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The Cl^- channel blocker niflumic acid releases Ca^{2+} from an intracellular store in rat pulmonary artery smooth muscle cells

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1 The effect of the Cl^- channel blockers niflumic acid (NFA), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), and anthracene-9-carboxylic acid (A-9-C), on Ca^{2+} signalling in rat pulmonary artery smooth muscle cells was examined. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored with either fura-2 or fluo-4, and caffeine was used to activate the ryanodine receptor, thereby releasing Ca^{2+} from the sarcoplasmic reticulum (SR).

2 NFA and NPPB significantly increased basal $[Ca^{2+}]_i$ and attenuated the caffeine-induced increase in $[Ca^{2+}]_i$. These Cl^- channel blockers also increased the half-time ($t_{1/2}$) to peak for the caffeine-induced $[Ca^{2+}]_i$ transient, and slowed the removal of Ca^{2+} from the cytosol following application of caffeine. Since DIDS and A-9-C were found to adversely affect fura-2 fluorescence, fluo-4 was used to monitor intracellular Ca^{2+} in studies involving these Cl^- channel blockers. Both DIDS and A-9-C increased basal fluo-4 fluorescence, indicating an increase in intracellular Ca^{2+}, and while DIDS had no significant effect on the $t_{1/2}$ to peak for the caffeine-induced Ca^{2+} transient, it was significantly increased by A-9-C.

3 In the absence of extracellular Ca^{2+}, NFA significantly increased basal $[Ca^{2+}]_i$, suggesting that the release of Ca^{2+} from an intracellular store was responsible for the observed effect.

4 Depleting the SR with the combination of caffeine and cyclopiazonic acid prevented the increase in basal $[Ca^{2+}]_i$ induced by NFA. Additionally, incubating the cells with ryanodine also prevented the increase in basal $[Ca^{2+}]_i$ induced by NFA.

5 These data show that Cl^- channel blockers have marked effects on Ca^{2+} signalling in pulmonary artery smooth muscle cells. Furthermore, examination of the NFA-induced increase in $[Ca^{2+}]_i$ indicates that it is likely due to Ca^{2+} release from an intracellular store, most probably the SR.
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Abbreviations: A-9-C, anthracene-9-carboxylic acid; CPA, cyclopiazonic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; NFA, niflumic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid

Introduction

In smooth muscle cells, the intracellular chloride (Cl^-) concentration is normally maintained at levels higher than its electrochemical equilibrium. It is widely accepted that this high intracellular Cl^- concentration plays an important role in regulating a number of cellular functions, including intracellular pH, cell volume, contractility, and membrane potential (for a review see Chipperfield & Harper, 2000). Many of these cellular effects of Cl^- are dependent upon the activities of Cl^- channels. For example, in smooth muscle, agonist-induced release of Ca^{2+} from intracellular stores can activate plasma membrane Cl^- channels resulting in membrane depolarization. This in turn leads to activation of voltage-gated Ca^{2+} channels and Ca^{2+} influx (for review see Large & Wang, 1996; Kitamura & Yamazaki, 2001). Chloride channels in the plasma membrane have been extensively studied using the patch-clamp technique, in vascular (Hogg *et al*., 1994a, b; Yuan, 1997), tracheal (Janssen & Sims, 1992), gastric (Wade *et al*., 1996), and urogenital (Cotton *et al*., 1997) smooth muscle preparations.

A number of studies, examining the effect of Cl^- channel blockers on the contractile activity of vascular smooth muscle, have supported the above role for Cl^- channels. Specifically, the Cl^- channel blocker niflumic acid (NFA) has been shown to reduce the magnitude of agonist-induced contractions in the rat aorta (Criddle *et al*., 1996; Lamb & Barna, 1998) and pulmonary artery (Wang *et al*., 1997; Yuan, 1997; Hyvelin *et al*., 1998), as well as the rabbit basilar (Uchida *et al*., 1990) and middle cerebral arteries (Gokina & Bevan, 2000). Other Cl^- channel blockers including 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and anthracene-9-carboxylic acid (A-9-C) have been shown to have similar inhibitory effects on vascular smooth muscle contraction (Lamb & Barna, 1998; Gokina & Bevan, 2000). However, establishing the role of Cl^- channels in cell physiology is often complicated by the fact that many of these Cl^- channel blockers also have nonspecific effects. For instance, NFA has been shown to block nonselective cation channels (Gogelein *et al*., 1990), L-type Ca^{2+} channels (Doughty *et al*., 1998), and also activate Ca^{2+}-

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activated K^+ channels (Greenwood & Large, 1995; Toma *et al*., 1996).

There is increasing evidence that Cl^- movement also plays an important role in counteracting electrogenic Ca^{2+} fluxes across the sarcoplasmic reticulum (SR) membrane, during contraction–relaxation cycles in cardiac (Meissner & McKinley, 1982), skeletal (Kourie *et al*., 1996a, b), and smooth muscle (Pollock *et al*., 1998). In saponin-permeabilized gastric smooth muscle cells from rabbit, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and indanyloxyacetic acid were found to almost completely block Ca^{2+} uptake by the SR, suggesting a role for Cl^- channels in SR Ca^{2+} handling (Pollock *et al*., 1998).

Despite a large number of studies examining the effect of Cl^- channel blockers on the contractile and electrophysiological properties of smooth muscle, there have been very few studies that systematically look at the effect of these drugs on intracellular Ca^{2+} signalling (Pon *et al*., 1993; Yuan, 1997; Hyvelin *et al*., 1998). Thus, the objective of the present study was to determine the effect of four different Cl^- channel blockers on caffeine-induced Ca^{2+} signalling in rat pulmonary artery smooth muscle cells (PASMCs). Caffeine was used as an agonist since it activates the ryanodine receptors in the SR membrane, thereby allowing the effects of Cl^- channel blockers on SR Ca^{2+} signalling to be examined simultaneously. Our results show that, in rat PASMCs, Cl^- channel blockers have significant effects on $[Ca^{2+}]_i$ signalling, while detailed study of the effect of NFA indicates that it releases Ca^{2+} from an intracellular store, probably the SR. A preliminary account of this work has been presented to the British Pharmacological Society (Cruickshank & Drummond, 2002).

