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RESEARCH PAPER

Mechanisms of U46619- and 5-HT-induced contraction of bovine pulmonary arteries: role of chloride ions

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Background and purpose: Thromboxane A_2 and 5-hydroxytryptamine (5-HT) are implicated in pulmonary hypertension. The involvement of chloride, voltage-operated calcium channels (VOCCs), store-operated calcium channels (SOCCs) and the Rho kinase in the contractile response of bovine pulmonary arteries (BPA) to the thromboxane A_2 mimetic U46619 and 5-HT was investigated.
Experimental approach: Endothelium-intact ring segments of BPA were mounted in Krebs/Henseleit buffer (37°C) under a tension of 2g and gassed with 95%O_2/5%CO_2.
Key results: Depletion or removal of extracellular chloride, inhibition of chloride and SOCC, Na:K:2Cl, Cl/HCO_3, Rho kinase inhibited contractions to U46619. Combining Rho kinase inhibition and chloride channel blockade (with NPPB) almost abolished the contractions to U46619. In contrast 5-HT-induced contraction was inhibited by verapamil and mibefradil. Depletion of stored calcium with caffeine almost abolished the response to U46619 but not 5-HT. The contraction by the sarco(endo)plasmic reticulum Ca^{2+}-ATPase inhibitor CPA was abolished by SOCC and chloride channel blockade (with NPPB) and by chloride depletion.
Conclusions and implications: This study suggests that the contractile response of BPA to U46619 involves Rho kinase together with a chloride-sensitive mechanism, which does not involve VOCC but may have a role in calcium release and calcium entry via SOCC. In contrast contraction of the BPA by 5-HT appears to involve verapamil- and mibefradil-sensitive VOCC. This study may indicate that the use of calcium channel blockers in the management of pulmonary hypertension may not always be effective and that Rho kinase and chloride channels may be targets for the development of new therapies.
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Keywords: Thromboxane A_2; U46619; 5-hydroxytryptamine; bovine pulmonary arteries; chloride; store-operated calcium channels; sarcoplasmic reticulum; Rho-kinase; voltage-operated calcium channels

Abbreviations: 9-AC, 9-anthracene carboxylic acid; 2-APB, 2-amino ethoxy diphenylborate; CPA, cyclopiazonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; NFA, niflumic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid; SKF96365, 1-[B-[3-(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl]-1*H*-imidazole; Y-27632, (*R*)-(+)*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide

Introduction

5-Hydroxytryptamine and thromboxane A_2 are pulmonary vasoconstrictors that are implicated in the development of pulmonary hypertension (Christman *et al.*, 1992; Watts, 2005). Agonist-induced vasoconstriction may involve an elevation of cytosolic free calcium arising from the release of calcium from storage organelles, mainly the sarcoplasmic reticulum (SR) (Laporte *et al.*, 2004), calcium entry through voltage-operated calcium channels (VOCCs), receptor-operated calcium channels (ROCCs) (McFadzean and Gibson, 2002) and store-operated calcium channels (SOCCs) also known as capacitative calcium entry channels and store-depletion-activated channels (Berridge, 1995). An increase in the conductance of SOCCs is associated with depletion of the calcium content of sarco(endo)plasmic reticulum (Parekh and Penner, 1997; Lewis, 1999), and although calcium entry through SOCCs is considered to be important in replenishing the calcium content of storage organelles following agonist-stimulated release, there is accumulating evidence indicating that this source of calcium can also contribute to cellular responses (reviewed in Parekh and Penner, 1997; Putney, 2001) including pulmonary artery vasoconstriction (McDaniel *et al.*, 2001; Ng and Gurney, 2001; Snetkov *et al.*,

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2003). In addition to increasing cytosolic-free calcium, some receptors that couple to the Rho A/Rho kinase pathway also increase the sensitivity of the contractile machinery to the available calcium. Rho kinases stimulate myosin activity through phosphorylation of the regulatory light chain and by inhibiting myosin light chain phosphatase (MLCP) (Fukata *et al*., 2001; Somlyo and Somlyo, 2003).

Although its role remains to be clearly defined there is increasing evidence that chloride is also important in smooth muscle contraction. In most smooth muscle $[Cl^-]_i$ is maintained above equilibrium by mechanisms that include the Na:K:2Cl co-transporter (Owen, 1984), the Cl/HCO_3 exchanger (Aickin and Brading, 1984), or 'pump III' (Chipperfield *et al*., 1993). Consequently, the chloride equilibrium potential (E_{Cl}) is 20–40 mV more positive than the resting membrane potential (Large and Wang, 1996; Chipperfield and Harper, 2000). It has been suggested therefore that agonists that increase the chloride conductance of the plasma membrane produce an inward depolarizing current capable of increasing the open state of VOCCs (Nelson, 1995; Criddle *et al*., 1996; Wang *et al*., 1997; Yuan, 1997) to increase cytosolic-free calcium and promote muscle contraction. Concomitant stimulation of chloride accumulating mechanisms maintain or potentiate $[Cl^-]_i$, E_{Cl} and a sustained depolarization (Chipperfield and Harper, 2000).

As well as a possible role in regulating calcium entry through VOCCs, chloride may also have an important role in calcium handling by the SR. SR membrane is highly permeable to chloride (Kasai and Kometani, 1979) and the presence of several chloride channels has been established by both electrophysiological (Kourie *et al*., 1996; Clark *et al*., 1997) and molecular (Jentsch *et al*., 2001; Nilius and Droogmans, 2003) techniques. Studies using reconstituted SR membrane (Al-Awqati, 1995; Kourie, 1997) and permeabilized smooth muscle cells (Pollock *et al*., 1998) have established that calcium accumulation and release from skeletal and smooth muscle is chloride dependent. The mechanism by which chloride influences calcium uptake and release by the SR remains to be clearly established.

The present study examined the involvement of chloride in the contractile response to the thromboxane A_2 mimetic U46619 and 5-HT in bovine pulmonary arteries The possible roles of the Rho A/Rho kinase pathway, VOCC and SOCC were also explored. A better understanding of the transduction pathways for agonists that increase pulmonary vascular resistance may provide new pharmacological targets for the management of pulmonary hypertension.

Methods

Tissue preparation

Bovine lungs were obtained from a local abattoir within 30 min of slaughter. Ring segments of conventional artery (3–4 mm in length, 3–5 mm diameter) were dissected from third- and fourth-generation branches.

Organ bath studies

Endothelium-intact artery rings were suspended between two stainless-steel wire hooks in 10 ml Linton vessel chambers containing Krebs/Henseleit physiological salt solution (PSS) of the following composition (mM): NaCl (119), KCl (4.7), $NaHCO_3$ (24.8), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $CaCl_2$ (2.5), glucose (11.1). Tissues were maintained at 37°C under a tension of 2 g, and gassed with a mixture of 95% O_2/5% CO_2. Changes in isometric tension were measured by force–displacement transducer (Grass Instruments, FT03). In experiments using lanthanum (La^{3+}), the CO_2-gassed HCO_3 buffering was replaced with air-gassed 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffering, to prevent precipitation of La^{3+}; this solution consisted of (mM): NaCl (119), KCl (4.7) MgCl (1.0), KH_2PO_4 (0.5), NaH_2PO_4 (0.5), $CaCl_2$ (2.5), glucose (11.1), HEPES (10), pH adjusted to 7.4 with NaOH.

