Ryanodine receptors decant internal Ca^{2+} store in human and bovine airway smooth muscle

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ABSTRACT: Several putative roles for ryanodine receptors (RyR) were investigated in human and bovine airway smooth muscle.

Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and membrane current were investigated in single cells by confocal fluorimetry and patch-clamp electrophysiology, respectively, whereas mechanical activity was monitored in intact strips with force transducers.

RyR released Ca^{2+} from the sarcoplasmic reticulum in a ryanodine- and chloroethyl phenol (CEP)-sensitive fashion. Neither ryanodine nor CEP inhibited responses to KCl, cholinergic agonists or serotonin, indicating no direct role for RyR in contraction; in fact, there was some augmentation of these responses. In tissues pre-contracted with carbachol, the concentration–response relationships for isoproterenol and salmeterol were unaffected by ryanodine; relaxations due to a nitric oxide donor were also largely unaffected. Finally, it was examined whether RyR were involved in regulating $[Ca^{2+}]_i$ within the subplasmalemmal space using patch-clamp electrophysiology as well as Ca^{2+} fluorimetry: isoproterenol increased $[Ca^{2+}]_i$- and Ca^{2+}-dependent K^+ current activity in a ryanodine-sensitive fashion.

In conclusion, ryanodine receptors in airway smooth muscle are not important in directly mediating contraction or relaxation. The current authors speculate instead that these allow the sarcoplasmic reticulum to release Ca^{2+} towards the plasmalemma (to unload an overly full Ca^{2+} store and/or increase the Ca^{2+}-buffering capacity of the sarcoplasmic reticulum) without affecting bronchomotor tone.

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In airway smooth muscle (ASM), activation of the contractile apparatus is initiated by agonist-induced mobilisation of internally sequestered Ca^{2+} from the sarcoplasmic reticulum (SR). Receptor-mediated activation of phospholipases triggers the cleavage of membrane-bound phosphatidylinositol 4,5-bisphosphate, liberating inositol-1,4,5-trisphosphate (IP_3), which in turn acts on specific ligand-activated Ca^{2+} channels on the SR (IP_3-receptors). Intracellular Ca^{2+} binds to and activates calmodulin, thereby triggering myosin phosphorylation *via* activation of myosin light chain kinase. The SR also expresses another class of Ca^{2+} channel, referred to as the ryanodine receptor (RyR) because of its sensitivity to that plant alkaloid. The endogenous ligand for this receptor is debated, with many proposing it to be cyclic adenosine diphosphate ribose (cADPR). The RyR also shows substantial sensitivity to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), being enhanced by a range of $[Ca^{2+}]_i$ above baseline and then

suppressed as $[Ca^{2+}]_i$ rises into potentially cytotoxic ranges. RyR, of which there are three subtypes, play a wide-ranging variety of roles in excitation–contraction (EC) coupling in smooth muscle (SM) of all types.

On the one hand, many view RyR as being important in contraction *via* a mechanism similar to that operative in cardiac muscle: *i.e.* a rise in $[Ca^{2+}]_i$ activates RyR on the SR, leading to a massive release of Ca^{2+}, a phenomenon referred to as Ca^{2+}-induced Ca^{2+} release (CICR) [1]. In cardiac muscle, the rise in $[Ca^{2+}]_i$ is secondary to Ca^{2+} influx through voltage-dependent Ca^{2+} channels and reverse-mode Na^+/Ca^{2+} exchange. The same mechanisms have been demonstrated in many SM preparations, particularly those of the vasculature [2].

On the other hand, it has been suggested that RyR can be involved in SM contraction *via* a skeletal muscle-like mechanism. In the latter,

membrane depolarisation activates voltage-dependent Ca^{2+} channels, which do not conduct a substantial influx of Ca^{2+} but rather interact directly/physically with RyR on the SR: the conformational change in the plasmalemmal channels produced by membrane depolarisation triggers activation of the RyR, culminating in Ca^{2+} release [1]. This mechanism has been proposed to be operative in ASM [3]. In a variation upon this theme, it has recently been suggested [4] that the voltage-gated Ca^{2+} channels in arterial myocytes can signal through a direct physical interaction with G proteins and activation of the phospholipase $C–IP_3$ pathway: Ca^{2+} released through IP_3-dependent channels of the SR then activates RyR to amplify the cytosolic Ca^{2+} signal.

Finally, some ascribe a role for RyR in relaxation. In particular, vasodilators are proposed to increase the activity of RyR, producing localised elevations of $[Ca^{2+}]_i$ known as ''sparks'', which in turn increase the activity of Ca^{2+}-dependent K^+ channels; the latter then mediate membrane hyperpolarisation and relaxation [5].

The role of RyR in ASM in particular is still not entirely clear, with some proposing its importance in bronchoconstriction [3, 6–11] or in bronchodilation [12, 13], and others finding no detectable role [14]. In the present study, the role of RyR in human and bovine ASM was investigated using Ca^{2+}-dye fluorimetry, mechanical tension and K^+ currents as indices of $[Ca^{2+}]_i$.

METHODS

Preparation of isolated tissues

All experimental procedures were approved by the McMaster University Animal Care Committee, the McMaster University Biosafety Committee and the St Joseph's Healthcare Research Ethics Board (all Hamilton, ON, Canada), and conformed to the guidelines set out by the Canadian Council on Animal Care (Ottawa, ON). Segments of donor (*i.e.* nondiseased) human main-stem bronchi were provided by the Lung Transplant Program (Toronto, ON). The overlying connective tissue, vasculature and thicker portions of the epithelium were removed and the SM was then cut into strips (~1 mm wide) parallel to the muscle fibres. Tracheas were obtained from cows weighing 200–500 kg euthanised at the local abattoir and transported to the lab (Firestone Institute for Respiratory Health, McMaster University, Hamilton) in ice-cold Krebs buffer. Upon receiving trachea in the lab, the epithelium was removed and tracheal ASM strips (~2–3 mm wide, ~10 mm long) were excised and used immediately or stored at 4°C for use up to 48 h later.

Cell isolation

Strips of SM were minced and placed in modified Hank's balanced salt solution (with $NaHCO_3$, without $CaCl_2$ and $MgSO_4$) containing collagenase (blend type F, 2 mg·mL^{-1}; Sigma-Aldrich Co., Oakville, ON, Canada) and elastase (type IV, 250 mg·mL^{-1}) and gently agitated for 20–40 min at 37°C. Cells were dispersed into solution by gentle mechanical agitation with a wide-bore Pasteur pipette. The cell suspension was then centrifuged to form a loose pellet that was resuspended in standard Ringer's solution.

