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THEMED SECTION: ENDOTHELIUM IN PHARMACOLOGY

RESEARCH PAPER

Mechanisms of U46619-induced contraction of rat pulmonary arteries in the presence and absence of the endothelium

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Background and purpose: Thromboxane A_2 and endothelial dysfunction are implicated in the development of pulmonary hypertension. The receptor-transduction pathway for U46619 (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$)-induced contraction was examined in endothelium-intact (E+) and denuded (E–) rat pulmonary artery rings.
Experimental approach: Artery rings were mounted on a wire myograph under a tension of 7–7.5 mN at 37°C and gassed with 95% O_2/5% CO_2. Isometric recording was made by using Powerlab data collection and Chart 5 software.
Key results: Both E+ and E– contractile responses were sensitive to Rho-kinase inhibition and the chloride channel blocker NPPB [5-nitro-2-(3-phenylpropylamino)benzoic acid]. The E+ response was sensitive to the store-operated calcium channel blockers SKF-96365 {1-[B-[3-(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl]-1H-imidazole hydrochloride} and 2-APB (2-amino ethoxy diphenylborate) (75–100 μmol·L^{-1}). The E– response was sensitive to 2-APB (10–30 μmol·L^{-1}), a putative IP_3 receptor antagonist, and the calcium and chloride channel blockers nifedipine, DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid) and niflumic acid but was insensitive to SKF-96365. Inhibiting K_V with 4-AP in E+ rings exposed a contraction sensitive to nifedipine, DIDS and niflumic acid, whereas inhibiting BK_{Ca} exposed a contraction sensitive to mibefradil, DIDS and niflumic acid. This indicates that removal of the endothelium allows the TP receptor to inhibit K_V, which may involve coupling to phospholipase C, because inhibition of phospholipase C with U73122 (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-y]amino]hexyl] – 1H-pyrrole-2,5-dione) switched the E– pathway to the E+ pathway.
Conclusions and implications: The results from this study indicate that distinct transduction pathways can be employed by the TP receptor to produce contraction and that the endothelium is able to influence the coupling of the TP receptor.
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Keywords: thromboxane A_2; U46619; rat pulmonary arteries; endothelium; chloride channels; store-operated calcium channels; Rho-kinase; voltage-operated calcium channels; potassium channels

Abbreviations: 4-AP, 4-aminopyridine; 2-APB, 2-amino ethoxy diphenylborate; ChTx, charybdotoxin; CPA, cyclopiazonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; NFA, niflumic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; NS1619, (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl) phenyl]-5-9trifluoromethyl)-2*H*-benzyimidazol-2-one; PNU37883, N-cyclohexyl-N′-tricyclo[3.3.1.13,7]dec-1-yl-4-morpholi necarboximidamine hydrochloride; SKF-96365, 1-[B-[3-(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl]-1H-imidazole hydrochloride; SOCCs, store-operated calcium channels; SR, sarcoplasmic reticulum; TRAM 34, 1-[(2-chlorophenyl)diphenylmethyl]-1H pyrazole; TXA_2, thromboxane A_2; U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-y]amino]hexyl] – 1H-pyrrole-2,5-dione; VOCCs, voltage-operated calcium channels; Y-27632, (-(R)+)trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride

Introduction

Thromboxane A_2 (TXA_2) has been implicated in the pathogenesis of many cardiovascular diseases (Fitzgerald *et al.*, 1987) including pulmonary hypertension (Christman *et al.*,

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1992). It contracts vascular smooth muscle by binding to specific G-protein-coupled receptors (TP receptors) that may increase cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) by releasing stored calcium, mainly from the sarcoplasmic reticulum (SR), or by causing entry of extracellular calcium through activation of channels in the plasma membrane. These include channels that are closely associated with the receptor, so called receptor-operated calcium channels, store-operated calcium channels (SOCCs), also known as capacitative calcium entry channels and store-depletion-activated channels, whose activation is triggered by depletion of the SR store following receptor-mediated calcium release and voltage-operated calcium channels (VOCCs) whose activation follows receptor-mediated membrane depolarization (Berridge, 1995; Parekh and Penner, 1997; Catterall, 2000; Putney, 2001; McFadzean and Gibson, 2002). In addition to elevating $[Ca^{2+}]_i$ TP receptor activation of the small GTPase RhoA via the $G_{12/13}$ family of heterotrimeric G-proteins leads to activation of Rho-kinase, which phosphorylates and inhibits myosin light chain phosphatase. This enhances the effect of the calcium-activated myosin light chain kinase by maintaining myosin in a phosphorylated state (Somlyo and Somlyo, 2000; Fukata *et al*., 2001).

Membrane depolarization and VOCC activation in smooth muscle can be achieved through regulation of K^+ and Cl^- conductance (Nelson and Quayle, 1995; Large and Wang, 1996; Chipperfield and Harper, 2000). This is because K^+ is the predominant conductance in the resting cell and the K^+ equilibrium potential (E_K) is more negative (around –89 mV) than the resting membrane potential (around –60 mV). Receptor-linked inhibition of the resting K^+ conductance would therefore increase the membrane potential. In contrast, because chloride conductance is low in the resting cell and the equilibrium potential (E_{Cl}) is more positive than the resting membrane potential, agonists that increase the plasma membrane chloride conductance produce an inward depolarizing current. Hence, membrane depolarization may be achieved by increased I_{Cl} and/or decreased I_K.

The plasma membrane chloride channels that have been characterized in arteries appear to be calcium-activated and sensitive to the chloride channel blocker niflumic acid (NFA) (Criddle *et al*., 1996; 1997; Wang *et al*., 1997; Yuan, 1997). As well as regulating membrane potential and calcium entry through VOCCs chloride channels in the SR membrane may be important in calcium movement across the SR (Kasai and Kometani, 1979; Clark *et al*., 1997; Kourie *et al*., 1997; Jentsch *et al*., 2001; Nilius and Droogmans, 2003). This has been demonstrated mainly in skeletal muscle SR, but more recently calcium uptake into the SR of permeabilized smooth muscle was found to be inhibited by the chloride channel blockers NPPB [5-nitro-2-(3-phenylpropylamino)benzoic acid] and IAA-94 but not NFA (Pollock *et al*., 1998).

In rat pulmonary arteries, Cogolludo *et al*. (2003) showed that the TXA_2 mimetic U46619 (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$)-induced tone was insensitive to Rho-kinase inhibition but sensitive to the VOCC blocker nifedipine and that inhibition of K_V, via a PKC-dependent mechanism sensitive to phospholipase C (PLC) and diglyceride, mediates membrane depolarization. Also in rat pulmonary arteries Snetkov *et al*. (2006) have demonstrated that U46619-induced tone is sensitive to the VOCC blocker diltiazem and this inhibitory effect is much greater when diltiazem was combined with 2-APB (2-amino ethoxy diphenylborate) (30 μmol·L^{-1}). In contrast, Alapati *et al*. (2007), using bovine pulmonary arteries, found that U46619-induced tone was sensitive to Rho-kinase inhibition but insensitive to VOCC blockade and although the response was sensitive to 2-APB at high concentrations (100 μmol·L^{-1}) as well as SKF-96365 {1-[B-[3-(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl]-1H-imidazole hydrochloride}, which are used to inhibit SOCC, the response was insensitive to low concentrations of 2-APB (30 μmol·L^{-1}) that are commonly used to inhibit the IP_3 receptor. This study, in addition to suggesting a role for SR calcium release coupled with entry of calcium through SOCC and Rho-kinase, also indicated an important role for chloride in the contractile response to U46619.

In addition to species differences, the experimental procedures performed in the bovine arteries were conducted with an intact endothelium, unlike the rat pulmonary arteries used by Cogolludo *et al*. (2003). Therefore, in the present study the involvement of Rho-kinase, chloride channels, potassium channels, calcium release, SOCC and VOCC in the U46619-induced contractile response of rat pulmonary artery was investigated in the presence and absence of the endothelium.

Methods

Tissue preparation

Male Wistar rats weighing 200–250 g were stunned and killed by cervical dislocation. Ring segments of 2–3 mm in external diameter and 3–4 mm in length were dissected mainly from the second and some third arterial generations. In endothelium-denuded preparations the endothelium was removed by gentle abrasion of the intimal surface. Successful removal of the endothelium was determined by the absence of endothelium-dependent relaxation to bradykinin.

Myograph studies

Endothelium-intact or denuded arterial rings were mounted on a small vessel wire myograph for isometric recording in Krebs physiological saline solution consisting of (in mmol·L^{-1}): NaCl 119, KCl 4.7, $NaHCO_3$ 24.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11. In some experiments 5.4 mmol·L^{-1} glucose was used. Tissues were maintained at 37°C under a tension of 7–7.5 mN and gassed with a mixture of 95% O_2/5% CO_2. Changes in isometric tension were recorded by using Powerlab data collection and Chart 5 software.

Experimental protocols

The tissues were allowed to equilibrate for 60 min before each experiment. Rings were initially contracted with KCl (60 mmol·L^{-1}). Agonists were added to the myograph chambers cumulatively in 0.5 log units to construct cumulative concentration–response curves.

The role of chloride in the contractile response to U46619

The involvement of chloride in the contractile response to U46619 was investigated by using the chloride channel

blockers NPPB (10–100 μmol·L^{-1}), NFA (30 μmol·L^{-1}) and DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid) (100 μmol·L^{-1}) (Large and Wang, 1996; Jentsch *et al*., 2001).

Experiments examining the role of voltage-operated calcium channels on the contractile response to U46619

The involvement of the L-type and T-type VOCCs in the contractile response to U46619 was examined by using the Ca_v 1.1 and 1.2 channel blocker nifedipine (1 μmol·L^{-1}) and the Ca_v 3.1–3.3 channel blocker mibefradil (10 μmol·L^{-1}, Alexander *et al*., 2008). In these experiments and all subsequent experiments involving nifedipine, darkened conditions were employed where myograph chambers were wrapped in foil and overhead lights switched off.