Experimental protocols

The tissues were allowed to equilibrate for 60 min before each experiment. Rings were initially contracted with KCl (60 mM). Agonists were added to the organ baths cumulatively in 0.5 log units to construct cumulative log concentration response curves. Using this approach both 5-HT- and U46619-induced monophasic contractions and each subsequent agonist addition was made, once a sustained plateau was attained. In most experiments, agonist responses are expressed as a percentage of the initial KCl response.

Chloride channel blockers and chloride transport inhibitors

The involvement of chloride channels in the contractile response to 5-HT and U46619 was investigated using the chloride channel blockers 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB, 50 μM), 9-anthracenecarboxylic acid (9-AC, 500 μM) and niflumic acid (30 μM) (Jentish *et al*., 2001; Large and Wang 1996). The involvement of chloride transport was examined using the Na:K:2Cl co-transport inhibitor bumetanide (100 μM), 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS, 100 μM (U46619) and 500 μM (5-HT), Aickin and Brading, 1984) an inhibitor of the Cl/HCO_3 exchanger and acetazolamide (1 mM), an inhibitor of the anion transporter pump III. All inhibitors were pre-incubated for 40 min before the addition of agonists. Control tissues were preincubated with the appropriate drug vehicle. All experiments involving bumetanide were conducted in organ baths wrapped in foil and overhead lights off.

Experiments investigating the effects of chloride depletion and chloride-free Krebs on the contractile response to U46619 and 5-HT

In these experiments, the chloride content of normal physiological salt solution (PSS) (129 mM) was modified to obtain either a chloride-free solution by replacing NaCl, KCl and $CaCl_2$ in an equimolar fashion with their corresponding gluconate salt or, in chloride depletion experiments, only NaCl was replaced with gluconate to produce a final $[Cl^-]_o$ ~10 mM. In these experiments, contractile responses were recorded in g/100 mg tissue wet weight ($[Cl^-]_o = 129$ mM).

Experiments investigating the effect of VOCC and SOCC blockers on the contractile response to U46619 and 5-HT

The involvement of VOCCs in the contractile response to 5-HT and U46619 was examined using the VOCC blockers nifedipine (1 μM), verapamil (10 μM), mibefradil (10 μM) (Alexander *et al*., 2004). The involvement of SOCCs in the contraction to U46619 and 5-HT was examined using the putative SOCC blockers 2APB (50 and 100 μM) and SKF96365 (100 μM) (Putney, 2001) preincubated for 40 min. The effect of these agents was also examined on the contraction induced by the reversible sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor cyclopiazonic acid (CPA, 10 μM) (Seidler *et al*., 1989). All experiments involving nifedipine were conducted in organ baths wrapped in foil and overhead lights off.

Experiments examining the involvement of Rho kinase in the contractile response to U46619 and 5-HT

In these studies, tissues were preincubated with the Rho kinase inhibitor Y-27632 (30 μM) (Ishizaki *et al*., 2000) or its vehicle in control tissue for 45 min before the addition of agonists. In some experiments, Rho kinase inhibition was combined with 2-APB to block SOCC or NPPB to block chloride channels.

Data analysis

All data was collected using chart for Windows (AD Instruments). Maximum contractile responses to agonists were calculated as a percentage of the contraction produced by KCl (60 mM) and were expressed as the means $\pm$ s.e.m. From experiments involving CPA, chloride-free and depleted PSS and calcium-free PSS, the contraction was expressed in g 100 mg^{-1} of tissue wet weight. The mean log concentration response curves to agonists were analysed by fitting to a four-parameter logistic equation (given below) using nonlinear regression (Graph Pad Prism),

$$Y = \text{bottom} + (\text{top} - \text{bottom})/1 + 10^{(\text{Log EC}_{50} - X)P}$$

where X is the logarithm of the molar concentration of agonist, Y is the response and P is the Hill slope. E_{max} is the maximum contraction and log EC_{50} is the agonist concentration that produces 50% of the maximum response. Comparison between mean sensitivity (pEC_{50}) or maximum contraction (R_{max}) was carried out using Student's *t*-test and $P<0.05$ is considered significant.

Chemicals

U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$) was purchased from Biomol Research Laboratories Inc. 5-Hydroxytryptamine creatinine sulphate (5-HT), nifedipine, verapamil hydrochloride, mibefradil dihydrochloride, CPA, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid disodium salt hydrate (DIDS), bumetanide and niflumic acid (NFA) were purchased from Sigma-RBI. 5-Nitro-2-(3-phenylpropyl-amino)benzoic acid (NPPB), 9-anthracenecarboxylic acid (9-AC), (*R*)-(+)*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride (Y-27632), 1-[B-[3-(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl]-1*H*-imidazole hydrochloride (SKF96365) and 2-amino ethoxy diphenylborate (2APB) were purchased from Tocris Bioscience.

Niflumic acid, NPPB and CPA were dissolved in dimethyl sulphoxide. U46619, 9-AC, bumetanide and nifedipine were dissolved in ethanol. DIDS was dissolved in 0.1 M potassium bicarbonate. All other drugs were dissolved in deionized water. In all cases, the appropriate drug vehicle was included in the control tissue.

Results

Effect of the chloride channel blockers on 5-HT- and U46619-induced contractions

NPPB (50 μM), 9-AC (500 μM) and niflumic acid (30 μM) had no effect on the concentration response curve for 5-HT (1 nM–300 μM) (Figure 1a). NPPB and 9-AC but not niflumic acid shifted the concentration–response curve for U46619 (1 nM–3 μM)-induced contraction to the right and reduced the maximum response (Figure 1b and Table 1).

The effect of chloride transport inhibitors, chloride-depleted and chloride-free PSS on the concentration–response curve to 5-HT and U46619

The chloride transport inhibitors bumetanide (100 μM, Na:K:2Cl co-transporter), DIDS (100 μM (U46619) and 500 μM (5-HT), Cl/HCO_3 exchange) and acetazolamide (1 mM, pump III) did not affect the concentration–response curve to 5-HT (Figure 1c). The concentration–response curve to U46619 was shifted to the right by DIDS (100 μM) and bumetanide but not acetazolamide (Figure 1d, Table 1).

Neither chloride depletion nor chloride-free PSS altered the baseline tone. The concentration–response curve for 5-HT was unaffected by chloride depletion ($[Cl^-]_o = 10$ mM), but was shifted to the left and the maximum response increased in chloride-free ($[Cl^-]_o = 0$) PSS compared with normal ($[Cl^-]_o = 129$ mM) PSS (Figure 2a). In contrast the concentration–response curve for U46619 was shifted to the right and the maximum response reduced in both chloride-depleted and chloride-free PSS although chloride depletion produced a slightly greater rightward shift than chloride-free PSS (Figure 2b and Table 2).

Effect of the Rho kinase inhibitor Y-27632 on the concentration–response curve to 5-HT and U46619

The Rho kinase inhibitor Y-27632 (30 μM) had no effect on the concentration–response curve for the 5-HT (Figure 3a), but shifted the concentration–response curve for U46619 to the right and reduced the maximum response (Figure 3b and Table 4).

