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Caffeine inhibits $InsP_3$ responses and capacitative calcium entry in canine pulmonary arterial smooth muscle cells

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Abstract

Caffeine is a well described and characterized ryanodine receptor (RyR) activator. Previous evidence from independent research studies also indicate caffeine inhibits $InsP_3$ receptor functionality, which is important to activation of capacitative Ca^{2+} entry (CCE) in some cell types. In addition, RyR activation elicits excitatory-coupled Ca^{2+} entry (ECCE) in skeletal muscle myotubes. Recent studies by our group show that canine pulmonary arterial smooth muscle cells (PASMCs) have functional $InsP_3$ receptors as well as RyRs, and that CCE is dependent on $InsP_3$ receptor activity. The potential for caffeine to activate ECCE as well as inhibit $InsP_3$ receptor function and CCE was examined using fura-2 fluorescent imaging in canine PASMCs. The data show caffeine causes transient as well as sustained cytosolic Ca^{2+} increases, though this is not due to CCE or ECCE activity as evidenced by a lack of an increase in Mn^{2+} quench of fura-2. The experiments also show caffeine reversibly inhibits 5-HT elicited – $InsP_3$ mediated Ca^{2+} responses with an IC_{50} of 6.87×10^{-4} M and 10 mM caffeine fully inhibits CCE. These studies provide the first evidence that caffeine is an inhibitor of $InsP_3$ generated Ca^{2+} signals and CCE in PASMCs.

Keywords

Fura-2; pulmonary arterial smooth muscle; intracellular calcium; ryanodine receptor; $InsP_3$ receptor

1.1 Introduction

The sarcoplasmic reticulum of smooth muscle cells have two types of Ca^{2+} release channels that can be differentiated on their functionality and pharmacology. These include inositol 1,4,5-triphosphate ($InsP_3$) sensitive Ca^{2+} channels that are activated by $InsP_3$ and ryanodine sensitive channels (RyRs) that are activated by cytosolic Ca^{2+} increases. Functionally, $InsP_3$ receptors are activated downstream of neural or humoral stimulation of G-protein coupled or tyrosine coupled membrane bound receptors (Bootman and Berridge, 1995). We find 5-Hydroxytryptamine (5-HT) is a potent activator of $InsP_3$ receptor mediated Ca^{2+} responses in

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canine pulmonary arterial smooth muscle cells (PASMCs) (Wilson et al., 2005) with responses being mediated through 5-HT_{2A} receptors. Caffeine elicits Ca^{2+} responses with similar temporal characteristics; however it selectively activates RyRs as evidenced by the inhibition of responses by ryanodine, dantrolene, and FLA 365 (Janiak et al., 2001;Ng et al., 2007;Ostrovskaya et al., 2007;Wilson et al., 2002).

Pharmacological discrimination of ryanodine and $InsP_3$ receptor activity is important because a number of reports show these receptors influence the activity of one another (Lamont and Wier, 2004;MacMillan et al., 2005;McCarron et al., 2003;Ng et al., 2007;Zhang et al., 2003;Zhang et al., 2004;Zheng et al., 2005). Determining the extent of communication between $InsP_3$ receptors and RyRs is significant because these Ca^{2+} release channels are important mediators of excitation-contraction coupling in smooth muscle. Unfortunately, common blockers of $InsP_3$ and RyRs have non-selective effects on intracellular Ca^{2+} signaling, which makes interpreting their roles and activities difficult. For example, 2-Amino-biphenyl borate (2-APB) is a commonly used and commercially available $InsP_3$ receptor antagonist (Birnbaumer et al., 2000;Boulay et al., 1999;Ma et al., 2000;Wu et al., 2000), and yet it also inhibits non-selective cation channels important to SR Ca^{2+} store refilling (Boulay et al., 1999;Ma et al., 2000;Prakriya and Lewis, 2001;Wilson et al., 2005). 2-APB therefore not only directly blocks $InsP_3$ generated Ca^{2+} responses but also may limit RyR or $InsP_3$ Ca^{2+} signals by reducing SR store refilling. Ryanodine is a widely used RyR antagonist, however, its binding characteristics are time and dose dependent. What is more, ryanodine binding often locks the RyR into a sub-conductance state, which ultimately depletes the SR Ca^{2+} store (Janiak et al., 2001;Wilson et al., 2002;Zucchi and Ronca-Testoni, 1997). Related to this, ryanodine administration and subsequent SR Ca^{2+} stores depletion often activates capacitative Ca^{2+} entry, which elevates the cytosolic [Ca^{2+}] (Ng et al., 2007;Wilson et al., 2002).

RyR stimulation activates excitation-coupled calcium entry pathways (ECCE) in skeletal muscle myotubes, which share common pharmacological and functional properties with CCE (Cherednichenko et al., 2004;Hurne et al., 2005). Like CCE, ECCE may also be important to repletion of emptied SR Ca^{2+} stores, and yet they are activated through a distinctive coupling mechanism. ECCE in skeletal muscle myotubes is dependent on functional L-type Ca^{2+} channels and RyRs while CCE is reliant on release or depletion of the SR (Cherednichenko et al., 2004; Putney, Jr., 2005;Wilson et al., 2002). This novel excitatory-coupled Ca^{2+} entry pathway in skeletal muscle is therefore likely to be important to skeletal muscle excitation-contraction coupling. Given the expression and importance of RyRs to smooth muscle excitability, it is plausible ECCE also exists in arterial myocytes. Even still, there is no information regarding the presence of ECCE in smooth muscle.

Previous contractile and Ca^{2+} imaging studies from our laboratory demonstrated that structural and functional differences exist in the SR Ca^{2+} stores of pulmonary arterial smooth muscle cells (Jabr et al, 1997; Janiak et al, 2001; Wilson et al, 2002; Wilson et al, 2005; Ng et al, 2007). Phenylephrine contracted pulmonary arterial rings through release of $InsP_3$ sensitive stores (Jabr et al, 1997). This initial contraction could be inhibited without affecting subsequent contraction due to release of ryanodine sensitive stores with caffeine, when the $InsP_3$ stores were depleted in the presence of the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA) blocker cyclopiazonic acid. Similarly, stimulating ryanodine-treated cells with caffeine depleted the ryanodine Ca^{2+} stores, but did not affect subsequent contraction due to release of the $InsP_3$ sensitive stores (Jabr et al, 1997). These contractile experiments were substantiated with Ca^{2+} imaging experiments on isolated pulmonary arterial myocytes (Janiak et al, 2001; Wilson et al, 2002). These experiments provided good evidence that in canine pulmonary arterial smooth muscle cells (PASMCs) the $InsP_3$ and ryanodine sensitive Ca^{2+} stores were functionally independent. Additional experiments performed in isolated PASMCs determined that capacitative Ca^{2+} entry could only be activated through simultaneous depletion

of the caffeine-ryanodine and $InsP_3$-related Ca^{2+} stores (Wilson et al, 2002), and that active $InsP_3$ receptors were important to this process (Ng et al, 2007).