Muscle bath technique

ASM strips were mounted vertically in organ baths using silk suture (Ethicon 4-O; Ethicon Inc., Somerville, NJ, USA) tied to a Grass FT.03 force transducer (Astro-Med Industrial Park, West Warwick, RI, USA) on one end and to a glass rod, which served as an anchor, on the other end. These were bathed in Krebs–Ringer's buffer containing indomethacin (10 µM) and N^{ω}-nitro-L-arginine (L-NNA; 10^{-4} M), bubbled with 95% O_2 and 5% CO_2, and maintained at 37°C. Tissues were passively stretched to impose a preload tension of ~1 g in bovine tracheal SM (TSM) or ~0.5 g in human bronchi. Isometric changes in tension were digitised (2 samples·s^{-1}) and recorded online (DigiMed System Integrator; MicroMed, Louisville, KY, USA) for plotting on the computer. Tissues were equilibrated for 1 h before commencing the experiments, during which time they were challenged with 60 mM KCl three times, to assess the functional state of each tissue.

Ca^{2+} fluorimetry

Isolated tracheal ASM cells were incubated with fluo-4 acetoxymethyl ester dye (2 mM, containing 0.1% pluronic F-127; Invitrogen Canada Inc., Burlington, ON, Canada) for 30 min at 37°C. Cells were then placed in a Plexiglas recording chamber, allowed to adhere for 10 min, then superfused with Ringer's solution for a period of 30 min prior to experimentation to allow for complete dye hydrolysis. Agonists were delivered within the bathing solution or *via* a micropipette (Picospritzer^{TM} II; General Valve, Fairfield, NJ, USA) brought into close proximity to the cell. Confocal microscopy was performed at room temperature (21–23°C) using a custom-built apparatus based on an inverted Nikon Eclipse TE2000-4 microscope using a $40 \times$ S Fluor oil objective. Briefly, 488-nm illumination from a photodiode laser was scanned across an isolated cell in X- and Y-planes using two mirrors oscillating perpendicularly at 8 kHz and 30 Hz, respectively. The emitted fluorescence (>500 nm; F_{510}) was detected by a photomultiplier. The signal was then digitised and the images were generated (1 frame·s^{-1}, 480×400 pixels), and these were stored in TIF stacks of several hundred frames using image-acquisition software (Video Savant 4.0; IO Industries, London, ON, Canada). Image files were then imported into Scion (Scion Corporation, Frederick, MD, USA) for subsequent analysis, using a custom-written macro designed to determine average fluorescence intensity over a defined non-nuclear region of interest. Fluorescence intensities were expressed as a fraction of the baseline fluorescence at the beginning of the experiment (F/F_0).

Patch-clamping experiments

Electrophysiological responses were tested in single ASM cells that were phase dense and appeared relaxed. Membrane K^+ currents were recorded at room temperature using the nystatin perforated patch configuration (0.3 mg·mL^{-1}). Micropipettes (tip resistance 3–5 MΩ) were made using a programmable puller (model P-87; Sutter Instrument Co., Novato, CA, USA) and then heat polished. Access resistance ranged from 9 to 32 MΩ and whole cell capacitance ranged from 11 to 46 pF. Membrane currents were filtered at 1 kHz and sampled at 2 kHz. Current signals were converted from analogue to digital format (DigiData 1200; Axon Instruments, Foster City, CA, USA). Acquisition and analysis of data were accomplished using an Axopatch 200B amplifier and pCLAMP8 software
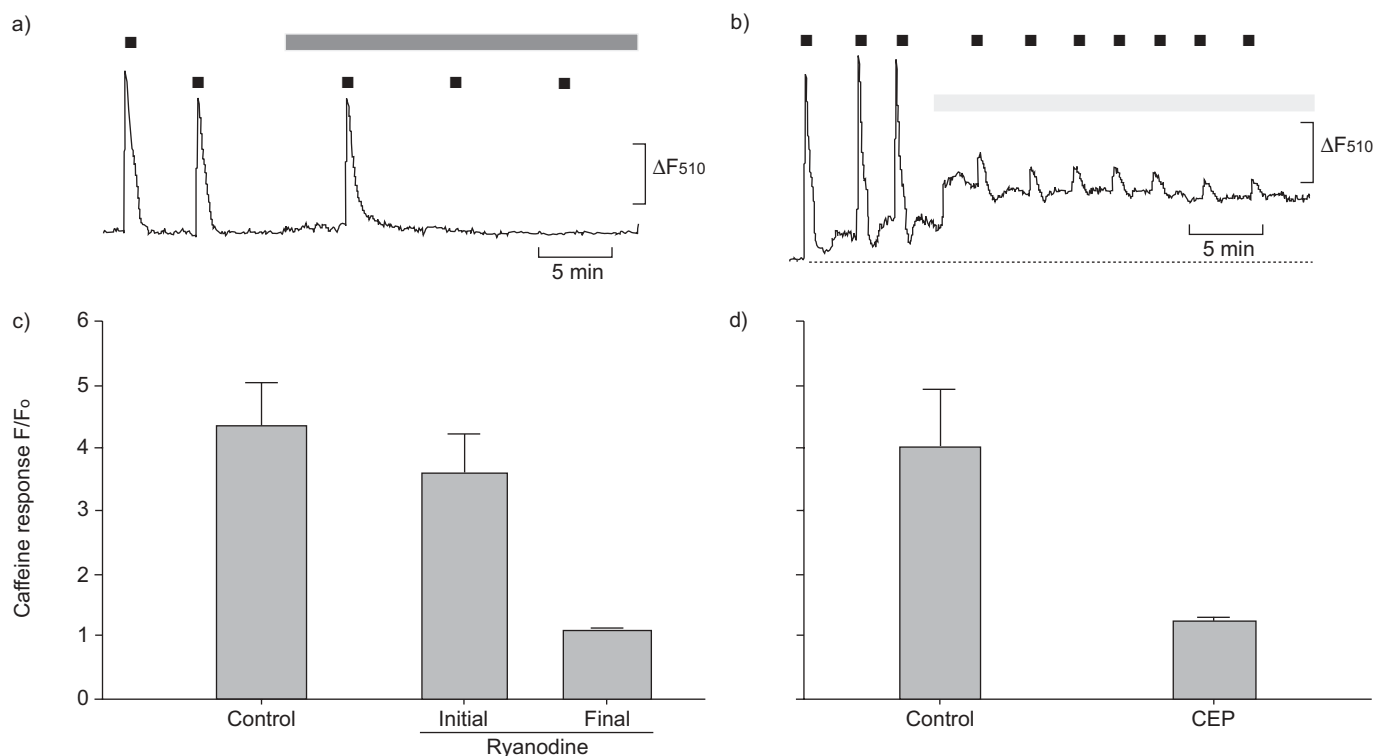
FIGURE 1.   Ryanodine receptors are functional. Representative recordings of Ca^{2+} transients evoked by caffeine (10 mM in application pipette; ■) before and during application of a) ryanodine (10^{-5} M; ■) or b) chloroethyl phenol (CEP; 10^{-4} M; ■) in the tissue bath. ·········: level of activity before the interventions were imposed. Mean $\pm$ SEM peak values of the Ca^{2+} transients, expressed as a fraction of the baseline fluorescence at the beginning of the experiment (F/F0) for c) ryanodine-treated cells (n=8) and d) CEP-treated cells (n=9). F_{510}: emitted fluorescence.

