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# Involvement of Rho-kinase in contraction of guinea-pig aorta induced by prostanoid $EP_3$ receptor agonists

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**1** The mechanism of contraction of guinea-pig isolated aorta induced by the prostanoid $EP_3$ receptor agonist sulprostone (0.1–300 nM) has been investigated. In 60% of the experiments, the sulprostone log concentration–response curve (maximum = 15–40% of 100 nM U-46619 response; *low-responders*) was unaffected by the removal of extracellular $Ca^{2+}$, blockade of L-type $Ca^{2+}$ channels with nifedipine and depletion of internal $Ca^{2+}$ stores. In the remaining preparations (35–65% of 100 nM U-46619 response; *high-responders*), contractions to higher sulprostone concentrations showed a nifedipine-sensitive component, which was enhanced by charybdotoxin.

**2** In $Ca^{2+}$-free Krebs solution, established contractions to 300 nM sulprostone were abolished by the Rho-kinase inhibitors H-1152, Y-27632 and HA-1077 ($IC_{50}$ values = 190, 770 and 2030 nM). The PKA/Rho-kinase inhibitor H-89 (10 nM–10 $\mu$M) caused enhancement progressing to inhibition. The selective PKC inhibitor Ro 32-0432 (3 $\mu$M) had no effect, while staurosporine, recently shown to be a potent Rho-kinase inhibitor, abolished sulprostone responses ($IC_{50}$ ~47 nM), but its action was slow. The MAP kinase inhibitors SB 202190, SB 203580 and PD 80958 produced little inhibition.

**3** In normal Krebs solution, H-1152 and Y-27632 abolished established contractions to 300 nM sulprostone and 1 $\mu$M phenylephrine, and partially inhibited 10 $\mu$M phenylephrine and 50 mM $K^+$ responses.

**4** The results are discussed in relation to the reported potencies of the protein kinase inhibitors in enzyme assays. Activation of the Rho-kinase pathway appears to be a primary mechanism of contraction induced by $EP_3$ receptor agonists in guinea-pig aorta.
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**Keywords:** Vascular smooth muscle; prostanoid $EP_3$ receptor; sulprostone; nifedipine; $Ca^{2+}$-sensitization; Rho-kinase; H-1152; Y-27632; HA-1077; staurosporine

**Abbreviations:** $BK_{Ca}$, large conductance calcium-activated $K^+$ channel; CPA, cyclopiazonic acid; c/nPKC, conventional/novel protein kinase C; CTX, charybdotoxin; DAG, 1,2-diacylglycerol; $EC_{50}$, concentration producing half-maximal response; $InsP_3$, inositol 1,4,5-trisphosphate; $PGE_2$, prostaglandin $E_2$; MLC, myosin light chain; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; Rho-kinase, Rho-associated kinase; SOCC, store-operated $Ca^{2+}$ channel; SR, sarcoplasmic reticulum

## Introduction

Prostaglandin $E_2$ ($PGE_2$) is usually considered to be a vasodilator; the receptors involved are prostanoid $EP_2$ and/or $EP_4$ subtypes, which couple efficiently to Gs (see Coleman *et al*., 1994). However, $PGE_2$ and its close analogues also contract vascular smooth muscle, and over the past few years we have examined these actions in isolated blood vessels from several species, including the human pulmonary artery (Qian *et al*., 1994), guinea-pig aorta (Jones *et al*., 1998), pig carotid artery, and rabbit mesenteric artery and saphenous vein (Jones & Chan, 2001). In each case, an $EP_3$ receptor was identified based on the high potency of the PGE analogue SC 46275, a selective $EP_3$ agonist (Savage *et al*., 1993), and the potency ranking SC 46275 > sulprostone > $PGE_2$ > 17-phenyl-$\omega$-trinor $PGE_2$, which is reversed for the $EP_1$ receptor. The $EP_3$ agonists typically induced weak contraction, which synergized with contractions induced by other vasoconstrictors such as the $\alpha_1$-adrenoceptor agonist phenylephrine and the prostanoid TP receptor agonist U-46619.