Caffeine is a widely used RyR activator, but previous research indicates it blocks $InsP_3$ receptor functionality at concentrations commonly used to activate RyRs (Bezprozvanny et al., 1994;Parker and Ivorra, 1991). Because caffeine is used extensively in vascular biology we wished to examine the potential for caffeine to activate ECCE or CCE, and its ability to inhibit $InsP_3$ receptor function. The functional separation of the caffeine-ryanodine and $InsP_3$ related Ca^{2+} stores, the well characterized Ca^{2+} responses to caffeine and to agonists that generate $InsP_3$, and the influence that RyR and $InsP_3R$ activation has to CCE makes canine PASMCs an ideal preparation to test the hypotheses that caffeine inhibits $InsP_3$ related Ca^{2+} responses, and modulates the activity of CCE and ECCE (Janiak et al., 2001;Ng et al, 2007; Ostrovskaya et al., 2007;Wilson et al., 2005;Wilson et al., 2002).

2.1 Experimental/Materials and methods

2.2 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 sacrificed with pentobarbital sodium (45 mg kg^{-1} I.V.) and ketamine (15 mg kg^{-1} I.V.), as approved by the University of Nevada at 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 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 hours. To disperse cells, tissue was placed in low-Ca^{2+} PSS containing 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 hours 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 hours until experiments were performed.

2.3 Cell Culture

HEK 293 cells (ATCC, Manassas, VA) were obtained and cultured in low glucose Dubelco's modified Eagles media (Invitrogen, San Diego, CA) with 10% fetal bovine serum. Cells were passed weekly and fresh media applied every 2–3 days.

2.4 Fluorescence Imaging

2.4.1 Global Ca^{2+} measurements—The cytosolic [Ca^{2+}] was measured in canine PASMCs and HEK 293 cells loaded with the ratiometric Ca^{2+} sensitive dye fura-2 AM (Molecular Probes, Eugene, OR) using a dual excitation digital Ca^{2+} imaging system (IonOptix Inc., Milton, MA) equipped with an intensified CCD as previously described (Janiak et al., 2001;Wilson et al., 2005;Wilson et al., 2002). The imaging system was mounted on an inverted microscope (Nikon) outfitted with a 40X (NA 1.3, Nikon Inc., Melville, NY) oil immersion objective. Fura-2 AM was dissolved in 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) 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 capacitative Ca^{2+} entry 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 EGTA. Cells were illuminated with a xenon arc lamp at 340 ± 15 and 380 ± 12 nm (Omega Optical, Brattleboro, VT) and emitted light was collected from regions that encompassed single cells with a CCD at 510 nm (Nikon Inc., Melville, NY). 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 ratio of fluorescence excited at 340 and 380 nm (R) as described by Grynkiewicz et al (1985) based on the relation $[Ca^{2+}]_I = Kd * (Sf_2/Sb_2) * (R-R_{min})/(R_{max}-R)$, where the K_D for Ca^{2+} binding to fura-2 was assumed to be 224 nM (Grynkiewicz et al., 1985) and the values for F_{380} in the absence of extracellular Ca^{2+} (Sf_2), F_{380} in the presence of 10 mM extracellular Ca^{2+} (Sb_2), minimum ratio (R_{min}), and maximum ratio (R_{max}) were determined from in situ calibrations of fura-2 for each cell after applying 1 μM ionomycin. To determine the maximum ratio, R_{max}, cells were perfused with a balanced salt solution that contained 10 mM Ca^{2+}, while the minimum ratio, R_{min}, was obtained after applying balanced salt solution that did not have any added Ca^{2+} and contained 10 mM EGTA. These calibration procedures are based on those described previously by us (Ng et al, 2005; Ng et al, 2007; Wilson et al, 2002; Wilson et al, 2005). During the Ca^{2+} calibration, 5 mM 2,3 butane dione monoxime was added to the bathing solution to inhibit smooth muscle contraction (Waurick et al., 1999).

2.4.2 Mn^{2+} quench—Mn^{2+} is a commonly used probe for studying Ca^{2+} influx changes because many Ca^{2+} selective channels are permeable to Mn^{2+} and because it quenches the fura-2 fluorescence emission (Missiaen et al., 1990). Also, Mn^{2+} cannot be transported out of the cytosol into intracellular compartments or extruded from the cell (Gomes and Madeira, 1986). The rate of Mn^{2+} quench of fura-2 was determined by regression analysis of fluorescent intensity over time for canine PASMCs excited at 357 ± 10 nm as previously described (Janiak et al., 2001;Wilson et al., 2005;Wilson et al., 2002). Cells were analyzed only if the rate of fura-2 quench by Mn^{2+} in the presence of ionomycin was at least 4 fold greater than the basal rate. Background fluorescence was collected automatically and subtracted from the acquired fluorescence video images during each experiment. The Ca^{2+} free balanced salt solution used in the Mn^{2+} quench experiments did not have any added Ca^{2+} or EGTA. Experimental temperature was 22–25° C.

2.5 Chemicals and drugs

Ionomycin free acid was purchased from Calbiochem (San Diego, CA), Fura-2 from molecular probes (Eugene, OR) and all other chemicals were purchased from Sigma (St. Louis, MO).

2.6 Statistical analysis

All data are presented as mean ± S.E.M. Statistical difference between groups was assessed with a One-Way Analysis of Variance (ANOVA) with a Newman-Keuls multiple comparison procedure or a Friedman repeated measures ANOVA on ranks with SNK multiple comparison procedure. 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. A Hill equation (eq. 1)

$$Y = A_1 + A_2 - A_1/(1+10 \wedge (\log(xo - x))^{*}p) \quad (1)$$

was used to determine the half-maximum inhibition of agonist mediated Ca^{2+} increases by pharmacological blockers, where A_1 = bottom asymptote, A_2 = top asymptote, Log xo = IC_{50}, p = hill slope. The n values reported reflect the total number of cells tested. Multiple trials were performed on cells isolated from multiple dogs for most experimental paradigms with the specific number of cells being listed in the figure legends.

3.1 Results

Figure 1 shows the influence of 10 mM caffeine on estimated cytosolic [Ca^{2+}] in canine PASMCs. Figure 1A shows that 10 mM caffeine elicited a rapid increase in cytosolic [Ca^{2+}] of 93 nM, which then relaxed and stabilized ~ 40 nM above basal values in the continued presence of the agonist. This caffeine-mediated increase in cytosolic [Ca^{2+}] is somewhat lower than the average response of 166 ± 21 nM above resting levels shown in Figure 1B, but well within the normal range of variability for caffeine-elicited Ca^{2+} responses in canine PASMCs (Janiak et al, 2001;Ng et al, 2007;Ostrovskaya et al; 2007;Wilson et al, 2002;Wilson et al, 2005). In the continued presence of 10 mM caffeine, cytosolic [Ca^{2+}] was substantially lower but remained 26 ± 3 nM above basal values in these same cells.

