of external Ca\(^{2+}\) markedly attenuated the responses to CCh in TSM but not in BSM (Fig. 6). Whereas it might be argued that this is related to the differing diffusional barriers in TSM strips vs. BSM rings, we find that the responses to CCh or CPA in both tissues develop rapidly and are easily washed out, suggesting that molecules as large as these can diffuse freely into and out of the tissues. Previously, our laboratory (13) showed that these tissues differ markedly in their responses to CPA under basal conditions (i.e., not when Ca\(^{2+}\) release is being enhanced by CCh): BSM tissues respond with a large contraction, whereas TSM tissues exhibited little or no response. These data are consistent with a continuous SERCA-mediated uptake of Ca\(^{2+}\) into the sarcoplasmic reticulum (inhibition of which leads to elevation of [Ca\(^{2+}\)]\(_i\) and contraction), which is much more efficient in BSM than in TSM; this hypothesis would also account for the differing sensitivity to Ca\(^{2+}\)-deficient media between these tissues. These functional differences between TSM and BSM are very important, because it is the bronchial airways that are more sensitive to inflammation and that play a greater role in determining resistance to airflow than the trachealis (24). This calls into question the clinical relevance of studies done using TSM tissues.

Finally, we investigated other putative signaling pathways that might be involved in excitation-contraction coupling, because substantial tone could still be seen in the presence of CPA (Fig. 1); previously, our laboratory found that we could completely deplete the sarcoplasmic reticulum of Ca\(^{2+}\) (as indicated by complete loss of Cl\(^{-}\) currents) and prevent voltage-dependent Ca\(^{2+}\) influx using voltage-clamp techniques, and yet contractions in those very same cells were unaffected (15)! Diacylglycerol is also generated concurrently with IP\(_3\) and leads to activation of protein kinase C; however, the importance of this latter pathway to contraction in ASM per se is debated. Other more recently described effector pathways include Rho kinase-mediated suppression of myosin light chain phosphatase (3, 4, 26, 32, 39), extracellular regulated kinase-mediated phosphorylation of caldesmon/calponin (37), phosphorylation of cytoskeletal proteins (9), and tyrosine kinase activity (34). The relative contributions of these and other pathways at different extremes of muscarinic stimulation have not yet been investigated. We found that inhibition of Rho kinase produces a much more profound effect (>75% inhibition) on the lower end of the CCh dose-response relationship than that seen during maximal cholinergic stimulation (<25%) (Fig. 7); submaximal cholinergic contractions are more relevant physiologically and clinically than maximal cholinergic excitation. Tyrosine kinase seems to be important in BSM but not TSM (Fig. 7A); others have found tyrosine kinase to be important for serotonin-induced Ca\(^{2+}\) release (34) and for refilling of the Ca\(^{2+}\) store (20).

In conclusion, there are several diverse mechanisms underlying excitation-contraction coupling in ASM, the relative importance of which seem to vary, depending on the spasmoden used (muscarinic vs. histamine), the degree of stimulation (threshold vs. maximal), the tissue (trachea vs. bronchi), and possibly also the species. It may be that there is a change in the relative contributions of these pathways during pathological conditions such as airway inflammation and asthma; this change might account in part for the increased airway hyperresponsiveness seen in these clinical conditions.

REFERENCES


