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RESEARCH PAPER

Role of $InsP_3$ and ryanodine receptors in the activation of capacitative Ca^{2+} entry by store depletion or hypoxia in canine pulmonary arterial smooth muscle cells

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Background and purpose: Experiments were performed to determine if capacitative Ca^{2+} entry (CCE) in canine pulmonary arterial smooth muscle cells (PASMCs) is dependent on $InsP_3$ receptors or ryanodine receptors as induction of CCE is dependent on simultaneous depletion of the functionally separate $InsP_3$- and ryanodine-sensitive sarcoplasmic reticulum (SR) Ca^{2+} stores in these cells.
Experimental approach: Myocytes were isolated from canine pulmonary arteries using enzymatic procedures and were used within 8 h of preparation. Measurements of cytosolic Ca^{2+} were made by imaging fura-2 loaded individual myocytes that were perfused with physiological buffered saline solution with or without Ca^{2+}.
Key results: Treating myocytes with 10 μM cyclopiazonic acid (CPA), removing extracellular Ca^{2+}, and briefly applying 10 mM caffeine and 10 μM 5-hydroxytryptamine (5-HT) depleted SR Ca^{2+} stores. Extracellular Ca^{2+} reintroduction caused cytosolic [Ca^{2+}] to elevate above baseline signifying CCE. The $InsP_3$ receptor inhibitors 2-aminobiphenylborate (50-75 μM; 2-APB) and xestospongin-C (20 μM; XeC) abolished CCE. Yet, CCE was unaffected by 10 μM or 300 μM ryanodine or 10 μM dantrolene, which modify ryanodine receptor activity. Higher dantrolene concentrations (50 μM), however, can inhibit both ryanodine receptors and $InsP_3$ receptors, did reduce CCE. In contrast, CCE activated by hypoxia was unaffected by XeC (20 μM).
Conclusions and implications: The results provide evidence that CCE activated by depletion of both $InsP_3$ and ryanodine SR Ca^{2+} stores in canine PASMCs is dependent on functional $InsP_3$ receptors, whereas the activation of CCE by hypoxia appears to be independent of functional $InsP_3$ receptors.
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Keywords: fura-2; sarcoplasmic reticulum; xestospongin-C; 5-hydroxytryptamine; 2-aminobiphenylborate; dantrolene; cyclopiazonic acid; hypoxia

Abbreviations: 2-APB, 2-aminobiphenylborate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CCE, capacitative Ca^{2+} entry; CIF, calcium influx factor; CPA, cyclopiazonic acid; CRAC, calcium release-activated channels; DMSO, dimethyl sulphoxide; HPV, hypoxic pulmonary vasoconstriction; $InsP_3$, inositol 1,4,5-triphosphate; I_{SOC}, store-operated currents; PASMCs, pulmonary arterial smooth muscle cells; PSS, physiological saline solution; SERCA, sarcoplasmic–endoplasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum; TRPC, canonical transient receptor potential; XeC, xestospongin-C

Introduction

Two types of Ca^{2+} release channels are located on the sarcoplasmic reticulum (SR) of smooth muscle cells: ryanodine-sensitive channels (ryanodine receptors that are activated by rises in Ca^{2+}), and inositol 1,4,5-triphosphate ($InsP_3$)-sensitive Ca^{2+} channels that are activated by $InsP_3$, which is produced downstream of neural or humoral stimulation of G-protein or tyrosine-coupled membrane bound receptors (Bootman and Berridge, 1995). Recent contractile and Ca^{2+} imaging studies from our laboratory demonstrated that functional differences exist in the SR Ca^{2+} stores of acutely isolated canine pulmonary arterial smooth muscle cells

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(PASMCs) (Jabr *et al*., 1997; Janiak *et al*., 2001). The contractile data showed that in canine pulmonary arterial rings phenylephrine caused contraction through release of Ca^{2+} from $InsP_3$-sensitive stores (Somlyo and Somlyo, 1994). The α_1 adrenoceptor-mediated contraction could be inhibited when the $InsP_3$-sensitive Ca^{2+} stores were depleted of their Ca^{2+} in the presence of the sarcoplasmic–endoplasmic reticulum Ca^{2+} ATPase (SERCA) blocker cyclopiazonic acid (CPA) (Goeger *et al*., 1988) without affecting subsequent contraction due to release of ryanodine-sensitive Ca^{2+} stores with caffeine (Jabr *et al*., 1997). Similarly, depletion of ryanodine-sensitive Ca^{2+} stores by exposing cells to caffeine in the presence of ryanodine did not affect subsequent contraction due to release of the $InsP_3$-sensitive Ca^{2+} stores. These experiments along with Ca^{2+} imaging experiments on canine PASMCs (Janiak *et al*., 2001) provided good evidence that in these cells, the $InsP_3$- and ryanodine-sensitive Ca^{2+} stores were independent.

5-Hydroxytryptamine (5-HT) is a potent mediator of pulmonary hypertension by stimulating contraction and proliferation of PASMCs (McGoon and Vlietstra, 1984; MacLean *et al*., 2000) and its effects on intracellular Ca^{2+} and contractility are similar to those of phenylephrine. We have recently shown that Ca^{2+} release and contractility elicited by 5-HT is dependent on activation of $InsP_3$ receptors, L-type Ca^{2+} channels as well as tonic Ca^{2+} entry pathways (Wilson *et al*., 2005). 5-HT, however, does not promote pulmonary arterial contraction through activation of ryanodine receptors (Wilson *et al*., 2005).

Depletion of SR Ca^{2+} stores in many cell types activates Ca^{2+} permeable store-operated currents (I_{SOC}) on the plasma membrane, which replenish the empty stores through a process known as 'capacitative Ca^{2+} entry' (CCE) (Putney, 1986). Recently, we reported that CCE (Wilson *et al*., 2002) as well as I_{SOC} (Wilson *et al*., 2005) in canine PASMCs are activated in parallel with the organization of the SR Ca^{2+} stores; CCE and I_{SOC} can be activated only with simultaneous $InsP_3$ and ryanodine SR Ca^{2+} store depletion. 5-HT exposure alone was also not sufficient to activate CCE or I_{SOC} in canine PASMCs (Wilson *et al*., 2005).

The mechanisms linking SR Ca^{2+} store depletion to CCE activation are diverse. CCE can be activated through a coupling of SR-bound $InsP_3$ receptors (Ma *et al*., 2000) or possibly ryanodine receptors (Bennett *et al*., 1998; Kiselyov *et al*., 2001; Cherednichenko *et al*., 2004; Hurne *et al*., 2005) to store-operated channels. Decreases in the luminal Ca^{2+} content can also activate CCE independent of direct $InsP_3$ receptor stimulation (Hofer *et al*., 1998). Alternatively, depletion of the SR Ca^{2+} stores may lead to the production and release of a calcium influx factor (CIF) (Trepakova *et al*., 2000) or STIM proteins (Roos *et al*., 2005; Zhang *et al*., 2005; Spassova *et al*., 2006) that activates CCE. A better understanding of the process of CCE activation in PASMCs is particularly important, since release of SR Ca^{2+} stores and induction of CCE pathways in canine (Jabr *et al*., 1997; Ng *et al*., 2005) and rat (Robertson *et al*., 2000; Wang *et al*., 2005; Weigand *et al*., 2005) have been implicated in the unique vasoconstrictor response of pulmonary arteries to hypoxia (HPV). Because CCE in canine PASMCs can be activated by simultaneous depletion (Wilson *et al*., 2002) of the functionally independent $InsP_3$- and ryanodine-sensitive SR Ca^{2+} stores (Janiak *et al*., 2001), the present study was designed to examine whether store depletion *per se* is required for CCE activation in canine PASMCs or whether there is an additional involvement of $InsP_3$ receptors or ryanodine receptors. We also tested whether functional $InsP_3$ receptors are required for activation of CCE by hypoxia. An involvement of one or more of these receptors may provide evidence in support of a conformational coupling model of CCE. The findings indicate that functional $InsP_3$ receptors, but not ryanodine receptors, are required for store depletion-induced CCE activation in canine PASMCs, whereas activation of CCE by hypoxia is independent of functional $InsP_3$ receptors.