Experiments investigating the effect of IP_3 receptor antagonism and SOCC blockade on the contractile response to U46619

The involvement of IP_3-mediated calcium release and Ca^{2+} influx through SOCCs was examined by using the putative IP_3 receptor antagonist and SOCC blocker 2-APB at a range (1–100 μmol·L^{-1}) of concentrations (Putney, 2001; Bootman *et al*., 2002). In addition the effect of SKF-96365 (10 μmol·L^{-1}) was also used as a putative SOCC blocker (Putney, 2001).

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

In these studies, tissues were pre-incubated with the Rho-kinase inhibitor Y-27632 [(-(R)+)trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride] (Ishizaki *et al*., 2000). In most experiments Y-27632 was used at a supra-maximal concentration (30 μmol·L^{-1}), which was determined from initial studies examining a range of concentrations. In some experiments Rho-kinase inhibition was combined with 2-APB (30 μmol·L^{-1} or 100 μmol·L^{-1}), NPPB, NFA, DIDS, SKF-96365 or nifedipine.

Experiments examining the effect of IP_3 receptor antagonism, SOCC blockade and chloride channel blockade on the contractile response to the sarco(endo)-plasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor cyclopiazonic acid

Arterial rings were constricted with cyclopiazonic acid (CPA) (10 μmol·L^{-1}) in the absence and presence of NPPB, NFA, DIDS, SKF-96365 and 2-APB (30 μmol·L^{-1} or 100 μmol·L^{-1}). All drugs were pre-incubated for 45 min before the addition of CPA.

Experiments examining the effect of potassium channel blockade on the contractile response to U46619

The effect of the K^+ channel blockers 4-AP (4-aminopyridine) (1 mmol·L^{-1}, K_V), charybdotoxin (ChTx, 100 nM, BK_{Ca} and IK_{Ca}), TRAM 34 (1-[(2-chlorophenyl)diphenylmethyl]-1H pyrazole) (10 μmol·L^{-1}, IK_{Ca}) (Félétou and Vanhoutte, 2006) and PNU37883 (N-cyclohexyl-N′-tricyclo[3.3.1.13,7]dec-1-yl-4-morpholi necarboximidamine hydrochloride) (K_{ATP}, 10 μmol·L^{-1}, Cui *et al*., 2003) was investigated on endothelium-intact rings. The effects of nifedipine, mibefradil, NFA and DIDS on the U46619-induced concentration–response curve in the presence of 4-AP or ChTx were also examined.

Experiments examining the effect of nitric oxide and prostaglandin inhibition on the contractile response to U46619

The influence of endogenous nitric oxide and prostaglandins on the response to U46619 in endothelium-intact tissue was investigated by using the combination of nitric oxide synthase inhibitor, L-NAME (100 μmol·L^{-1}) with the nitric oxide chelator, hydroxycobolamine (200 μmol·L^{-1}; Danser *et al*., 2000). Indomethacin (10 μmol·L^{-1}) was also included to inhibit cyclooxygenase and endogenous prostanoids. The sensitivity of the response in the absence of nitric oxide and prostanoids to nifedipine and 2-APB (30 μmol·L^{-1}) was also investigated.

Experiments examining the involvement of PLC in the contractile response to U46619

The role of PLC on the TP receptor transduction was investigated by examining the effect of the PLC inhibitor U73122 (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-y]amino]hexyl] – 1H-pyrrole-2,5-dione) (1 μmol·L^{-1}, Smith *et al*., 1990) on the sensitivity of the U46619-induced contractile response in E– (endothelium-denuded) to 2-APB (30 μmol·L^{-1}) and nifedipine or 2-APB (100 μmol·L^{-1}) and SKF-96365. The effect of 2-APB (100 μmol·L^{-1}) was also examined in E+ (endothelium-intact) in the absence and presence of U73122.

Effect of the chloride channel blocker NFA and DIDS on tone induced by KCl or U46619

To establish whether NFA and DIDS have a direct inhibitory action on the L-type VOCC their effect on KCl-induced contraction was compared with that of nifedipine. To establish whether NFA or DIDS increase I_{BK} their effect on U46619-induced tone in endothelium-denuded vessels in the presence of nifedipine was compared with that of the BK_{Ca} activator NS1619 [(1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-9trifluoromethyl)-2*H*-benzyimidazol-2-one] (Edwards *et al*., 1994).

Drug incubation

Unless otherwise stated all drugs, or an equal volume of the drug vehicle in control tissues, were incubated for approximately 40 min before the addition of U46619.

Data analysis

All data were collected by using chart for Windows (ADInstruments).

Maximum contractile responses to agonists were calculated as a percentage of the contraction produced by KCl (60 mmol·L^{-1}) and are expressed as the means ± s.e.mean. Experiments involving CPA-induced contraction are expressed as a percentage of the maximum response. The mean log concentration–response curves to agonists were

analysed by fitting to a four-parameter logistic equation (given below) using non-linear regression (Graph Pad Prism):

$$Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10^{(\log EC50 - X)nH})$$

Where X is the logarithm of the molar concentration of agonist, Y is the response and nH is the Hill slope. Top is the maximum contraction (E_{max}), and log EC_{50} is the agonist concentration that produces 50% of the maximum response. Bottom is the resting tone in the absence of any contractile agonist. Comparisons between mean sensitivity (pEC_{50}) or maximum contraction (R_{max}) were carried out by using Student's unpaired *t*-test and Bonferroni *post* test. $P < 0.05$ was considered significant.

Chemicals and materials

U46619 was purchased from Biomol Research Laboratories Inc. Nifedipine, mibefradil dihydrochloride, CPA, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), U73122, SKF-96365, DIDS, NS1619, NFA, indomethacin and hydroxocobalamin (acetate salt) were purchased from Sigma-RBI. NPPB, Y-27632, TRAM 34, 4-AP, ChTx, PNU37883 and 2-APB were purchased from Tocris Bioscience.

Stock concentrations of nifedipine, NPPB, U73122 and CPA were dissolved in dimethyl sulphoxide while U46619, PNU37883, NS1619, indomethacin, TRAM 34 and NFA were dissolved in ethanol. DIDS was dissolved in 0.1 M potassium bicarbonate. All other drugs were dissolved in deionized water at room temperature except hydroxycobalamin, which was dissolved at 90°C for 5 min on the day of the experiment.

The small vessel wire myograph was from Danish Myotech (Denmark).

Results

Effect of the Rho-kinase inhibitor Y-27632, the VOCC blocker nifedipine, the IP_3 receptor/SOCC blocker 2-APB and the SOCC blocker SKF-96365 on the concentration–response curve for U46619 in endothelium-intact rings (E+)

Y-27632 (1–30 μmol·L^{-1}) produced a concentration-dependent inhibition of the U46619-induced concentration–response curve with 10 μmol·L^{-1} and 30 μmol·L^{-1} producing similar inhibition (Figure 1A, Table 1). Nifedipine (1 μmol·L^{-1}) and 2-APB (30 μmol·L^{-1}) had no effect on the concentration–response curve for U46619; however, 75 μmol·L^{-1} and 100 μmol·L^{-1} 2-APB as well as SKF-96365 (10 μmol·L^{-1}) produced a similar inhibition, and the inhibition produced by the combination of 2-APB (100 μmol·L^{-1}) and SKF-96365 was no greater than either drug alone (Figure 1B, Table 1).

Effect of the chloride channel blockers NPPB, NFA and DIDS on the concentration–response curve for U46619 in E+ rings

NPPB (10–100 μmol·L^{-1}) shifted the concentration–response curve for U46619 to the right and reduced the maximum response (Figure 2A, Table 1). This effect was concentration-dependent with a maximum inhibition at 30 μmol·L^{-1}. NFA (30 μmol·L^{-1}) and DIDS (100 μmol·L^{-1}) had no effect on the U46619-induced response (Figure 2B).

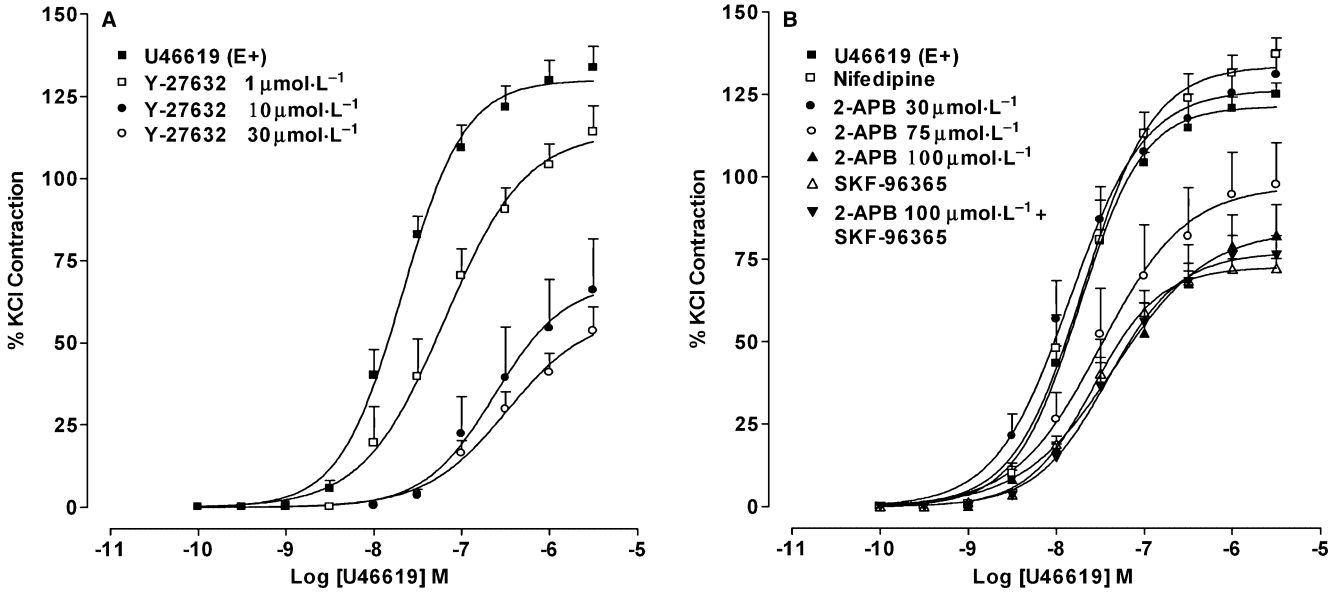

Figure 1 Effect of the Rho-kinase inhibitor Y-27632, the VOCC blocker nifedipine, the SOCC blocker SKF-96365 and the SOCC blocker/IP_3 receptor antagonist 2-APB on the concentration–response curve for U46619-induced contraction of endothelium-intact (E+) rat pulmonary arteries. (A) Response to U46619 in the absence and presence of Y-27632 at 1 μmol·L^{-1}, 10 μmol·L^{-1} and 30 μmol·L^{-1}; (B) response to U46619 alone and in the presence of nifedipine (1 μmol·L^{-1}), 2-APB at 30 μmol·L^{-1}, 75 μmol·L^{-1} and 100 μmol·L^{-1}, SKF-96365 (10 μmol·L^{-1}) and SKF-96365 at 10 μmol·L^{-1} combined with 2-APB at 100 μmol·L^{-1}. Results are the means, with vertical lines showing s.e.mean, from five to seven experiments. 2-APB, 2-amino ethoxy diphenylborate; SKF-96365, 1-[B-[3-(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl]-1H-imidazole hydrochloride; SOCC, store-operated calcium channel; U46619, 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$; VOCC, voltage-operated calcium channel; Y-27632, (-(R)+)trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride.