The $EP_3$ receptor has attracted the attention of molecular biologists due to its existence in multiple isoforms, numbering eight for the human subtype (Namba *et al*., 1993; Pierce & Regan, 1998; Breyer *et al*., 2001). The variation occurs only beyond the first 10 amino-acid residues of the C-terminal cytoplasmic tail and is due to alternative mRNA splicing. The isoforms, which appear to be diffe-rentially expressed in tissues, interact similarly with agonists but show quite different coupling modes. All of the isoforms couple to $G_i$ resulting in inhibition of adenylate cyclase, while some also couple to $G_s$ to activate adenylate cyclase and/or $G_q$ to activate phospholipase C (PLC). A further option is seen in PC12 cells where agonist activation of the cow recombinant $EP_{3B}$ isoform leads to $G_{13}$

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coupling, activation of the small GTPase Rho, activation of Rho-kinase and eventually neurite retraction (see Hatae *et al*., 2002). The Rho-kinase pathway is also involved in vascular smooth muscle contraction, where activated Rho-kinase inhibits myosin phosphatase, causing $Ca^{2+}$-sensitization, that is, enhanced contraction without a change in the level of cytosolic-free $Ca^{2+}$ and myosin light chain (MLC) kinase activity (see Somlyo & Somlyo, 2000; Fukata *et al*., 2001; Somlyo, 2002). This pathway has to a large extent taken over the established role of protein kinase C (PKC) in $Ca^{2+}$-sensitization (Walsh *et al*., 1996; Hori & Karaki 1998; Arner & Pfitzer, 1999). The development of selective Rho-kinase inhibitors, such as Y-27632 (Uehata *et al*., 1997) and H-1152 (Ikenoya *et al*., 2002; Sasaki *et al*., 2002), has provided important tools for investigating the role of these Rho-kinase pathways. The current work examines the effect of Rho-kinase and PKC inhibitors on the contractile action of the $EP_3$ agonist sulprostone on guinea-pig isolated aorta. Sulprostone was preferred to SC 46275, since the latter is a C1-methyl ester and may require de-esterification within the tissue to achieve full biological activity.

The TP receptor, which is widely distributed in the vascular system, activates the classical $G_q$/phospholipase C$\beta$ ($G_q$/PLC$\beta$) pathway (see Narumiya *et al*., 1999). In the continued presence of the TP agonist, $Ca^{2+}$ influx through both voltage-operated (usually L-type) $Ca^{2+}$ channels and other $Ca^{2+}$-permeable channels contributes to strong sustained contraction (Fukuo *et al*., 1986; Mené *et al*., 1988; Tosun *et al*., 1998). In addition, an involvement of p38 mitogen-activated protein (MAP) kinase has been proposed in rat mesenteric resistance arteries based partly on the inhibitory action of the selective p38 MAP kinase inhibitor SB 203580 (Bolla *et al*., 2002); MAP kinases are also involved in TP agonist actions on guinea-pig coronary artery (Morinelli *et al*., 1994) and human uterus smooth muscle cells (Miggin & Kinsella, 2001). In the current study, we have also examined the action of several MAP kinase inhibitors on the contractile activity of U-46619 and sulprostone on guinea-pig aorta.

$EP_3$ receptor-mediated contraction also occurs *in vivo*, as shown by the recent studies of Audoly *et al*. (1999), where deletion of the $EP_3$ receptor gene increased the depressor effect of intravenous $PGE_2$ in male mice, but not in female mice. In this situation, there may be synergism between the cellular events elicited by $PGE_2$ acting on $EP_3$ receptors and noradrenaline released onto $\alpha_1$-adrenoceptors at sympathetic postganglionic synapses. One of our aims is to investigate the nature of this synergism.