(Axon Instruments). Drug application was achieved by a micropuffer (Picospritzer™ II) or by addition to the perfusion medium.

Pipettes were sealed to the cell using a negative pressure at 0 mV; holding potential was then set to -70 mV. Cell break-in was achieved by the pore-forming antibiotic nystatin. Access resistance was compensated using 70% prediction and 70% correction. In most experiments, K^+ current–voltage relationships were recorded before and after drug application. The K^+ currents were also measured during step depolarisation to +30 mV (delivered at intervals of 10–15 s), to record the time-course of the drug effect. The data were later plotted and analysed using Clampfit 8.0 (Molecular Devices, Sunnyvale, CA, USA) and SigmaPlot 2000 (Jandel Scientific, San Rafael, CA, USA) software. Currents were standardised according to membrane area if applicable.

Solutions and chemicals
Tissues were studied using Krebs–Ringer's buffer containing 116 mM NaCl, 4.2 mM KCl, 2.5 mM $CaCl_2$, 1.6 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 22 mM $NaHCO_3$ and 11 mM D-glucose, bubbled with 95% O_2 and 5% CO_2 to maintain pH at 7.4. L-NNA (10^{-4} M) and indomethacin (10 μM) were also added to prevent generation of nitric oxide and of cyclo-oxygenase metabolites of arachidonic acid, respectively. Single cells were studied in Ringer's buffer containing 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM hydroxyethyl piperazine

ethane sulphonic acid and 10 mM D-glucose (pH adjusted to 7.4 using NaOH).

All chemicals were obtained from Sigma-Aldrich Co. and prepared as 10 mM stock solutions, either as aqueous solutions (caffeine, KCl and chloroethyl phenol (CEP)) or in dimethyl sulphoxide (DMSO; ryanodine and cyclopiazonic acid). Aliquots were then added to the muscle baths. The final bath concentration of solvents did not exceed 0.1%, which has been found elsewhere to have little or no effect on mechanical activity.

Data analysis
Contractions were expressed as a percentage of the response to 60 mM KCl added during the equilibration period (immediately before onset of the experiment). Data are reported as mean $\pm$ SEM and n refers to the number of animals. Statistical comparisons were made using unpaired t-tests, with $p < 0.05$ being considered statistically significant.

RESULTS
RyR are functional in ASM
Using Ca^{2+}-indicator dye fluorimetry, the present authors first showed that RyR are functional within the ASM. Single cells loaded with fluo-4 and challenged with caffeine exhibited a sharp spike-like elevation in $[Ca^{2+}]_i$ that reached a peak within 10 s and decayed to a much lower level even as the agent continued to be applied, quickly returning to baseline levels within seconds after application of the drug had ended (fig. 1).
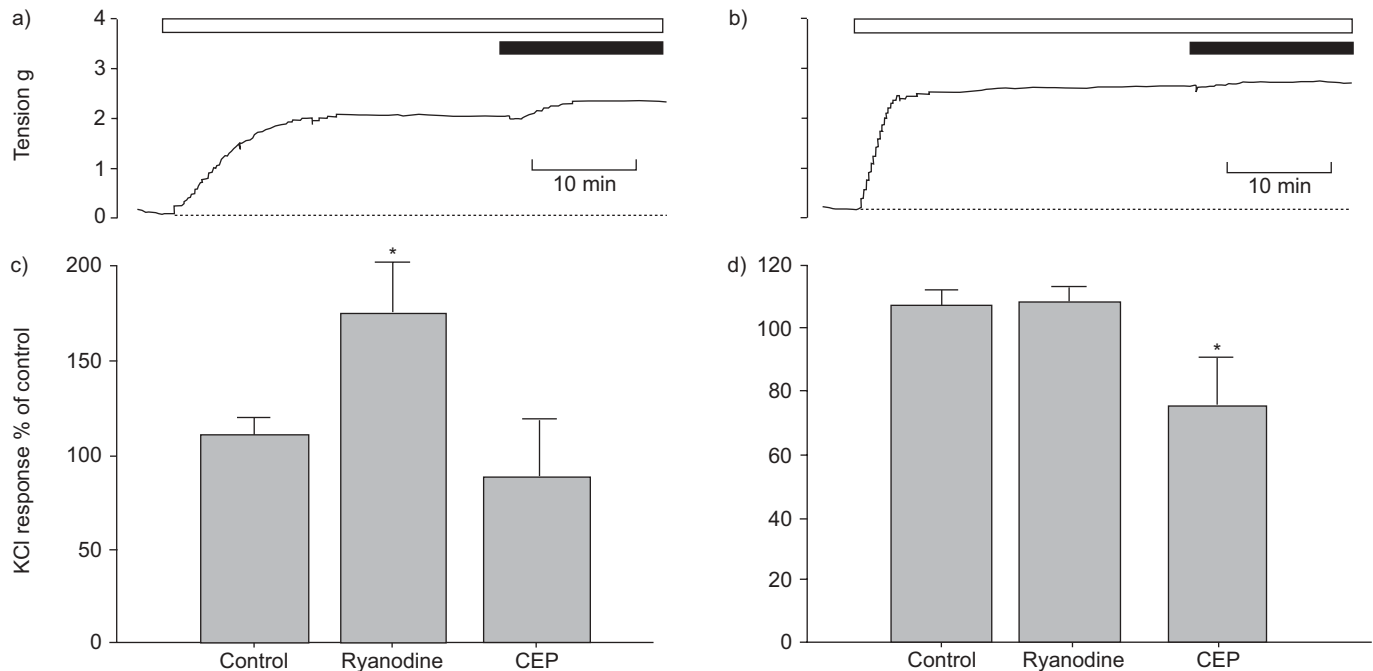
FIGURE 2. Ryanodine receptors and skeletal muscle-type coupling. Representative tracings showing the effect of ryanodine (10^{-5} M; ■) on contractions evoked by KCl (□) at a) 30 mM and b) 60 mM. ··········: level of activity before the interventions were imposed. Mean±SEM changes in KCl-evoked tone produced by ryanodine (10^{-5} M) or chloroethyl phenol (CEP; 10^{-4} M) using KCl at c) 30 mM and d) 60 mM. Four or five samples in each group. *: $p < 0.05$.