Table 1 The effects of the chloride channel blocker NPPB, the SOCC/IP_3 blocker 2-APB and putative SOCC blocker SKF-96365 and the Rho-kinase inhibitor Y-27632 on the concentration–response curve for U46619 in endothelium-intact arterial rings

	pEC_{50}	R_{max} %	n
Control	7.84 ± 0.04	119.6 ± 4.3	8
+NPPB (50 μmol·L^{-1})	6.39 ± 0.11	63.8 ± 5.5	5#
Control	7.97 ± 0.04	121.6 ± 2.3	5
+2-APB (75 μmol·L^{-1})	7.5 ± 0.17	97 ± 8.6	5#,γ
+2-APB (100 μmol·L^{-1})	7.34 ± 0.17	84 ± 7	5#,γ
+SKF-96365	7.5 ± 0.17	72.5 ± 1	5#
+2-APB (100 μmol·L^{-1}) + SKF-96365	7.42 ± 0.08	76.9 ± 3	5
Control	7.67 ± 0.05	130 ± 3.4	5
+Y-27632 (10 μmol·L^{-1})	6.62 ± 0.27	68 ± 14	5#,*
+Y-27632 (30 μmol·L^{-1})	6.48 ± 0.18	58.6 ± 8	5#,*
Control	7.84 ± 0.04	121.7 ± 2.3	6
+Y-27632 (30 μmol·L^{-1}) + 2-APB (100 μmol·L^{-1})	6.5 ± 0.06	26.5 ± 1.5	5#
+Y-27632 (30 μmol·L^{-1}) + SKF-96365	6.53 ± 0.13	21.2 ± 2.3	5#

2-APB, 2-amino ethoxy diphenylborate; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; SKF-96365, 1-[B-[3-(4-methoxyphenyl) propoxy]-4-methoxy-phenethyl]-1H-imidazole hydrochloride; SOCC, store-operated calcium channel; U46619, 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$; Y-27632, (-(R)+)trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride.
#Significantly different from control ($P < 001$).
γ, *, no significant difference ($P > 0.05$) between concentrations (Bonferroni *post* test).

Effect of NPPB, 2-APB (100 μmol·L^{-1}) and SKF-96365 on the concentration–response curve for U46619 in E+ rings in the presence of Rho-kinase inhibition

The contractile response to U46619 in the presence of Y-27632 (30 μmol·L^{-1}) was reduced to a similar extent by 2-APB (100 μmol·L^{-1}) and SKF-96365 (10 μmol·L^{-1}) and abolished by NPPB (50 μmol·L^{-1}) (Figure 3, Table 1).

Effect of 2-APB, SKF-96365, NPPB, DIDS and NFA on the contractile response induced by the SERCA inhibitor CPA

Cyclopiazonic acid (10 μmol·L^{-1})-induced contractions were abolished by NPPB (50 μmol·L^{-1}), SKF-96365 (10 μmol·L^{-1}) and 2-APB (100 μmol·L^{-1}) but were unaffected by NFA (30 μmol·L^{-1}), DIDS (100 μmol·L^{-1}) and 2-APB (30 μmol·L^{-1}) (Figure 4).

The concentration–response curve for U46619 in the absence of endothelium (E–) and the effects of Y-27632, nifedipine and 2-APB on this response. For comparison the effect of 2-APB and/or nifedipine was examined on the concentration–response curve for U46619 in E+ vessels in the presence of the nitric oxide synthase inhibitor L-NAME, the nitric oxide scavenger hydroxycobalamin and the cyclooxygenase inhibitor indomethacin

L-NAME (100 μmol·L^{-1}), hydroxycobalamin (200 μmol·L^{-1}) and indomethacin (10 μmol·L^{-1}) or removal of the endothelium increased the tissue sensitivity and maximum response to U46619 (Figure 5A,D, Tables 1 and 2).

In E– nifedipine (1 μmol·L^{-1}) shifted the curve to the right and reduced the maximum response (Figure 5B, Table 2). The response was unaffected by 2-APB at 1 μmol·L^{-1} but 10 μmol·L^{-1} and 30 μmol·L^{-1} produced similar inhibition (Figure 5B, Table 2). The combination of nifedipine and 2-APB (30 μmol·L^{-1}) produced a marked inhibition of the concentration–response curve (Figure 5B, Table 2).

In E– Y-27632 (30 μmol·L^{-1}) shifted the concentration–response curve to the right and reduced the maximum response (Figure 5C, Table 2) and Y-27632 together with nifedipine and 2-APB (30 μmol·L^{-1}) abolished the contractile response to U46619 (Figure 5C).

In E+ in the presence of L-NAME, hydroxycobalamin and indomethacin, neither nifedipine (1 μmol·L^{-1}) nor 2-APB (30 μmol·L^{-1}) alone or in combination had any effect on the concentration–response curve (Figure 5D).

The effect of the PLC inhibitor U73122 on the concentration–response curve to U46619 in E+ and E– vessels and the effect of nifedipine, 2-APB and SKF-96365 on these responses

U73122 (1 μmol·L^{-1}) had no affect on the concentration–response curve to U46619 in either E+ (Figure 6A) or E– (Figure 6B). In E– the response in the presence of U73122 was unaffected by nifedipine and 2-APB (30 μmol·L^{-1}) (Figure 6B), but both 2-APB (100 μmol·L^{-1}) and SKF-96365 (10 μmol·L^{-1}) produced a similar rightward shift and reduction in the maximum response (Figure 6C), and in E+ the inhibitory effect of 2-APB (100 μmol·L^{-1}) and SKF-96365 was unaffected by U73122 (Figure 6A).

Effect of NPPB, NFA and DIDS on the concentration–response curve for U46619 in E– rings in the absence and presence of nifedipine, 2-APB and Y-27632

NPPB (50 μmol·L^{-1}) caused a substantial rightward shift of the concentration–response curve for U46619 and a reduction of the maximum response (Figure 7A, Table 2). The response in the presence of NPPB was unaffected by nifedipine (1 μmol·L^{-1}), 2-APB (30 μmol·L^{-1}), NFA (30 μmol·L^{-1}) or DIDS (100 μmol·L^{-1}) but was abolished by the addition of Y-27632 (30 μmol·L^{-1}) (Figure 7A). Both NFA and DIDS produced a similar rightward shift of the concentration–response curve and reduction in the maximum response (Figure 7B,C, Table 2), and in combination the inhibition was no greater than either drug alone (Figure 7C). Both 2-APB and Y-27632, but not nifedipine, caused a further inhibition of the concentration–response curve in the presence of NFA or DIDS (Figure 7B,C, Table 2), and the response in the presence of NFA or DIDS together with 2-APB (30 μmol·L^{-1}) was abolished by the further addition of Y-27632 (Figure 7B,C).

The effect of nifedipine, NFA and DIDS on the contractile response induced by KCl

The KCl (60 mmol·L^{-1})-induced contraction was abolished by preincubation with nifedipine (1 μmol·L^{-1}) but was unaffected by NFA (30 μmol·L^{-1}) or DIDS (100 μmol·L^{-1}) (Figure 8A).

The effect of NFA, DIDS and the BK_{Ca} activator NS1619 on U46619-preconstricted E– rings

In E– rings in the presence of nifedipine and contracted with U46619 (50 nmol·L^{-1}) neither NFA (30 μmol·L^{-1}) nor DIDS (100 μmol·L^{-1}) induced relaxation whereas NS1619 (10 μmol·L^{-1}) substantially reduced the contraction (Figure 8B).