Effect of VOCC blockade and depleting stored calcium with caffeine on the concentration–response curve to 5-HT and U46619

The concentration–response curve for 5-HT was shifted to the right and the maximum response reduced by both

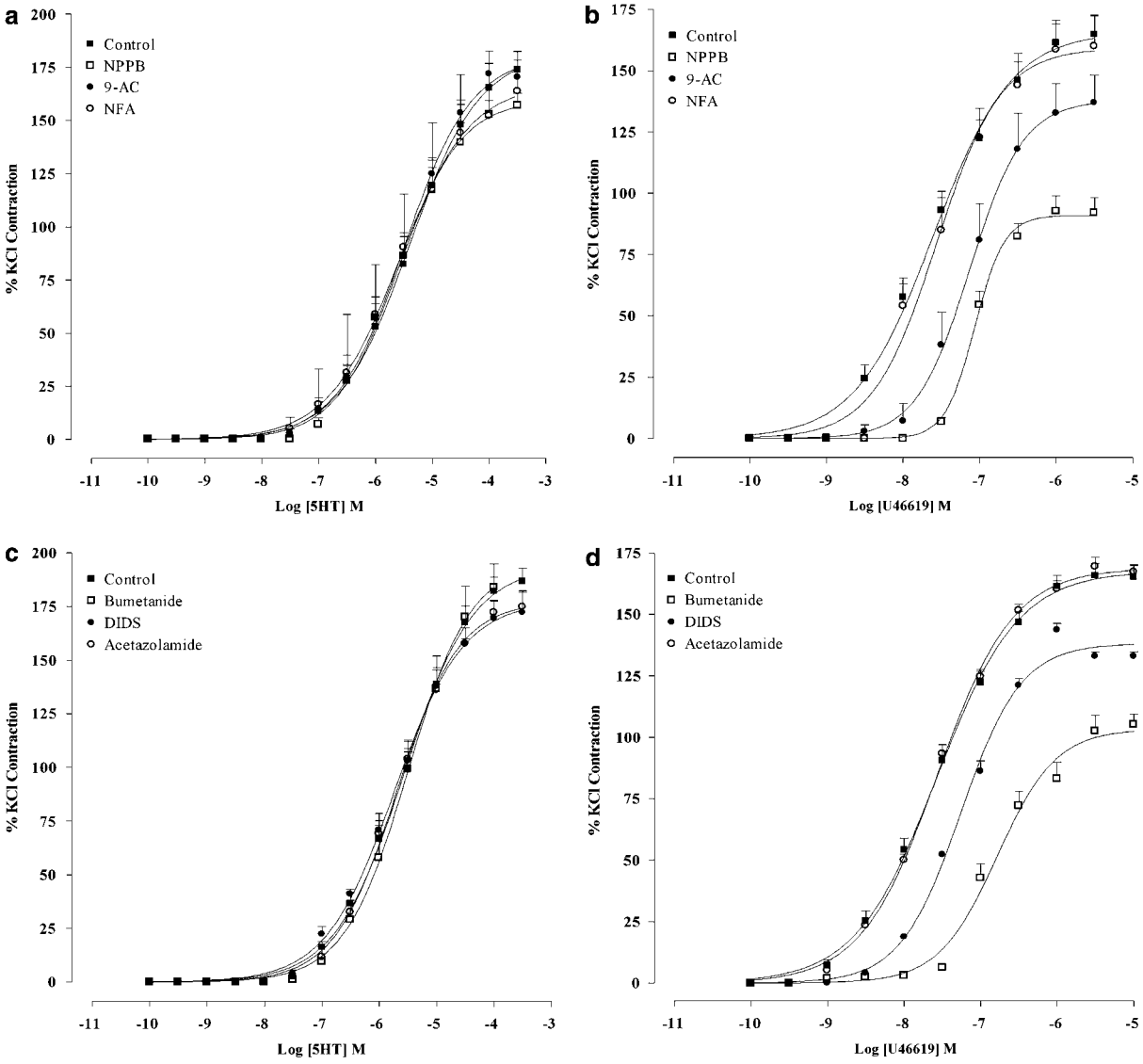

Figure 1 Effect of chloride channel blockade and chloride transport inhibition on the concentration response–curve for contraction to 5-HT and U46619. Control responses and the effect of NPPB (50 μM), 9-AC (500 μM) and niflumic acid (NFA, 30 μM) are shown in (**a**) (5-HT) and (**b**) (U46619) and bumetanide (100 μM), DIDS (500 μM, 5-HT and 100 μM, U46619) and acetazolamide (1mM) are shown in (**c**) (5-HT) and (**d**) (U46619). Results are the means$\pm$s.e.m. from 5–16 experiments.

Table 1 Effect of the chloride channel blockers and chloride transport inhibitors on the concentration response curve for U46619

U46619	*pEC$_{50}$*	$R_{max\ \%}$	n
Control	7.6$\pm$0.07	169$\pm$5	6
NPPB	6.56$\pm$0.03*	91$\pm$2*	5
9-AC	6.6$\pm$0.09*	138$\pm$8*	6
NFA	7.59$\pm$0.1	161$\pm$8	6
Bumetanide	6.84$\pm$0.13*	99$\pm$10*	5
DIDS	6.61$\pm$0.03*	169$\pm$4	5
Acetazolomide	7.12$\pm$0.09	157$\pm$9	6

Abbreviations: DIDS, 4,4′ diisothiocyanostilbene-2,2′-disulphonic acid; NFA, niflumic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid.
*$P<0.001$.

verapamil and mibefradil (both 10 μM) but not by nifedipine (1 μM). The combination of verapamil and mibefradil substantially reduced the response (Figure 4a and Table 3). The concentration response curve for U46619 was unaffected by nifedipine (1 μM), verapamil (10 μM), mibefradil (10 μM) (Figure 4b). The addition of caffeine caused a small transient contraction. Thereafter caffeine caused a rightward shift of the 5-HT concentration–response curve and reduced the maximum response (Figure 3c), (pEC_{50} and R_{max} values: 5-HT control, 5.4$\pm$0.07, 171$\pm$6%, $n=4$; caffeine, 5.1$\pm$0.04, 94$\pm$2%. $P<0.001$, $n=4$) but almost abolished contractions to U46619 (Figure 3d) pEC_{50} and R_{max} values: U46619 control, 7.8$\pm$0.1, 152$\pm$7%, $n=4$; caffeine, 6.8$\pm$0.2, 42$\pm$4%. $P<0.001$, $n=4$.

Effect of the putative SOCC blockers SKF96365 and 2-APB on the concentration response curve to U46619

SKF96365 (100 μM) and 2-APB (100 μM) shifted the concentration response curve for the U46619-induced contraction