The caffeine-evoked responses were eliminated by 10^{-5} M ryanodine (n=8; fig. 1a and c). Note, however, that the first response evoked in the presence of ryanodine was little affected, consistent with the known tendency for ryanodine to bind preferentially to open RyR rather than those in the resting (closed) state. Conversely, CEP (10^{-4} M), a compound that mimics the ability of ryanodine to open RyR but without the inhibitory effects on RyR channel function [15, 16], increased baseline fluorescence (mean±SEM F/F0 of 2.4±0.3; n=9) and reduced the magnitude of the caffeine-evoked transients (fig. 1b and d). This was interpreted as indicating activation of RyR by CEP, thereby raising $[Ca^{2+}]_i$ and depleting the SR. Interestingly, as previously described [17], neither ryanodine nor CEP evoked any increase in tension (data not shown).

RyR and skeletal muscle-type EC coupling
The current authors examined whether RyR were directly involved in mediating contraction of ASM *via* the skeletal muscle-type mechanism that has been proposed to be operative in ASM [3, 4]. Tissues were depolarised using KCl (30 or 60 mM for 60 min) then challenged with agents that would inhibit or activate RyR (ryanodine 10^{-5} M and CEP 10^{-4} M, respectively) or with vehicle control (DMSO). KCl-evoked contractions were sustained, reaching a stable plateau within 30 min. Contrary to what would be predicted if skeletal-type coupling was operative in these tissues, the KCl-evoked contractions were not significantly decreased by ryanodine (fig. 2): in fact, the submaximal contractions evoked by 30 mM KCl were significantly augmented by ryanodine. Activation of RyR using CEP, however, significantly reduced tension evoked by 60 mM KCl (fig. 2).

RyR and cardiac muscle-type EC coupling
Next, the possibility was examined that contractions were dependent upon release of Ca^{2+} through RyR, in turn triggered by influx of external Ca^{2+} (CICR) *via* voltage-dependent Ca^{2+} channels and/or reverse-mode Na^+/Ca^{2+} exchange. Bovine tissues (n=4) were pretreated with ryanodine (10^{-5} M) before assessing their responsiveness to KCl (15–60 mM), acetylcholine (ACh; 10^{-9}–10^{-4} M) or serotonin (10^{-9}–10^{-4} M). In human tissues (n=3), the effect of ryanodine upon contractions evoked by carbachol (Cch) was assessed.

As shown previously [17], ryanodine significantly augmented responses to submaximal KCl stimulation but not to maximally effective concentrations of KCl (fig. 3a). Conversely, neither the cholinergic nor serotonergic concentration–response relationships were influenced by pretreatment with ryanodine, clearly ruling out a central role for CICR. Likewise, in human airway tissues, ryanodine did not suppress cholinergic responsiveness (fig. 3d). Qualitatively identical results were obtained when an excessively high concentration of ryanodine of 10^{-4} M was used (n=4; data not shown).

Since the temporal aspects of the excitatory response to an agonist are not adequately assessed when examining its concentration–response relationships in this way, another set of tissues was pretreated with ryanodine (10^{-6} or 10^{-5} M) or vehicle for 15 min then challenged with a single concentration of Cch (10^{-7} M) instead of ACh, due to the hydrolysable nature of the latter. The hypothesis was that any contribution of RyR in these contractions would be reflected in a decrease in the rate-of-rise (the first derivative of the contraction trace) of the cholinergic contraction and/or in the relative magnitudes of peak *versus* plateau responses. At the concentration used, Cch
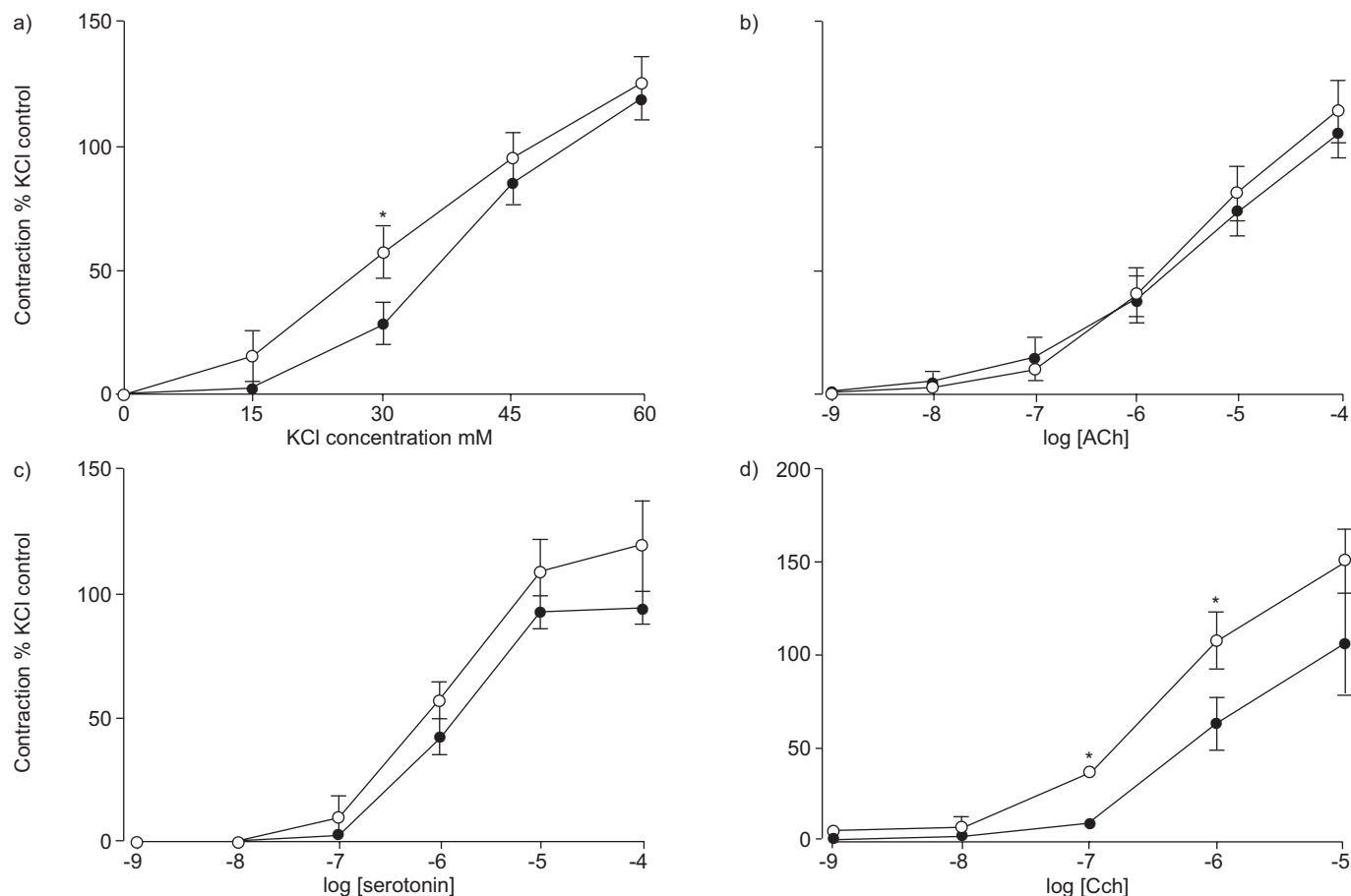
FIGURE 3. Role for ryanodine receptors in airway smooth muscle contraction in general. Mean concentration–response relationships for a) KCl, b) acetylcholine (ACh) or c) serotonin in bovine tracheal smooth muscle in the absence (●) *versus* presence of ryanodine (10^{-5} M; ○; n=4). d) Corresponding mean concentration–response relationship for carbachol (Cch) in human main stem bronchi (n=3). *: p<0.05.