## Methods

### *Isolated tissue preparations*

All experimental procedures were performed under licence issued by the Government of the Hong Kong SAR and endorsed by the Animal Research Ethics Committee of the Chinese University of Hong Kong. Male Dunkin–Hartley guinea-pigs, weighing 400–500 g, were killed by cervical dislocation and exsanguination. The descending thoracic aorta was excised and placed in Krebs–Henseleit solution: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.18, $NaHCO_3$ 25, glucose 10 mM. Adherent fat and connective tissue were removed. After cutting 3 mm-wide rings, the interior surface of each was gently rubbed with a wooden toothpick to remove the endothelium. In each experiment, the second to seventh rings of aorta rostral to the diaphragm were used, since they gave similar contractile responses to single concentrations of both U-46619 (100 nM) and phenylephrine (10 $\mu$M) ($n=12$, one-factor ANOVA, $P>0.5$ for all pairwise comparisons of means). The first ring of the aorta was always discarded, since it gave responses to U-46619 and phenylephrine about 30% larger than those of other rings ($P<0.05$). Each ring was suspended in a 10 ml organ bath containing Krebs–Henseleit solution at 37°C and aerated with 95% $O_2$/5% $CO_2$ to maintain pH at 7.4. Washout of the bathing solution was by upward displacement and overflow. Isometric tension changes were recorded with Grass FT03 force transducers linked to a MacLab 4/Macintosh PowerMac computer system (sampling rate 40/min). The bathing solution contained 1 $\mu$M indomethacin to suppress endogenous prostaglandin production.

### *Experimental protocols*

At the start of each experiment, tension was repeatedly applied to the ring until the resting tension remained steady at 1.0 g. Following an equilibration period of 1 h, the preparations were checked for reproducible contractile responses to 40 mM KCl; the rare preparation that showed weak responses to KCl was discarded. Each preparation was then exposed to 100 nM U-46619. After washout of U-46619 and a rest period of 1 h, phenylephrine (10 $\mu$M) was added, followed by acetylcholine (10 $\mu$M); relaxation of less than 5% was taken to indicate adequate removal of endothelium; 25–60% relaxation was seen when the endothelium was nominally intact. The TP receptor antagonist GR 32191 (0.2 $\mu$M) was present in the bathing fluid except when U-46619 was tested.

In inhibitor pretreatment studies, a single concentration of inhibitor was applied for 20 min before starting the cumulative agonist series. Since near-maximal responses to sulprostone and U-46619 were slow to reverse on washout, only one cumulative concentration series was applied to each preparation. For prolonged pretreatment with staurosporine, Krebs solution containing staurosporine or vehicle was perfused through the organ bath at 2 ml min$^{-1}$ for 2 h. In the $Ca^{2+}$ modulation study, six treatments were applied starting 90 min before agonist addition: normal bathing fluid (control); bathing fluid lacking $CaCl_2$ and containing 50 $\mu$M EGTA ($Ca^{2+}$-free); three challenges with 10 $\mu$M phenylephrine at 20 min intervals under $Ca^{2+}$-free conditions ($Ca^{2+}$ store depletion (Phe)); 10 $\mu$M cyclopiazonic acid (CPA); 10 $\mu$M CPA under $Ca^{2+}$-free conditions ($Ca^{2+}$ store depletion (CPA)); 10 $\mu$M CPA followed by three challenges with 10 $\mu$M phenylephrine under $Ca^{2+}$-free conditions ($Ca^{2+}$ store depletion (CPA and Phe)).

In inhibitor post-treatment studies, a stable response to the contractile agent was first established, followed by cumulative addition of the inhibitor. In order to complete the inhibitor series within 1 h, only three or four doses of inhibitor were applied to each preparation, say 10, 100, 1000 nM on one preparation, and 30, 300 and 3000 nM on a second preparation from the same animal. Control (vehicle-treated) preparations were used in each experiment. A balanced design with respect to ring location was employed.