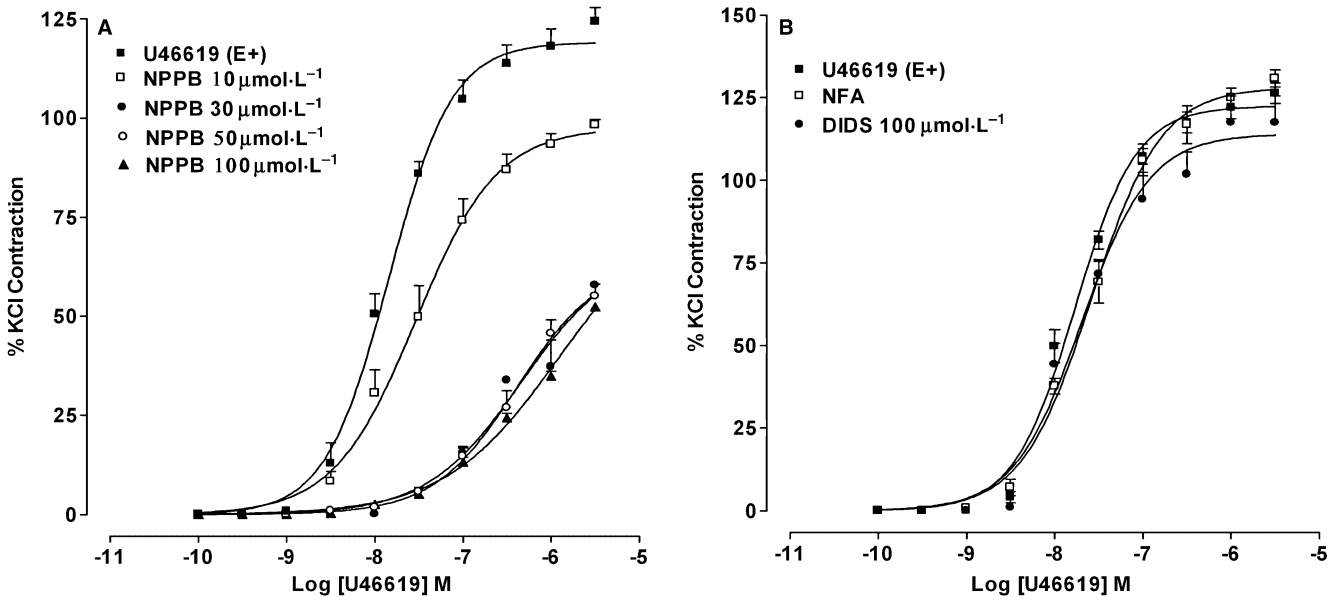

Figure 2 Effect of the chloride channel blockers NPPB, NFA and DIDS on the concentration–response curve for U46619-induced contraction of (E+) rat pulmonary arteries. (A) Response to U46619 in the absence and presence of NPPB at 10 μmol·L^{-1}, 30 μmol·L^{-1}, 50 μmol·L^{-1} and 100 μmol·L^{-1}; (B) response to U46619 in the absence and presence of NFA (30 μmol·L^{-1}) or DIDS (100 μmol·L^{-1}). Results are the means, with vertical lines showing s.e.mean, from four to five experiments. NFA, niflumic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; U46619, 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$.

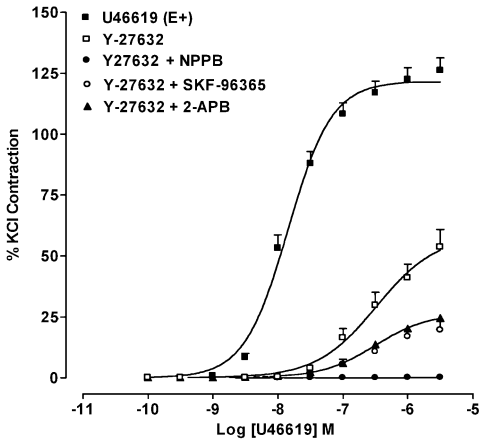

Figure 3 Effect of Rho-kinase inhibition on the concentration–response curve for U46619 in (E+) rat pulmonary arteries in the absence and presence of the chloride channel blocker NPPB or the SOCC blockers 2-APB and SKF-96365. Responses to U46619 are shown in the absence and presence of Y-27632 (30 μmol·L^{-1}) alone or combined with NPPB (30 μmol·L^{-1}), SKF-96365 (10 μmol·L^{-1}) or 2-APB (100 μmol·L^{-1}). Results are the means, with vertical lines showing s.e.mean, from five experiments. 2-APB, 2-amino ethoxy diphenylborate; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; SKF-96365, 1-[B-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride; SOCC, store-operated calcium channel; U46619, 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$; Y-27632, (-(R)+)trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride.

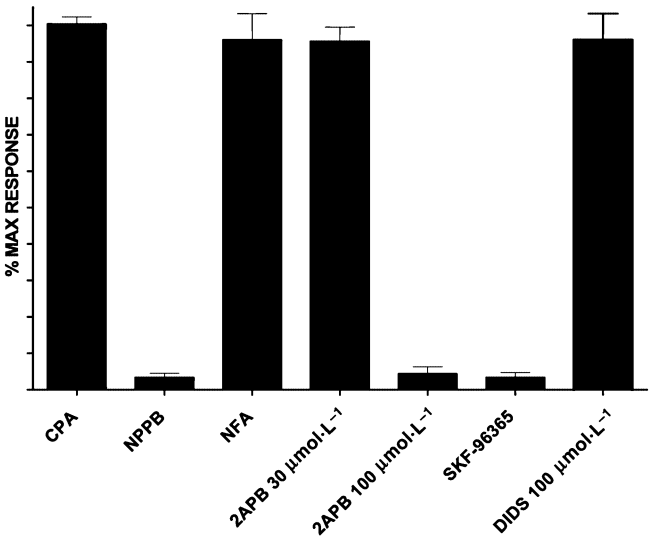

Figure 4 The contractile response to the SERCA inhibitor CPA (10 μmol·L^{-1}) alone in (E+) rat pulmonary arteries and in the presence of NPPB (30 μmol·L^{-1}), NFA (30 μmol·L^{-1}), DIDS (100 μmol·L^{-1}), SKF-96365 (10 μmol·L^{-1}) and 2-APB at 30 μmol·L^{-1} and 100 μmol·L^{-1}. Results are the means, with vertical lines showing s.e.mean, from five experiments. 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; SKF-96365, 1-[B-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride.

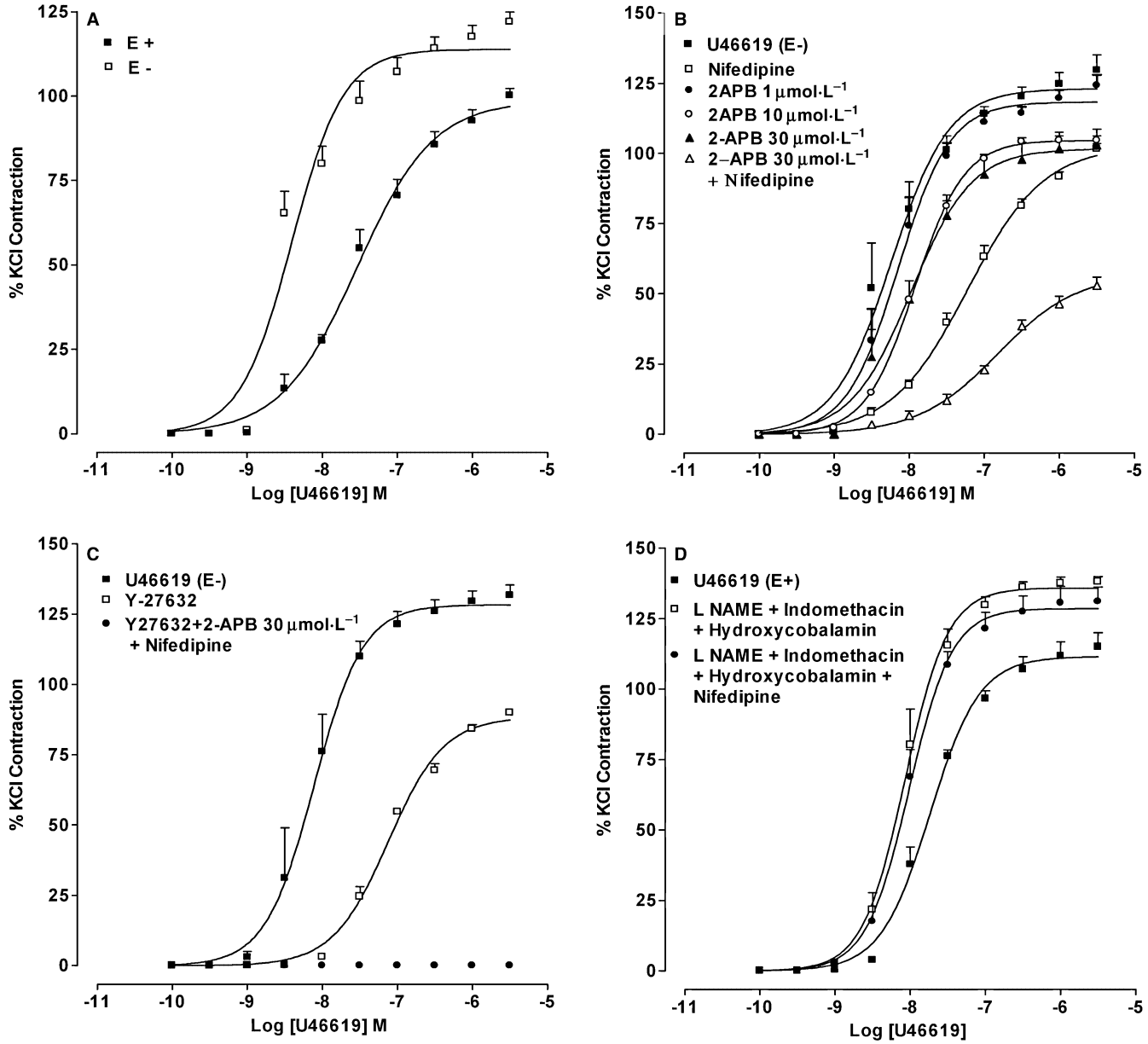

Figure 5 The concentration–response curve for U46619 in the presence and absence of the endothelium and in E– the effect of low concentrations of 2-APB alone (IP_3 receptor blockade) and in combination with the VOCC blocker nifedipine in the absence and presence of Rho-kinase inhibition. For comparison the effect of nifedipine and 2-APB on the concentration–response curve to U46619 in E+, in the absence of endogenous nitric oxide and prostanoids, is shown. (A) Response to U46619 in E+ and E–. (B) Response to U46619 in E– in the absence and presence of nifedipine (1 μmol·L^{-1}), 2-APB at 1 μmol·L^{-1}, 10 μmol·L^{-1} and 30 μmol·L^{-1}, and nifedipine (1 μmol·L^{-1}) combined with 2-APB (30 μmol·L^{-1}). (C) Response to U46619 in E– in the absence and presence of Y-27632 (30 μmol·L^{-1}) and Y-27632 with both nifedipine and 2-APB (30 μmol·L^{-1}). (D) Response to U46619 in E+ in the absence and presence of L-NAME (100 μmol·L^{-1}), indomethacin (10 μmol·L^{-1}) and hydroxycobalamine (200 μmol·L^{-1}) together or in combination with nifedipine (1 μmol·L^{-1}). Results are the means, with vertical lines showing s.e.mean, from five experiments. 2-APB, 2-amino ethoxy diphenylborate; U46619, 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$; VOCC, voltage-operated calcium channel; Y-27632, (-(R)+)trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride.