Methods

Cell isolation

Smooth muscle cells were isolated from high-resistance canine pulmonary arteries as previously described (Janiak *et al*., 2001; Wilson *et al*., 2002). Mongrel dogs of either sex were killed with pentobarbital sodium (45 mg kg^{-1} intravenously) and ketamine (15 mg kg^{-1} intravenously), as approved by the University of Nevada, Reno Institutional Animal Care and Use Committee. The heart and lungs were excised *en bloc*. The third and fourth branches of pulmonary arteries were dissected at 5°C to decrease cellular metabolic activity. Pulmonary artery isolations and smooth muscle cell dispersions were made in a low-Ca^{2+} physiological saline solution (PSS) containing in mM: 125 NaCl; 5.36 KCl; 0.336 Na_2HPO_4; 0.44 K_2HPO_4; 11 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES); 1.2 $MgCl_2$; 0.05 $CaCl_2$; 10 glucose; pH 7.4 (adjusted with Tris), osmolarity 300 mosM. Arteries were cleaned of connective tissue, cut into small pieces and placed in a tube containing fresh PSS. Tissue was immediately digested or cold stored in the refrigerator (5°C) up to 24 h. To disperse cells, tissue was placed in low-Ca^{2+} PSS enzymes containing (in mg ml^{-1}): 0.5 collagenase type XI; 0.03 elastase type IV and 0.5 bovine serum albumin (fat-free) for 14–16 h at 5°C. The tissue was then washed several times with 5°C low-Ca^{2+} PSS solution and triturated with a fire-polished Pasteur pipette. The resulting dispersed PASMCs were cold stored at 5°C up to 8 h until experiments were performed. Approximately, 30% of the cells used in this study were dispersed from overnight storage of tissues and the remainder of the cells were dispersed from tissues isolated on the same day.

Fluorescence imaging

Cytosolic [Ca^{2+}] was measured in canine PASMCs loaded with the ratiometric Ca^{2+}-sensitive dye fura-2 AM (Molecular Probes, Eugene, OR, USA) using a dual excitation digital Ca^{2+} imaging system (IonOptix Inc., Milton, MA, USA) equipped with an intensified charge-coupled device (CCD). The imaging system was mounted on an inverted microscope (Nikon Inc., Melville, NY, USA) outfitted with a magnification of ×40 (NA 1.3, Nikon Inc.) oil immersion objective. Fura-2 AM was dissolved in dimethyl sulphoxide (DMSO) and added from a 1 mM stock to the cell suspension at a final

concentration of 10 μM. Cells were loaded with fura-2 AM for 20–30 min in a perfusion chamber (Warner Instruments, Hamden, CT, USA) at room temperature in the dark. Cells were then washed for 30 min to allow for dye esterification at 2 ml min^{-1} with a balanced salt solution of the following composition (mM): 126 NaCl; 5 KCl; 0.3 NaH_2PO_4; 10 HEPES; 1 $MgCl_2$; 2 $CaCl_2$; 10 glucose; pH 7.4 (adjusted with NaOH) 285–305 mosM. Measurements of cytosolic [Ca^{2+}] before and during CCE and pharmacological manipulation were made once the fura-2 fluorescence ratio stabilized. The Ca^{2+}-free balanced salt solution was prepared by substituting $MgCl_2$ for $CaCl_2$ and adding 1 mM ethylene glycol tetraacetic acid (EGTA). Cells were illuminated with a xenon arc lamp at 340 $\pm$ 15 and 380 $\pm$ 12 nm (Omega Optical, Brattleboro, VT, USA), and emitted light was collected from regions that encompassed single cells with a CCD at 510 nm (Nikon Inc.). In most experiments, images were acquired at 1 Hz and stored on either compact disk or magnetic media for later analysis. Although it is difficult to precisely measure the intracellular calcium concentration ([Ca^{2+}]$_i$) (Baylor and Hollingworth, 2000), estimates were made from the relation $[Ca^{2+}]_i = K_d(Sf_2/Sb_2)(R-R_{min})/(R_{max}-R)$, where R_{min} and R_{max} are the F_{380}/F_{340} ratios of Ca^{2+}-free and Ca^{2+}-saturated fura-2, respectively. Sf_2 is the F_{380} of Ca^{2+}-free fura-2 and Sb_2 is F_{380} of Ca^{2+}-bound fura-2. The values of Sf_2 and R_{min} were determined by bathing cells in a balanced salt solution that did not have any added Ca^{2+} and contained 10 mM EGTA and 1 μM ionomycin. The values of Sb_2 and R_{max} were determined by bathing cells in a balanced salt solution contained 10 mM Ca^{2+} and 1 μM ionomycin. The K_d for fura-2 was assumed to be 224 nM (Grynkiewicz *et al*., 1985). During the Ca^{2+} calibration, 5 mM 2,3-butanedione monoxime was added to the bathing solution to inhibit smooth muscle contraction (Waurick *et al*., 1999). Experimental temperature was 22–25°C.

In experiments where the effect of hypoxia was investigated, hypoxia was induced by switching normoxic balanced salt solution to hypoxic balanced salt solution, which continuously superfused the cells in the recording chamber as previously described (Ng *et al*., 2005). Hypoxic solution was prepared by continuous gassing with uncertified gas mixtures containing 95% N_2 and 5% CO_2 (Sierra Welding, Sparks, NV, USA). The uncertified gas mixture contained minimal amounts of oxygen, which equilibrated with the solution to avoid exposure of cells to anoxic condition. All solutions were saturated with either normoxic or hypoxic gas mixtures for at least 30 min before the start of perfusion, and maintained at pH 7.4. The P_{O_2}, measured in preliminary experiments with an O_2-sensitive electrode (MI-730; Microelectrodes Inc., Bedford, NH, USA), was 145 $\pm$ 1 mm Hg during normoxic PSS perfusion and fell to 15 $\pm$ 1 mm Hg within 79 $\pm$ 2 s of hypoxic exposure. The P_{O_2} of hypoxic solutions was measured at the end of each experiment and was found to be 15–18 mm Hg, ensuring that the P_{O_2} did not approach anoxia during recording of each experiment.