Previous reports show that activation of ECCE or CCE pathways enhances the rate of Mn^{2+} quench of Fura-2 (Cherednichenko et al., 2004;Hurne et al., 2005;Ng et al., 2005;Wilson et al., 2005;Wilson et al., 2002). The potential for caffeine activation of ECCE pathways was therefore examined in canine PASMCs by measuring the rate of Mn^{2+} quench of fura-2. Figure 2 shows the results of these studies. Figure 2A shows the fluorescence intensity over time measured at 510 nm at an excitation wavelength of 357 nm in a single PASMC. Removal of extracellular Ca^{2+} did not cause any decline in the fluorescence intensity. However, 100 μM Mn^{2+} caused the fluorescence intensity to decrease at a rate of $-0.065\ s^{-1}$. The quench rate by Mn^{2+} was not appreciably influenced by 10 mM caffeine remaining at $-0.055\ s^{-1}$. Figure 2B summarizes these results showing that 10 mM caffeine does not alter Mn^{2+} permeability. Exposure to 10 mM caffeine did not significantly alter the Mn^{2+} quench of fura-2, which was $-0.029 \pm 0.003\ s^{-1}$ before and $-0.029 \pm 0.004\ s^{-1}$ during caffeine. Subsequent exposure to 1 μM ionomycin shows these cells were viable as it caused a 19-fold increase in the quench rate. This lack of an influence of caffeine on the Mn^{2+} quench rate is similar to our finding that 5-HT stimulation also does not increase Mn^{2+} entry across the plasma membrane (Wilson et al, 2005). In comparison to the lack of effect of caffeine, our previous studies show the Mn^{2+} quench rate doubles when the intracellular Ca^{2+} stores are depleted (Wilson et al, 2002; Ng et al, 2005; Ng et al, 2007).

Given the previous reports of caffeine inhibition of $InsP_3$ receptors, we examined the effects of sustained caffeine exposure on 5-HT elicited cytosolic [Ca^{2+}] responses. Figure 3 shows the results of these studies. Figure 3A shows that a 30 s 10 μM 5-HT exposure caused a rapid, transient elevation in the cytosolic [Ca^{2+}] of 247 nM that fell back to baseline with agonist washout. 10 mM caffeine caused a rapid transient increase in cytosolic [Ca^{2+}] of 404 nM that fell toward baseline in the continuous presence of agonist, stabilizing 35 nM above basal values. After ~3 minutes in the continued presence of 10 mM caffeine, a 30 s 10 μM 5-HT exposure elicited a reduced cytosolic [Ca^{2+}] increase of 80 nM.

Figure 3B shows the change in estimated cytosolic [Ca^{2+}] from baseline for a cell stimulated with 10 μM 5-HT in the absence and presence of 500 μM and then 5 mM caffeine. Ten-micromolar 5-HT elicited a cytosolic [Ca^{2+}] increase that was minimally affected by 500 μM caffeine. However, Figure 3B also shows increasing the caffeine concentration to 5 mM markedly reduced the 5-HT mediated [Ca^{2+}] response.

Figure 3C shows a normalized dose-response curve for caffeine inhibition of 5-HT elicited cytosolic [Ca^{2+}] increases for cells that were stimulated with 10 μM 5-HT in the absence and then presence of different concentrations of caffeine. This concentration of 5-HT was used as it elicits the maximal cytosolic [Ca^{2+}] response (Wilson et al, 2005). 5-HT-mediated cytosolic [Ca^{2+}] increases were not affected by 100 μM caffeine, which also illustrates the Ca^{2+}-responses to 5-HT stimulation do not desensitize. However, the cytosolic [Ca^{2+}] increases to 5-HT were fully blocked by 5–10 mM caffeine. These data were fit to a simple binding equation (eq. 1, solid line) with an estimated $IC_{50} = 6.87 \times 10^{-4}$ M.

Caffeine inhibition of 5-HT elicited cytosolic [Ca^{2+}] increases is comparable to the effects of known $InsP_3$ receptor antagonists (Wilson et al, 2005). Wilson et al, 2005 (Figure 2) shows the $InsP_3$ receptor inhibitors 2-APB and xestospongin C inhibited 5-HT-mediated Ca^{2+} responses. Importantly, Ng et al, 2007 (Figure 1) shows 2-APB and xestospongin C did not reduce caffeine elicited Ca^{2+} transients. Thus, the caffeine-induced decrements in the 5-HT elicited Ca^{2+} responses shown in Figure 3 are not because caffeine caused prior activation of $InsP_3$ receptors and release or depletion of the $InsP_3$-portion of the sarcoplasmic reticulum.

One important pharmacological consideration is the ability to recover from antagonism. This property was examined by stimulating cells with 10 μM 5-HT in the absence as well as presence of 1 mM caffeine, which was chosen as it is close to the IC_{50} value. Figure 4A shows the estimated cytosolic [Ca^{2+}] in a single myocyte. Exposing the cell to 10 μM 5-HT for 30 s caused a rapid transient increase in cytosolic [Ca^{2+}] of 134 nM, which decayed back to basal values. Exposure of this cell to 1 mM caffeine for ~ 3 min caused basal cytosolic [Ca^{2+}] to increase roughly 30 nM, in accordance with ryanodine receptor activation (Janiak et al, 2001). 1 mM caffeine reduced the cytosolic [Ca^{2+}] increase due to 10 μM 5-HT approximately one-third, being 99 nM. When caffeine was removed from the bathing solution the cytosolic [Ca^{2+}] relaxed to baseline values, showing further evidence for RyR activation by 1 mM caffeine. The cytosolic [Ca^{2+}] increase due to 10 μM 5-HT also returned to control levels following caffeine washout in this cell, being 163 nM. Figure 4B summarizes the findings showing caffeine reversibly inhibits 5-HT elicited cytosolic [Ca^{2+}] increases in pulmonary arterial myocytes. Before 1 mM caffeine, 10 μM 5-HT caused a 205 ± 16 nM cytosolic [Ca^{2+}] increase. In the presence of 1 mM caffeine, however, the response was reduced nearly two-thirds, being 72 ± 14 nM in these same cells. Once caffeine was washed from the bath, 10 μM 5-HT caused a cytosolic [Ca^{2+}] elevation of 153 ± 23 nM, which was not significantly different than the control value.

Even though caffeine appears to reduce the 5-HT mediated Ca^{2+} response it is important to know that 5-HT mediated Ca^{2+} responses do not desensitize. Figures 3 and 4 as well as our previous studies provide evidence that 5-HT can repeatedly cause Ca^{2+} responses of similar magnitude (Wilson et al, 2005; Ostrovskaya et al, 2007). The overlaid traces in Figure 3B clearly show that 10 μM 5-HT cause repeated Ca^{2+} increases of similar magnitude, even in the presence of a low caffeine concentration. The dose-response curve shown in Figure 3C substantiates this finding, and shows summary data that also indicates 10 μM 5-HT can cause multiple cytosolic Ca^{2+} increases of similar magnitude in the presence of low caffeine concentrations. Figure 4 provides additional support as it shows recovery of the 5-HT-mediated Ca^{2+} response once caffeine is washed from the bathing solution. These findings are similar to Ostrovskaya et al, 2007 (Figures 6C and 7A), which show results from specific control experiments; where canine PASMCs were repeatedly stimulated with 10 μM 5-HT without any decrement in the elicited Ca^{2+}-response. In Wilson et al, 2005 (Figure 5A), we also show that continuous 5-HT application caused robust and maintained Ca^{2+} signals in canine PASMCs.