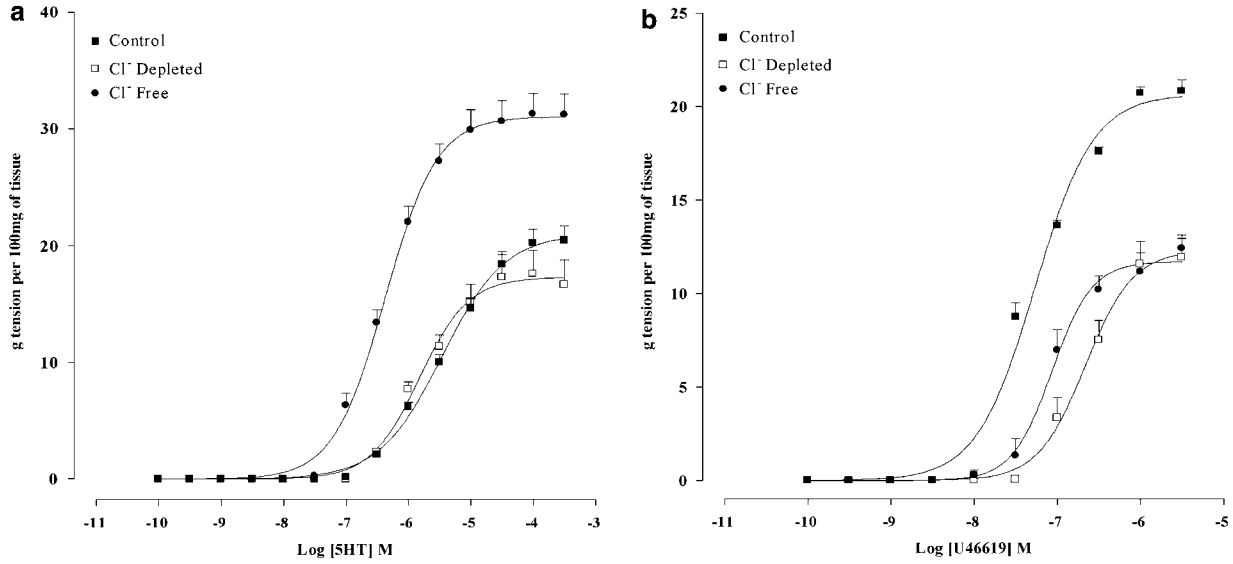

Figure 2 Effect of chloride-depleted and chloride-free PSS on the concentration–response curve for contraction to 5-HT (**a**) and U46619 (**b**). Control responses, $[Cl^-]_o = 129$ mM; Cl^--depleted, $[Cl^-]_o = 10$ mM; chloride-free. Results are the means ± s.e.m. from 5 to 6 experiments.

Table 2 Effect of changing the extracellular chloride content on the concentration–response curve for U46619

U46619		*pEC_{50}*	*$R_{max}g$ (100 mg^{-1})*	n
Control $[Cl]_o = 129$ mM	6	7.27 ± 0.04	20.67 ± 0.47	6
$[Cl]_o = 10$ mM	6	6.66 ± 0.07	12.31 ± 0.72*	6
$[Cl]_o = 0$ mM	5	7.06 ± 0.05	11.71 ± 0.45*	6

*$P < 0.001$.

to the right and reduced the maximum response (Figure 5 and Table 4). 2-APB (50 μM) had no effect on the concentration–response curve for the U46619-induced contraction (data not shown).

Effect of air-gassed HEPES buffering and La^{3+} on the concentration–response curve to U46619 and 5-HT

The concentration–response curve for 5-HT was unaffected by changing from the CO_2-gassed HCO_3 buffering to air-gassed HEPES buffering (Figure 6a). In contrast, the concentration–response curve for U46619 was shifted to the right and the maximum response reduced by air-gassed HEPES buffering and this response was unaffected by 100 μM La^{3+} (Figure 6b) pEC_{50} and R_{max} values: CO_2/HCO_3-buffered PSS, 7.42 ± 0.08, 36 ± 4; air/HEPES-buffered PSS, 7.05 ± 0.07, 20 ± 3; La^{3+}, 7.07 ± 0.07, 18 ± 2.0, $n = 5$, in each group.

Effects of combining Rho kinase inhibition with SOCC or chloride channel blockade on the concentration–response curve for U46619

In the presence of Y-27632 the SOCC blocker 2-APB (100 μM) produced a further inhibition of the maximum response, but did not alter tissue sensitivity to U46619 in the presence of Rho kinase inhibition (Figure 7a and Table 4). Also in the presence of Y-27632, NPPB substantially reduced the maximum contractile response without altering the sensitivity of the tissue to U46619 (Figure 7b and Table 4).

Effect of the chloride channel blocker NPPB and the Rho kinase inhibitor on the concentration–response curve for U46619 in nominally free Ca^{2+}

In nominally Ca^{2+}-free PSS, the concentration–response curve for U46619-induced contraction was shifted to the right and the maximum response reduced. The addition of either NPPB or Y27632 almost abolished the response to U46619 (Figure 8 and Table 5).

Effect of the SOCC blockers SKF96365 and 2-APB and chloride channel blockers, NPPB and niflumic acid, on the contraction to the SERCA inhibitor CPA

CPA (10 μM)-induced contractions (70 ± 4% KCl, $n = 7$) were abolished by 2-APB (100 μM, $n = 7$), SKF 96365 (100 μM, $n = 7$), chloride depletion and NPPB (50 μM, $n = 7$) but not niflumic acid (30 μM, $n = 8$) (Figure 9).

Discussion

The present study shows that the contractile response of bovine pulmonary arteries to the thromboxane A_2 mimetic U46619, but not 5-HT, is sensitive to the Cl^- channel blockers NPPB and 9-AC but not niflumic acid suggesting that an NPPB and 9-AC-sensitive, niflumic acid-insensitive chloride conductance plays an important role in the contraction to U46619, but not 5-HT. Although 9-AC and niflumic acid can also increase potassium conductance in smooth muscle (Greenwood and Large, 1998; Toma *et al.*, 1996) it is unlikely that this action could account for the inhibitory effect of 9-AC since its action is agonist specific.

In smooth muscle, estimates of $[Cl^-]_i$ range between 31 and 51 mM, which means that E_{Cl} −35 to −18 mV, is relatively positive in relation to the membrane potential (Chipperfield and Harper, 2000) and has the potential to depolarize the membrane sufficiently to activate VOCC and

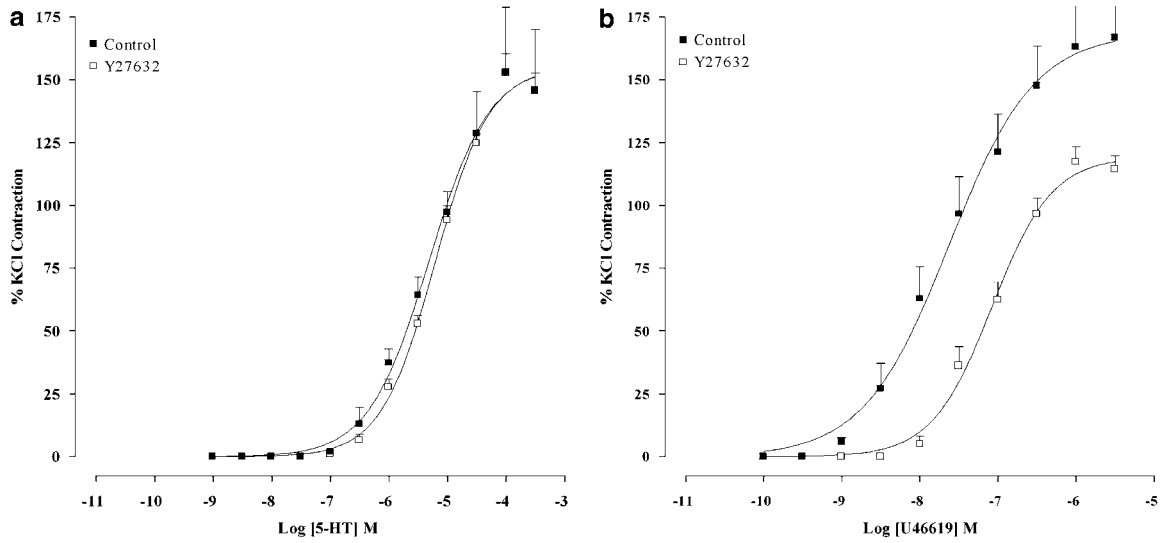

Figure 3 Effect of the Rho kinase inhibitor Y27632 on the concentration–response curve for contraction to 5-HT (**a**) and U46619 (**b**). Control responses, Y-27632, 30 μM. Results are the means$\pm$s.e.m. from 3 to 7 experiments.