Methods

Cell isolation

Experiments were carried out on smooth muscle cells freshly isolated from rat pulmonary and mesenteric arteries. Male Sprague–Dawley rats (200–300 g) were killed by cervical dislocation in accordance with current UK legislation. For PASMC isolation, the heart and lungs were removed *en bloc* and placed in a dissecting solution of the following composition (in mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, glucose 5.5, $NaHC0_3$ 25, HEPES 10, pH adjusted to 7·4 with NaOH. Thereafter, intrapulmonary arteries (400–800 μm outside diameter) were dissected out and PASMCs isolated using the method previously described by Drummond & Tuft (1999). Briefly, arterial ring segments ($\sim$1 mm in length) were placed in a dissociation solution of the following composition (mM): NaCl 128, KCl 5.4, KH_2PO_4 0.95, Na_2HPO_4 0.35, $MgSO_4$ 1.17, glucose 10, sucrose 2.9, $NaHCO_3$ 4.16, HEPES 10, pH adjusted to 7.3 with NaOH. Papain (1.5 mg ml^{-1}) and DL-dithiothreitol (1 mg ml^{-1}) were added to the solution and the tissue was maintained at 4°C for 60 min in a refrigerator. The solution containing the tissue was then transferred to a water bath at 37°C for 6 min. Thereafter, the arterial rings were transferred to fresh dissociation solution containing collagenase (Sigma type VIII, 1.5 mg ml^{-1}) and incubated in the water bath for a further 5 min at 37°C. Gentle trituration of the tissue with a fire-polished Pasteur pipette yielded single smooth muscle cells, which remained viable for up to 6 h. A similar procedure was used to isolate cells from small branches of the mesenteric artery (200–500 μm outside diameter), with the exception that the incubation period in papain was 15 min, and 10 min in collagenase.

Measurement of $[Ca^{2+}]_i$

To measure $[Ca^{2+}]_i$, PASMCs were incubated with 5 μM fura-2 AM for 40 min at room temperature. Fura-2 fluorescence was monitored using a PTI DeltaRam microfluorimeter (PTI, Lawrenceville, NJ, U.S.A.), coupled to a Nikon Eclipse TE200 microscope with an $\times$40 NA 1.3 objective lens. Ratio measurements of fura-2 were made at 20 Hz and converted to $[Ca^{2+}]_i$ using the method described by Grynkiewicz *et al*. (1985), with an assumed Ca^{2+}-fura-2 K_d of 200 nM. R_{max}, R_{min} and β were calculated as follows: R_{max} is the fluorescence ratio in saturating Ca^{2+} (340 nm/380 nm excitation); R_{min} is the fluorescence ratio in the absence of Ca^{2+} (340 nm/380 nm excitation); and β is the fluorescence ratio for dye excited at 380 nm (Ca^{2+}-free/saturating Ca^{2+}), as described in Moore *et al*. (1990). Both DIDS and A-9-C were found to affect fura-2 fluorescence (see Figure 2); therefore, in studies using DIDS and A-9-C, fluo-4 AM (5 μM) was used to monitor intracellular Ca^{2+}.

Caffeine (20 mM), which activates the ryanodine receptor to release SR Ca^{2+}, was applied to a single cell *via* a pressure-ejection pipette, positioned about 100 μm from the cell, using a Picospritzer II (General Valve, Fairfield, NJ, U.S.A.). Cells were bathed in an extracellular solution of the following composition (in mM): NaCl 150, KCl 5.4, $MgCl_2$ 1.2, $CaCl_2$ 1.8, HEPES 10, glucose 10, pH adjusted to 7.4 with NaOH. The Ca^{2+}-free extracellular solution had the following composition (mM): NaCl 150, KCl 5.4, $MgCl_2$ 3.0, HEPES 10, EGTA 1, glucose 10, and the pH adjusted to 7.4 with NaOH. All experiments were carried out at room temperature (20–25°C).

Reagents and data analysis

Cyclopiazonic acid (CPA) and ryanodine (both Calbiochem, CN Biosciences, Nottingham, U.K.), NFA, DIDS, NPPB, and A-9-C (all from Sigma, Sigma-Aldrich Ltd., Poole, U.K.) were prepared as stock solutions in DMSO and diluted as required. Unless otherwise stated, all drugs were bath applied. Bath concentrations of DMSO did not exceed 0.2% and had no effect on Ca^{2+} signalling. Fura-2 AM and fluo-4 AM were obtained from Molecular Probes (Cambridge Bioscience, Cambridge, U.K.).

Following caffeine application, the half-time ($t_{1/2}$) for recovery of $[Ca^{2+}]_i$ to its basal level was determined. Data are presented as the mean$\pm$s.e.m., and n = number of cells studied. For graphical clarity in the $[Ca^{2+}]_i$ records, not all data points are shown. Statistical tests of difference were made using Student's paired t-test, with $P<0.05$ being considered statistically significant.

Results

Caffeine-induced changes in $[Ca^{2+}]_i$ in PASMCs and the effect of ryanodine

The application of 20 mM caffeine for 5 s produced a transient increase in $[Ca^{2+}]_i$, from a basal value of 80$\pm$2 nM to a peak

value of 752 ± 44 nM ($n=6$), in rat PASMCs. The half-time ($t_{1/2}$) to peak, for the caffeine-induced increase in $[Ca^{2+}]_i$, was 0.5 ± 0.1 s after the onset of caffeine application, with $[Ca^{2+}]_i$ returning to basal levels over the subsequent 20–40 s period. The $t_{1/2}$ for recovery of $[Ca^{2+}]_i$ following the application of caffeine was 8 ± 0.6 s. In order to evoke reproducible responses, a 5–6 min interval was allowed between caffeine applications (Figure 1a).

Incubating PASMCs in 50 μM ryanodine for 5 min reduced the magnitude of the caffeine-induced $[Ca^{2+}]_i$ transient; however, subsequent responses to caffeine were completely abolished (Figure 1b). This indicates that the caffeine-induced $[Ca^{2+}]_i$ transients in PASMCs are entirely dependent upon Ca^{2+} release from the SR *via* the ryanodine receptor.

Effect of NFA, NPPB, DIDS, and A-9-C on fura-2 excitation spectra

The excitation spectra for fura-2 was recorded in the presence of either NFA, NPPB, DIDS or A-9-C, in order to determine whether any of these agents affected fura-2 fluorescence. Figure 2a and b show that neither NFA (50 μM) nor NPPB (10 μM) affected fura-2 fluorescence, in either Ca^{2+}-containing or Ca^{2+}-free solution. However, both 100 μM DIDS and 50 μM A-9-C were found to affect fura-2 fluorescence, when excited between 320 and 380 nm. This was observed in both Ca^{2+}-containing and Ca^{2+}-free solutions (Figure 2c, d). Consequently, studies with DIDS and A-9-C used fluo-4 to monitor intracellular Ca^{2+}, as neither had an effect on fluo-4 fluorescence (not shown).