### *Data analysis*

Responses were measured as tension ($g$) generated relative to the resting state. In the inhibitor post-treatment experiments, responses were corrected for the change in tension of the control preparation in each experiment. The results were expressed as mean ± s.e.m., where $n$ represents the number of preparations used (each from a different animal). GraphPad Prism software was used to fit the following sigmoid equation to log concentration–response data:

$$\text{Response} = \text{lower asymptote} + \frac{\text{upper asymptote} - \text{lower asymptote}}{1 + 10^{n(\log EC_{50} - \log A)}}$$

where $A$ is the molar concentration of agent, $EC_{50}$ (or $IC_{50}$) is the molar concentration of agent eliciting a 50% maximal response and $n$ is the slope factor (Hill's factor). The lower asymptote was constrained to the tension immediately prior to the first dose of contractile agent in pretreatment experiments and to 100% in post-treatment experiments.

Statistical analyses were performed with SuperANOVA software (Abacus Concepts Inc., U.S.A.) on tension ($g$) data. The effects of pretreatments on cumulative agonist sequences were determined by repeated-measures two-factor ANOVA coupled with equally weighted planned orthogonal contrasts of cell means (Glass & Hopkins, 1995); the repeated-measures compact included any postaddition nifedipine data. The effects of treatments on established responses were determined by repeated-measures one-factor ANOVA with planned contrasts. All tests were two-tailed and the limit of statistical significance was set at $P = 0.05$.

### *Drugs and solutions*

The following compounds were gifts: sulprostone from Schering AG, Germany; GR-32191 (9α-(biphenylyl)-methoxy-11$\beta$-hydroxy-12$\beta$-(*N*-piperidinyl)-$\omega$1-octanor-prost-4Z-enoic acid) from Glaxo Group Research, U.K.; Y-27632 ((+)-*R*-*trans*-4(1-aminoethyl)-*N*-(4-pyridyl)cyclohexane carboxamide dihydrochloride monhydrate) from Yoshitomi Pharmaceutical Industries, Japan. The following compounds were purchased: PMA (phorbol-12-myristate-13-acetate), Ro 32-0432 (*S*-3-[8-(dimethylaminomethyl)-6,7,8,9-tetrahydropyrido[1,2-a]indol-10-yl]-4-(1-methyl-3-indoly)-1H-pyrrole-2,5-dione, HCl), HA-1077 ((5-isoquinolylsulphonyl)homopiperazine, 2HCl), H-89 (*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulphonamide, 2HCl), SB 202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-imidazole), SB 203580 (4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)1H-imidazole), PD 98059 (2′-amino-3′-methoxyflavone) and staurosporine from Calbiochem Co., Germany; U-46619 (11,9-epoxymethano $PGH_2$) from Cayman Chemical Co., U.S.A.; charybdotoxin (CTX), EGTA, indomethacin, nifedipine, phenylephrine hydrochloride from Sigma-Aldrich, U.S.A. H-1152 ((+)-*S*-2-methyl-1-[(4-methyl-5-isoquinolyl)-sulphonyl]-homopiperazine) was synthesized in Professor Sasaki's laboratory.

Concentrations and solvents for primary stock solutions were as follows: sulprostone, U-46619 and nifedipine (all 10 mM), and indomethacin (20 mM) in absolute ethanol; CPA, PMA, Ro 32-0432, H-89, HA-1077, PD 98059, SB 202190, SB 203580 (all 10 mM) and staurosporine (1 mM) in DMSO; CTX (10 $\mu$M) in 0.9% NaCl solution; phenylephrine (100 mM), H-1152 (1 mM) and Y-27632 (10 mM) in distilled water; EGTA (500 mM) in 10% $NaHCO_3$ solution. All substocks were prepared with 0.9% NaCl solution.

## Results

### *Two components of sulprostone-induced contraction of guinea-pig aorta*

*Variability in sulprostone responses and effects of nifedipine* The selective TP receptor antagonist GR 32191 (Lumley *et al*., 1989) was present at a concentration of 0.2 $\mu$M in the bathing fluid, except when initial near-maximal responses to 100 nM U-46619 and concentration-response relationships for U-46619 were obtained. The expected dose ratio for this concentration is 300–500 based on reported $pA_2$ values of 9.4 and 9.15 for guinea-pig aorta (Ogletree & Allen, 1992; Jones *et al*., 1998).