elicited contractions that were comparable in magnitude to those evoked by 60 mM KCl; these reached a peak within 10 min then decayed partially (<10%) towards a stable plateau. In the bovine ASM tissues, pretreatment with ryanodine caused a significant increase, rather than a decrease, in the mean magnitude of the peak response, but had no effect on the mean magnitude of the plateau response or on the peak rate-of-rise of these contractions (fig. 4a). Likewise, in the human ASM tissues, there was no significant effect whatsoever of ryanodine (fig. 4b).

RyR and relaxation of ASM
To test whether RyR are involved in bronchodilation (*e.g.* through the stimulation of Ca^{2+}-dependent K^+ channels, as is the case in vascular SM [18, 19]), agonist-evoked relaxations were examined in the tissues that had been pretreated with ryanodine (10^{-6} or 10^{-5} M) or vehicle for 15 min then preconstricted with Cch (10^{-7} M) for 30 min (*i.e.* the tissues represented in fig. 4). In the bovine tissues, the full concentration–response relationships for isoproterenol (n=6; fig. 5a) and *S*-nitroso-*N*-acetyl-penicillamine (SNAP; n=6; fig. 5b) were tested, but only the half-maximally (10^{-9} M) and maximally (10^{-7} M) effective concentrations of salmeterol were tested (n=6; fig. 5d), due to the considerably slower time-course of action of this long-acting

β-agonist. In one set of human tissues (n=5), the effects of 10^{-6} or 10^{-5} M ryanodine were tested upon salmeterol-evoked relaxations in a similar manner (fig. 5c). In another set of tissues (n=3), however, only the effect of 10^{-5} M ryanodine was tested upon isoproterenol-evoked relaxations (fig. 5e).

No effect was seen for any of these pretreatment conditions upon the isoproterenol concentration–response relationship in bovine TSM; likewise, relaxations evoked by isoproterenol in human bronchi were not significantly affected. Salmeterol-evoked relaxations in human ASM appeared to be decreased by these conditions, although this effect was not found to be statistically significant. There was no effect upon salmeterol relaxations in bovine TSM. With respect to relaxations evoked in bovine TSM by SNAP, there was no effect of 10^{-6} M ryanodine on any portion of the SNAP concentration–response relationship. Likewise, there was no effect of 10^{-5} M ryanodine upon the lower portion (10^{-8} or 10^{-7} M) or the uppermost portion (10^{-5} M) of the SNAP concentration–response relationship. However, the response to 10^{-6} M SNAP was significantly decreased by 10^{-5} M ryanodine.

Interactions between RyR and plasmalemma
Finally, the present authors examined whether RyR were involved in regulating $[Ca^{2+}]_i$ within the subplasmalemmal
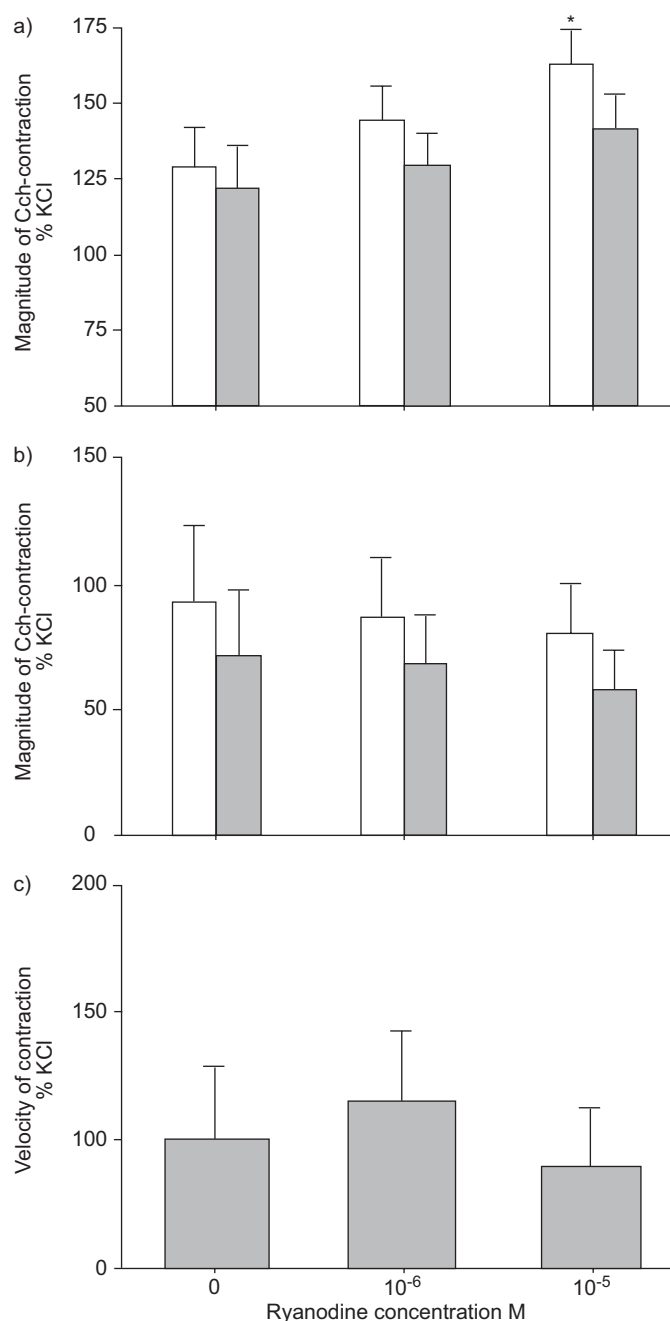
FIGURE 4. Ryanodine receptors and temporal aspects of cholinergic contractions. Mean magnitudes of contractions evoked by carbachol (Cch; 10^{-7} M), with peak (□) and sustained (■) components, following pretreatment with ryanodine (10^{-6} or 10^{-5} M) or vehicle in a) bovine tracheal smooth muscle or b) human main stem bronchi. c) Rate of Cch-evoked contractions in the bovine tissues, standardised as the percentage of the rate of the KCl-evoked contraction within each tissue. Six or seven samples in each group. *: $p < 0.05$.