Effect of NFA, NPPB, DIDS or A-9-C on caffeine-induced Ca^{2+} transients in PASMCs

After obtaining a control $[Ca^{2+}]_i$ transient to caffeine, a 5 min recovery period was allowed before the addition of NFA (50 μM) to the extracellular solution. Following a 2 min equilibration period, NFA was found to significantly increase basal $[Ca^{2+}]_i$ from 87 ± 15 to 253 ± 45 nM ($n=6$, $P<0.05$, Figure 3a and b). In the presence of NFA, the peak $[Ca^{2+}]_i$ attained during application of caffeine was reduced from 1017 ± 143 to 642 ± 54 nM, showing a significant reduction in the change in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) ($P<0.05$, Figure 3c). NFA also increased the $t_{1/2}$ to peak for the caffeine-induced increase in $[Ca^{2+}]_i$ from 0.6 ± 0.1 to 1.5 ± 0.3 s ($P<0.05$, Figure 3d), and increased the $t_{1/2}$ for recovery of $[Ca^{2+}]_i$ from 7.2 ± 1.3 to 17.4 ± 3.3 s ($P<0.05$, Figure 3e).

In a separate series of experiments, NPPB (10 μM) was also found to significantly increase basal $[Ca^{2+}]_i$ from 104 ± 3 to 167 ± 9 nM ($n=5$, $P<0.05$) after a 2 min equilibration period (Figure 4a and b). The peak $[Ca^{2+}]_i$ attained during application of caffeine was reduced from 712 ± 72 to 415 ± 74 nM in

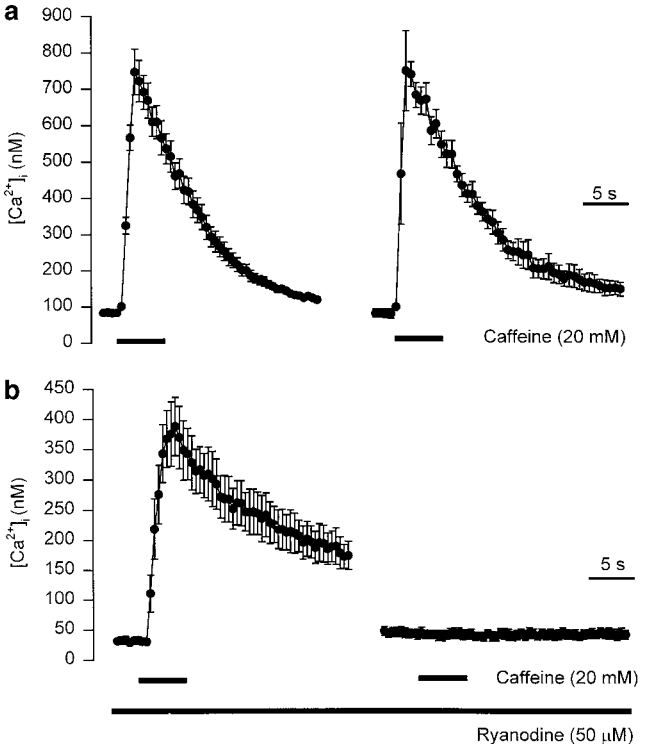

Figure 1 Caffeine increases $[Ca^{2+}]_i$ *via* a ryanodine-sensitive Ca^{2+} store. Caffeine (20 mM) was applied to single PASMCs *via* a pressure-ejection pipette positioned approximately 100 μm from the cell. Reproducible $[Ca^{2+}]_i$ transients were obtained when a 5–6 min recovery period was allowed between caffeine applications (a). The break between transients represents the recovery period during which time recordings were not made. Cells were incubated with ryanodine (50 μM) for 5 min and caffeine re-applied. Subsequent caffeine-induced $[Ca^{2+}]_i$ transients were abolished (b). Data are shown as mean values $\pm$ s.e.m. ($n=6$).

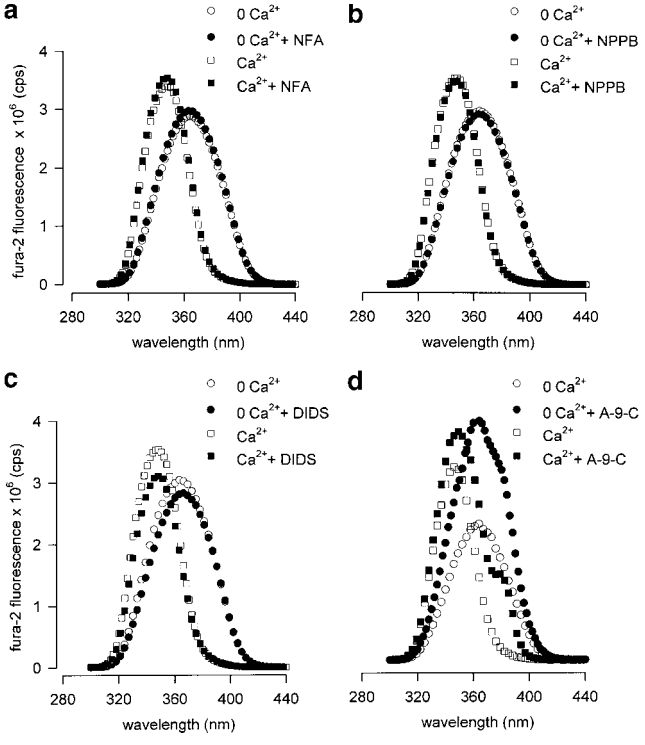

Figure 2 Effect of the Cl^- channel blockers NFA, NPPB, DIDS, and A-9-C on fura-2 excitation spectra. Excitation spectra for 1 μM fura-2 in Ca^{2+}-free and Ca^{2+}-containing solution in the absence or presence of NFA (50 μM) (a). Excitation spectra for 1 μM fura-2 in Ca^{2+}-free and Ca^{2+}-containing solution in the absence or presence of NPPB (10 μM) (b). Excitation spectra for 1 μM fura-2 in Ca^{2+}-free and Ca^{2+}-containing solution in the absence or presence of DIDS (100 μM) (c). Excitation spectra for 1 μM fura-2 in Ca^{2+}-free and Ca^{2+}-containing solution in the absence or presence of A-9-C (50 μM) (d). Emission was measured at 510 nm with a 10 nm bandpass filter.