To test if sustained caffeine exposure causes a general inhibition of $InsP_3$ receptor induced cytosolic [Ca^{2+}] increases or whether the elevation in cytosolic 6[Ca^{2+}] negatively influences $InsP_3$ activation analogous experiments were performed on HEK 293 cells. HEK 293 cells are known to express functional $InsP_3$ receptors (Luo et al., 2000), while functional ryanodine receptors, such as can be activated by caffeine, are diminutive (Querfurth et al., 1998) or non-existent (Ostrovskaya et al., 2007;Rossi et al., 2002). Figure 5A shows the estimated cytosolic [Ca^{2+}] in a single HEK 293 cell. Exposing the cell to 100 μM carbachol for 30 s, which releases Ca^{2+} from the endoplasmic reticulum stores in these cells, caused a rapid transient increase in cytosolic [Ca^{2+}] of 149 nM. Following the rapid increase, the cytosolic [Ca^{2+}] decayed back to basal values. Exposure of this cell to 10 mM caffeine for ~ 3 min did not alter basal cytosolic [Ca^{2+}] from the control values of 85–95 nM. Although this does not conclusively show a lack of molecular expression, it shows these cells lack functional RyRs as caffeine activates all known RyR isoforms (Zucchi and Ronca-Testoni, 1997). Subsequently, 100 μM carbachol for 30 s in the continued presence of 10 mM caffeine elicited a 14 nM cytosolic [Ca^{2+}] increase. The cytosolic [Ca^{2+}] responses to 100 μM carbachol recovered after removing caffeine, being 170 nM. Figure 5B summarizes the data showing caffeine reversibly inhibits carbachol induced cytosolic [Ca^{2+}] increases in HEK 293 cells. Prior to 10 mM caffeine, 100 μM carbachol elicited a 169 ± 15 nM increase in cytosolic [Ca^{2+}]. In the presence of 10 mM caffeine, however, 100 μM carbachol caused a small, 9 ± 4 nM, yet significant ($P<0.05$, paired *t*-test) increase in cytosolic [Ca^{2+}] in these same cells. Following caffeine washout, 100 μM carbachol caused cytosolic [Ca^{2+}] elevations equivalent to control, being 184 ± 15 nM.

We recently described $InsP_3$ receptor inhibition blocks CCE in canine PASMCs (Ng et al., 2007). This finding led us to examine whether or not caffeine inhibits CCE, and to compare these findings with our earlier studies (Ng et al., 2007). We also performed separate control experiments as caffeine exposure for significant periods inhibits phosphodiesterase function and increases cAMP. This is an important consideration as cAMP dependent kinase activity has recently been shown to inhibit CCE (Ay et al., 2006, Smani et al., 2007, Zhang et al., 2007), and store operated channels (Liu et al., 2005). In these control experiments, cells were exposed to the membrane permeable cAMP analogue 8-Br-cAMP (1 mM) for similar lengths of time as those treated with caffeine, which was a total of 5 – 10 minutes, and thus sufficiently long enough to increase cytosolic cAMP levels. This concentration of 8-Br-cAMP was chosen because it should provide a saturating level of intracellular cAMP (Wilson et al, 2000), and thus provide an adequate measure for the influence of cAMP dependent kinases on CCE.

Figure 6 summarizes the effects of caffeine and 8-Br-cAMP on CCE. Figure 6A shows that capacitative Ca^{2+} entry was activated by depleting the SR Ca^{2+} stores, and then reintroducing extracellular Ca^{2+} as we have done previously (Ng et al., 2005;Ng et al., 2007;Wilson et al., 2002). These experiments were performed by perfusing the cells with a Ca^{2+} free bathing solution in the continuous presence of 10 μM CPA and exposing the cells twice for 30 s to 10 mM caffeine and 10 μM 5-HT. Subsequent to this, 2 mM-extracellular Ca^{2+} was added to the bathing solution in the continued presence of 10 μM CPA. This procedure elevates cytosolic Ca^{2+} above basal values, and is due to activation of CCE pathways (Ng et al., 2005;Ng et al., 2007;Wilson et al., 2002). Figure 6A shows a representative recording of the estimated cytosolic [Ca^{2+}] from a canine pulmonary arterial myocyte following depletion of the intracellular Ca^{2+} stores and during re-addition of extracellular Ca^{2+}. Following Ca^{2+} readdition, the cytosolic [Ca^{2+}] increased 43 nM above basal levels, which is consistent with activation of CCE in canine PASMCs (Ng et al., 2005;Ng et al., 2007;Wilson et al., 2002). Figure 6B shows the influence of 1 mM 8-Br-cAMP on the cytosolic [Ca^{2+}] with Ca^{2+} readdition following depletion of the sarcoplasmic reticulum Ca^{2+} stores. Following Ca^{2+} readdition, the cytosolic [Ca^{2+}] increased 31 nM above basal levels in this cell, which is similar to the control cell shown in Figure 6A and also indicative of CCE. Figure 6D provides a summary of the results, showing that incubation with 1 mM 8-Br-cAMP did not prevent CCE

activation. In the 8-Br-cAMP treated cells, the cytosolic [Ca^{2+}] increased 33 ± 12 nM, which was equivalent to the development of CCE in untreated cells (54 ± 12 nM) from the same animals.

In comparison to the lack of effect of 8-Br-cAMP on CCE, Figure 6C shows sustained exposure to 10 mM caffeine prevented CCE activation, which builds on the finding that it fully inhibits 5-HT elicited cytosolic [Ca^{2+}] responses in canine PASMCs (Figure 3). Figure 6D summarizes the CCE mediated cytosolic [Ca^{2+}] responses in the presence of 10 mM caffeine, which were 4 ± 5 nM below basal Ca^{2+} levels. The decrement in the CCE response by 10 mM caffeine is similar to the depression in CCE due to pharmacological inhibition of the store-operated channels responsible for CCE as shown in Wilson et al., 2002 (Figures 1, 2, 7 and 8), or due to $InsP_3$ receptor inhibition as shown in Ng et al, 2007(Figures 3 and 5). In Wilson et al., 2002, CCE was diminished by reducing extracellular Ca^{2+} entry through extracellular Ca^{2+} removal (6 ± 6 nM below basal [Ca^{2+}] values) or by 10 mM Ni^{2+} (70 ± 14 nM below basal [Ca^{2+}] values). Similarly CCE was reduced in Ng et al., 2007 through $InsP_3R$ inhibition with 50 μM 2-APB (19 ± 15 nM below basal [Ca^{2+}] values), 20 μM xestospongin C (4 ± 10 nM below basal [Ca^{2+}] values), or 50 μM dantrolene (0 ± 13 nM).

The inhibition of CCE by caffeine is markedly dissimilar from modifiers of RyR activity used in Ng et al., 2007 (Figures 4 and 5). In these previous studies, we showed CCE was not reduced by 10 or 300 μM ryanodine or by 10 μM dantrolene. This is important because it indicates that the influence of caffeine on CCE is not due to ryanodine receptor activation.