The effect of the potassium channel blockers PNU37883 (K_{ATP}), ChTx (BK_{Ca} and IK_{Ca}), TRAM 34 (IK_{Ca}) and 4-AP (K_V) on basal tone and on the concentration–response curve to U46619 in E+ rings in the absence and presence of the VOCC blockers nifedipine and mibefradil, the chloride channel blockers NFA and DIDS or the putative IP_3 receptor antagonist 2-APB

In E+ the concentration–response curve to U46619 was insensitive to the T-type calcium channel blocker mibefradil (10 μmol·L^{-1}) (Figure 9A). Neither ChTx (100 nmol·L^{-1}), TRAM 34 (10 μmol·L^{-1}), 4-AP (1 mmol·L^{-1}) nor PNU37883 (10 μmol·L^{-1}) affected the resting tone of the vessels (data not shown). ChTx and 4-AP but not PNU37883 or TRAM 34 caused a leftward shift of the concentration–response curve and an increase in the maximum response (Figure 9A–D, Table 3). Nifedipine (1 μmol·L^{-1}), NFA (30 μmol·L^{-1}) and DIDS (100 μmol·L^{-1}) but not 2-APB (30 μmol·L^{-1}) or mibefradil,

Table 2 The effects of the Rho-kinase inhibitor Y-27632, the chloride channel blockers NPPB, NFA and DIDS, the VOCC blocker nifedipine, the SOCC/IP_3 blocker 2-APB and the SOCC blocker SKF-96365 alone or in combination on the concentration–response curve for U46619 in endothelium-denuded arterial rings

E–	pEC_{50}	R_{max} %	n
Control	8.4 ± 0.05	128 ± 3.9	5
+Y-27632 (30 μmol·L^{-1})	7.12 ± 0.03	88.4 ± 1.8	4#
Control	8.19 ± 0.06	125 ± 3.6	6
+NFA	7.3 ± 0.04	99.8 ± 2.0	5#
+DIDS	7.2 ± 0.04	100 ± 2.5	5
+Nifedipine	7.23 ± 0.04	102.7 ± 2.4	5#
+Nifedipine + NFA	7.28 ± 0.04	100 ± 2.3	4#
+Nifedipine + DIDS	7.14 ± 0.07	103.1 ± 4.3	4
+DIDS + NFA	7.3 ± 0.05	99 ± 3.1	5
Control	8.28 ± 0.06	128.4 ± 3	6
+NPPB (50 μmol·L^{-1})	6.56 ± 0.15	56.7 ± 5.6	5#
Control	8.3 ± 0.06	123 ± 2.8	8
+2-APB (10 μmol·L^{-1})	8.08 ± 0.04	108 ± 2.0	5#,*
+2-APB (30 μmol·L^{-1})	7.97 ± 0.07	101.1 ± 3.4	5#,*
+Nifedipine + 2-APB (30 μmol·L^{-1})	6.82 ± 0.07	57.3 ± 3	5#

2-APB, 2-amino ethoxy diphenylborate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; NFA, niflumic acid; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; SKF-96365, 1-[B-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride; SOCC, store-operated calcium channel; U46619, 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$; VOCC, voltage-operated calcium channel; Y-27632, (-(R)+)trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride.
#Significantly different from control ($P > 0.001$).
*no significant difference between concentrations (Bonferroni *post* test).

produced a similar inhibition of the concentration–response curve seen in the presence of 4-AP (Figure 9A,B, Table 3), and the inhibitory effect of combining nifedipine with NFA was no greater than either drug alone (data not shown). Mibefradil, NFA and DIDS but not nifedipine produced a similar inhibition of the contractile response in the presence of ChTx (Figure 9C, Table 3). The inhibitory effect of combining mibefradil and NFA was no greater than either drug alone (data not shown). 4-AP did not affect the concentration–response curve for U46619 in E– (data not shown).

Effect of lowering the glucose concentration from 11 mmol·L^{-1} to 5.4 mmol·L^{-1} on the concentration–response curve to U46619 and the sensitivity of rings to nifedipine in the absence and presence of endothelium

In E+ the concentration–response curve to U46619 in 5.4 mmol·L^{-1} glucose was not different from that in 11 mmol·L^{-1} and was equally insensitive to nifedipine. Removal of the endothelium produced a nifedipine-sensitive leftward shift of the curve that was no different from that seen in 11 mmol·L^{-1} (Figure S1A,B).

Discussion

Conflicting observations reported for the transduction pathway for the TP receptor in pulmonary arteries led us to investigate differences between endothelium-intact and denuded arteries. In the present study the involvement of Rho-kinase, chloride channels, SOCC and VOCC in U46619-induced contractile response of rat pulmonary artery was investigated in the presence and absence of the endothelium. The data described herein indicate that distinct receptor-transduction pathways mediate the contractile response in endothelium-intact and denuded arteries.

Endothelium-intact arteries

In E+ rings nifedipine did not affect the U46619-induced response, which indicates that calcium entry via a nifedipine-sensitive L-type VOCC is not involved in the contraction to U46619.

2-amino ethoxy diphenylborate has been shown to act as both an IP_3 receptor antagonist and SOCC blocker (Bootman *et al.*, 2002; Peppiatt *et al.*, 2003). The concentration–response curve in E+ was unaffected by low concentrations of 2-APB (30 μmol·L^{-1}) but was inhibited by high concentrations (75 and 100 μmol·L^{-1}). Because the inhibitory effect of 2-APB (at high concentrations) occurred in the presence of PLC inhibition it seems unlikely that its action is due to antagonism of the IP_3 receptor. Moreover SKF-96365, a putative SOCC blocker, produced a similar inhibition to 2-APB, and both SKF-96365 and 2-APB (100 μmol·L^{-1}) abolished the contraction induced by the SERCA inhibitor CPA. By inhibiting calcium accumulation by the SR, CPA, in the presence of passive calcium release, causes depletion and calcium entry via SOCCs and in some smooth muscle this produces a contractile response (McFadzean and Gibson, 2002). These observations indicate that the inhibitory action of 2-APB (100 μmol·L^{-1}) and SKF-96365 is due to blockade of calcium entry via SOCC. That the contractile response was also sensitive to Rho-kinase inhibition suggests that calcium sensitization, together with calcium release, store-depletion and calcium entry via SOCC, is important in the TP-mediated contractile response in E+ vessels.

In E+ the contractile response to U46619 was also inhibited by the chloride channel blocker NPPB but not by NFA or DIDS. This observation is similar to that obtained for bovine pulmonary arteries (Alapati *et al.*, 2007), where inhibition of the U46619-induced contraction by NPPB was similar to that produced by chloride depletion/chloride-free conditions, suggesting that an NPPB-sensitive NFA/DIDS-insensitive chloride conductance may play an important role in the contraction to U46619 in both bovine and rat pulmonary arteries. We have suggested that NPPB inhibits calcium release from the SR, which in turn would prevent store-depletion and calcium entry through SOCC (see Alapati *et al.*, 2007). This was based on the observation that chloride appears to be important in calcium movement across the SR (Al-Awqati, 1995; Kourie *et al.*, 1997) and that NPPB but not NFA prevents calcium accumulation in smooth muscle SR (Pollock *et al.*, 1998). This view is supported by the observation in bovine pulmonary arteries that both NPPB and chloride depletion as well as the putative SOCC blockers SKF-96365 and 2-APB (100 μmol·L^{-1}) abolish tone induced by the SERCA inhibitor CPA (Alapati *et al.*, 2007). That NPPB, 2-APB and SKF-96365 also inhibit CPA-induced tone in the rat would suggest that it has a similar action in the rodent. Thus NPPB would be expected to prevent calcium release, SR depletion and calcium entry through SOCC, which could explain the observation that in the presence of Rho-kinase inhibition it abolished the contractile response.