induce contraction arising from influx of calcium through the VOCC. The transporters that have been reported to maintain $[Cl^-]_i$ above the Donnan equilibrium for chloride (approx. 2.4 mM) include the Na:K:2Cl co-transporter (Owen, 1984), the Cl/HCO_3 exchanger (Aickin and Brading, 1984), or the anion transporter 'pump III' (Chipperfield *et al.*, 1993). That bumetanide, an inhibitor of Na:K:2Cl co-transporter (Russell, 2000) and DIDS, an inhibitor of Cl/HCO_3 exchange (Hoffmann and Simonsen, 1989) but not acetazolomide, which is reported to block pump III (Chipperfield *et al.*, 1993), inhibited the contraction to U46619 but not 5-HT is consistent with a role for chloride in the contraction to U46619 and this may indicate that in bovine pulmonary arteries chloride is accumulated by Na:K:2Cl co-transporter and possibly also by Cl/HCO_3 exchange. However, since DIDS also blocks Cl^- channels (Kourie, 1997; Greenwood and Large, 1998) its inhibitory effect may arise from reduced conductance and/or inhibition of Cl/HCO_3 exchange.

Criddle *et al.* (1996) proposed that calcium-dependent chloride channels (Cl_{Ca}) in the plasma membrane are activated by an agonist-induced release of calcium from intracellular stores. In this scheme, contractile responses should display a similar sensitivity to Cl_{Ca} blockade and VOCC blockade and this was demonstrated in the rat aorta for noradrenaline and the rat perfused mesenteric bed for both noradrenaline and 5-HT (Criddle *et al.*, 1996). Reducing the extracellular chloride concentration (chloride depletion) has also been used to demonstrate this role for chloride since this manoeuvre would increase E_{Cl} and therefore the magnitude of the depolarization and subsequent contraction (Lamb and Barna, 1998). This has been demonstrated in rat aorta where the contractile response to noradrenaline and 5-HT is enhanced when $[Cl^-]_o$ is reduced to 10 mM (Lamb and Barna, 1998).

In the BPA, the lack of sensitivity of the U46619-induced contraction to VOCC blockade indicates that the scheme outlined above does not appear to operate in the BPA for the thromboxane (TP) receptor. This view is also supported by the finding that chloride depletion, which had no effect on the concentration–response curve to 5-HT produced an inhibition rather than potentiation of U46619-induced contraction similar to those reported for some other vascular tissues (Gould and Hill, 1996; Jensen *et al.*, 1997). Moreover, removal of $[Cl^-]_o$, which would be expected to abolish E_{Cl}, also reduced the maximum contraction similar to chloride depletion. Why it produced less of a rightward shift than chloride depletion is unclear. These findings suggest that the contractile response mediated through activation of the TP receptor does not involve the scheme outlined in Criddle *et al.* (1996). However, it is noteworthy that vasomotion in this tissue was sensitive to the chloride channel blockers used in this study including niflumic acid and nifedipine (unpublished observations).

Although the contractile response to 5-HT was unaffected by chloride depletion it was substantially increased when chloride was removed. The reason for the enhanced contraction is unclear.

In contrast to U46619, the contractile response to 5-HT was sensitive to the VOCC blockers, verapamil and mibefradil but not nifedipine. Verapamil inhibits the L-type channels $Ca_v1.1$, 1.2 and 1.3 (Alexander *et al.*, 2004). Since nifedipine, which is selective for the high-voltage activated L-type channels Ca_v 1.1 and 1.2 (Alexander *et al.*, 2004), did not affect the response to 5-HT this suggests that the response mediated by 5-HT involves the activation of the low-medium voltage-activated L-type channel Ca_v 1.3. Mibefradil has a high specificity for each of the T-type channels (Ca_v 3.1–3.3), which are low-voltage activated channels (Catterall, 1995). Since the inhibition produced by the combination of verapamil and mibefradil was additive and substantially reduced the contractile response to 5-HT this suggests that their actions are on distinct channels and therefore that 5-HT activates both an L- and T-type channel to evoke contraction in this vessel. In this tissue, the contractile response to 60 mM KCl can be completely