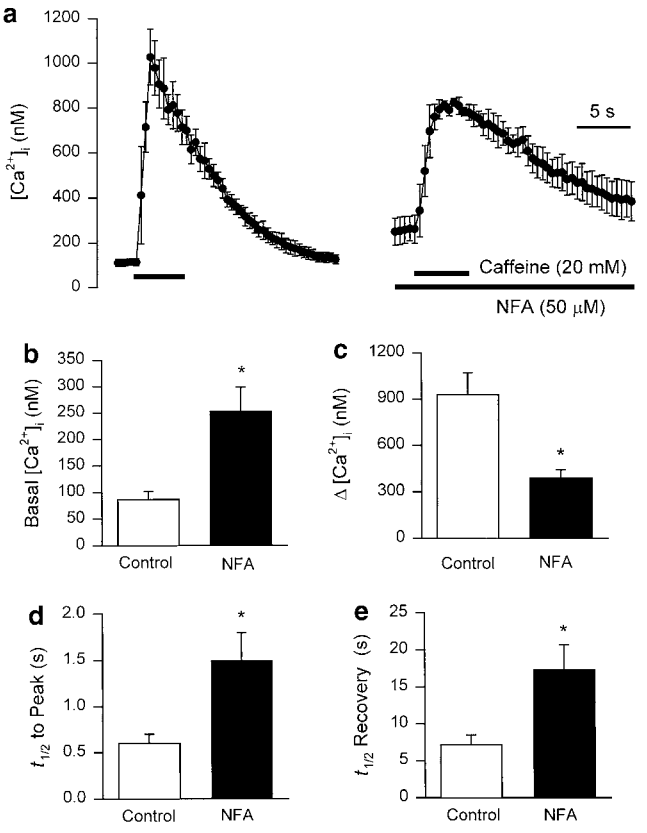

Figure 3 Effect of NFA on caffeine-induced $[Ca^{2+}]_i$ transients in rat PASMCs. Averaged caffeine-induced $[Ca^{2+}]_i$ transients from six cells, before and after the addition of 50 μM NFA (a). $[Ca^{2+}]_i$ was not recorded during the preincubation period (2 min) with NFA. Summary data showing: the effect of NFA on basal $[Ca^{2+}]_i$ (b); the change in $[Ca^{2+}]_i$ produced by caffeine ($[Ca^{2+}]_i$) (c); the half-time ($t_{1/2}$) to peak for the caffeine-induced $[Ca^{2+}]_i$ transient (d); and the half-time ($t_{1/2}$) for Ca^{2+} removal following caffeine application (e). Data are shown as mean values $\pm$ s.e.m. ($n=6$) and $^*P<0.05$.

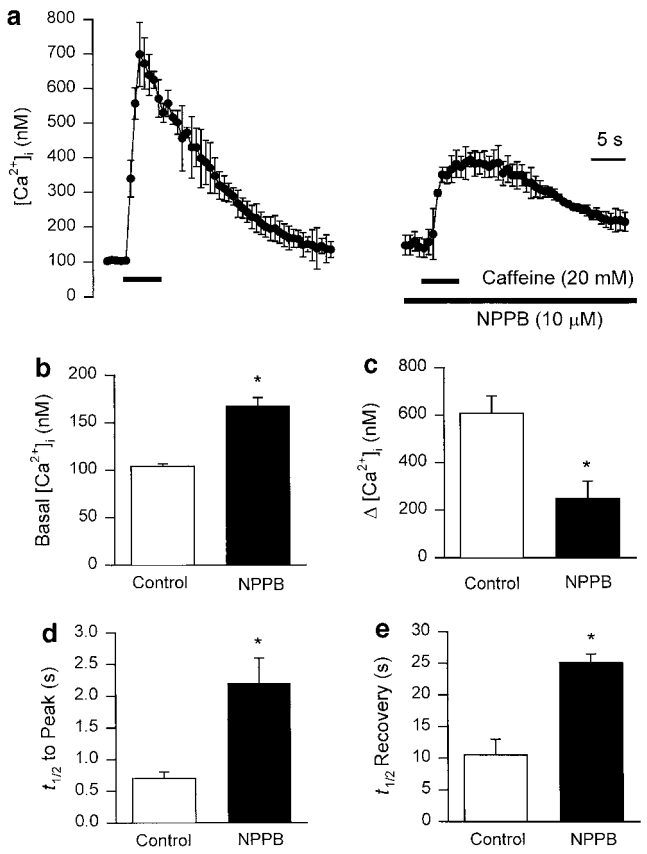

Figure 4 Effect of NPPB on caffeine-induced $[Ca^{2+}]_i$ transients in rat PASMCs. Averaged caffeine-induced $[Ca^{2+}]_i$ transients from five cells, before and after the addition of 10 μM NPPB (a). $[Ca^{2+}]_i$ was not recorded during the preincubation period (2 min) with NPPB. Summary data showing: the effect of NPPB on basal $[Ca^{2+}]_i$ (b); the change in $[Ca^{2+}]_i$ produced by caffeine ($[Ca^{2+}]_i$) (c); the half-time ($t_{1/2}$) to peak for the caffeine-induced $[Ca^{2+}]_i$ transient (d); and the half-time ($t_{1/2}$) for Ca^{2+} removal following caffeine application (e). Data are shown as mean values $\pm$ s.e.m. ($n=5$) and $^*P<0.05$.

the presence of NPPB, again indicating a significant reduction in $\Delta[Ca^{2+}]_i$ ($P<0.05$, Figure 4c). NPPB also increased the $t_{1/2}$ to peak for the caffeine-induced increase in $[Ca^{2+}]_i$ from 0.7 ± 0.1 to 2.2 ± 0.4 s ($P<0.05$, Figure 4d), and increased the $t_{1/2}$ for recovery of $[Ca^{2+}]_i$ following stimulation from 10.5 ± 2.5 to 25.2 ± 1.3 s ($P<0.05$, Figure 4e).

Since DIDS and A-9-C were found to affect the fura-2 fluorescence, fluo-4 was used to monitor intracellular Ca^{2+}. Due to uncertainties regarding the calibration of the fluo-4 signal, fluo-4 fluorescence was not converted to $[Ca^{2+}]_i$. Following a 2 min incubation period with DIDS (100 μM), fluo-4 fluorescence increased by $23\pm1\%$ ($n=6$, $P<0.05$), which most likely reflects an increase in $[Ca^{2+}]_i$ (Figure 5a and b). DIDS did not affect the magnitude of the caffeine-induced increase in fluo-4 fluorescence (Figure 5c), suggesting that it had no effect on the amount of Ca^{2+} released from the SR. Furthermore, DIDS had no effect on the $t_{1/2}$ to peak for the caffeine-induced increase in fluo-4 fluorescence (0.8 ± 0.2 for control and 1 ± 0.3 s in the presence of DIDS) (Figure 5d), nor did it affect the $t_{1/2}$ for recovery of fluo-4 fluorescence (4.4 ± 0.5 s for control and 5.5 ± 0.6 s in the presence of DIDS) (Figure 5e).