4.1 Discussion

Our results show the widely used RyR activator caffeine is also a rapid, yet reversible, inhibitor of 5-HT elicited and presumably $InsP_3$ receptor mediated cytosolic [Ca^{2+}] increases in both acutely isolated smooth muscle cells and cultured mammalian cells. Caffeine inhibited 5-HT mediated Ca^{2+} increases with an IC_{50} similar to its direct effects on $InsP_3$ receptors (Bezprozvanny et al., 1994). Further to this, the antagonism of 5-HT elicited Ca^{2+} responses by caffeine in canine PASMCs was akin to the actions of the $InsP_3$ receptor inhibitors 2-APB and xestospongin C (Wilson et al, 2005) and similar to caffeine antagonism of $InsP_3$ related Ca^{2+} responses in Xenopous oocytes (Parker and Ivorra, 1991). Capacitative calcium entry was also reduced by caffeine concentrations that fully inhibit $InsP_3$ receptors (Bezprozvanny et al., 1994) but not by 8-Br-cAMP.

Caffeine-dependent depression of 5-HT mediated Ca^{2+} responses may be due to either direct or indirect inhibition of $InsP_3$ receptor signaling. The basis of the present studies was to assess if caffeine inhibits $InsP_3$-related Ca^{2+} signaling events, and the experiments accomplished this goal. The simplest explanation is that caffeine reduces 5-HT elicited Ca^{2+} responses by directly inhibiting $InsP_3$ receptor activation as has been previously observed (Bezprozvanny et al., 1994;Parker and Ivorra, 1991). Support for this is the observation that the IC_{50} for caffeine-inhibition of the 5-HT dependent Ca^{2+} responses is similar to its antagonism of $InsP_3$ receptor function (Bezprozvanny et al., 1994;Parker and Ivorra, 1991). However, as a methylxanthine, caffeine also enhances cAMP formation through phosphodiesterase inhibition, and this cAMP elevation can antagonize $InsP_3$ receptor activation (Pauvert et al, 2003; Pauvert et al, 2004; Bai and Sanderson, 2006). We attempted to minimize this cAMP generation by only applying caffeine for short periods prior to 5-HT stimulation. The reversibility of caffeine inhibition of 5-HT elicited Ca^{2+} responses also suggests there was not long-lived cAMP accumulation. Even still, this cannot refute the potential that caffeine-mediated cAMP formation reduces 5-HT dependent Ca^{2+} responses. Elucidating the specific mechanism of caffeine inhibition of 5-HT mediated Ca^{2+} responses is an important consideration, and thus warrants future investigation.

Caffeine- dependent inhibition of 5-HT mediated Ca^{2+} responses is not likely due to a direct effect on L-type Ca^{2+} channels. Although L-type Ca^{2+} channels are inhibited by millimolar caffeine (Zholos et al., 1991), the IC_{50} is roughly 20-fold higher (Martin et al., 1989) than its ability to inhibit 5-HT mediated Ca^{2+} increases. Further support is that dihydropyridine inhibition of L-type Ca^{2+} channels does not reduce the magnitude of 5-HT-dependent Ca^{2+} responses in canine PASMCs (Wilson et al, 2005; Ostrovskaya et al, 2007).

Caffeine does not likely directly activate CCE or ECCE pathways in canine PASMCs. Evidence for this is that caffeine failed to increase the fura-2 quench rate by Mn^{2+}, which compares with the increase in Mn^{2+} quench due to depletion of the intracellular Ca^{2+} stores (Ng et al., 2005;Ng et al., 2007;Wilson et al., 2002). This inability of caffeine to enhance Mn^{2+}-permeability mimics that of 5-HT, which also does not activate CCE (Wilson et al., 2005). The lack of ECCE was anticipated as the coupling between L-type Ca^{2+} channels and RyRs in skeletal and smooth muscle are distinct from one another. In skeletal muscle, these proteins are physically associated and allow for classic conformational-coupling (Hurne et al., 2005). Comparatively, RyR activity in smooth muscle is loosely coupled to L-type Ca^{2+} channels, where extracellular Ca^{2+} influx excites RyRs (Collier et al., 2000). Importantly, ECCE activity in skeletal muscle is dependent on L-type Ca^{2+} channel and RyR expression, where RyR mutations alter ECCE gating (Hurne et al., 2005). This suggests there is a physical association between the RyR and the channel responsible for ECCE in skeletal muscle, an association that may not exist in smooth muscle.

Caffeine inhibition of CCE was not surprising as we recently showed $InsP_3$ receptors but not RyRs are important to CCE activity in canine PASMCs (Ng et al., 2007). The finding that caffeine inhibits CCE also builds on several previous studies showing $InsP_3$ receptor activity is important to CCE (Birnbaumer et al., 2000;Ma et al., 2000;Wang et al., 2001). However, as caffeine can directly inhibit $InsP_3$ receptors (Bezprozvanny et al., 1994;Parker and Ivorra, 1991) as well as L-type Ca^{2+} channels (Zholos et al., 1991) it is possible that caffeine may also block other Ca^{2+} permeable ion channels, such as store-operated channels. The potential for caffeine inhibition of store-operated channels will require additional studies, as we were unable to find any reports supporting or refuting this alternative.

The finding that 8-Br-cAMP did not influence capacitative calcium entry is revealing as several recent reports indicate cAMP inhibits store-operated Ca^{2+} entry in airway smooth muscle (Ay et al., 2006), coronary arterial myocytes (Smani et al., 2007), and human intrapulmonary arterial smooth muscle cells (Zhang et al., 2007), as well as store-operated channel currents in portal vein myocytes (Liu et al., 2005). We are unsure of why our findings in canine PASMCs are distinct from the results found in other myocytes. One possibility is that the magnitude of the CCE response in the canine cells is relatively small and the heterogeneity between individual cells may be sufficiently high to mask a small change in capacitative calcium entry due to cAMP, or it may be the result of differences in the experimental conditions. For example, Zhang et al., 2007 preincubated their cells for 30 minutes with agents that increased cyclic nucleotide levels while we treated our cells for only 5–10 minutes. On closer examination of the data presented by Zhang and co-workers they observed that forskolin and IBMX only partially reduced the capacitative calcium entry response, and that this effect was dependent on the administration time, which was 30 min or 4 hours. Based on the data we present in Figure 6, there may be a trend for cAMP to reduce capacitative calcium entry, however this depression in cytosolic calcium is small and within the observed variability. The potential modulation of capactiative calcium entry by cAMP is important as this pathway is essential during the development of hypoxic induced pulmonary vasoconstriction (Ng et al., 2005; Ng et al., 2007), a process that regulates blood flow distribution and gas exchange in the lung, and because these pathways are important for cell growth and proliferation (Sweeney et al., 2002). This may also be therapeutically relevant as agents that increase cyclic nucleotides, such

as the PDE5 inhibitor sildenafil and the prostacylin analogue iloprost cause pulmonary arterial vasorelaxation and thus are useful in the treatment of pulmonary hypertension (Gessler et al., 2008; Wilkins et al., 2008).