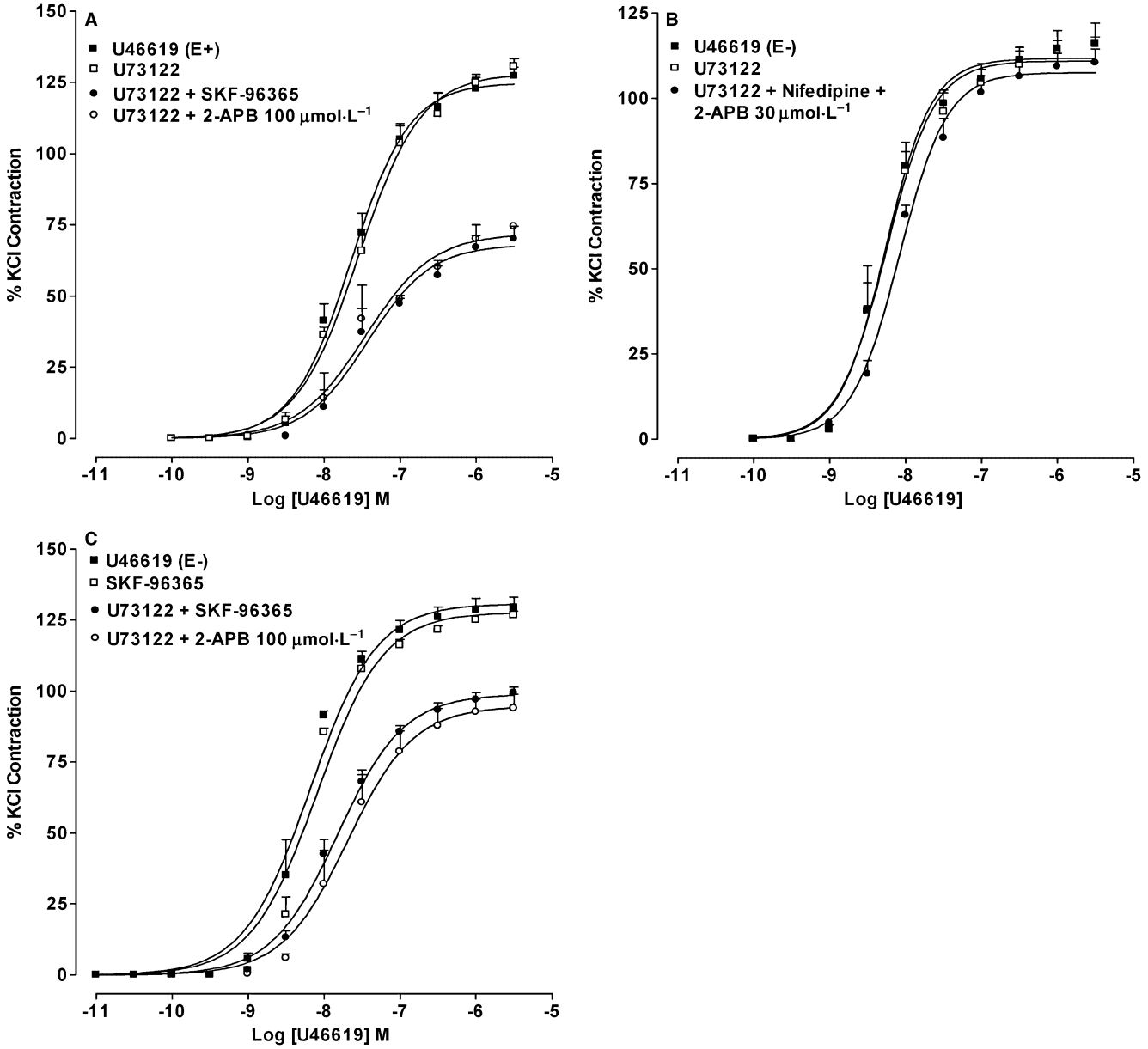

Figure 6 Effect of phospholipase C inhibition with U73122 on the concentration–response curve for U46619 in E+ and E– and its effect on the sensitivity of the response in E– to nifedipine (VOCC blockade) and low concentrations of 2-APB (IP_3 receptor blockade) or to SOCC blockade with SKF-96365 or high concentrations of 2-APB. (A) Response to U46619 in E+ in the absence and presence of U73122 (1 μmol·L^{-1}) alone or in combination with SKF-96365 (10 μmol·L^{-1}) or with 2-APB (100 μmol·L^{-1}). (B) Response to U46619 in E– in the absence and presence of U73122 (1 μmol·L^{-1}) alone or combined with nifedipine (1 μmol·L^{-1}) and 2-APB (30 μmol·L^{-1}). (C) Response to U46619 in E– in the presence of SKF-96365 (10 μmol·L^{-1}) and the response to U46619 with SKF-96365 (1 μmol·L^{-1}) or 2-APB (100 μmol·L^{-1}) in the presence of U73122 (1 μmol·L^{-1}). Results are the means, with vertical lines showing s.e.mean, from five experiments. 2-APB, 2-amino ethoxy diphenylborate; SKF-96365, 1-[B-[3-(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl]-1H-imidazole hydrochloride; SOCC, store-operated calcium channel; U46619, 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$; U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-y]amino]hexyl] – 1H-pyrrole-2,5-dione; VOCC, voltage-operated calcium channel.

Endothelium-denuded arteries

Removal of the endothelium increased the sensitivity and the maximum contractile tension to U46619 and, as this response was insensitive to SKF-96365 but was inhibited by nifedipine, this suggests that the TP receptor couples to a nifedipine-sensitive VOCC but not SOCC when the endothelium is removed. Cogolludo *et al.* (2003) also demonstrated a nifedipine-sensitive response in endothelium-denuded rat pulmonary arteries. Because the contractile response in E– was sensitive to low concentrations of 2-APB (10 μmol·L^{-1} and 30 μmol·L^{-1}) that had no effect in the endothelium-intact vessels and did not inhibit the CPA-induced contraction and, because the inhibitory effect of low concentrations of 2-APB was not seen in the presence of PLC inhibition, this suggests that the action of 2-APB in E– vessels could be related to inhibition of the IP_3 receptor. This indicates that in E– TP

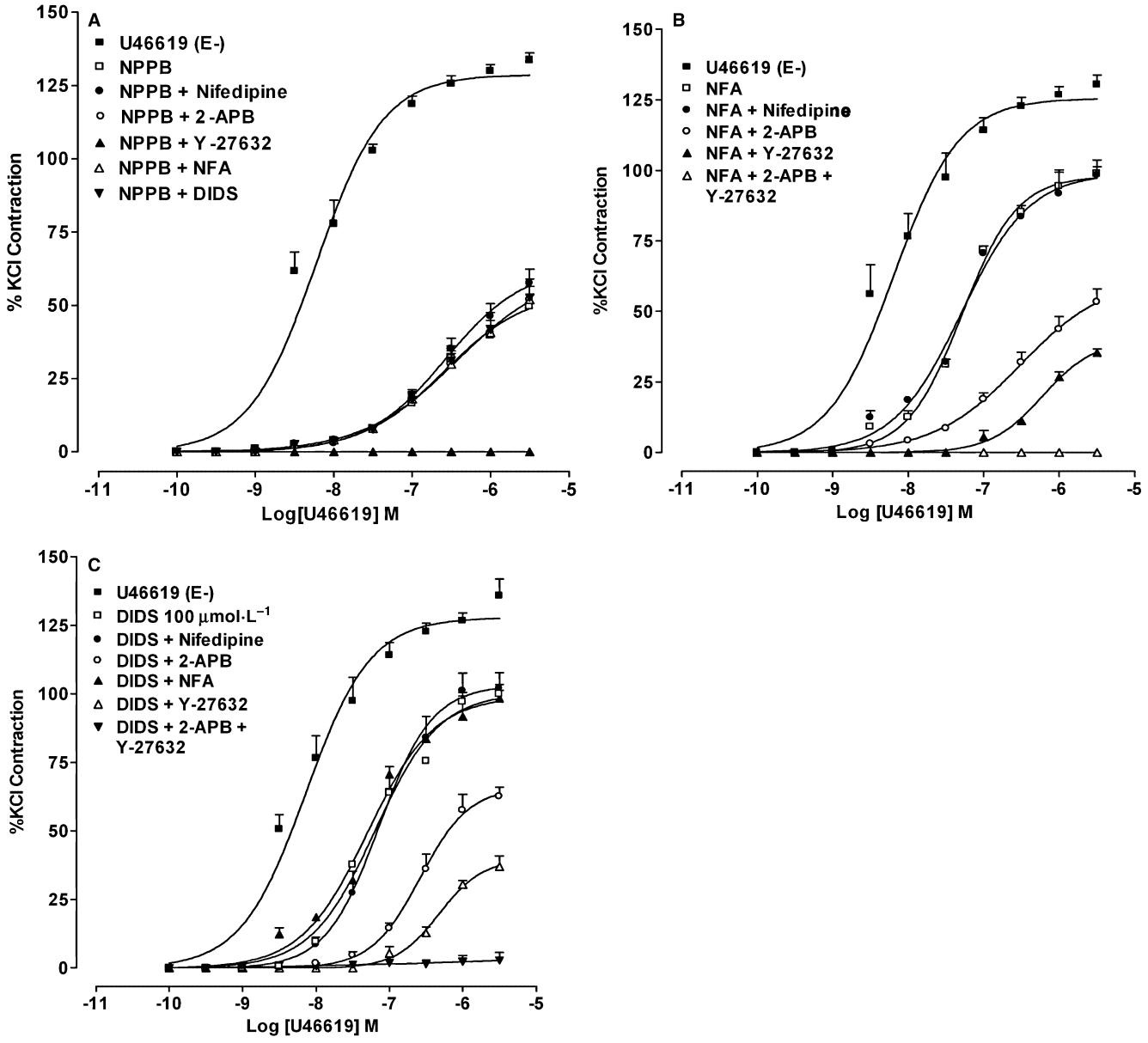

Figure 7 Effect of VOCC blockade with nifedipine, IP_3 receptor antagonism with 2-APB and Rho-kinase inhibition with Y-27632 on the concentration–response curve for U46619 in E– in the presence of the chloride channel blockers NPPB (30 μmol·L^{-1}), NFA (30 μmol·L^{-1}) or DIDS (100 μmol·L^{-1}). (A) Response to U46619 in E– in the absence and presence of NPPB alone (50 μmol·L^{-1}) or in combination with nifedipine (1 μmol·L^{-1}), 2-APB (30 μmol·L^{-1}), Y-27632 (30 μmol·L^{-1}), NFA (30 μmol·L^{-1}) or DIDS (100 μmol·L^{-1}). (B) Response to U46619 in E– in the absence and presence of NFA alone or in combination with nifedipine (1 μmol·L^{-1}), 2-APB (30 μmol·L^{-1}), Y-27632 (30 μmol·L^{-1}) or 2-APB with Y-27632. (C) Response to U46619 in E– in the absence and presence of DIDS (100 μmol·L^{-1}) alone or in combination with nifedipine (1 μmol·L^{-1}), 2-APB (30 μmol·L^{-1}), NFA (10 μmol·L^{-1}), Y-27632 (30 μmol·L^{-1}) or 2-APB with Y-27632. Results are the means, with vertical lines showing s.e.mean, from four to six experiments. 2-APB, 2-amino ethoxy diphenylborate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; NFA, niflumic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; VOCC, voltage-operated calcium channel; U46619, 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$; Y-27632, (-(R)+)trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride.

receptor stimulation activates a transduction pathway that causes calcium release via IP_3 receptor-linked channels.

The observation that nifedipine and 2-APB (30 μmol·L^{-1}) substantially reduced the contraction is similar to the findings obtained by Snetkov *et al*. (2006). The finding that the additional inhibition of Rho-kinase abolished the contraction indicates that in E– vessels TP receptor-mediated contraction involves calcium entry via a nifedipine-sensitive VOCC, possibly an IP_3-mediated calcium release and calcium sensitization via Rho-kinase. The involvement of Rho-kinase is in contrast to the conclusion reached in the study by Cogolludo *et al*. (2003). The present study shows that 10 μmol·L^{-1} Y-27632 produced a maximal effect in these arteries and that even 1 μmol·L^{-1}, the concentration used by Cogolludo *et al*. (2003), produced a significant reduction of the contractile response.