After a 2 min incubation period with A-9-C (500 μM), basal fluo-4 fluorescence was increased by $8\pm0.2\%$ ($n=7$, $P<0.05$), which most likely reflects an increase in $[Ca^{2+}]_i$ (Figure 6a and b). The magnitude of the caffeine-induced increase in fluo-4 fluorescence, as assessed by $\Delta F/F_0$, was reduced from 0.99 ± 0.05 to 0.83 ± 0.06 in the presence of A-9-C ($P<0.05$, Figure 6c). The $t_{1/2}$ to peak for the caffeine-induced increase in fluo-4 fluorescence was increased from 1.1 ± 0.2 s to 1.7 ± 0.2 s in the presence of A-9-C (Figure 6d). However, the $t_{1/2}$ for recovery was not affected (6.8 ± 0.8 s for control and 6.2 ± 0.5 s in the presence of A-9-C) (Figure 6e).

Effect of reducing extracellular Cl^- on $[Ca^{2+}]_i$ in PASMCs

To examine the effect of reducing the extracellular Cl^- concentration on Ca^{2+} homeostasis, PASMCs were bathed in extracellular medium in which NaCl had been replaced with equimolar sodium glutamate. In reduced Cl^- extracellular medium, basal $[Ca^{2+}]_i$ was 80 ± 10 nM, which was not different from that observed in regular extracellular medium (78 ± 14 nM, $n=4$, $P>0.05$). Furthermore, caffeine-induced $[Ca^{2+}]_i$ transients were not affected in reduced Cl^- extracellular medium, where the peak increase in $[Ca^{2+}]_i$ of 525 ± 22 nM was not different from that observed in regular extracellular medium (573 ± 29 nM, $n=4$, $P>0.05$).

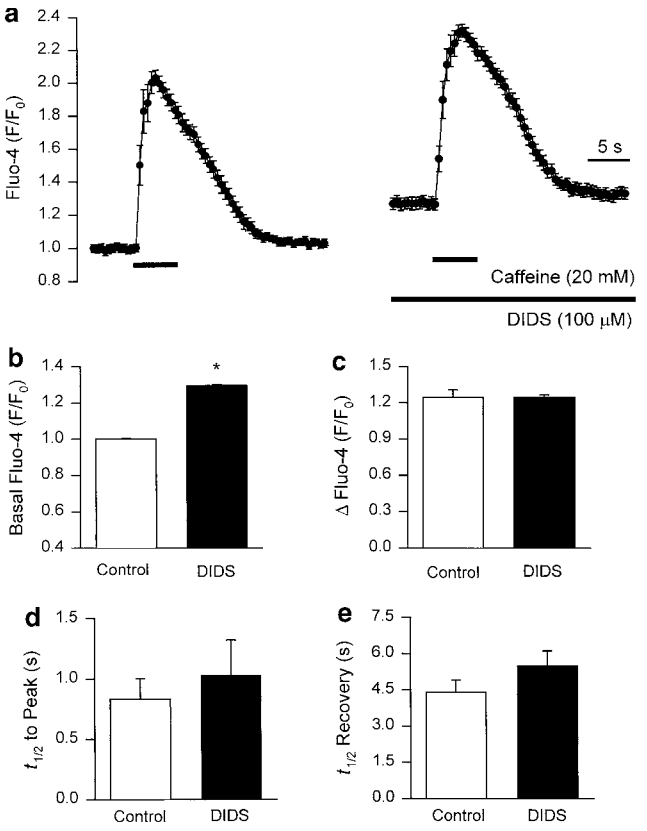

Figure 5 Effect of DIDS on caffeine-induced changes in fluo-4 fluorescence in rat PASMCs. Averaged caffeine-induced changes in fluo-4 fluorescence, recorded from six cells, before and after the addition of 100 μM DIDS (a). Fluorescence was not recorded during the preincubation period (2 min) with DIDS. Summary data showing the effect of DIDS on basal fluo-4 fluorescence (b); the change in fluorescence produced by caffeine (Δfluo-4) (c); the half-time ($t_{1/2}$) to peak for the caffeine-induced fluorescence transient (d); and the half-time ($t_{1/2}$) for Ca^{2+} removal following caffeine application (e). Data are shown as mean values $\pm$ s.e.m. ($n=6$) and $^*P<0.05$.

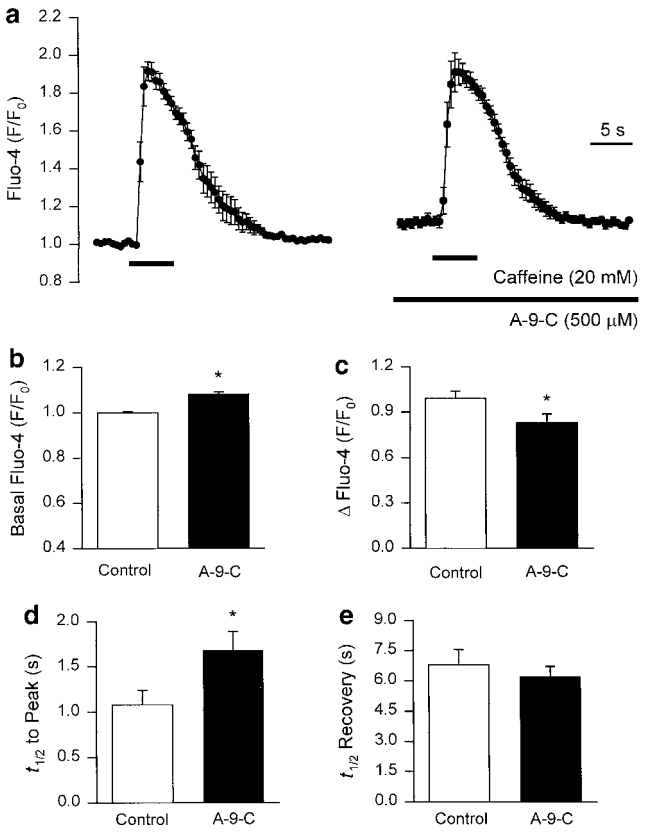

Figure 6 Effect of A-9-C on caffeine-induced changes in fluo-4 fluorescence in rat PASMCs. Averaged caffeine-induced changes in fluo-4 fluorescence, recorded from seven cells, before and after the addition of 500 μM A-9-C (a). Fluorescence was not recorded during the preincubation period (2 min) with A-9-C. Summary data showing the effect of A-9-C on basal fluo-4 fluorescence (b); the change in fluorescence produced by caffeine (Δfluo-4) (c); the half-time ($t_{1/2}$) to peak for the caffeine-induced fluorescence transient (d); and the half-time ($t_{1/2}$) for Ca^{2+} removal following caffeine application (e). Data are shown as mean values $\pm$ s.e.m. ($n=7$) and $^*P<0.05$.