Near-maximal responses to sulprostone (300 nM) on the aorta showed two phases, a rapid, essentially hyperbolic rise in tension followed by a slow linear rise (Figure 1a). In about 60% of the experiments, responses after 30 min contact were equivalent to 15–48% of the 100 nM U-46619 response, and subsequent addition of 1 $\mu$M nifedipine had no obvious effect, although there were small downward drifts in some preparations (Figure 1a,b). In the remaining experiments, sulprostone responses were equivalent to 38–75% of the 100 nM U-46619 response and nifedipine produced clear partial relaxations. Nifedipine (10 nM–10 $\mu$M) inhibited established contractions to 50 mM $K^+$ by a maximum of 73 ± 4% with an $IC_{50}$ value of 58 nM ($n = 6$, data not shown). Figure 1a shows that 1 $\mu$M nifedipine was sufficient to abolish $K^+$-induced contraction attributable to opening of L-type $Ca^{2+}$ channels. The results agree with our previous study (Jones *et al*., 1998), where sulprostone-induced contraction was partially inhibited by nifedipine in about one-third of the experiments. Preparations having a sulprostone maximum within the lower range and insensitivity to nifedipine have been termed *low-responders*, while preparations having a sulprostone maximum within the higher range and a nifedipine-sensitive component have been termed *high-responders*.

Figure 1c shows the corresponding effects of pretreatment with 1 $\mu$M nifedipine on log concentration–response curves for sulprostone. In low-responders, the mean response to 300 nM sulprostone was about 27% of the 100 nM U-46619 response and nifedipine had no effect on the sulprostone curve ($P > 0.05$ at each concentration level, repeated-measures two-factor ANOVA). In high-responders, the mean response to 300 nM sulprostone was 59% and nifedipine inhibited responses to 30, 100 and 300 nM sulprostone only.

Log concentration–response curves for U-46619 (0.3–1000 nM) and phenylephrine (0.01–100 $\mu$M) (Figure 1d) were highly consistent between preparations in a single experiment and between experiments. Treatment of aorta preparations with 1 $\mu$M nifedipine had little inhibitory effect on the log concentration–response curves for either U-46619 or phenylephrine. Post-addition of 1 $\mu$M nifedipine induced distinct small reductions (9–10%) of the U-46619 response in three