evoking a large outward current. As has been shown previously [23–26], these represent a mixture of 4-aminopyridine-sensitive voltage-dependent K^+ currents as well as Ca^{2+}-dependent K^+ currents. Figure 6a shows the mean magnitudes of a series of consecutive K^+ current pulses. More importantly, this trace shows a marked augmentation of the Ca^{2+}-dependent K^+ current upon application of 10^{-6} M isoproterenol. Overall, these currents were augmented $71.6 \pm 25.8\%$ (n=5) by isoproterenol.

Many studies have characterised spontaneous transient outward currents in SM, including ASM [27–31], and shown these to represent instantaneous elevation of $[Ca^{2+}]_i$ at the plasmalemma due to activation of RyR, with consequent activation of a cluster of Ca^{2+}-dependent K^+ channels in their immediate proximity. These were not characterised in detail in the present study; however, isoproterenol (10^{-6} M) was also found to augment the frequency of these spontaneous K^+ currents (fig. 6b).

Finally, in another set of cells, the magnitude of K^+ currents at various steady-state potentials was examined in the presence and absence of both isoproterenol (10^{-6} M) and ryanodine (10^{-5} M). Figure 6c shows a typical recording, including the augmentation of these K^+ currents by isoproterenol and then marked suppression upon further addition of ryanodine. Overall, ryanodine suppressed isoproterenol-enhanced K^+ current measured at +30 mV by $35.9 \pm 16.2\%$ (n=4).

Collectively, these data suggest that isoproterenol elevates $[Ca^{2+}]_i$ underneath the plasmalemma. To corroborate this, Ca^{2+} fluorimetry was used. Cells were loaded with fluo-4 and challenged with 65 mM KCl (to clamp the membrane potential at ~0 mV and, thus, circumvent complications due to K^+ channel activation), then challenged with isoproterenol (10^{-5} M). Figure 7 shows that the latter intervention further elevated $[Ca^{2+}]_i$: the mean effect of isoproterenol was an increase in F/F_0 of $28 \pm 11\%$ (n=3).

DISCUSSION
In the present study, the contributions of RyR to EC coupling and Ca^{2+} handling in human and bovine ASM were examined.

Using the muscle bath technique to probe the contribution of RyR to excitatory responses to KCl, cholinergic agonists (ACh or Cch) or serotonin, it was shown that RyR play little or no causal role in skeletal-like or cardiac-like EC coupling in these tissues (figs 2, 3 and 4). The lack of a role for RyR in excitatory responses in ASM contradicts a number of other studies that have examined this question in murine [6, 7], rat [3, 9], rabbit [11] and canine [8] tissues; this might otherwise indicate species-related differences in the role of RyR in ASM. Another study found 0.2 mM ryanodine to abolish asynchronous Ca^{2+} waves in human airways (third and fourth order bronchi) [10], although its effects on force production were not reported (despite having described both types of effect for several other agents). The dissociation constant for ryanodine at RyR, as determined in membrane-free preparations, is in the low micromolar range [1]. However, the plasmalemma represents a substantial diffusional barrier for this hydrophilic agent at its intracellular target (the RyR), and for this reason many groups, including those cited, often use 10^{-4} M concentrations and/or prolonged treatment periods when using these agents. In the present study, the current authors do not feel that inadequate penetration of ryanodine is the explanation for the lack of

space. K^+ currents have already been characterised in detail in these tissues [20–22] and were not re-examined in detail in the present study; however, they were employed as biomarkers of $[Ca^{2+}]_i$ immediately underneath the plasmalemma.

In one experiment, bovine TSM cells were voltage-clamped at -70 mV and stepped at 10-s intervals to +30 mV, each time
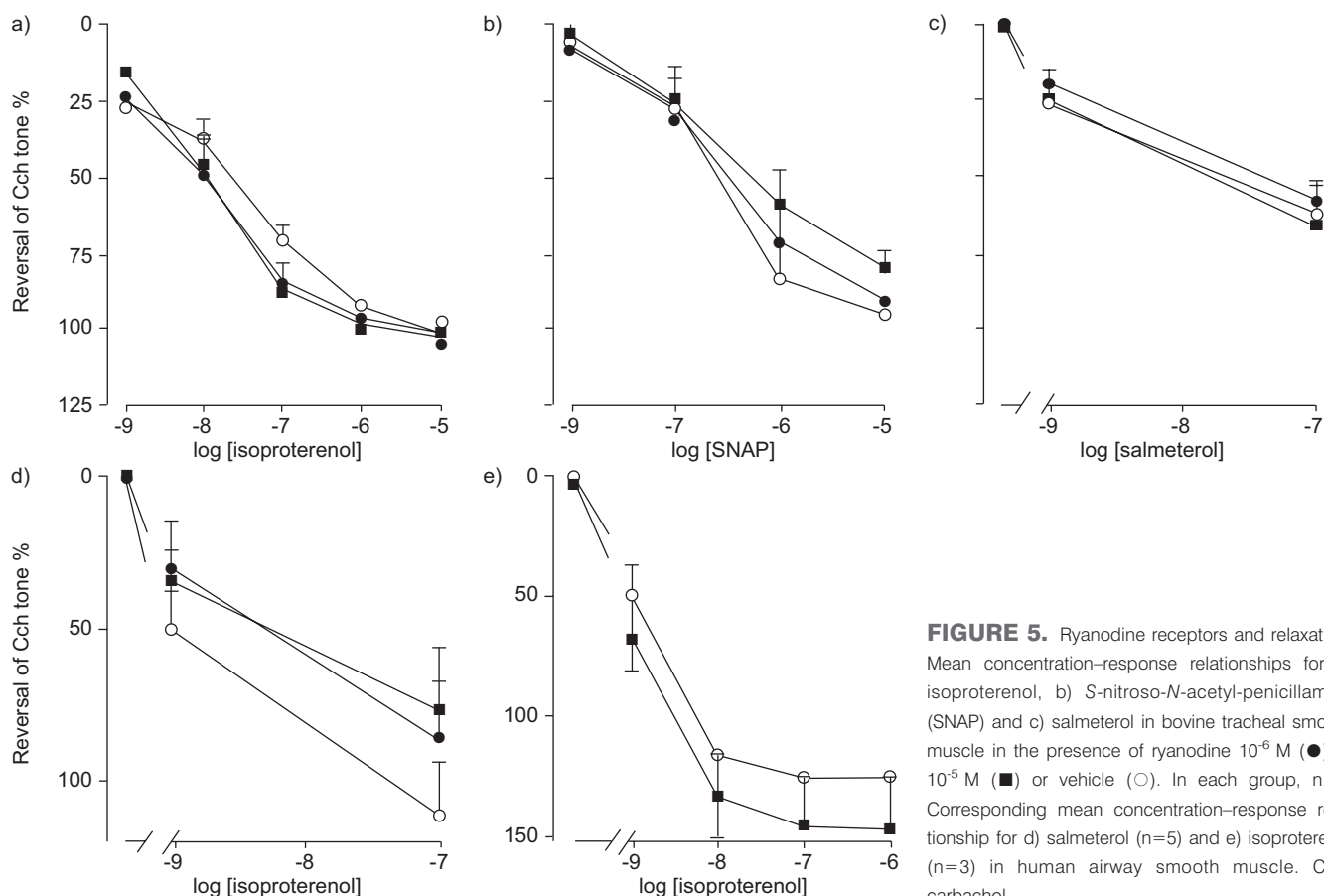
FIGURE 5. Ryanodine receptors and relaxation. Mean concentration–response relationships for a) isoproterenol, b) *S*-nitroso-*N*-acetyl-penicillamine (SNAP) and c) salmeterol in bovine tracheal smooth muscle in the presence of ryanodine 10^{-6} M (●) or 10^{-5} M (■) or vehicle (○). In each group, n=6. Corresponding mean concentration–response relationship for d) salmeterol (n=5) and e) isoproterenol (n=3) in human airway smooth muscle. Cch: carbachol.