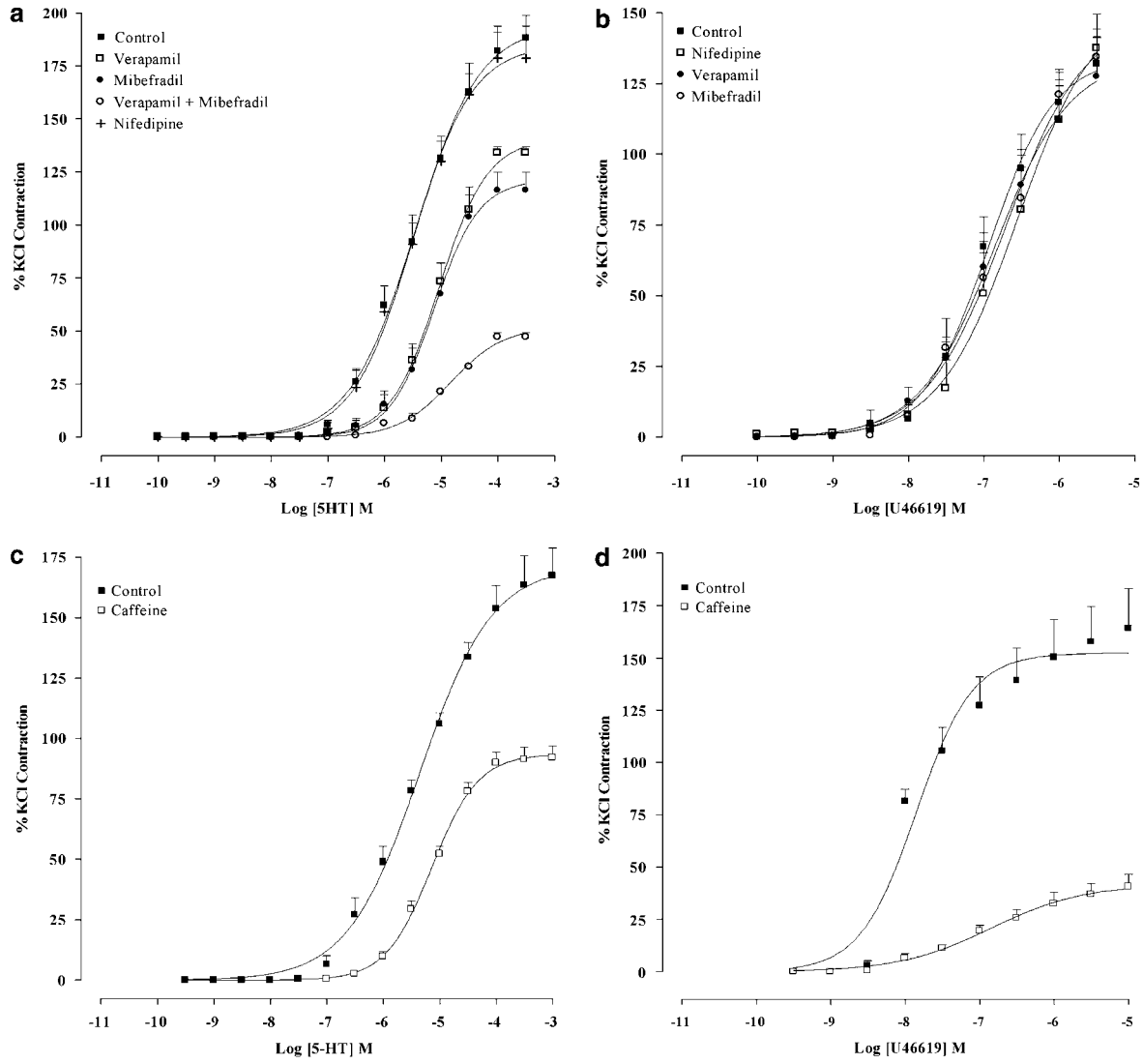

Figure 4 Effect of voltage-operated calcium channels blockade and depletion of stored calcium with caffeine on the concentration–response curve for contractions to 5-HT and U46619. Control responses and the effect of nifedipine (1 μM); verapamil (10 μM); mibefradil (10 μM); are shown in (**a**) (5-HT) and (**b**) (U46619). In addition the combined effect verapamil + mibefradil on 5-HT is shown in (**a**). The effect of caffeine (1 mM) is shown in (**c**) (5-HT) and (**d**) (U46619). Results are the means ± s.e.m. from 5 to 6 experiments.

Table 3 Effect of VOCC blockers on the concentration–response curve for 5-HT

5-HT	pEC_{50}	R_{max}*(%, KCl)*	n
VOCC control	5.47 ± 0.07	195 ± 7	7
Nifedipine	5.51 ± 0.09	186 ± 9	4
Verapamil	*5.04 ± 0.05	141 ± 5	5
Mibifradil	*5.11 ± 0.09	121 ± 7	6
Verapamil + Mibifradil	*4.83 ± 0.05	52 ± 12	5

Abbreviation: VOCC, voltage-operated calcium channels.
*$P < 0.001$.

abolished by nifedipine (1 μM) (unpublished observation) which clearly demonstrates the presence of a high-voltage activated L-type channel Ca_v 1.1 and/or 1.2. Since this channel is not activated by 5-HT this might imply that 5-HT induces a membrane depolarization, which is sufficient to activate Ca_v 1.3 as well as Ca_v 3.1 and/or 3.3 but is not great enough to activate Ca_v 1.1/1.2. The lack of effect of chloride channel blockers and chloride depletion would indicate that 5-HT does not produce depolarization by increasing chloride conductance.

The observation that the Rho kinase inhibitor Y-27632 also inhibited the U46619-induced contraction but not 5-HT, is consistent with the accepted view that the TP receptor couples, in part, to the Rho A/Rho kinase pathway (Somlyo and Somlyo, 2003). Activation of this pathway is associated with an increase in the sensitivity of the contractile machinery to the available calcium and is achieved through phosphorylation of the regulatory light chain and by inhibiting MLCP (Fukata *et al.*, 2001; Somlyo and Somlyo, 2003). It seems unlikely that chloride is involved in the transduction pathway between the TP receptor and Rho kinase since chloride channel blockade (with NPPB) and Rho kinase inhibition together produced an additional inhibition compared with Rho kinase inhibition alone.

In this study, caffeine had a much greater inhibitory effect on the contraction to U46619 than 5-HT. Since one of the actions of caffeine is to deplete the SR calcium store (Laporte *et al*., 2004), which is consistent with the transient contraction observed on the addition of caffeine, this observation may indicate that TP receptor activation is in part coupled to release of intracellular calcium. The much smaller inhibitory effect of caffeine on the 5-HT response is consistent with the observation that this response is mainly sensitive to verapamil and mibefradil, suggesting that calcium influx via VOCC underlies the contractile response and that 5-HT does not utilize intracellular calcium to the same extent as TP receptor activation. It is also possible that part of the action of caffeine is due to its ability to inhibit phosphodiesterase activity.

As depletion of the SR calcium content is associated with activation of SOCCs (Berridge, 1995; Parekh and Penner, 1997; Putney, 2001) the observation that the putative SOCC blockers 2-APB and SKF96365 inhibited U46619-, but not 5-HT-induced tone, may be consistent with a transduction mechanism for the TP receptor that involves depletion of stored calcium and calcium entry via SOCCs, which contributes to the contraction.

While SKF96365 can also inhibit non-SOC channels (McFadzean and Gibson, 2002) it is possible that the inhibitory effect of 2-APB and SKF 96365 may be due to this action. La^{3+}, which has been reported to inhibit SOCC in pulmonary smooth muscle and may distinguish between

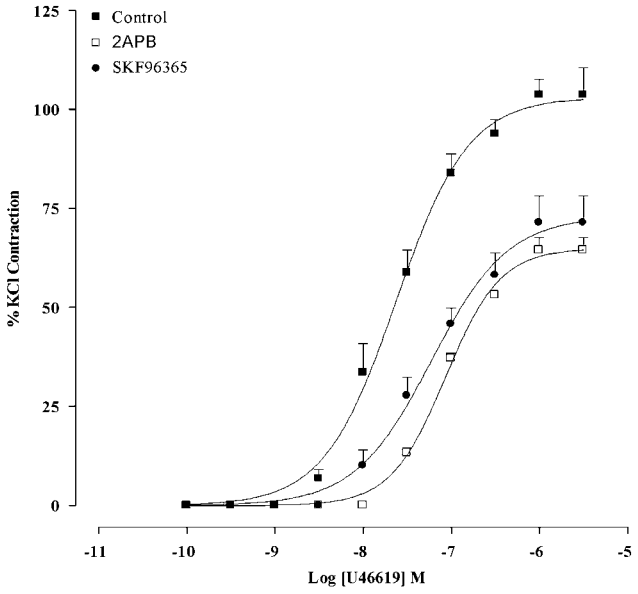

Figure 5 The effect of SOCC blockade on the concentration response curve for contraction to U46619. Control response and the effect of 2-APB (100 μM) and SKF96365 (100 μM). Results are the means$\pm$s.e.m. from 4 to 5 experiments.

Table 4 Effect of the Rho kinase inhibitor Y27632, the SOCC blockers 2-APB and SKF 96365 and the chloride channel blocker NPPB alone and the combination of Rho kinase inhibition with 2-APB or NPPB on the concentration response curve for U46619

U46619	pEC_{50}	R_{max} (%)	n
Control	7.67$\pm$0.16	172$\pm$13	5
Y27632	7.09$\pm$0.06*	120$\pm$5*	5
SKF96365	7.34$\pm$0.12*	73$\pm$6*	4
2-APB	7.07$\pm$0.03*	63$\pm$2*	5
Y27632 + 2-APB	7.05$\pm$0.07*	73$\pm$3*	5
NPPB	6.62$\pm$0.07*	96$\pm$6*	4
NPPB + Y27632	6.46$\pm$0.03*	41$\pm$1*	4

Abbreviations: 2-APB, 2-amino ethoxy diphenylborate; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; SKF96365, 1-[B-[3-(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl]-1*H*-imidazole; SOCC, store-operated calcium channels.
*$P<0.001$.