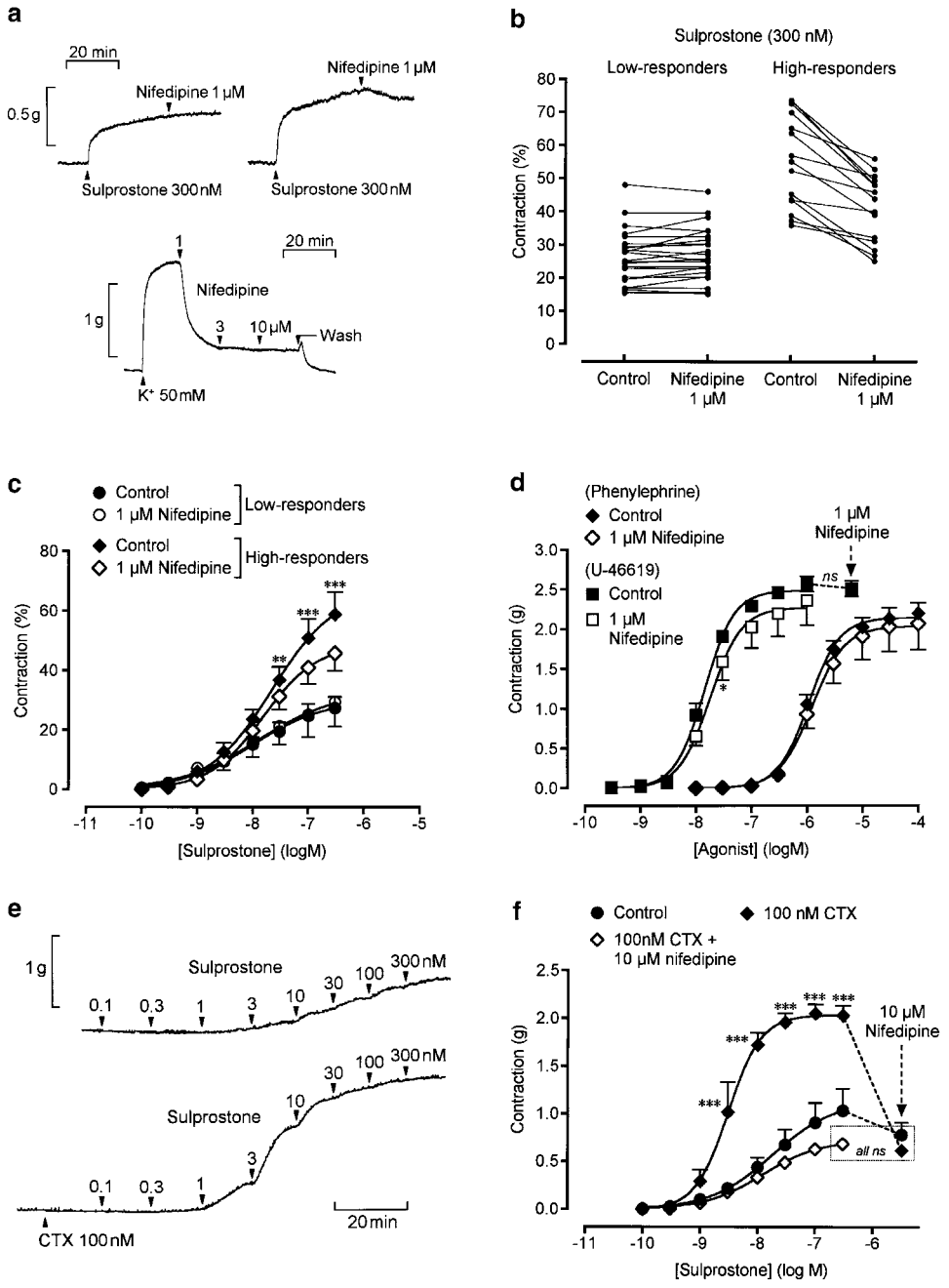


**Figure 1** Characteristics of sulprostone-induced contraction of guinea-pig aorta in normal Krebs solution, (a), (b) and (c) Low/high-responder status defined by lower/higher responses to sulprostone coupled with insensitivity/sensitivity to nifedipine ($n=5$ in panel c). An experimental record of the effect of nifedipine on $K^+$-induced contraction is also shown. (d) Effect of nifedipine on log concentration–response curves for phenylephrine and U-46619 ($n=6$). (e) Experimental record showing enhancement of sulprostone-induced contraction by CTX in preparations from the same guinea-pig. (f) Effect of nifedipine on the log concentration – response curve for sulprostone enhanced by CTX. Data were analyzed by repeated-measures two-factor ANOVA: panels (c), (d) and (f), $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ for comparisons of control and corresponding nifedipine or CTX data, $P>0.05$ for the remaining comparisons; panel (d), *ns* indicates $P>0.05$ for comparison of pre- and post-nifedipine data; panel (f), *all ns* indicates $P>0.05$ for the three contrasts within the box. In panels (b) and (c), contraction is expressed as a percentage of the 100 nM U-46619 response. Values are means ± s.e.m.

preparations and had no effect in the remaining three preparations (Figure 1d).

On both low- and high-responders, increasing the sulprostone concentration to 1 and 3 $\mu$M resulted in small additional contractions, and reversal of response was not seen (data not shown). Runs of low- or high-responders often occurred over periods of 1–3 weeks, and generally this meant that particular treatments in a group of experiments were often assessed on an apparently uniform subset. Low/high-responder status could not be attributed to either the season of the year, changes in husbandry, or differences in body weight of the guinea-pigs, which were all young adult males.