effect of this hydrophilic agent, since they too tested concentrations up to 10^{-4} M and pretreatment periods extending up to 60 min and did not find an inhibitory effect on the mechanical responses. It is hard to conclude that RyR play an important causal role in cholinergic EC coupling in human/bovine ASM, given that 10^{-5} M ryanodine was found to be sufficient to abolish caffeine-evoked Ca^{2+} transients (fig. 1) and to augment contractions evoked by submaximally effective KCl (fig. 2) without inhibiting cholinergic contractions (figs 3 and 4).

Conversely, RyR can play an important role in relaxation of (vascular) SM through a mechanism based solely upon electromechanical coupling: *i.e.* release of Ca^{2+} through RyR leads to activation of K^+ channels, membrane hyperpolarisation and closure of voltage-dependent Ca^{2+} channels. In the present study, isoproterenol did appear to promote Ca^{2+} release through RyR (figs 6 and 7); this was also documented using the nitric oxide donor SNAP [32], and the same response was found upon stimulation with isoproterenol [13] and interleukin-4 [12]. Nonetheless, contraction of ASM is normally poorly dependent upon electromechanical coupling and, in the present study, the bronchodilatory action of isoproterenol was found to be largely unaffected by inhibition of RyR. Although relaxations to submaximally effective concentrations of SNAP were partially suppressed by ryanodine (fig. 5), the physiological relevance of this is not clear.

However, effects of ryanodine and CEP were observed that suggest RyR are involved in regulating $[Ca^{2+}]_i$ within the cytosolic space between the plasmalemma and the SR. These observations include the facts that: 1) caffeine and CEP evoke substantial elevations in $[Ca^{2+}]_i$ (fig. 1) without corresponding contractions; 2) blocking RyR enhanced, rather than suppressed, KCl-evoked (figs 2 and 3) and cholinergic (fig. 4) contractions, consistent with an action on the superficial buffer barrier as previously described [17]; and 3) isoproterenol increased $[Ca^{2+}]_i$ (fig. 7) and Ca^{2+}-dependent K^+ currents (fig. 6) in a ryanodine-sensitive fashion. Altogether, the current data indicate that RyR are important in directing Ca^{2+} from the SR towards the plasmalemma (rather than towards the contractile apparatus), allowing for unloading of Ca^{2+} from the SR in a manner that does not directly have an impact on mechanical activity. That is, constant uptake of Ca^{2+} by sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) may lead to a situation in which the SR must partially decant (through RyR) to prevent overfilling. Also, bronchodilators may act in part by promoting partial emptying of the SR and, thus, allowing for greater Ca^{2+} uptake from the deep cytosol (*i.e.* increasing the buffering capacity of the SR). Others have described a close apposition of the SR with the plasmalemma. This arrangement provides an ideal setting for the kind of differential regulation of SR loading state and activity of the contractile apparatus that the current authors propose: preferentially directed release of Ca^{2+} through RyR, aimed towards Ca^{2+}-extrusion mechanisms in the plasmalemma (Ca^{2+} pump and Na^+/Ca^{2+} exchange), will allow for efficient SR unloading without contraction. This arrangement is also consistent with the finding that the most intense staining for RyR (RyR1) was primarily distributed around the periphery of
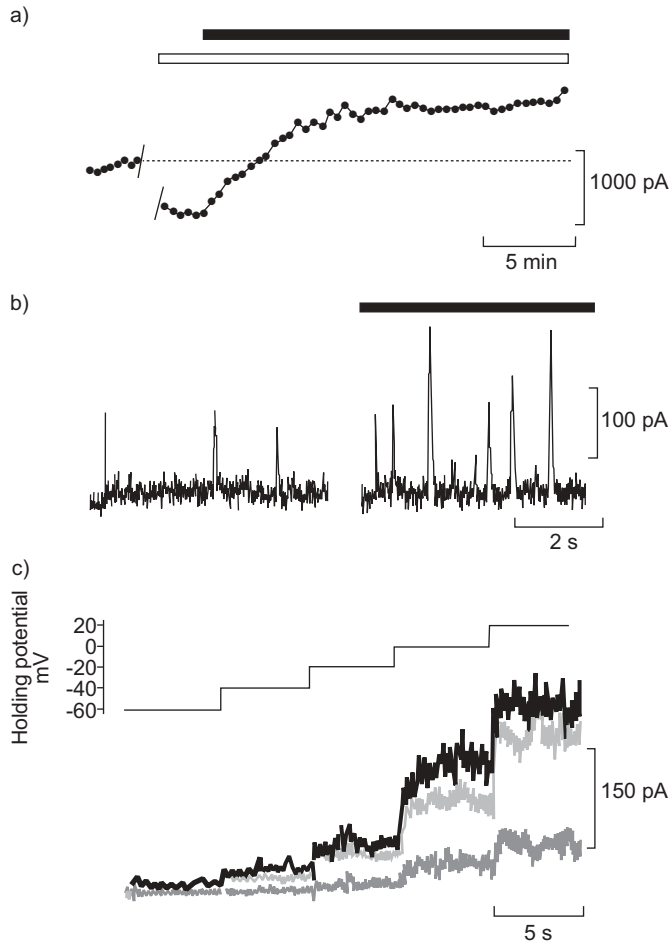
a)