Statistical analysis

All data are presented as mean $\pm$ s.e.m. Statistical difference within groups was determined with a two-tailed paired Student's *t*-test. Statistical difference between groups was determined with an unpaired Student's *t*-test. In cases where the data were not normally distributed, a Wilcoxon signed-rank sum test was used to test for differences within groups. A one-way analysis of variance with a Neuman–Keuls multiple comparison procedure was used to test for differences between groups. The specific test used for each data set is noted in the legend for each figure. A *P*-value <0.05 was accepted as statistically significant. The *n* values reported reflect the total number of cells tested. Multiple trials were performed and cells isolated from several animals for most experimental paradigms.

Chemicals and drugs

Ionomycin free acid and Xestospongin-C (XeC) were purchased from Calbiochem (San Diego, CA, USA), Fura-2 AM from Molecular Probes, ryanodine from Calbiochem LC Laboratories (Woburn, MA, USA) or Alomone Laboratories (Tel Aviv, Israel), and all other chemicals were purchased from Sigma (St Louis, MO, USA).

Results

Recently, we have shown that 50–75 μM 2-APB as well as 20 μM XeC significantly attenuated the cytosolic Ca^{2+} increases elicited by 5-HT (Wilson *et al*., 2005). To test the specificity of these agents, we now initially examined whether 2-APB or XeC might also inhibit CAF-induced ryanodine receptor-mediated cytosolic [Ca^{2+}] increases as well. Figure 1a shows that a 30-s 10 mM caffeine exposure caused a rapid, transient increase in cytosolic [Ca^{2+}] of 537 nM and this was not affected by exposure to 50 μM 2-APB, where 10 mM caffeine caused a 557 nM cytosolic [Ca^{2+}] increase. Figure 1b summarizes the results from nine cells and shows clearly that 50 μM 2-APB did not appear to inhibit ryanodine receptor activity. Very similar results were observed with XeC (Figure 1c). The cytosolic Ca^{2+} increase induced by caffeine (10 mM) was not affected by the addition of XeC (20 μM).

Role of $InsP_3$ receptors and ryanodine receptors in store depletion-induced CCE

Experiments were carried out to address the possibility that $InsP_3$ receptors are functionally coupled to CCE in canine PASMCs, as previously demonstrated in HEK 293 cells expressing transfected canonical transient receptor potential (TRPC) TRPC3 channels (Ma *et al*., 2000). Figure 2 shows the effects of the SERCA inhibitor CPA on the cytosolic [Ca^{2+}]. CPA, at the concentration used in the present studies, has been used to deplete intracellular Ca^{2+} stores in many smooth muscle cells including rabbit portal vein myocytes (Albert and Large, 2002) and rat uterine myocytes (Shmigol *et al*., 1999) as well as canine pulmonary arteries (Jabr *et al*., 1997) and smooth muscle cells (Janiak *et al*., 2001; Wilson *et al*., 2002). Specifically, CPA selectively depletes the $InsP_3$-sensitive Ca^{2+} stores in canine pulmonary arteries, as caffeine can still elicit ryanodine-sensitive Ca^{2+} release and

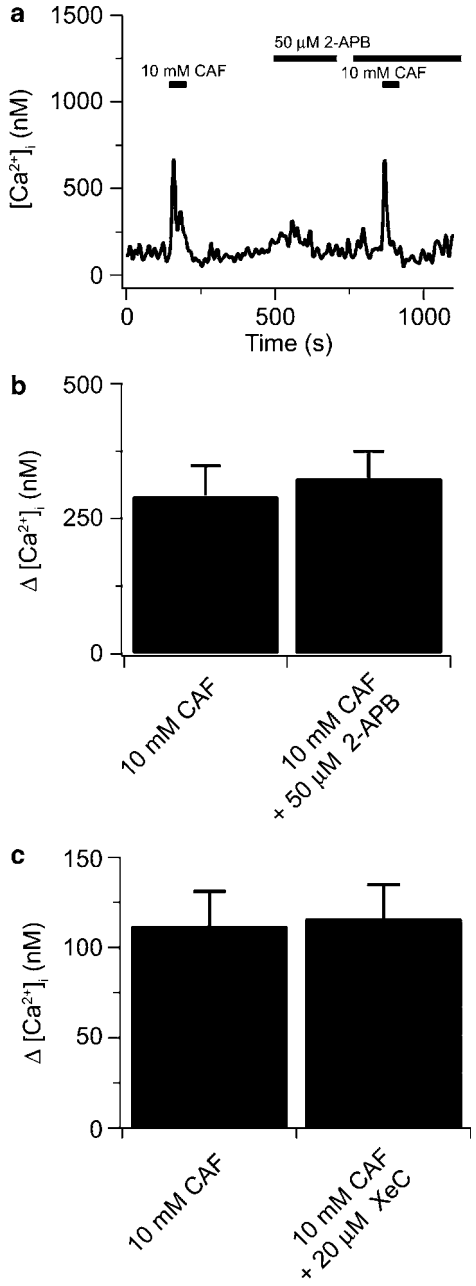

Figure 1 2-APB and XeC does not inhibit caffeine-elicited cytosolic Ca^{2+} increases in canine PASMCs. (**a**) 10 mM caffeine (CAF) induced Ca^{2+} transients in the absence then the presence of 50 μM 2-APB. (**b**) Bars show the magnitude of the peak cytosolic Ca^{2+} increase elicited by 10 mM caffeine before and during 50 μM 2-APB. There was no significant difference between the two groups using a two-tailed paired *t*-test ($n=9$). (**c**) Bars show the magnitude of the peak cytosolic Ca^{2+} increase elicited by 10 mM caffeine before and during 20 μM XeC. There was no significant difference between the two groups using a two-tailed paired *t*-test ($n=23$). Bars represent means$\pm$s.e.m. 2-APB, 2-aminobiphenylborate; PASMCs, canine pulmonary arterial smooth muscle cells; XeC, xestospongin-C.

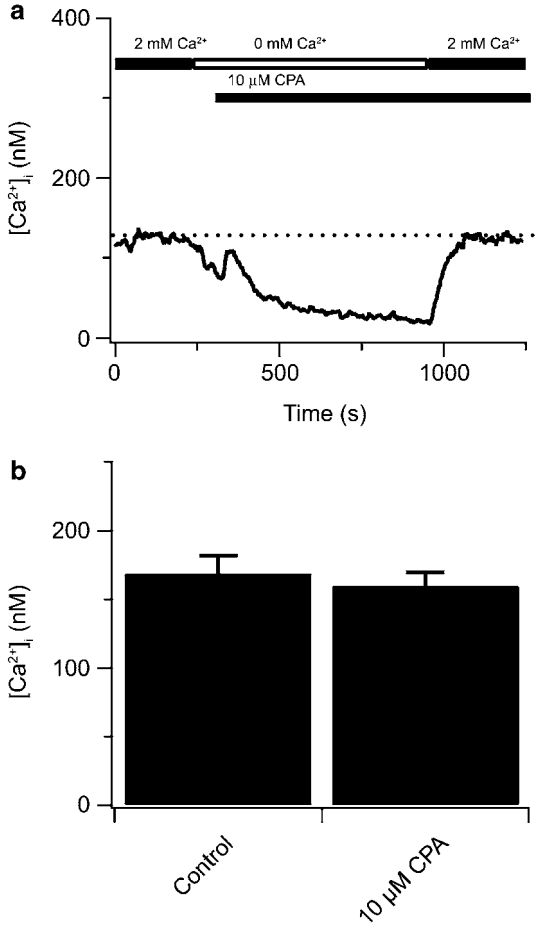

Figure 2 Cyclopiazonic acid (CPA) alone does not effectively activate CCE in canine PASMCs. (**a**) Effect of 10 μM CPA and extracellular Ca^{2+} removal on cytosolic [Ca^{2+}] during extracellular Ca^{2+} re-addition. (**b**) Bars indicate the cytosolic [Ca^{2+}]. Dashed line shows resting cytosolic [Ca^{2+}]. There was no significant difference between the basal [Ca^{2+}] measurements and those during CPA using a two-tailed paired *t*-test ($n=27$). Bars represent means$\pm$s.e.m. CCE, capacitative Ca^{2+} entry; PASMCs, canine pulmonary arterial smooth muscle cells.