CTX (10–100 nM), a blocker of large-conductance calcium-sensitive $K^+$ ($BK_{Ca}$) channels, enhanced sulprostone responses

in both low- and high-responders. Figure 1e,f shows the effect of 100 nM CTX on the sulprostone log concentration–response relationship in high-responders; the enhancement was abolished by either pre- or post-addition of 10 $\mu$M nifedipine. A high concentration of nifedipine was used in these experiments to ensure complete block of L-type $Ca^{2+}$ channels.

*Removal of external $Ca^{2+}$ and depletion of internal $Ca^{2+}$ stores* High- and low-responder status as defined previously correlated with the effect of removal of external $Ca^{2+}$. For example, in a series of experiments on high-responder preparations, extracellular $Ca^{2+}$ was depleted by omission of $CaCl_2$ from the bathing fluid together with addition of 50 $\mu$M EGTA. Under this *$Ca^{2+}$-free* condition, responses to 100 and 300 nM sulprostone were partially reduced, while responses to lower concentrations were unaffected (Figure 2a). In the same high-responder experiments, removal of extracellular $Ca^{2+}$ partially inhibited responses to phenylephrine and U-46619 over their entire concentration ranges (Figure 2b). Removal of external $Ca^{2+}$ had no effect on sulprostone responses in low-responders, while U-46619 and phenylephrine responses were again partially suppressed over their entire concentration ranges (data not shown).

Two protocols for depleting sarcoplasmic reticulum (SR) $Ca^{2+}$ stores were also employed in the high-responder experiments shown in Figure 2. Firstly, each preparation was challenged three times with 10 $\mu$M phenylephrine under $Ca^{2+}$-free conditions. At the third phenylephrine challenge, the initial phasic response, which is probably due to the activation of PLC$\beta$/$InsP_3$-induced release of internal $Ca^{2+}$, had disappeared, and the tonic response was reduced to 8.4±0.6% of control (Figure 2c). The subsequent log concentration–response curve for sulprostone was not significantly different from that recorded under $Ca^{2+}$-free conditions ($n=12$, $P>0.05$ at each concentration level, Figure 2a). In the second method of $Ca^{2+}$ store depletion, the SR $Ca^{2+}$ pump inhibitor CPA (10 $\mu$M) was used in $Ca^{2+}$-free medium. In normal bathing fluid, CPA induced small contractions (0.04±0.01 g, 2.8% of 100 nM U-46619 response, $n=12$), and also markedly enhanced responses to sulprostone (Figure 2d). CPA had no effect on resting tension in the absence of external $Ca^{2+}$. The log concentration–response curve for sulprostone following depletion of SR $Ca^{2+}$ stores with CPA was not significantly different from that obtained under $Ca^{2+}$-free conditions (compare unfilled squares in Figure 2d with unfilled circles in Figure 2a). Combining both $Ca^{2+}$ store-depletion protocols (treatment order = removal of extracellular $Ca^{2+}$, CPA addi-