The finding that removal of endogenous nitric oxide and prostanoids also increased the tissue sensitivity and maximum response indicates that endogenous nitric oxide

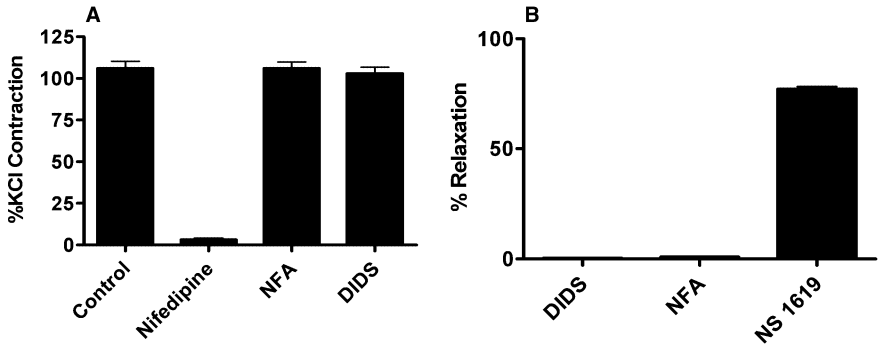

Figure 8 (A) The effect of VOCC and chloride channel blockade on KCl-induced constriction and (B) relaxation of U46619-induced tone by the chloride channel blockers DIDS and NFA compared with the BK_{Ca} activator NS1619. (A) E+, the effect of nifedipine (1 μmol·L^{-1}), NFA (30 μmol·L^{-1}) or DIDS (100 μmol·L^{-1}) on the KCl (60 mmol·L^{-1})-induced constriction; (B) E–, the effect of DIDS (100 μmol·L^{-1}), NFA (30 μmol·L^{-1}) or NS1619 (10 μmol·L^{-1}) on U46619 (50 nmol·L^{-1})-induced tone in the presence of nifedipine (1 μmol·L^{-1}). Results are the means, with vertical lines showing s.e.mean, from four to five experiments. DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; NFA, niflumic acid; NS1619, (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-9trifluoromethyl)-2*H*-benzyimidazol-2-one; U46619, 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$; VOCC, voltage-operated calcium channel.

and prostanoids normally attenuate the contractile response to U46619. As this response was unaffected by nifedipine or low concentrations of 2-APB, it is unlikely that the switch to the nifedipine- and 2-APB-sensitive pathway induced by removal of the endothelium is mediated by the loss of nitric oxide or prostanoids.

Endothelial cell function has been shown to be sensitive to the concentration of D-glucose (Graier *et al.*, 1996). It is possible that the pathway exposed by removal of the endothelium is suppressed by 11 mmol·L^{-1} D-glucose, the concentration used in this study. Because the concentration–response curve for U46619 was unchanged and removal of the endothelium still exposed the nifedipine-sensitive pathway when D-glucose was reduced to 5.4 mmol·L^{-1}, then the finding that the nifedipine-sensitive pathway was not seen in E+ is unrelated to the glucose concentration.

When the endothelium was absent the contractile response was sensitive to NFA and DIDS as well as NPPB. This suggests that an additional chloride conductance sensitive to NFA and DIDS is associated with the contractile response in E–. A calcium-activated chloride conductance (Cl_{Ca}) sensitive to NFA has been characterized in the plasma membrane in a number of blood vessels including rat pulmonary artery (Criddle *et al.*, 1996; 1997; Wang *et al.*, 1997; Yuan, 1997), and because the chloride equilibrium potential is more positive than the resting membrane potential (Chipperfield and Harper, 2000), activation of Cl_{Ca} would result in an inward depolarizing current. Because the inhibition of the U46619-induced contraction produced by nifedipine, DIDS or NFA was similar and no additional inhibition was observed when these compounds were added together, it is likely that TP receptors activate a NFA and DIDS-sensitive chloride conductance to produce a depolarization that is important in the activation of the high voltage-activated L-type channels.

This interpretation of the data is clouded by more recent studies showing that although NFA is a potent inhibitor of Cl_{Ca} it can also directly inhibit the L-type VOCC, and both NFA and DIDS have been found to increase $I_{K(Ca)}$ (reviewed in Greenwood and Leblanc, 2007). Such actions could account for the inhibitory effect of NFA in the present study. At the concentrations used in the present study direct inhibition of the L-type VOCC seems unlikely, because KCl-induced contractions were abolished by nifedipine but not NFA or DIDS. Also, the finding that neither NFA nor DIDS were inhibitory in E+, suggests they do not increase $I_{K(Ca)}$ to evoke a hyperpolarizing event. However, because the action of NFA (against Cl_{Ca}) is voltage-dependent (Hogg *et al.*, 1994a), it could be argued that its effects may only be exposed with membrane depolarization. This may well be the case for NFA; however, in contrast to the BK_{Ca} activator NS1619, neither NFA nor DIDS, whose inhibitory effect (on Cl_{Ca}) is voltage-independent (Hogg *et al.*, 1994b), induced relaxation of E– vessels contracted with U46619 in the presence of nifedipine. In addition NFA has also been shown to activate Cl_{Ca} (Greenwood and Leblanc, 2007), perhaps by increasing $[Ca^{2+}]_i$ (Cruickshank *et al.*, 2003). It is difficult however, to see how such an action could account for the inhibitory effects observed in the present study. Consequently, the effects of NFA and DIDS in the present study appear to be consistent with inhibition of Cl_{Ca}.

In E– vessels the inhibition produced by NPPB was greater than that produced by NFA or DIDS but was similar to the inhibition produced by the combined action of 2-APB (30 μmol·L^{-1}) and nifedipine and, in the presence of NPPB, neither 2-APB nor nifedipine altered the response. In the presence of NFA or DIDS, 2-APB but not nifedipine produced a further inhibition of the U46619-induced contraction. This supports the view that NFA and DIDS inhibit a chloride conductance in the plasma membrane (Cl_{Ca}) preventing an inward depolarizing current and subsequent VOCC activation leaving IP_3-mediated calcium release, which could explain the further inhibition by 2-ABP in the presence of NFA or DIDS. If NPPB also inhibits the NFA/DIDS-sensitive chloride conductance in the plasma membrane it would prevent the nifedipine-sensitive component, and if it additionally inhibits calcium movement across the SR it would also be expected to prevent calcium release via IP_3. This could explain the similar inhibition by NPPB and the combination of nifedipine and 2-APB. This view is further supported by the finding that, when

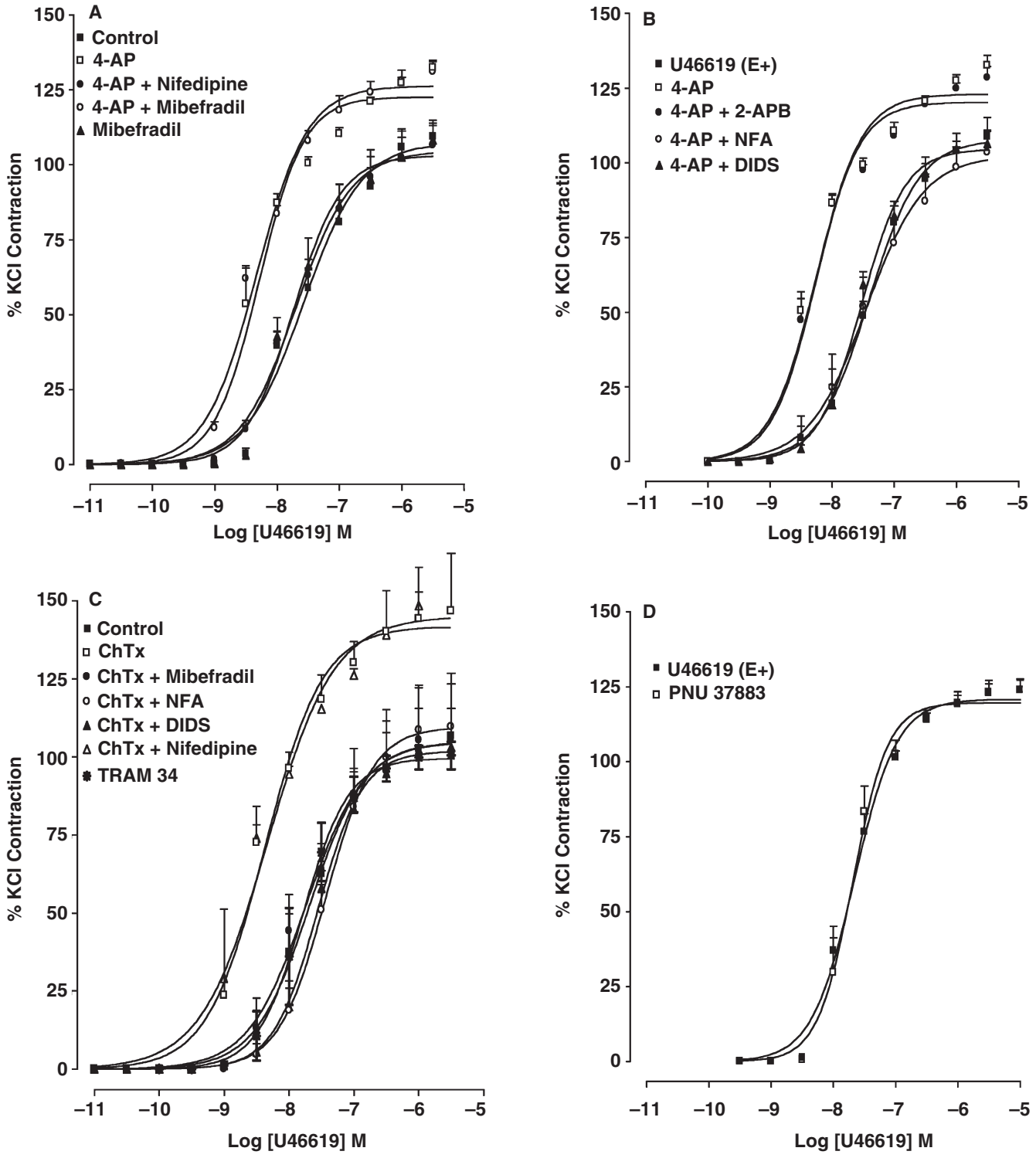

Figure 9 The effect of the potassium channel blockers 4-AP (1 mmol·L^{-1}, K_V), charybdotoxin (BK_{Ca} and $IK_{Ca,}$ 100 nmol·L^{-1}), TRAM 34 (IK_{Ca},10 μmol·L^{-1}) and PNU37883 (10 μmol·L^{-1}, K_{ATP}), on the concentration–response curve for U46619 in E+. (A) Response to U46619 in E+ in the absence and presence of 4-AP alone or combined with nifedipine (1 μmol·L^{-1}), mibefradil (10 μmol·L^{-1}) or mibefradil alone. (B) Response to U46619 in E+ in the absence and presence of 4-AP alone or in combination with 2-APB (30 μmol·L^{-1}), NFA (30 μmol·L^{-1}) or DIDS (100 μmol·L^{-1}). (C) Response to U46619 in E+ in the absence and presence of charybdotoxin alone or combined with mibefradil, NFA, DIDS, nifedipine, TRAM 34 alone. (D) Response to U46619 in E+ in the absence and presence of PNU37883. Results are the means, with vertical lines showing s.e.mean, from five experiments. 4-AP, 4-aminopyridine; 2-APB, 2-amino ethoxy diphenylborate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; NFA, niflumic acid; PNU37883, N-cyclohexyl-N′-tricyclo[3.3.1.13,7]dec-1-yl-4-morpholi necarboximidamine hydrochloride; TRAM 34, 1-[(2-chlorophenyl)diphenylmethyl]-1H pyrazole; U46619, 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$.