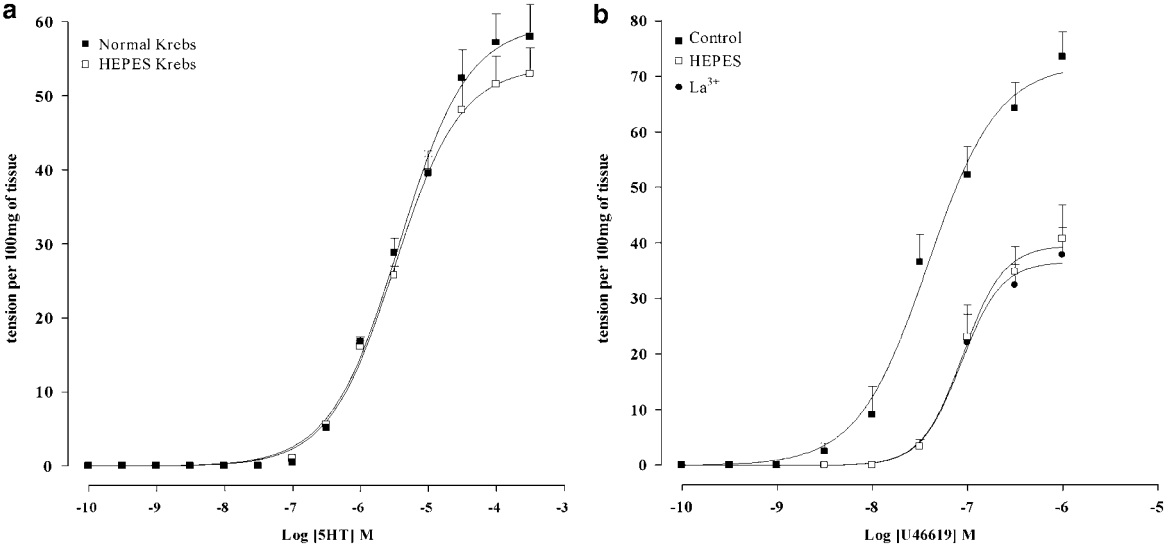

Figure 6 Comparison between the contractile responses of bovine pulmonary arteries to U46619 and 5-HT in CO_2-gassed HCO_3-buffered PSS and air-gassed 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered PSS and the effect of La^{3+} on the response to U46619 in air-gassed HEPES PSS. Results are the means$\pm$s.e.m. from 4 to 5 experiments.

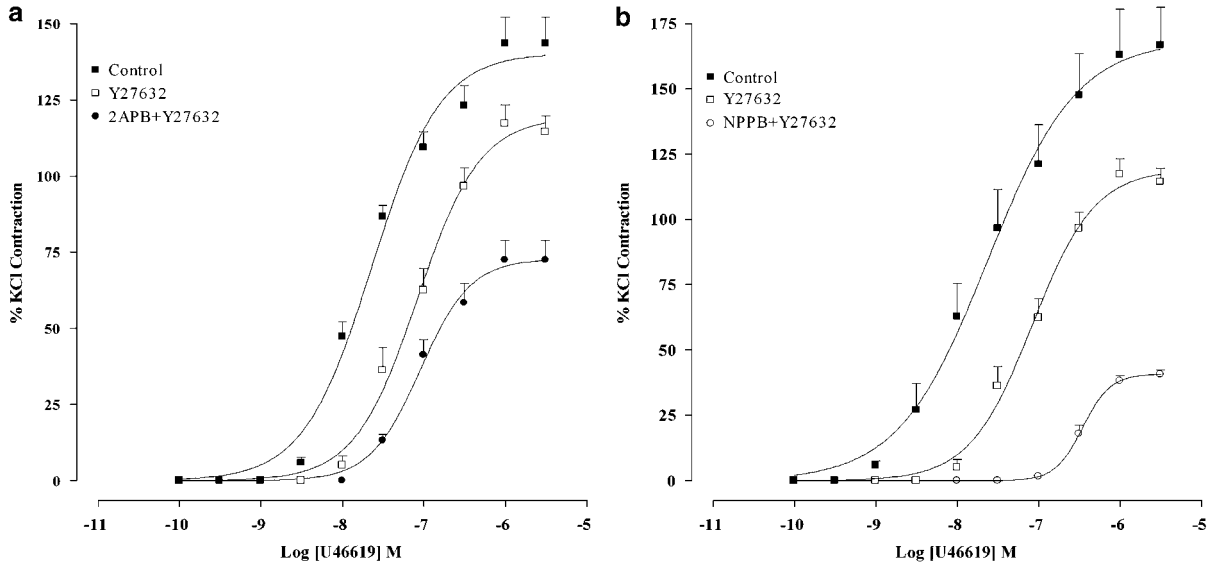

Figure 7 The effect of store-operated calcium channels (SOCC) blockade or chloride channel blockade on the concentration–response curve to U46619 in the presence of Rho kinase inhibition. Control responses in the absence and presence of Rho kinase inhibition with Y27632 (30 μM) are shown and the combined effect of Rho kinase inhibition with (**a**) 2-APB (100 μM) or (**b**) NPPB (50 μM). Results are the means$\pm$s.e.m. from 4 to 6 experiments.

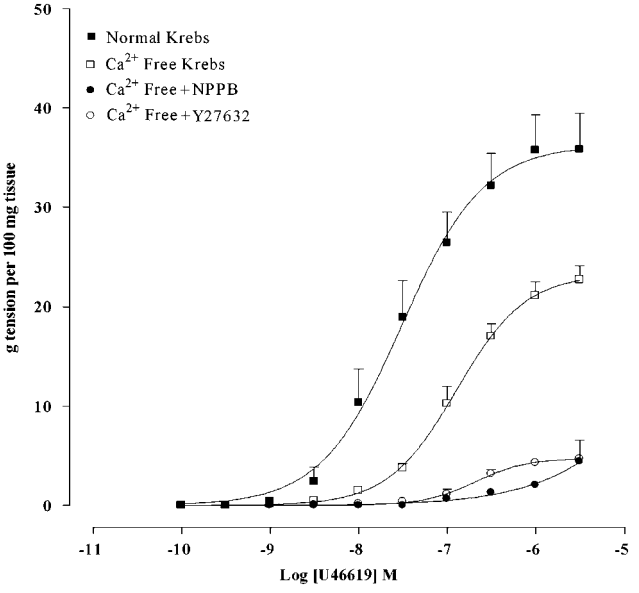

Figure 8 Effect of chloride channel blockade or Rho kinase inhibition on the concentration response curve for contraction to U46619 in nominally Ca^{2+}-free PSS. Control responses in normal and Ca^{2+}-free PSS are shown and the effect of NPPB (50 μM) or Y27632 (30 μM) on the response in Ca^{2+}-free PSS. Results are the means$\pm$s.e.m. from 4 to 8 experiments.

Table 5 The effects of nominally free $[Ca^{2+}]_o$ in the absence and presence of the chloride channel blocker NPPB and the Rho kinase inhibitor Y27632 on the concentration–response curve for U46619

U46619	*pEC_{50}*	R_{max} *(100 mg^{-1})*	n
$[Ca^{2+}]_o = 2.5$ mM	7.53$\pm$0.13	36.57$\pm$2.45	8
$[Ca^{2+}]_o = 0$	6.89$\pm$0.07	23.23$\pm$1.07	6
$[Ca^{2+}]_o = 0$ + NPPB	4.04$\pm$13.63	4.27$\pm$1.02*	4
$[Ca^{2+}]_o = 0$ + Y27632	6.67$\pm$0.07	4.47$\pm$0.28*	4

Abbreviation: NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid.
*$P<0.001$.