contractility in the presence of CPA (Jabr *et al.*, 1997; Janiak *et al.*, 2001). As demonstrated previously, CPA exposure is only expected to activate CCE in canine PASMCs when combined with ryanodine-sensitive Ca^{2+} store depletion. Figure 2a shows that Ca^{2+} re-addition following 10 μM CPA for $\sim$5 min in a Ca^{2+}-free bathing did not cause any rise in cytosolic [Ca^{2+}] above basal values in a single PASMC, with cytosolic [Ca^{2+}] remaining at 123 nM. Figure 2b summarizes data illustrating that CPA exposure in a Ca^{2+}-free bathing solution does not elicit any increase in Ca^{2+} above basal values with Ca^{2+} re-addition.

Figure 3 shows the effects of fully depleting both SR Ca^{2+} stores on the cytosolic [Ca^{2+}] and the actions of 2-APB and XeC on CCE in canine PASMCs. We have recently shown that 2-APB as well as XeC inhibit 5-HT-elicited Ca^{2+} increases (Wilson *et al.*, 2005), consistent with inhibition of $InsP_3$ receptors (Gafni *et al.*, 1997; Wu *et al.*, 2000; Ta *et al.*, 2005). Figure 3a shows that in a single PASMC the SR Ca^{2+} stores could be fully depleted by perfusing with a Ca^{2+}-free bathing solution in the continuous presence of 10 μM CPA and 10 μM ryanodine, and exposing the cell twice for 30 s to 10 mM caffeine and 10 μM 5-HT. We have used protocols similar to this one to simultaneously deplete $InsP_3$- and ryanodine-sensitive intracellular Ca^{2+} stores (Janiak *et al.*,

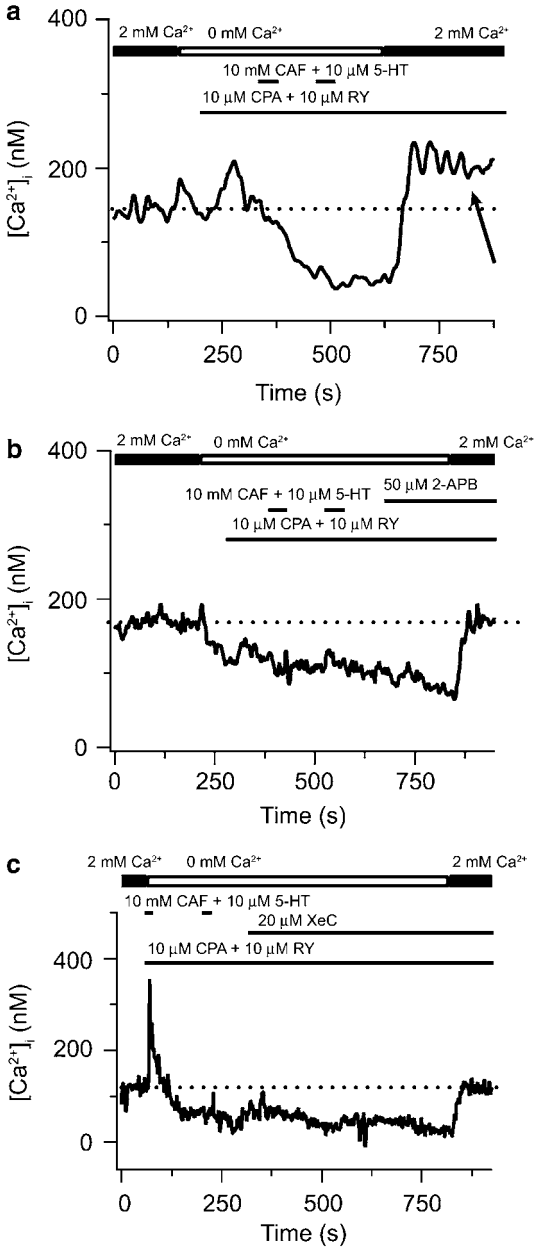

Figure 3 2-APB and XeC inhibit store depletion-induced CCE in canine PASMCs. (**a**) Effect of extracellular Ca^{2+} removal, 10 μM CPA, 10 μM ryanodine (RY), and sequential exposure to 10 mM caffeine (CAF) and 10 μM 5-HT on cytosolic [Ca^{2+}] during extracellular Ca^{2+} re-addition. (**b**) Effect of 50 μM 2-APB on cytosolic [Ca^{2+}] during extracellular Ca^{2+} re-addition following depletion of the SR Ca^{2+} stores. (**c**) Effect of 20 μM XeC on cytosolic [Ca^{2+}] during extracellular Ca^{2+} re-addition following depletion of the SR Ca^{2+} stores. Dashed line shows resting cytosolic [Ca^{2+}]. 2-APB, 2-aminobiphenylborate; 5-HT, 5-hydroxytryptamine; CCE, capacitative Ca^{2+} entry; CPA, cyclopiazonic acid; PASMCs, canine pulmonary arterial smooth muscle cells; SR, sarcoplasmic reticulum; XeC, xestospongin-C.

2001) and activate CCE in canine PASMCs (Wilson *et al*., 2002; Ng *et al*., 2005). Subsequent addition of 2 mM extracellular Ca^{2+} while in the continued presence of 10 μM CPA and 10 μM ryanodine caused cytosolic [Ca^{2+}] to rise 66 nM above basal values (arrow), indicating the activation of CCE. Figure 3b shows results from a similar experiment, except following complete store depletion where 50 μM 2-APB was added to the bathing solution in the absence and then the presence of 2 mM extracellular Ca^{2+}. Subsequent addition of 2 mM extracellular Ca^{2+} in the continued presence of 50 μM 2-APB, 10 μM CPA and 10 μM ryanodine did not elicit any rise in cytosolic [Ca^{2+}] above basal levels. Figure 3c shows results from an experiment where 20 μM XeC was added to the bathing solution following depletion of the $InsP_3$- and ryanodine-sensitive Ca^{2+} stores in the absence and then presence of 2 mM extracellular Ca^{2+}. Addition of 2 mM extracellular Ca^{2+} in the continued presence of 20 μM XeC, 10 μM CPA and 10 μM ryanodine did not cause any rise in cytosolic Ca^{2+} above basal levels. The mean data for these experiments from a number of cells are shown in Figure 5a.