5.1 Conclusions

This manuscript shows the following:

1. Caffeine inhibits 5-HT elicited - $InsP_3$ – mediated Ca^{2+} responses and CCE in smooth muscle.
2. The data provide correlative support for our previous studies that suggest $InsP_3$ receptors are important to the activation of CCE in canine PASMCs.
3. ECCE pathways may not exist in canine PASMCs.
4. The role of $InsP_3$ receptor activity may be difficult to assess when caffeine is used in tandem to activate RyRs.
5. There is a fundamental need for the development and characterization of RyR activators that do not interfere with $InsP_3$ receptor activity.

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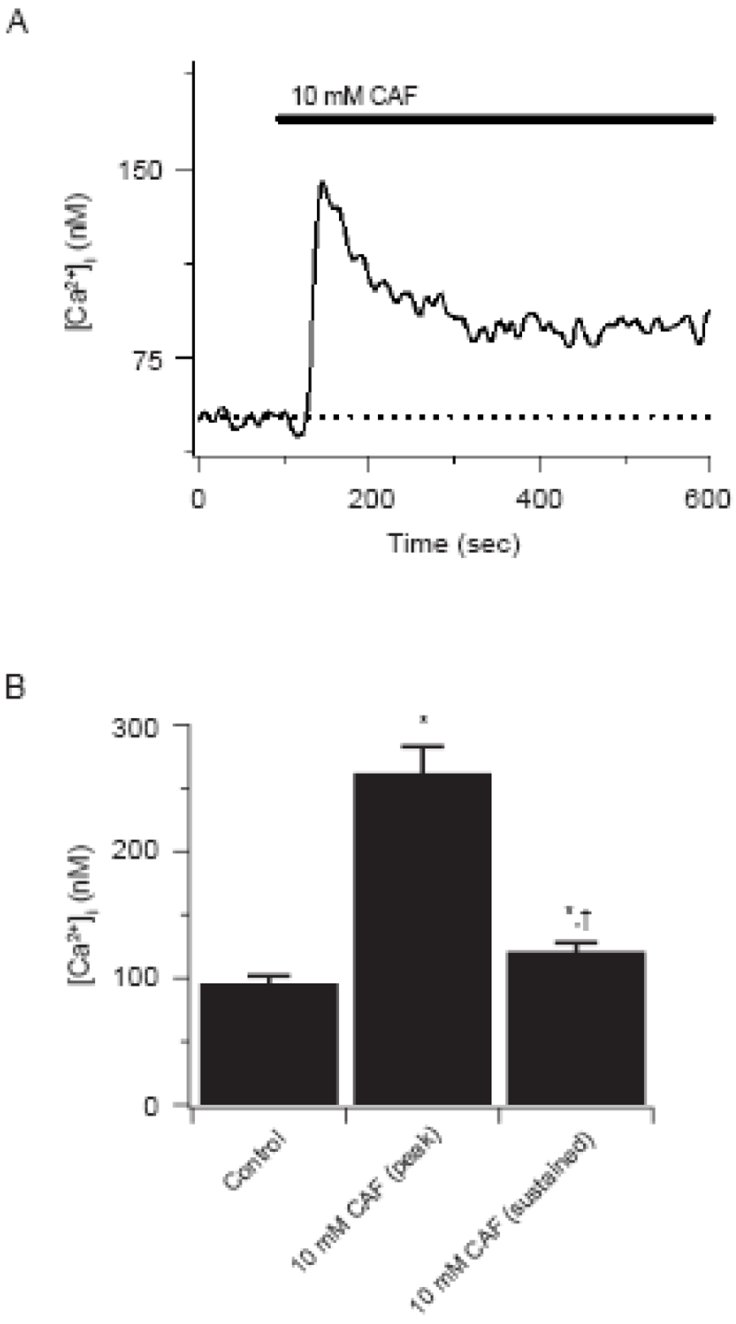

Figure 1.

Caffeine elicits cytosolic [Ca^{2+}] ncreases in PASMCs. (A) Caffeine induced Ca^{2+} transient. Caffeine was present at times shown by the horizontal bar. Dashed line shows resting cytosolic [Ca^{2+}]. (B) Bars indicate the cytosolic [Ca^{2+}] before and during caffeine. Error bars represent ± S.E.M for 53 cells * Denotes significant difference to control while † denotes difference as compared to peak caffeine conditions using Friedman repeated measures ANOVA on ranks with SNK multiple comparison procedures (P<0.05).