NFA-induced increase in basal $[Ca^{2+}]_i$

In order to further characterize the NFA-induced increase in $[Ca^{2+}]_i$ in smooth muscle cells, the Cl^- channel blocker was applied to a single cell using a pressure ejection pipette. This enabled the kinetics of the NFA-induced increase in $[Ca^{2+}]_i$ to be determined, as well as allowing investigation of the source of Ca^{2+} being mobilized to produce the response.

When NFA (0.1–1000 μM) was applied to single smooth muscle cells, it produced a concentration-dependent increase in $[Ca^{2+}]_i$, with the effect being half maximal at approximately 65 μM (Figure 7a). Therefore, in further studies, we continued to use 50 μM NFA. Following application of NFA (50 μM), $[Ca^{2+}]_i$ increased from 51 ± 1 to 89 ± 2 nM ($n=4$, $P<0.05$), reaching a plateau 71 ± 4 s after beginning the drug application. When the application of NFA was terminated, $[Ca^{2+}]_i$ recovered to the original basal level.

To establish whether the increase in $[Ca^{2+}]_i$ was dependent upon extracellular Ca^{2+}, NFA was applied to cells bathed in Ca^{2+}-free extracellular medium. In the absence of extracellular Ca^{2+}, NFA was still capable of increasing $[Ca^{2+}]_i$ from 53 ± 3 to 99 ± 10 nM ($n=6$, $P<0.05$), reaching a plateau 58 ± 16 s after beginning the drug application (Figure 7b). This finding suggested that NFA was releasing Ca^{2+} from an intracellular store.

NFA was also found to cause an increase in $[Ca^{2+}]_i$ in mesenteric artery smooth muscle cells. Specifically, application of NFA *via* a pressure-ejection pipette increased $[Ca^{2+}]_i$ from 80 ± 3 to 126 ± 9 nM ($n=8$, $P<0.05$). When NFA was applied to cells bathed in Ca^{2+}-free extracellular medium, $[Ca^{2+}]_i$ increased from 64 ± 4 to 78 ± 5 nM ($n=6$, $P<0.05$).

SR depletion prevents the NFA-induced increase in basal $[Ca^{2+}]_i$

In order to identify the intracellular source of Ca^{2+}, PASMCs were treated with the Ca^{2+}-ATPase inhibitor CPA (10 μM), and the SR was depleted by application of caffeine. Normally, after 1 min treatment with CPA, caffeine still produced an increase in $[Ca^{2+}]_i$ although the kinetics of the caffeine-induced $[Ca^{2+}]_i$ transient differed between control and CPA-treated cells (compare Figure 1a to Figure 8). A second application of caffeine in the continued presence of CPA did not produce a $[Ca^{2+}]_i$ transient, indicating that the SR was depleted of Ca^{2+}. When 50 μM NFA was then applied to cells, no increase in $[Ca^{2+}]_i$ was observed (Figure 8). These

data suggest that the NFA-induced increase in $[Ca^{2+}]_i$ is due to Ca^{2+} release from the SR.

Further support of this notion is provided by the studies with ryanodine. Application of NFA produced reproducible increases in basal $[Ca^{2+}]_i$. Cells were then incubated with 50 μM ryanodine for 5 min and caffeine applied to activate the ryanodine receptor. Subsequent application of NFA, in the continued presence of ryanodine, did not produce any increase in basal $[Ca^{2+}]_i$. Taken together, the data from Figures 8 and 9 suggest that NFA releases Ca^{2+} from a ryanodine-sensitive intracellular store, most likely the SR.

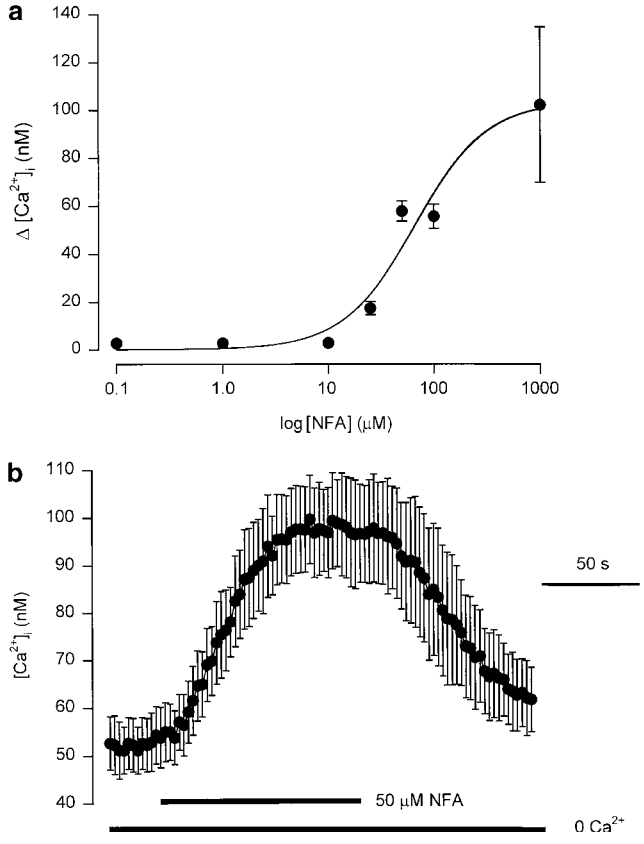

Figure 7 Effect of NFA on $[Ca^{2+}]_i$ in PASMCs. Concentration–response curve showing the magnitude of the increase in $[Ca^{2+}]_i$ when NFA was applied to single PASMCs using a pressure-ejection pipette. Data are shown as mean values ± s.e.m., and each point is representative of 5–7 different cells (a). PASMCs were incubated in Ca^{2+}-free extracellular medium (containing 1 mM EGTA) for 1 min prior to application of NFA (50 μM). NFA was applied to a single PASMC, using a pressure-ejection pipette positioned approximately 100 μm from the cell. Data are shown as mean values ± s.e.m. ($n = 5$).