Experiments were then performed to determine the involvement of ryanodine receptors in store depletion-induced CCE activation. Figure 4a shows that in a single canine PASMC, the SR Ca^{2+} stores could be fully depleted by perfusing with a Ca^{2+}-free bathing solution in the continuous presence of 10 μM CPA and exposing the cell three times for 30 s to 10 mM caffeine and 10 μM 5-HT. Subsequent addition of 2 mM extracellular Ca^{2+} while in the continued presence of 10 μM CPA activated CCE. In this cell, cytosolic [Ca^{2+}] increased to 65 nM above basal values. Figure 4b shows results from a similar experiment, except the protocol was designed to alter the conformation of the ryanodine receptors. The efficacy of ryanodine binding to ryanodine receptors is dependent on activation and opening of ryanodine receptors as well as the ryanodine concentration and exposure time. Thus, following depletion of the $InsP_3$- and ryanodine-sensitive Ca^{2+} stores, 300 μM ryanodine was added in the absence of extracellular Ca^{2+} and the cell was exposed twice for 30 s to 10 mM caffeine to promote ryanodine binding. Based on this ryanodine concentration and the duration of exposure, it is predicted that the ryanodine receptors would either be locked partially open, in a sub-conductance state, or would be in a closed inactivated state (Lattanzio *et al*., 1987; Humerickhouse *et al*., 1993). Subsequent addition of 2 mM extracellular Ca^{2+} in the continued presence of 300 μM ryanodine and 10 μM CPA still elicited a rise in cytosolic [Ca^{2+}] of 45 nM above basal values.

Figure 5a summarizes results illustrating that $InsP_3$ receptor but not ryanodine receptor activity is important for store depletion-induced CCE activation in canine PASMCs. Depletion of the intracellular Ca^{2+} stores in the presence of CPA and in the absence or presence of 10 μM or 300 μM ryanodine all resulted in CCE, while $InsP_3$ receptor inhibition abolished CCE. Subsequent to depletion of the $InsP_3$- and ryanodine-sensitive Ca^{2+} stores addition of 2 mM Ca^{2+} to the bathing solution containing 10 μM CPA elicited a significant elevation in cytosolic [Ca^{2+}] above basal value. Cells that were exposed to 10 μM ryanodine, as well as CPA, had similar significant cytosolic [Ca^{2+}] increases. Raising the ryanodine concentration to 300 μM, ryanodine did not affect the cytosolic [Ca^{2+}] increase (Figure 5a). These SR Ca^{2+} store depletion-induced rises in cytosolic Ca^{2+} were prevented by adding 20 μM XeC, or 50 μM 2-APB (Figure 5a).

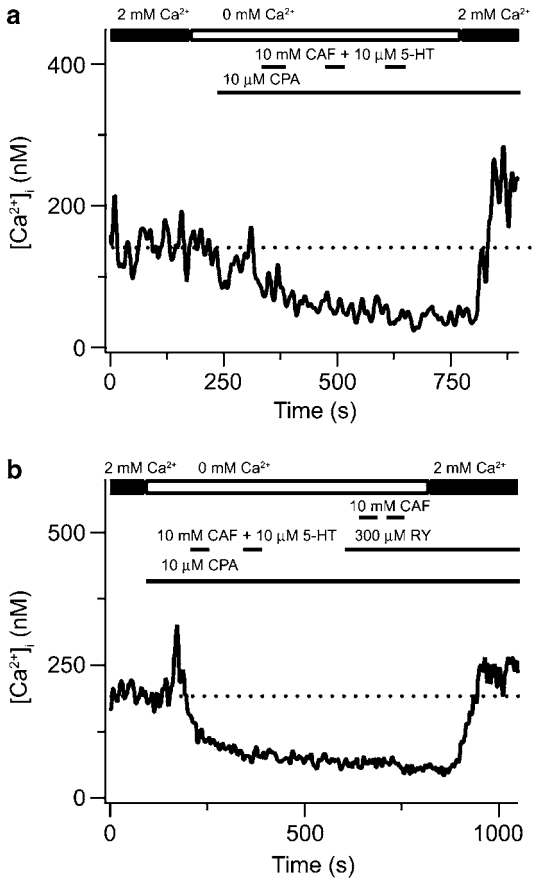

Figure 4 Ryanodine does not inhibit store depletion-induced CCE in canine PASMCs. (**a**) Effect of extracellular Ca^{2+} removal, 10 μM CPA, and sequential exposure to 10 mM caffeine (CAF) and 10 μM 5-HT on cytosolic [Ca^{2+}] during extracellular Ca^{2+} re-addition. (**b**) Effect of 300 μM ryanodine (RY) on cytosolic [Ca^{2+}] during extracellular Ca^{2+} re-addition following depletion of the SR Ca^{2+} stores. CCE, capacitative Ca^{2+} entry; CPA, cyclopiazonic acid; PASMCs, canine pulmonary arterial smooth muscle cells; SR, sarcoplasmic reticulum.

Using ryanodine as a tool to assess the role of ryanodine receptors in CCE could be problematic since it may be difficult to separate its ability to block ryanodine receptors from its ability to promote store depletion. We therefore performed a separate series of experiments using the ryanodine receptor inhibitor, dantrolene (Zucchi and Ronca-Testoni, 1997; Laporte *et al*., 2004; MacMillan *et al*., 2005; Zheng *et al*., 2005). Dantrolene was also chosen because at higher concentrations, it significantly reduces $InsP_3$ receptor-induced Ca^{2+} responses (MacMillan *et al*., 2005). Thus, it can be used to confirm the finding that $InsP_3$ receptors are important for store depletion-induced CCE in canine PASMCs. Control experiments were performed using the same protocol as in Figure 4a, whereas experiments involved dantrolene were performed using the same protocol as in Figure 4b and the results are summarized in Figure 5b. Depletion of the intracellular Ca^{2+} stores in the presence of 10 μM CPA and in the presence or absence of 10 μM dantrolene resulted in CCE, whereas 50 μM dantrolene abolished CCE. In control experiments, there was a significant elevation in cytosolic [Ca^{2+}] above basal values, and