Table 3 The effects of the potassium channel blockers 4-AP (K_V) and charybdotoxin (BK_{Ca}) alone and in the presence of the VOCC blockers nifedipine or mibefradil or the chloride channel blocker niflumic acid on the concentration–response curve to U46619 in endothelium-intact rings

	pEC_{50}	R_{max} %	*n*
Control	7.58 ± 0.07	107.5 ± 4	5
+4-AP	8.26 ± 0.47	123 ± 2.6	5#
+4-AP + Nifedipine	7.35 ± 0.07	98.6 ± 3.3	5
+4-AP + Mibefradil	8.33 ± 0.04	126 ± 2	5#
Control	7.41 ± 0.08	107.7 ± 5	5
+4-AP + NFA	7.45 ± 0.1	102 ± 6	5
+4-AP + DIDS	7.53 ± 0.04	104.5 ± 2.3	4
+4-AP + 2-APB	8.26 ± 0.05	120.2 ± 5	5#
Control	7.68 ± 0.05	104.9 ± 3	6
+Charybdotoxin	8.37 ± 0.06	142 ± 3.6	6#
+Charybdotoxin + Nifedipine	8.33 ± 0.05	145 ± 3	5#
+Charybdotoxin + Mibefradil	7.74 ± 0.05	105 ± 3	6
+Charybdotoxin + NFA	7.44 ± 0.09	109.6 ± 6	5
+Charybdotoxin + DIDS	7.56 ± 0.03	102 ± 2	6

4-AP, 4-aminopyridine; 2-APB, 2-amino ethoxy diphenylborate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; NFA, niflumic acid; U46619, 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$; VOCC, voltage-operated calcium channel.
#Significantly different from control ($P < 0.001$).

Rho-kinase was inhibited, the response was abolished by either NPPB alone or the combinations of nifedipine and 2-APB, NFA and 2-APB or DIDS and 2-APB.

While an increased chloride conductance will lead to an inward depolarizing current, the extent of depolarization may be limited by the presence of voltage-sensitive potassium channels or potassium channels activated by voltage and calcium (Nelson and Quayle, 1995). Voltage-sensitive (K_V), voltage- and calcium-sensitive (BK_{Ca}) and ATP-sensitive (K_{ATP}) K^+ channels have been characterized in pulmonary artery smooth muscle cells (see Yuan *et al.*, 1998), and of these a low threshold non-inactivating K_V (Evans *et al.*, 1996; Yuan *et al.*, 1998) has been shown to be the main potassium conductance influencing the resting membrane potential. Pharmacological inhibition of K_V with 4-AP produces membrane depolarization (Nelson and Quayle, 1995; Shimoda *et al.*, 1998), and pulmonary vasoconstrictors, including endothelin (Shimoda *et al.*, 1998), TXA_2 (Cogolludo *et al.*, 2003) and 5-HT (Cogolludo *et al.*, 2006), inhibit a K_V, hence, it has been suggested that agonist-induced inhibition of K_V underlies VOCC activation.

In the present study the potassium channel blockers 4-AP (K_V), ChTx (BK_{Ca} and IK_{Ca}) and PNU37883 (K_{ATP}) did not alter the basal tone, indicating that inhibition of K_V, BK_{Ca} or K_{ATP} alone is not sufficient to activate a VOCC.

In E+ vessels both the K_V inhibitor 4-AP and the BK_{Ca} inhibitor ChTx, but not the selective IK_{Ca} inhibitor TRAM 34 or the K_{ATP} inhibitor PNU37883, produced an increased tissue sensitivity and maximum contractile tension to U46619, indicating that K^+ conductance through K_V and BK_{Ca} produce outward hyperpolarizing currents that normally attenuate the U46619-induced constriction.

The finding that the contractile response to U46619, in the presence of 4-AP, was sensitive to nifedipine indicates that pharmacological inhibition of K_V, in the presence of TP receptor activation, may produce a depolarization that will activate a nifedipine-sensitive VOCC. Because nifedipine blocks the high voltage-activated L-type channels (Alexander *et al.*, 2008) it is possible that the depolarization arising from inhibition of K_V could activate this VOCC. However, as 4-AP did not affect the response to U46619 in the presence of NFA or DIDS, this could indicate that a chloride conductance in addition to inhibition of K_V is required for activation of the nifedipine-sensitive VOCC. Chloride conductance in resting smooth muscle is low (Chipperfield and Harper, 2000), which is also likely to be the case in the pulmonary artery, because the predominant chloride conductance is calcium-activated (Salter and Kozlowski, 1995; Wang *et al.*, 1997; Yuan, 1997). Also, the finding that 4-AP did not alter the basal tone suggests that TP receptor activation in E+ vessels couples to activation of a NFA-sensitive chloride conductance. This view is consistent with previous observations in isolated pulmonary artery smooth muscle cells showing that histamine and noradrenaline in rabbit (Wang and Large, 1992) and endothelin and noradrenaline in rat (Salter and Kozlowski, 1995; Wang *et al.*, 1997) induce a simultaneous inward chloride and outward potassium current with little net change in membrane potential. Because both currents are calcium-activated, with the outward current sensitive to ChTx (Salter and Kozlowski, 1995), it seems likely that TP receptor activation, similar to other agonists, activates Cl_{Ca} and BK_{Ca}.

Conductance through BK_{Ca} is also suggested by the observation that ChTx produces a similar increase in sensitivity to 4-AP. As this response is insensitive to nifedipine but sensitive to the T-type VOCC blocker mibefradil, this indicates that inhibition of BK_{Ca} is associated with activation of the T-type VOCC. The mibefradil-sensitive response was also abolished in the presence of NFA or DIDS. As far as we are aware the effect of NFA and DIDS on the T-type channel has not been studied. However, it seems unlikely that NFA directly inhibits this channel because the 5-HT-induced contraction in bovine pulmonary artery is mediated, in part, by a mibefradil-sensitive mechanism and this contraction, which does not appear to involve chloride, is insensitive to NFA (Alapati *et al.*, 2007). This suggests that, in addition to inhibition of BK_{Ca}, activation of a Cl_{Ca} conductance is required to activate the T-type VOCC.

These observations suggest that in rat pulmonary arteries sensitization of the TP-mediated contraction by inhibition of BK_{Ca} or K_V in the presence of stimulated Cl_{Ca} is associated with activation of the T-type and L-type VOCC respectively. Because the L-type channels that are sensitive to nifedipine ($Ca_V1.1$–$Ca_V1.2$) are high voltage-activated and the mibefradil-sensitive T-type channels are low voltage-activated (Alexander *et al.*, 2008), it is possible that the depolarization arising from inhibition of K_V in the presence of BK_{Ca} conductance is greater than that caused by inhibition of BK_{Ca} in the presence of K_V.

The above discussion supports the view that activation of the nifedipine-sensitive VOCC requires inhibition of K_V together with activation of Cl_{Ca}. Because NFA, DIDS and nifedipine did not affect the concentration–response curve for U46619 in endothelium-intact vessels but inhibited the response in the presence 4-AP, it is likely that under *in vitro* experimental conditions TP receptor activation in the E+ vessel does not normally inhibit K_V. However, removal of the

endothelium permits inhibition of K_V by TP receptor activation, as in vessels with no endothelium the response was insensitive to 4-AP but sensitive to nifedipine and NFA.

In E– vessels the transduction pathway is clearly sensitive to nifedipine, low concentrations of 2-APB and Rho-kinase. However, when PLC is inhibited, the pathway reverts to that of the E+ vessel, sensitive to SKF-96365, high concentrations of 2-APB and Rho-kinase inhibition. This indicates that removal of the endothelium allows the TP receptor to couple to PLC to expose the nifedipine/2-APB-sensitive pathway. This view is consistent with the results of several other studies in endothelium-denuded vessels or isolated cells demonstrating that inhibition of K_V can be mediated through activation of PLC and PKC (Beech 1997; Quayle *et al*., 1997; Shimoda *et al*., 1998; Cogolludo *et al*., 2003).

Conclusions

The results of the present study suggest that TP receptor activation can produce a contractile response by coupling to two distinct transduction pathways and that the endothelium is able to influence the coupling pathway. One pathway appears to involve calcium release and calcium entry through SOCC. The second involves activation of PLC, possibly IP_3-mediated calcium release and calcium entry via a nifedipine-sensitive calcium channel. Inhibition of K_V together with activation of a chloride conductance underlies activation of the nifedipine-sensitive VOCC. In both pathways Rho-kinase contributes to the contractile response. These observations may be important when considering strategies for the management of pulmonary hypertension.

Conflict of interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The effect of lowering the glucose concentration from 11 mmol·L^{-1} to 5.4 mmol·L^{-1}. (A) 5.4 mmol·L^{-1} D-glucose; E+ alone, E– alone, E+ and nifedipine, E– and nifedipine. (B) 11 mmol·L^{-1} D-glucose; E+ alone, E– alone, E+ and nifedipine, E– and nifedipine. Results are the means, with vertical lines showing s.e.mean, from five experiments.

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