Discussion

The present study has shown that the Cl^- channel blockers NFA and NPPB cause an increase in $[Ca^{2+}]_i$ in rat PASMCs. While fura-2 could not be used to measure $[Ca^{2+}]_i$ in the studies involving DIDS or A-9-C, the increase in fluo-4 fluorescence observed is nevertheless indicative of them also causing an increase in intracellular Ca^{2+}. This increase in $[Ca^{2+}]_i$ was maintained for as long as the smooth muscle cells were exposed to the Cl^- channel blocker. NFA also produced an increase in $[Ca^{2+}]_i$ in mesenteric artery smooth muscle cells, suggesting that the effect is not unique to pulmonary vascular smooth muscle cells. Increases in basal $[Ca^{2+}]_i$ have previously been observed with both NFA and flufenamic acid in visceral smooth muscle cells (Nitecki *et al*., 1994), ST_{885} cells (Poronnik *et al*., 1992), epithelial cells (Schultheiss *et al*., 2000), and neurones (Shaw *et al*., 1995; Partridge & Valenzuela, 2000).

In order to establish whether Cl^- channels were having a direct influence on basal $[Ca^{2+}]_i$, experiments were carried out under conditions where extracellular NaCl was substituted with an impermeant anion in the form of sodium glutamate. Under these conditions, basal $[Ca^{2+}]_i$ was not affected, nor was the magnitude of the caffeine-induced $[Ca^{2+}]_i$ transient; thus, it seems unlikely that the effects observed in the present study are mediated by Cl^- channels *per se*. Normally, activation of Ca^{2+}-activated Cl^- channels in smooth muscle would produce membrane depolarization, leading to the opening of voltage-dependent Ca^{2+} channels and Ca^{2+} influx. Indeed, chloride channel blockers have been shown to cause membrane hyperpolarization and dilation of pressurized cerebral arteries (Nelson *et al*., 1997). Thus, the increase in $[Ca^{2+}]_i$ produced by the Cl^- channel blockers used in the present study was rather unexpected, and a more detailed investigation of the underlying mechanism was therefore carried out for NFA.

When NFA was applied to either pulmonary or mesenteric artery smooth muscle cells using a pressure ejection pipette, the increase in $[Ca^{2+}]_i$ occurred almost immediately upon drug application, and returned to basal levels after drug application was terminated. The increase in $[Ca^{2+}]_i$ induced by NFA was still observed when cells were bathed in Ca^{2+}-free extracellular medium, suggesting that NFA is capable of releasing Ca^{2+} from an intracellular store. While the magnitude of the NFA-induced increase in $[Ca^{2+}]_i$ in mesenteric artery smooth muscle

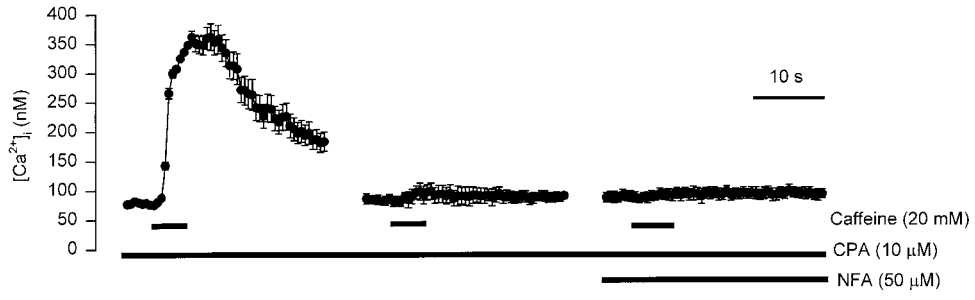

Figure 8 Effect of depleting the SR Ca^{2+} stores on NFA-induced increases in $[Ca^{2+}]_i$. Cells were incubated with the SR Ca^{2+} ATPase inhibitor CPA (10 μM) for 1 min prior to repeated caffeine (20 mM) application. Thereafter, NFA (50 μM) was applied. Data are shown as mean values ± s.e.m. ($n = 5$).

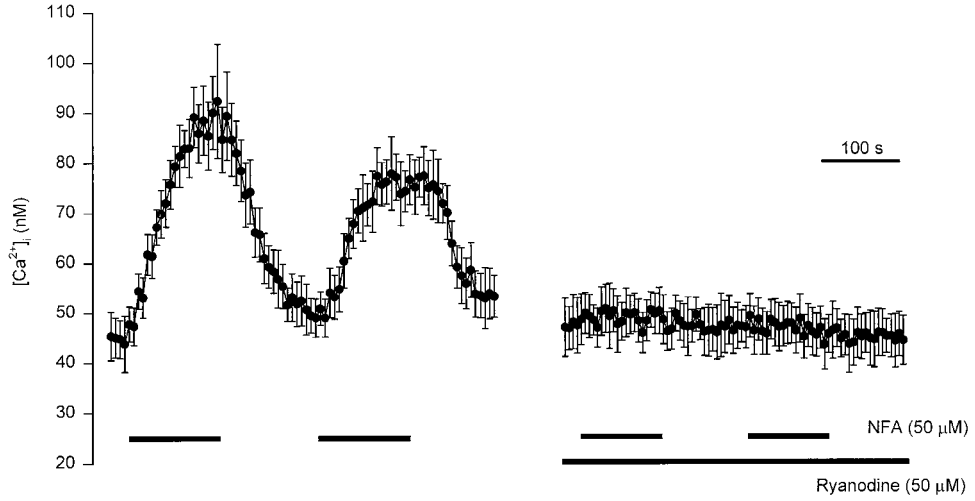

Figure 9 Effect of ryanodine on the NFA-induced increase in $[Ca^{2+}]_i$. Repeated application of NFA (50 μM) from a pressure-ejection pipette increased $[Ca^{2+}]_i$. Cells were then incubated with ryanodine (50 μM) and caffeine (20mM) was applied to activate the ryanodine receptor (*not shown*). Thereafter, NFA was applied. Data are shown as mean values $\pm$ s.e.m. ($n=3$).

cells was slightly reduced in Ca^{2+}-free extracellular medium, this may be due to the Ca^{2+} stores being depleted more rapidly in this preparation. After depleting the SR of Ca^{2+}, by repeatedly applying caffeine in the presence of the SR pump inhibitor CPA, the increase in $[Ca^{2+}]_i$ induced by NFA was completely abolished in PASMCs. This suggests that the NFA-induced increase in $[Ca^{2+}]_i$ is most likely due to Ca^{2+} release from the SR.

Further support for the involvement of the SR as the source of Ca^{2+} was provided by studies with ryanodine. When PASMCs were treated with ryanodine, at a concentration that completely abolished the caffeine-induced Ca^{2+} transient, the NFA-induced increase in $[Ca^{2+}]_i$ was also prevented. Additional, indirect support for an effect of Cl^- channel blockers on Ca^{2+} stores in smooth muscle is provided by studies where spontaneous transient outward currents (STOCs) have been studied. STOCs are due to activation of Ca^{2+}-activated potassium channels, that open in response to transient increases in $[Ca^{2+}]_i$ following spontaneous release from intracellular stores (Nelson *et al*., 1995). In portal vein smooth muscle cells, niflumic, flufenamic, and mefenamic acids reduced the frequency and amplitude of STOCs (Greenwood & Large, 1995), while NPPB completely abolished STOCs (Kirkup *et al*., 1996). Both groups suggested that a possible explanation for the observed effect was that the Cl^- channel blockers were modifying intracellular release from, or sequestration into, intracellular stores.