SOC and non-SOC channels in some tissues (McFadzean and Gibson, 2002), did not alter the CRC to U46619. A similar finding has been reported in rat pulmonary arteries (Snetkov *et al*., 2006). This may indicate the involvement of a non-SOC channel. However in air-gassed HEPES-buffered PSS, used to prevent La^{3+} precipitation, the concentration response curve to U46619 was markedly reduced compared with bicarbonate-buffered PSS. Since HEPES-buffered PSS did not alter the concentration response curve to 5-HT, this suggests that the omission of CO_2/HCO_3 buffering specifically inhibits the contractile response mediated by U46619. It is possible that one of the effects of CO_2/HCO_3 removal is to alter the function of the Cl/HCO_3 exchanger and hence $[Cl]_i$. Consequently, the lack of effect of La^{3+} may not be surprising and, without re-characterizing the response to U46619 in air-gassed HEPES-buffered PSS, a meaningful interpretation of the effect of La^{3+} is not possible.

In support of the involvement of SOCC in the U46619-induced contraction several studies have established that the SERCA inhibitors thapsigargin or CPA, by inhibiting calcium accumulation together with passive calcium release cause calcium entry via activation of SOCCs and, in some smooth muscle, this source of calcium produces an increase in tone (McFadzean and Gibson, 2002). In BPA, CPA induced a rapid increase in tone and, as this was abolished by 2-APB and SKF96365, suggests that calcium entry through SOCCs is entirely responsible for the CPA-induced tone. This supports the possibility that the 2-APB/SKF96365-sensitive component of the U46619-induced tone may be due to activation of SOCCs.

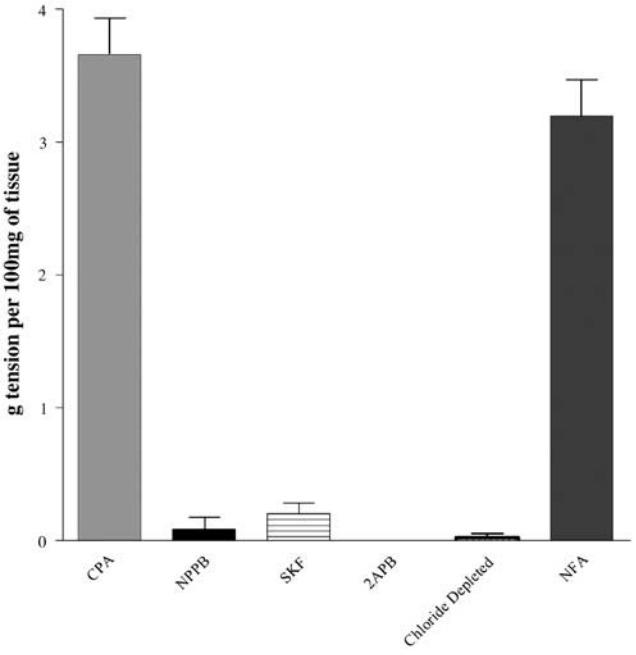

Figure 9 Effect of the putative store-operated calcium channels blockers SKF96365 (100 μM) and 2-APB (100 μM), chloride channel blockers NPPB (50 μM) and NFA (30 μM), and chloride depletion on the contraction to the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor cyclopiazonic acid (10 μM). Results are the means $\pm$ s.e.m. from 4 to 8 experiments.

That the CPA-induced tone was absent in chloride-depleted PSS indicates that chloride is important in the CPA-induced contraction and since this response was also abolished by NPPB but not niflumic acid suggests that an NPPB-sensitive chloride conductance is important in the CPA-induced activation of SOCC and that this action may also underlie its ability to inhibit U46619-induced contraction. Several reports have demonstrated that calcium uptake and release from the SR may be chloride dependent (Al-Awqati, 1995; Kourie, 1997). SR membrane is highly permeable to chloride (Kourie, 1997) and the presence of several chloride channels has been established by both electrophysiological (Kourie *et al*., 1996; Clark *et al*., 1997) and molecular techniques (Jentsch *et al*., 2001; Nilius and Droogmans, 2003).

The mechanism by which chloride influences calcium movement across the SR remains unclear. One possible link arises from the observations that the SERCA, which exchanges Ca^{2+} for H^+ (Yu *et al*., 1993), is electrogenic yet a membrane potential does not appear to be generated during accumulation or release of calcium (Russell *et al*., 1979; Pollock *et al*., 1998). It is suggested therefore that chloride movement accompanies calcium to maintain an electro-neutral accumulation/release of calcium by the SR and presumably therefore that calcium accumulation/release only occurs under electro-neutral conditions. While this model is based on studies using mainly skeletal muscle more recent evidence indicates that it also applies to smooth muscle since, and of relevance to the present study is the observation by Pollock *et al*. (1998), using saponin-permeabilized smooth muscle cells to access the SR, that Ca^{2+} uptake into the SR was blocked by NPPB and but not by (30 μM) niflumic acid.

It is possible that the inhibitory effect of altering chloride handling in the BPA arises from inhibition of calcium release and since this would prevent store depletion it would also prevent calcium influx through SOCCs. This scheme could explain the observation that the inhibition of the U46619-induced contraction by 2-APB and SKF96365 was significantly less than the inhibition produced by NPPB, chloride depletion or chloride-free conditions.

This would also explain the observation that Rho kinase inhibition combined with NPPB produced a much greater inhibition of U46619-induced contraction than Rho kinase and SOCC inhibition. This view is also supported by the observation that the concentration–response curve to U46619 in nominally free calcium, where calcium entry (through SOCCs) cannot contribute to the contraction, was abolished by NPPB. That the response in nominally free calcium was also abolished in the presence of Y-27632 suggests that the contraction in calcium-free medium is dependent on calcium release from the SR and calcium sensitization by Rho kinase.

Thus, the U46619-induced contraction of BPA may involve calcium release from the SR and entry through SOCCs together with activation of Rho kinase. The results of the present study contrast with reports in rat pulmonary arteries. In the study by Cogolludo *et al*. (2003) and by Snetkov *et al*. (2006), the contractile response to U46619 in rat pulmonary arteries was sensitive to L-type calcium channel blockers. In addition, Snetkov *et al*. (2006) reported that the response was sensitive to 2-APB at 30 μM and that the contractile response was substantially reduced by the combination of VOCC blockade and 2-APB (30 μM). In contrast, the present study found no effect of VOCC blockers on the contraction to U46619 and although the response was sensitive to high concentrations of 2-APB it was insensitive to low concentrations of 2-APB (30 μM). The present study used endothelium-intact arteries while the study by Cogolludo used endothelium-denuded arteries and although it is not stated whether endothelium-intact or denuded arteries were used in the Snetkov study, we believe that some of the differences between the present study and those by Cogolludo and Snetkov are due to the endothelium. We have reported preliminary data using rat pulmonary arteries showing that the contractile response to U46619 in endothelium-denuded, but not endothelium-intact, arteries is sensitive to nifedipine (Mckenzie *et al*., 2007).

In conclusion, the present study suggests that the contractile response of the BPA to the thromboxane A_2 mimetic U46619 involves Rho kinase together with a chloride-sensitive mechanism, which does not involve the activation of VOCC but may have a role in the release of calcium from the SR and calcium entry via SOCC. In contrast contraction of the BPA by 5-HT appears to involve calcium entry through verapamil- and mibefradil-sensitive VOCC. This study may indicate that the current use of calcium channel blockers in the management of pulmonary hypertension may not always be effective and that Rho kinase and chloride channels may be targets for the development of new therapies.

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