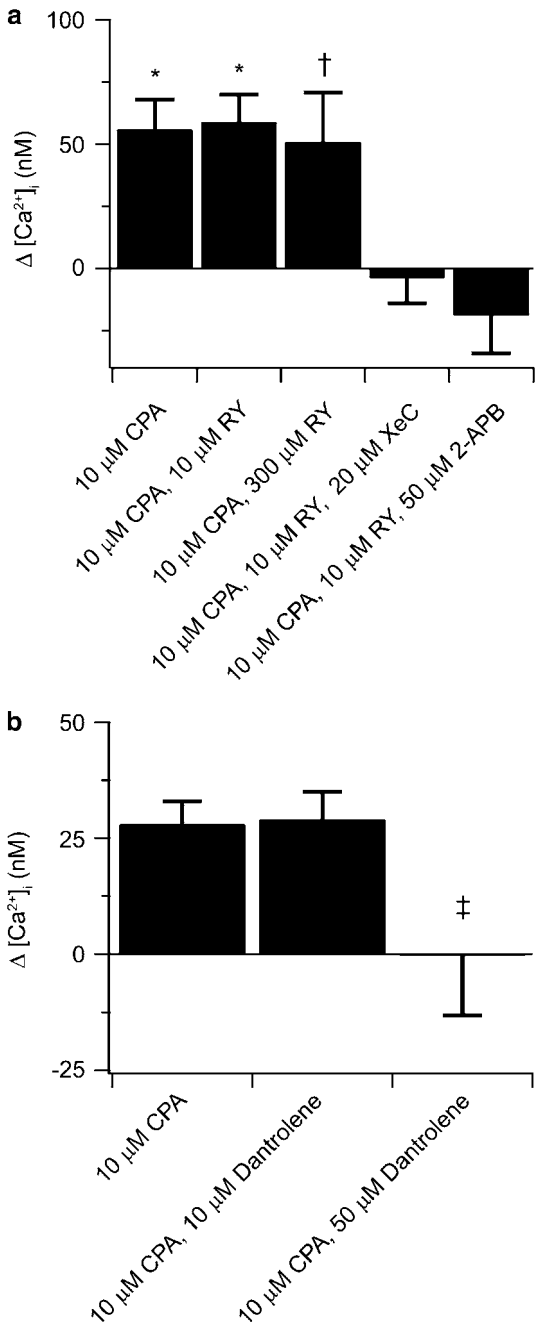

Figure 5 Effects of pharmacological manipulation of $InsP_3$ or ryanodine receptor activity on store depletion-induced CCE in canine PASMCs. (**a**) Summary of the cytosolic [Ca^{2+}] during extracellular Ca^{2+} re-addition following depletion of the SR Ca^{2+} stores under control conditions ($n=38$) and in the presence of 10 μM ($n=28$) or 300 μM ($n=8$) ryanodine, 20 μM XeC ($n=10$) or 50 μM 2-APB ($n=23$). (**b**) Summary of the cytosolic [Ca^{2+}] during extracellular Ca^{2+} re-addition following depletion of the SR Ca^{2+} stores under control conditions ($n=192$) and in the presence of 10 μM ($n=102$) or 50 μM ($n=81$) dantrolene. Bars indicate the change in cytosolic [Ca^{2+}] compared to the resting cytosolic [Ca^{2+}] and represent means $\pm$ s.e.m. Means significantly different from their controls by * two-tailed paired *t*-test ($P<0.001$), or † a signed-rank test, ‡ significantly different from other conditions (one-way ANOVA with Neuman–Keuls multiple comparison procedure; $P<0.05$). 2-APB, 2-aminobiphenylborate; ANOVA, analysis of variance; CCE, capacitative Ca^{2+} entry; $InsP_3$, inositol 1,4,5-triphosphate; PASMCs, canine pulmonary arterial smooth muscle cells; SR, sarcoplasmic reticulum; XeC, xestospongin-C.

cells that were exposed to 10 μM dantrolene had equivalent cytosolic [Ca^{2+}] increases. However, those exposed to the higher concentration of dantrolene (50 μM) did not have any cytosolic [Ca^{2+}] response. These results confirm that $InsP_3$ receptor, but not ryanodine receptor, activity is important for store depletion-induced CCE in canine PASMCs.

Alternatively, the apparent requirement for functional $InsP_3$ receptor activity for store depletion-induced CCE might be explained by the fact that in these experiments, an $InsP_3$ agonist, 5-HT, was used with CPA to facilitate $InsP_3$ store depletion, and the ability of 2-APB and XeC to inhibit CCE might rather reflect incomplete store depletion due to interference with the actions of 5-HT. To test this possibility, we repeated the experiments shown in Figure 3 in the absence of 5-HT, using CPA alone to deplete $InsP_3$-sensitive Ca^{2+} stores. As shown in Figures 6a–c, 20 μM XeC, was still capable of inhibiting store depletion-induced CCE even in the absence of $InsP_3$ receptor activation.

Role of $InsP_3$ receptors in hypoxia-induced CCE

In our earlier study (Ng *et al*., 2005), we found that pharmacological pre-depletion of intracellular Ca^{2+} stores prevented further activation of CCE by hypoxia, suggesting that hypoxic activation of CCE may simply be due to depletion of intracellular Ca^{2+} stores. We, therefore, examined whether activation of CCE by acute exposure to hypoxia would exhibit the same dependence on functional $InsP_3$ receptor activity as store depletion-induced CCE (cf Figure 5). Figure 7a illustrates an experiment in which hypoxia is shown to activate CCE in a canine PASMC. During exposure to a Ca^{2+}-free bathing solution, the cell was exposed to hypoxia, which elicited an initial transient rise in cytosolic [Ca^{2+}] due to Ca^{2+} release from intracellular stores (Ng *et al*., 2005). In the continued presence of hypoxia, subsequent addition of 2 mM extracellular Ca^{2+} caused cytosolic [Ca^{2+}] to rise above basal values, indicative of CCE activation. Nifedipine was used in these experiments to eliminate Ca^{2+} entry through voltage-dependent Ca^{2+} channels. This nifedipine-insensitive rise in cytosolic [Ca^{2+}] following addition of extracellular Ca^{2+} has been shown to be blocked by SKF 96365 and Ni^{2+}, inhibitors of CCE channels (Ng *et al*., 2005). Figure 7b illustrates the effects of 20 μM XeC on the hypoxia-induced activation of CCE. Exposure to XeC failed to block the hypoxia-induced activation of CCE. Figure 7c summarizes similar results from cells in the absence and presence of XeC.

Discussion and conclusion

The results of this study provide evidence that store depletion-induced CCE in canine PASMCs is dependent on functional $InsP_3$ receptor activity. Store depletion-induced CCE, however, does not appear to be critically dependent on ryanodine receptor activity. This work complements findings of other groups (Kiselyov *et al*., 1998, 1999; Ma *et al*., 2000), which illustrate a requirement of $InsP_3$ receptor activity as an important component of CCE and store-operated channel activity.