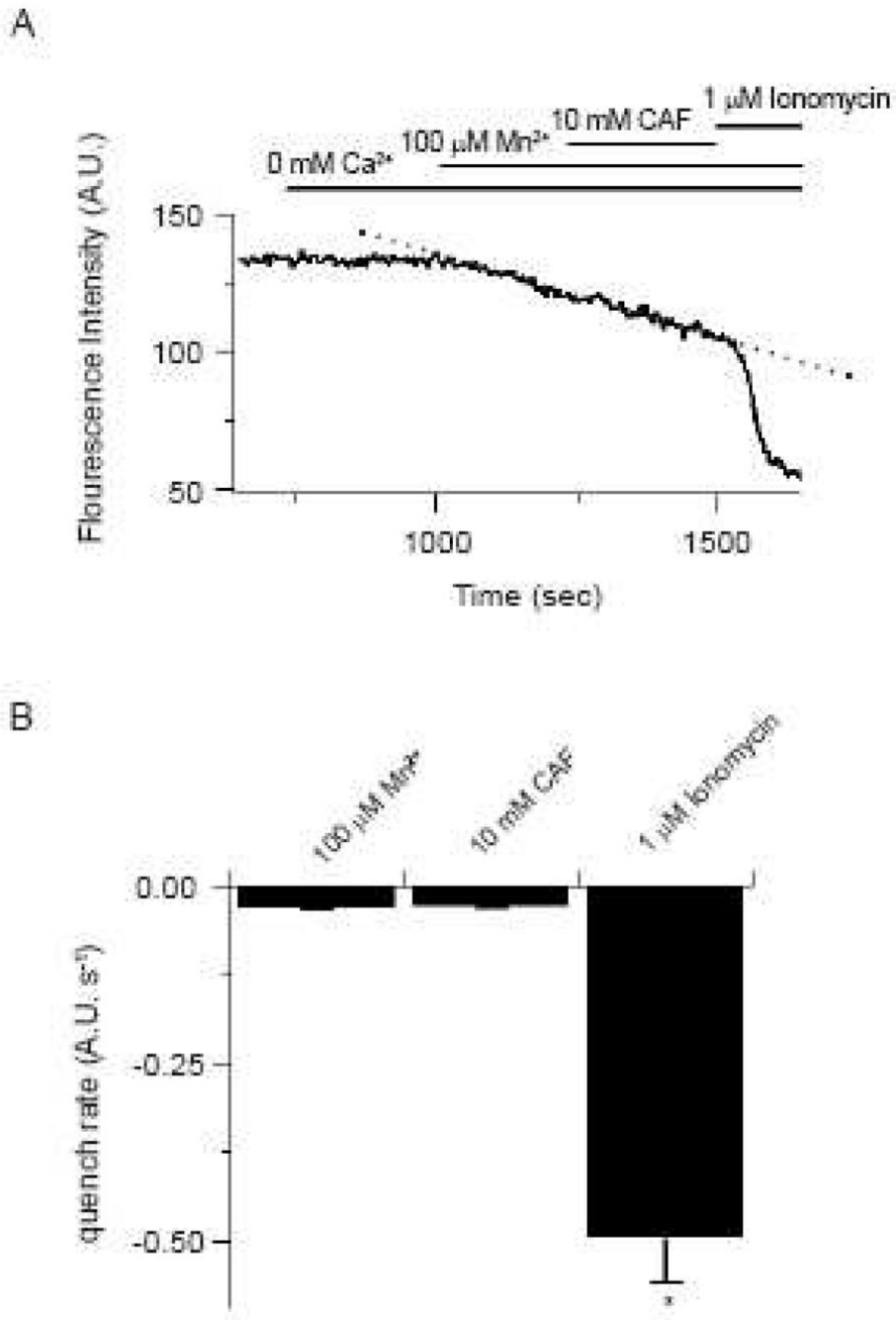

Figure 2.

Caffeine does not enhance Mn^{2+} quench of fura-2 in PASMCs. (A) 10 mM caffeine effect on the rate of fura-2 quench by 100 μM Mn^{2+}. Agonists were present at times shown by the horizontal bars. Dashed line shows the resting quench rate. (B) Bars show the fura-2 quench rate. Error bars represent ± S.E.M. for 23 cells. * Denotes significant difference to 100 μM Mn^{2+} and 100 μM Mn^{2+} + 10 mM CAF conditions using Friedman repeated measures ANOVA on ranks with SNK multiple comparison procedures (P<0.05).

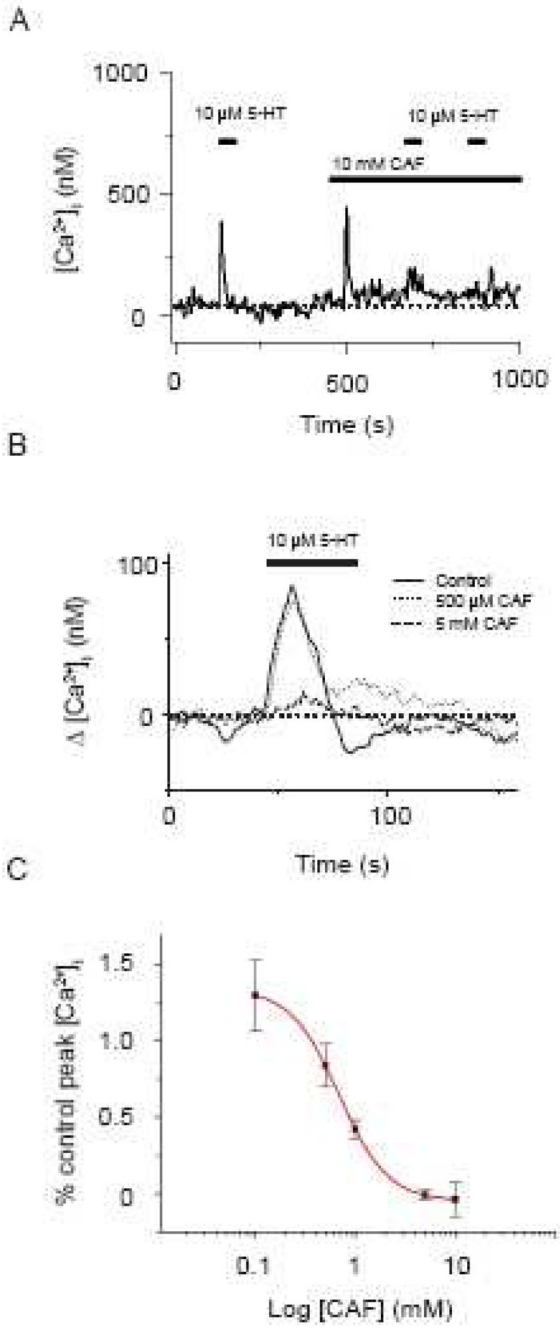

Figure 3.

Caffeine blocks 5-HT elicited cytosolic [Ca^{2+}] increases in PASMCs. (A) 10 μM 5-HT induced Ca^{2+} transient in the absence then presence of 10 mM caffeine. Agonists were present at times shown by the horizontal bars. Dashed line shows the resting cytosolic [Ca^{2+}]. (B) Dose dependent inhibition of 10 μM 5-HT induced Ca^{2+} transients by caffeine. Solid line is the resultant dose-response curve fitted to the data with a simple binding equation. Values are means of % peak Ca^{2+}. Error bars represent ± S.E.M for 8 to 10 cells at each concentration of caffeine evaluated.

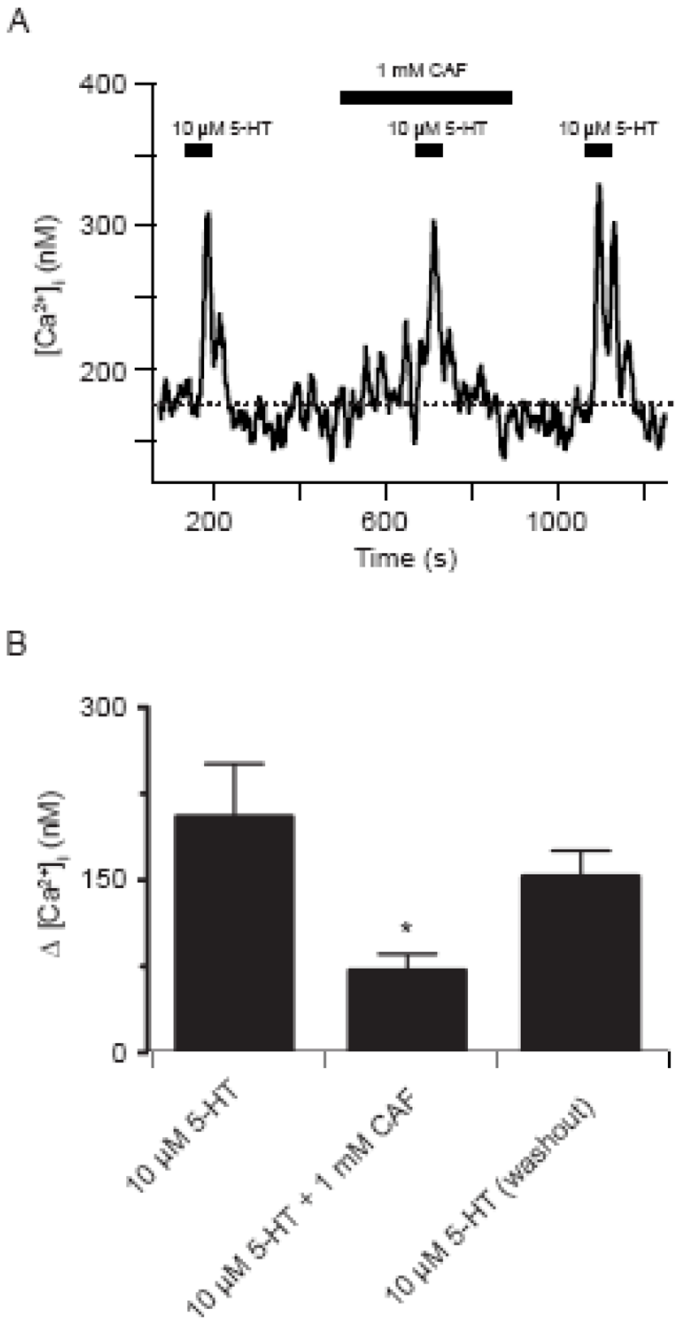

Figure 4.