1000 pA

5 min

b)

100 pA

2 s

c)

Holding potential
mV
20
0
-20
-40
-60

150 pA

5 s

FIGURE 6. Isoproterenol and ryanodine modulate K^+ currents. a) Magnitudes of K^+ currents evoked by step depolarisations (from -70 to +30 mV) in bovine tracheal smooth muscle under control conditions as well as during application of 1 mM 4-aminopyridine (AP; □) and then 10^{-6} M isoproterenol (■), both in the tissue bath. ⋯⋯⋯: level of activity before the interventions were imposed. b) Recordings of spontaneous transient outward currents evoked during voltage clamp at 0 mV in the absence and then presence of isoproterenol (10^{-6} M; ■) in the bath. c) Steady-state K^+ currents evoked at holding potentials of -60 to +20 mV before (■) *versus* during application of 10^{-6} M isoproterenol (■) and subsequent addition of 10^{-5} M ryanodine (■), both in the bath.

the SM cells (data not shown). In fact, the current authors hypothesise that RyR are found preferentially on the plasma-lemmal face of the SR compared with the opposite side of the SR, although light-based microscopy methods are not sufficient to discriminate this degree of spatial heterogeneity.

This mechanism also accounts for a set of paradoxical observations that are experimentally important (in that they may provide important insights into Ca^{2+} signalling) but perhaps physiologically irrelevant (in that they may never occur in nature): contraction of ASM is normally largely insensitive to Ca^{2+} channel blockers, but becomes acutely so under conditions in which SR function is disrupted [17, 30, 33–37]. It has been reviewed elsewhere [38] how these observations, as well as others showing refilling of the SR by voltage-dependent Ca^{2+} channels in a manner that does not involve uptake by SERCA [30, 33, 34],
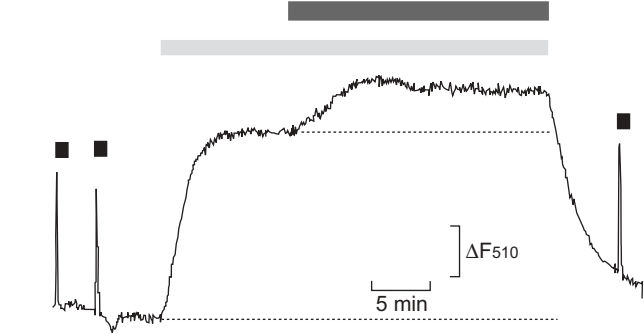

ΔF510

5 min

FIGURE 7. Isoproterenol elevates $[Ca^{2+}]_i$. Representative fluorimetric recording showing responses to caffeine (10 mM in application pipette; ■) as well as elevation of $[Ca^{2+}]_i$ upon addition of 65 mM KCl (■); replacing NaCl, in order to avoid hyperosmotic effects) and then subsequent addition of 10^{-6} M isoproterenol (■) in the tissue bath. F_{510}: emitted fluorescence; ⋯⋯⋯: level of activity before the interventions were imposed.

can be explained by retrograde influx of Ca^{2+} into the SR through RyR under conditions in which the driving force on Ca^{2+} has been reversed.

CEP exerted apparently paradoxical effects upon the KCl-evoked contractions: suppressing responses to 60 mM KCl without affecting those to 30 mM KCl (both compared with the control responses; fig. 2). CEP is reputed to mimic ryanodine's ability to open RyR, but without the inhibitory effects on RyR channel function [15, 16], and the observation of its ability to elevate $[Ca^{2+}]_i$ and abrogate fluorimetric responses to caffeine (fig. 1) is consistent with this. It should also, therefore, mimic ryanodine's ability to disrupt the superficial buffer barrier; however, responses to 30 mM and 60 mM KCl were both reduced in the presence of CEP relative to those obtained in the presence of ryanodine. Low *et al.* [39] were the first to examine the effect of CEP on SM and also described a direct Ca^{2+}-releasing effect of CEP (as reflected in fluorimetric recordings) accompanied by an inhibition of both agonist- and KCl-evoked contractions. Low *et al.* [39] suggested that this might indicate a direct effect of CEP on some post-receptor mechanism, such as Ca^{2+} interaction with calmodulin, the ability of Ca^{2+}/calmodulin to activate myosin light chain kinase, or the interaction of myosin and actin and cross-bridge cycling itself [39]. This would also explain the paradoxical effects of CEP against KCl in the present study: with 30 mM KCl, both CEP and ryanodine disrupted the superficial buffer barrier and, thus, augmented the KCl response, but CEP also added some other inhibitory effect that masked the net increase (fig. 2a). With 60 mM KCl, neither ryanodine nor CEP augmented the responses (because the superficial buffer barrier was overwhelmed at this level of Ca^{2+} influx) but CEP still exerted this additional inhibitory effect (fig. 2b). The current data do not allow further speculation on the nature of this second inhibitory effect of CEP.

Some studies have postulated that cADPR (produced by the ectoenzyme CD38) and/or nicotinic acid adenine dinucleotide phosphate modulate Ca^{2+} responses *via* an action on high-affinity binding sites coupled in some way to RyR [40–44] and may, therefore, represent the endogenous ligand(s) for RyR

[45]. It may be, then, that these molecules are important for regulation of the filling state of the SR in ASM.

In conclusion, ryanodine receptors are not directly important in mediating airway smooth muscle contraction (neither through skeletal-like coupling nor Ca^{2+}-induced Ca^{2+} release) or relaxation. Instead, the present data suggest that ryanodine receptors direct sarcoplasmic reticulum Ca^{2+} towards the plasmalemma and its associated extrusion mechanisms (Ca^{2+}-pump and Na^+/Ca^{2+} exchange), in order to decant an overloaded sarcoplasmic reticulum and/or increase sarcoplasmic reticulum Ca^{2+}-buffering capacity without necessarily evoking bronchoconstriction.

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