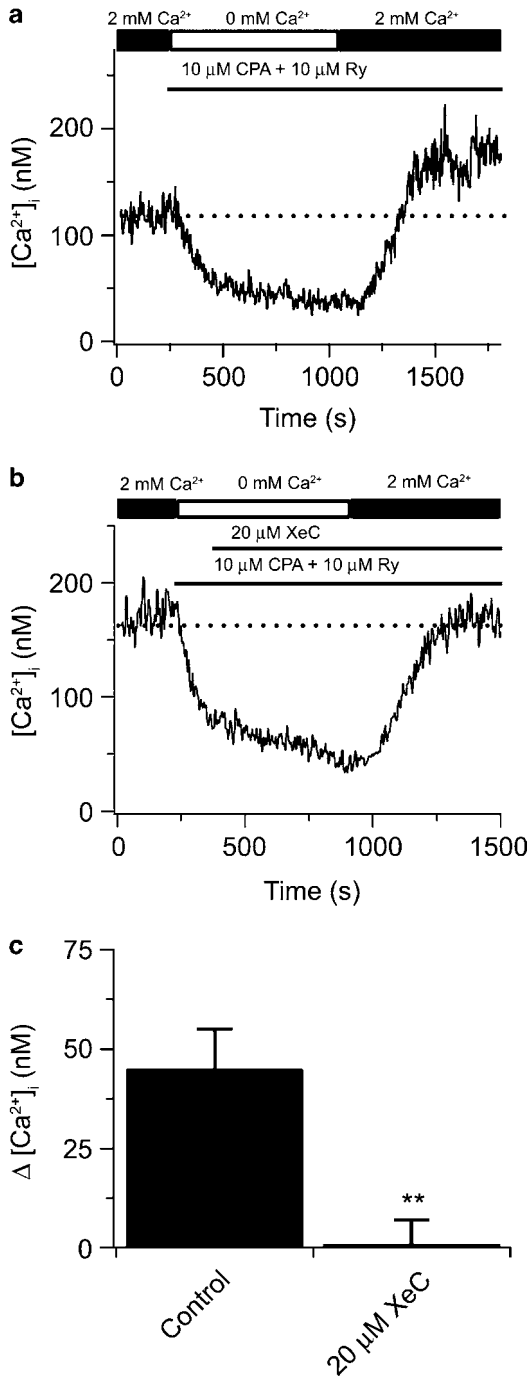

Figure 6 XeC inhibits store depletion-induced CCE in the absence of 5-HT in canine PASMCs. (**a**) Effect of extracellular Ca^{2+} removal, 10 μM CPA, 10 μM ryanodine on cytosolic [Ca^{2+}] during extracellular Ca^{2+} re-addition. (**b**) Effect of 20 μM XeC on cytosolic [Ca^{2+}] during extracellular Ca^{2+} re-addition following depletion of the SR Ca^{2+} stores in the absence of 5-HT. (**c**) Bars indicate the cytosolic [Ca^{2+}]. Dashed line shows resting cytosolic [Ca^{2+}]. There was a significant difference between the increase in cytosolic [Ca^{2+}] induced by store depletion, in the absence ($n=38$) and presence ($n=47$) of 20 μM XeC using an unpaired *t*-test (**$P<0.001$). Bars represent means$\pm$s.e.m. 5-HT, 5-hydroxytryptamine; CCE, capacitative Ca^{2+} entry; CPA, cyclopiazonic acid; PASMCs, canine pulmonary arterial smooth muscle cells; SR, sarcoplasmic reticulum; XeC, xestospongin-C.

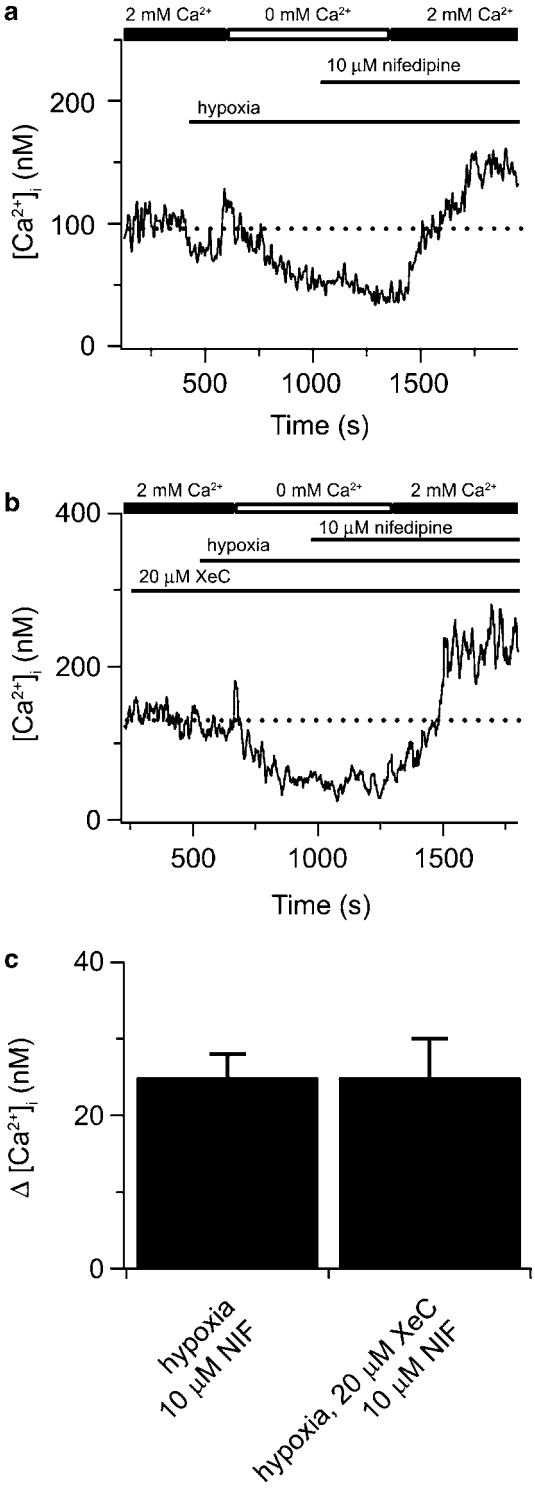

Figure 7 XeC does not inhibit hypoxia-induced CCE in canine PASMCs. (**a**) Effect of extracellular Ca^{2+} removal and hypoxia on cytosolic [Ca^{2+}] during extracellular Ca^{2+} re-addition in the presence of 10 μM nifedipine (NIF). (**b**) Effect of 20 μM XeC on cytosolic [Ca^{2+}] during extracellular Ca^{2+} re-addition following hypoxic stimulation in the presence of 10 μM nifedipine. (**c**) Bars indicate the cytosolic [Ca^{2+}]. Dashed line shows resting cytosolic [Ca^{2+}]. There was no significant difference between the increase in cytosolic [Ca^{2+}] induced by hypoxia, in the absence ($n=28$) and presence ($n=34$) of 20 μM XeC using an unpaired *t*-test. Bars represent means$\pm$s.e.m. CCE, capacitative Ca^{2+} entry; PASMCs, canine pulmonary arterial smooth muscle cells; XeC, xestospongin-C.

The mechanism of CCE activation in canine PASMCs is complex, requiring an integration of separate signals. CCE is dependent on $InsP_3$ receptors, as shown by the inhibition of CCE by 2-APB, XeC and high concentrations of dantrolene. While each of these pharmacological agents may not be entirely selective, we have capitalized on their common antagonism of the $InsP_3$ receptor. 2-APB is known to inhibit $InsP_3$ receptors as well as the activity of some store-operated channels (Prakriya and Lewis, 2001, 2002; Lievremont *et al*., 2005) and SERCA-dependent Ca^{2+} uptake (Missiaen *et al*., 2001). The dose–response relationship for 2-APB inhibition of 5-HT-mediated Ca^{2+} increases that we have recently reported (Wilson *et al*., 2005) match that shown for inhibition of CCE-mediated Ca^{2+} increases. However, since 2-APB may also block the non-selective cation channels responsible for CCE at concentrations similar to those that block $InsP_3$ receptors, one must be cautious of reaching more than a speculative conclusion. We therefore chose to also examine the effects of the $InsP_3$ receptor antagonist XeC (Gafni *et al*., 1997; Kiselyov *et al*., 1998; Ta *et al*., 2005) on 5-HT-elicited Ca^{2+} responses (Wilson *et al*., 2005) as well as CCE. Our previously reported data illustrate that XeC blocked 5-HT-induced Ca^{2+} increases with similar efficacy to its inhibition of muscarinic receptor-mediated Ca^{2+} increases in murine colonic smooth muscle (Bayguinov *et al*., 2001). This leads to the presumption that $InsP_3$ receptor activation mediates the 5-HT-induced Ca^{2+} responses. Moreover, XeC at these same concentrations inhibited store depletion-induced CCE in the present experiments. It therefore appears that the effects of XeC in our experiments can be attributed to inhibition of $InsP_3$ receptors and not due to direct interaction with non-selective cation channels responsible for CCE, since XeC had no effect on hypoxia-induced CCE. Our findings with dantrolene are comparable to those of MacMillan *et al*. (2005), who showed that 50 μM, but not 10 μM, dantrolene inhibit $InsP_3$-induced Ca^{2+} responses in colonic myocytes.