Caffeine reversibly inhibits 5-HT mediated cytosolic [Ca^{2+}] increases in PASMCs. (A) 10 μM 5-HT induced cytosolic [Ca^{2+}] transient in the absence and presence of 1 mM caffeine. Agonists were present at times shown by the horizontal bars. Dashed line shows the resting cytosolic [Ca^{2+}]. (B) Bars indicate the change in the cytosolic [Ca^{2+}] from basal values in the presence and absence of caffeine. Error bars represent ± S.E.M for 16 myocytes. * Denotes significant difference to 10 μM 5-HT and 10 μM 5-HT during washout conditions using a repeated measures ANOVA with a SNK multiple comparison procedure ($P<0.05$).

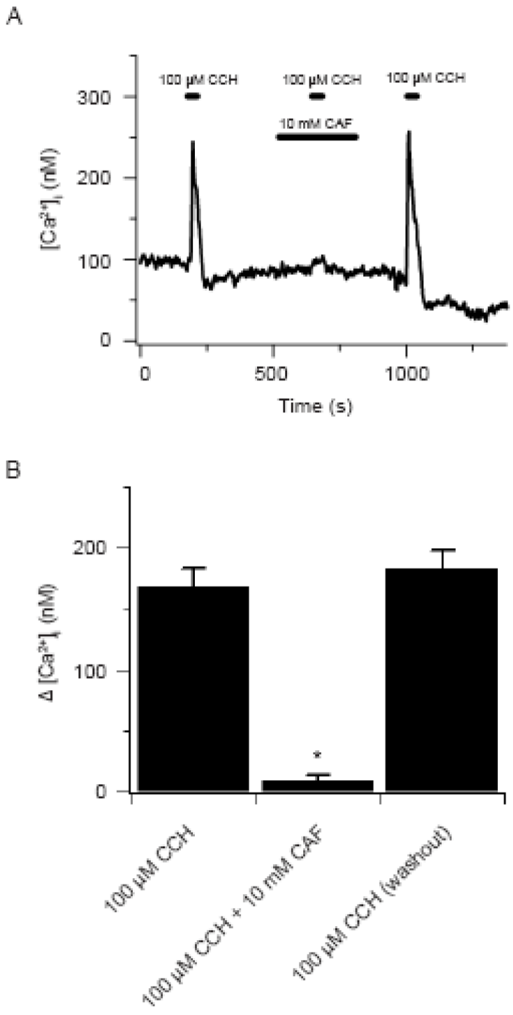

Figure 5.

Caffeine blocks carbachol elicited cytosolic [Ca^{2+}] increases in HEK 293 cells. (A) 100 μM carbachol induced Ca^{2+} transient in the absence and presence of 10 mM caffeine. Agonists were present at times shown by the horizontal bars. (B) Bars indicate the change in the cytosolic [Ca^{2+}] from basal values in the absence and presence of caffeine. Error bars represent ± S.E.M for 65 cells. * Denotes significant difference to 100 μM carbachol and 100 μM carbachol during washout conditions using a Friedman repeated measures ANOVA on ranks with a SNK multiple comparison procedure (P<0.05).

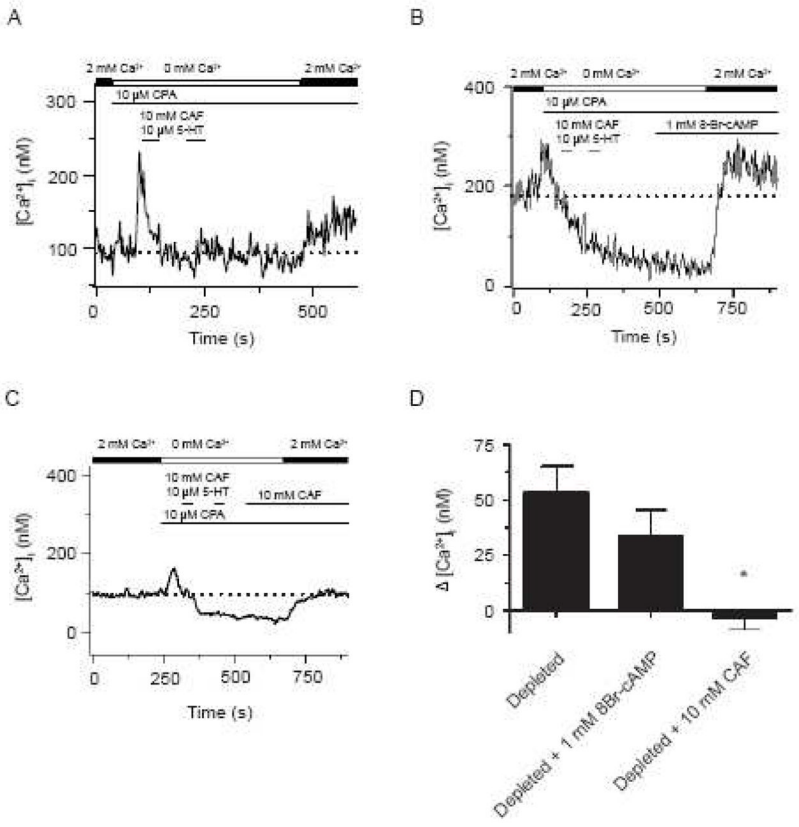

Figure 6.

Caffeine but not 8-Br-cAMP inhibits CCE in canine PASMCs. (A) Effect of extracellular Ca^{2+} removal, 10 μM CPA, sequential exposure to 10 mM caffeine and 10 μM 5-HT on cytosolic $[Ca^{2+}]$ with extracellular Ca^{2+} re-addition. Effects of (B) 1 mM 8-Br-cAMP and (C) 10 mM caffeine on the cytosolic $[Ca^{2+}]$ with depletion of the sarcoplasmic reticulum Ca^{2+} stores. Agonists were present at times shown by the horizontal bars. Dashed line shows the resting cytosolic $[Ca^{2+}]$. (D) Bars indicate the change in cytosolic $[Ca^{2+}]$ from resting levels for myocytes that were store depleted in the absence (n=9) or presence of 8-Br-cAMP (n=6) or caffeine (n=10). Error bars represent ± S.E.M. Mean value significantly different from other groups by a One-Way ANOVA with a Newman-Keuls Multiple Comparison Test ($P<0.05$).