In addition to increasing basal $[Ca^{2+}]_i$ in PASMCs, NFA and NPPB also reduced the magnitude of the caffeine-induced $[Ca^{2+}]_i$ transient. One explanation for the effects of NFA and NPPB on the caffeine-induced $[Ca^{2+}]_i$ transient in PASMCs is that these Cl^- channel blockers are actually releasing Ca^{2+} from the SR, thereby reducing the amount of Ca^{2+} that can subsequently be released by caffeine. A similar effect has been observed with NPPB on vasopressin-induced $[Ca^{2+}]_i$ transients in A7r5 cells (Pon *et al*., 1993). However, in contrast to these studies, NFA (50 μM) has been shown to have no effect on the endothelin-1-induced Ca^{2+} oscillations in freshly isolated rat PASMCs (Hyvelin *et al*., 1998), or on the 5-HT-induced $[Ca^{2+}]_i$ transients in cultured rat PASMCs (Yuan, 1997).

A number of studies have shown that Cl^- channel blockers are capable of activating the ryanodine receptor from skeletal (Kawasaki & Kasai, 1989; Oba, 1997) and cardiac muscle (Zahradníková & Zahradník, 1993; Hill & Sitsapesan, 2002). Specifically, in bilayer studies, DIDS and 4-acetoamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) have been shown to activate the skeletal muscle ryanodine receptor, and lock it in an open state without decreasing the single-channel conductance (Kawasaki & Kasai, 1989). NFA was found to have a dual effect on the skeletal muscle ryanodine receptor, increasing the open probability of the channel at 10 μM and blocking the channel at 100 μM (Oba *et al*., 1996). A recent study on cardiac ryanodine receptors has shown that DIDS increased single-channel conductance and open probability, and altered the voltage dependence of channel gating (Hill & Sitsapesan, 2002). Such an effect of Cl^- channel blockers on the ryanodine receptor *in situ* could result in the release of Ca^{2+} from the SR, thereby reducing the magnitude of the caffeine-induced $[Ca^{2+}]_i$ transient in PASMCs.

Although DIDS was found to increase basal fluo-4 fluorescence in the present study, it had no effect on either the magnitude or kinetics of the caffeine-induced fluo-4 fluorescence transient. Given that DIDS is known to have a direct effect on the ryanodine receptor, as alluded to above, this result may be considered somewhat surprising. However, it is known that DIDS is membrane impermeant (Cabantchik & Rothstein, 1974; Brayden *et al*., 1993), and, unless there is a specific transport process for getting DIDS inside the cell, it is unlikely to have any effect on the ryanodine receptor in an intact cell. DIDS is known to be capable of inhibiting the plasma membrane Ca^{2+} pump (Niggli *et al*., 1982), so this could explain the increase in basal fluo-4 fluorescence observed; however, this would need to be confirmed in future studies. While A-9-C also increased basal fluo-4 fluorescence, its effect was markedly reduced compared to that of DIDS. Unlike the other Cl^- channel blockers used in the present study, the ability of A-9-C to activate the ryanodine receptor does not appear to have been investigated, and, although A-9-C is known to be membrane permeable, the mechanism responsible for the increase in basal fluo-4 fluorescence is

unclear at the present time. A-9-C also reduced the magnitude of the change in fluo-4 fluorescence in response to caffeine, as well as increasing the $t_{1/2}$ to peak. The slowed release of Ca^{2+} from the SR in the presence of A-9-C could account for the reduced magnitude of the fluo-4 fluorescence transient, as Ca^{2+}-removal processes would be more effective at removing Ca^{2+} from the cytosol.

As with A-9-C, NFA and NPPB were also found to increase the $t_{1/2}$ to peak for SR Ca^{2+} release in PASMCs, following stimulation with caffeine. Recent hypotheses have suggested that Cl^- flux across the SR membrane plays an important role in counteracting any charge build-up that occurs during Ca^{2+} release from the SR (Pollock *et al*., 1998), that would otherwise inhibit SR Ca^{2+} release (Kargacin *et al*., 2001). Janssen (2002) has hypothesized that Cl^- channels in the plasma membrane also increase Ca^{2+} release from the SR by maintaining a high SR to cytosol Cl^- gradient, promoting Cl^- and hence Ca^{2+} release from the SR. Inhibiting Cl^- channels, with for example NFA, could prevent Cl^- efflux such that Ca^{2+} release from the SR is reduced. Thus, the increased time to peak could be due to inhibition of anion channels in both the SR and plasma membrane, resulting in impaired release of Ca^{2+} from the SR.

It has also been shown that certain Cl^- channel blockers, including NPPB and indanyloxyacetic acid 94, inhibit SR Ca^{2+} uptake in permeabilized gastric smooth muscle cells, while they had no effect on SR Ca^{2+} uptake in permeabilized cardiac cells (Pollock *et al*., 1998). It is therefore possible that the slowing of intracellular Ca^{2+} removal following caffeine stimulation in the present study, may in part be due to SR Ca^{2+} uptake being inhibited. However, as indicated above, activation of the SR ryanodine receptor would also impair the ability of the SR to sequester Ca^{2+}, thereby having the apparent effect of slowing the rate of Ca^{2+} removal. This notion is supported by the observation that ryanodine had a marked effect on Ca^{2+} removal (Figure 1b), most likely because the SR was 'leaky' and therefore unable to function as a Ca^{2+} store.

In conclusion, we have presented evidence that four different Cl^- channel blockers have marked effects on $[Ca^{2+}]_i$ signalling in PASMC. A consistent finding was that all the four Cl^- channel blockers increased basal $[Ca^{2+}]_i$, while a more detailed examination of the NFA-induced increase in $[Ca^{2+}]_i$ indicated that this was due to release of Ca^{2+} from a ryanodine-sensitive store. This ability of NFA to release Ca^{2+} from the SR could explain why this Cl^- channel blocker has been found to attenuate the magnitude of agonist or caffeine-induced contractions (e.g. Gokina & Bevan, 2000), and also inhibit STOCs, which are due to the activation of K^+ channels *via* SR Ca^{2+} release (Greenwood & Large, 1995). Clearly, direct monitoring of SR Ca^{2+} in intact cells would enable a greater understanding of the effect of Cl^- channel blockers on SR Ca^{2+} release and uptake.

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