Our experimental evidence demonstrates that altering the ryanodine receptor open state by promoting ryanodine binding or through inhibition by 10 μM dantrolene does not affect store depletion-induced CCE. The expectation was that if ryanodine receptors were as critically involved in the process of CCE activation in smooth muscle as they are in excitation-coupled Ca^{2+} entry pathways described in skeletal myocytes (Cherednichenko *et al*., 2004; Hurne *et al*., 2005), there should have been gross changes in CCE magnitude. Our data therefore do not support a model of tight coupling between the ryanodine receptor and the store-operated channels underlying store depletion-induced CCE in canine pulmonary arterial smooth muscle. Instead, the data lend support to the theory that this form of CCE activation requires coupling of $InsP_3$ receptors to the store-operated channels that are responsible for CCE and concomitant depletion of the functionally separate $InsP_3$- and ryanodine-sensitive Ca^{2+} stores in these canine PASMCs. Thus, there appears to be regulatory feedback from the SR Ca^{2+} stores to the store-operated channels through both passive depletion of the SR Ca^{2+} stores and the activity of $InsP_3$ receptors. Our data, however, does not rule out a role of coupling of ryanodine receptors and the store-operated

channels responsible for hypoxia-induced CCE, since hypoxic-mediated increases in contractile tension and cytosolic [Ca^{2+}] in canine pulmonary arterial smooth muscle are significantly attenuated by pretreatment with ryanodine and caffeine (Jabr *et al*., 1997).

There are a number of possible explanations for why depletion of the ryanodine-sensitive Ca^{2+} stores is necessary to induce CCE even though ryanodine receptor activation is not. An obvious possibility is that functional ryanodine-sensitive SR Ca^{2+} stores buffer enhanced Ca^{2+} influx. This is highly unlikely, since our data shows that CCE (Wilson *et al*., 2002) and I_{SOC} (Wilson *et al*., 2005) are activated only when the functionally independent $InsP_3$- and ryanodine-sensitive SR Ca^{2+} stores are depleted. Specifically, neither CCE (Wilson *et al*., 2002) nor I_{SOC} (Wilson *et al*., 2005) are active when the ryanodine-sensitive SR Ca^{2+} stores are filled, and thus they cannot operate as a Ca^{2+} sink. More generally, functional Ca^{2+} stores should alter the time to reach the steady-state cytosolic [Ca^{2+}], but not the final steady-state cytosolic [Ca^{2+}], which is dependent on the balance of the inward and outward Ca^{2+} fluxes across the plasma membrane (Smith *et al*., 1996). A more likely possibility is that, in canine PASMCs, a passive Ca^{2+} leak pathway from the ryanodine-sensitive SR Ca^{2+} store coordinates with the function of $InsP_3$ receptors to regulate the activity of the store-operated channels responsible for CCE.

Depletion of the SR Ca^{2+} stores may regulate the activity of intracellular signaling pathways in canine PASMCs. The decreased luminal Ca^{2+} concentration could induce activation of Ca^{2+} independent phospholipase A_2 (Smani *et al*., 2003), which may be involved with CIF generation and store-operated channel and CCE activation in vascular smooth muscle cells (Trepakova *et al*., 2000). CCE could also be due to translocation of STIM1 from the SR to the plasma membrane, which activates calcium release-activated channels (CRAC) in T lymphocytes (Roos *et al*., 2005; Zhang *et al*., 2005; Spassova *et al*., 2006) and TRPC1 (Huang *et al*., 2006; Lopez *et al*., 2006).

Capacitative calcium entry has been linked to various ion channels. Orai1 are novel transmembrane proteins that may be the molecular correlate for CRAC in T lymphocytes and other cells (Peinelt *et al*., 2006). Yet, Orai1 works in concert with STIM proteins through an unresolved mechanism to regulate store depletion Ca^{2+} entry and I_{CRAC} (Mercer *et al*., 2006; Peinelt *et al*., 2006; Soboloff *et al*., 2006). Other candidate channel proteins responsible for CCE in canine PASMCs include TRPC channels, which are a class of non-selective cation channels that have also been proposed as molecular correlates of CCE in some cells, including smooth muscle (Beech, 2005). The sensitivity of CCE in canine PASMCs to pharmacological inhibitors and the biophysical characteristics of I_{SOC} differs from that of CRAC channels in lymphocytes and is more typical of some TRPC channels. TRPC channels are expressed in canine PASMCs (Walker *et al*., 2001). Depletion of intracellular Ca^{2+} stores activates TRPC4 (McKay *et al*., 2000), whereas stimulation of G-protein-coupled receptors and diacylglycerols activates TRPC6 and TRPC7, independent of Ca^{2+} store depletion (Hofmann *et al*., 1999; Okada *et al*., 1999; McKay *et al*., 2000; Beck *et al*., 2006; Maruyama *et al*., 2006; Vazquez *et al*., 2006). TRPC4 has an $InsP_3$ receptor binding site (Boulay *et al*., 1999) and activation of TRPC3 is coupled to $InsP_3$ receptor function (Ma *et al*., 2000). Most relevant to pulmonary arterial function, it has been suggested that TRPC1 and TRPC6 may mediate store-operated Ca^{2+} entry and receptor-operated Ca^{2+} entry in rat PASMCs, respectively, and expression of both were significantly elevated in chronic hypoxia (Lin *et al*., 2004).

It is interesting that the functionally independent $InsP_3$- and ryanodine-sensitive SR Ca^{2+} stores in canine PASMCs must be simultaneously depleted to activate CCE (Wilson *et al*., 2002) and I_{SOC} (Wilson *et al*., 2005). Our original prediction was that CCE and I_{SOC} activation would be graded and dependent on the size of the $InsP_3$- and ryanodine-sensitive SR Ca^{2+} stores and the extent of store depletion. Possibly, a constitutive Ca^{2+} entry pathway provides sufficient Ca^{2+} entry to refill an empty Ca^{2+} store when the other store is full, which then inhibits CCE activity. This is supported by our previously reported Mn^{2+} quench data (Wilson *et al*., 2002, 2005), which illustrates there is a tonic Ca^{2+} influx pathway that contributes to pulmonary arterial contractility elicited by 5-HT (Wilson *et al*., 2005).

Several previous studies (Jabr *et al*., 1997; Robertson *et al*., 2000; Ng *et al*., 2005; Wang *et al*., 2005; Weigand *et al*., 2005) have provided evidence that hypoxic increases in intracellular Ca^{2+} in PASMCs may well involve the activation of CCE. The underlying mechanism(s) involved is unknown, but it may be due to a direct effect of hypoxia on CCE Ca^{2+} entry pathways, the ability of hypoxia to directly deplete intracellular Ca^{2+} stores or due to hypoxic facilitation of signal transduction pathways linking store depletion to CCE activation. The observation that store depletion-induced CCE in canine PASMCs is dependent on functional $InsP_3$ receptor activity, whereas hypoxia-induced CCE is not, suggests that while hypoxia-induced CCE may involve store depletion, other mechanisms as well are likely to be involved.

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Conflict of interest

The authors state no conflict of interest.

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