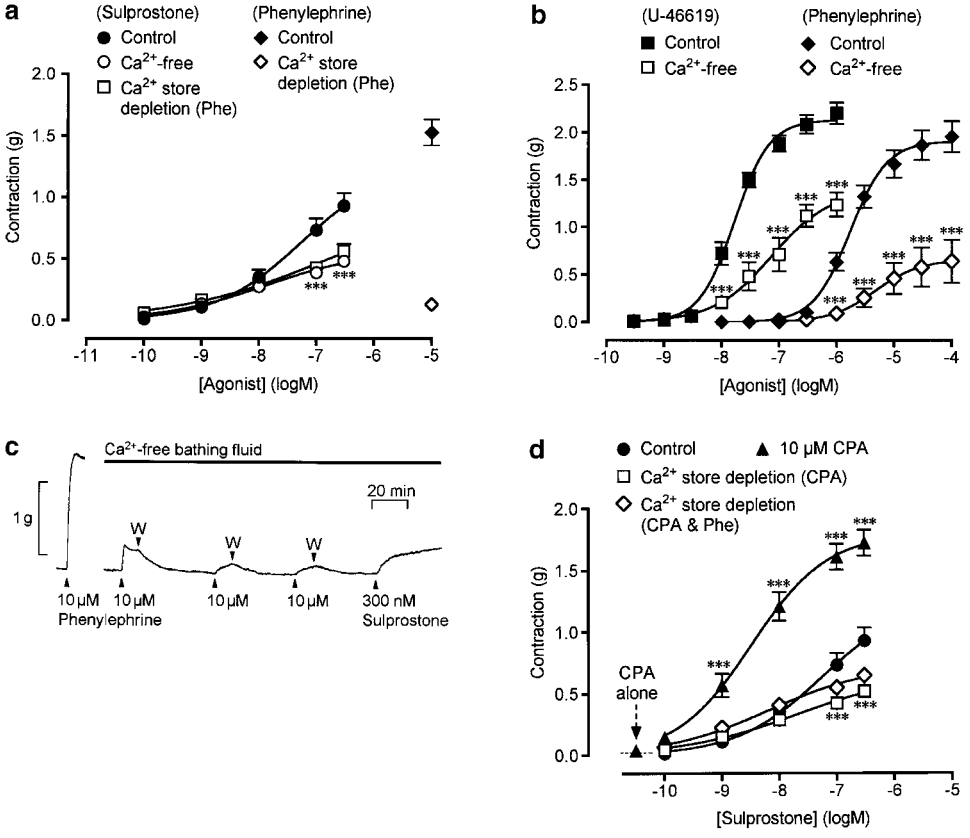


**Figure 2** Effects of $Ca^{2+}$ modulation on agonist-induced contraction of high-responder guinea-pig aorta preparations. (a) Log concentration–response curves for sulprostone under control, $Ca^{2+}$-free and $Ca^{2+}$ store depletion (phenylephrine, Phe) conditions ($n=12$). The control response to 10 $\mu$M phenylephrine and the corresponding third response during the $Ca^{2+}$ store depletion (Phe) procedure are also shown ($n=12$). (b) Log concentration–response curves for U-46619 ($n=4$) and phenylephrine ($n=9$) under control and $Ca^{2+}$-free conditions. (c) Experimental tracing showing the $Ca^{2+}$ store depletion (Phe) protocol and a subsequent response to sulprostone. (d) Log concentration–response curves for sulprostone under control (same as panel a), CPA treatment, $Ca^{2+}$ store depletion (CPA), and $Ca^{2+}$ store depletion (CPA and Phe) conditions ($n=12$). ***$P<0.001$ for comparsion with control data (repeated-measures two-factor ANOVA). Values are means±s.e.m.

tion, repeated phenylephrine) also gave a sulprostone curve similar to that under $Ca^{2+}$-free conditions.

Rings from different locations in the thoracic aorta appeared to be affected similarly by the different calcium treatments shown in Figure 2. Thus for sulprostone, two-factor ANOVAs applied separately to data for each concentration showed that statistical significance ($P>0.05$) was not achieved for either the ring-location factor (six levels) or the ring-location/treatment interaction ($6\times6$ levels).

At this point, we had identified a component of sulprostone-induced contraction that appears to be independent of both $Ca^{2+}$ influx and release of internal $Ca^{2+}$, and is present in all aorta preparations.

### *Effects of protein kinase inhibitors on established contraction to sulprostone in the absence of external $Ca^{2+}$*

Protein kinase inhibitors were added cumulatively to aorta preparations (both low- high-responders) contracted with 300 nM sulprostone in $Ca^{2+}$-free bathing fluid; $IC_{50}$ values are presented in Table 1.

*PKC inhibitors* The PKC activator phorbol myristate acetate (PMA) at 10 $\mu$M slowly contracted aorta preparations, requiring 60–90 min to achieve a stable response of 0.12–0.4 g in both normal and $Ca^{2+}$-free Krebs solution. Preliminary experiments in normal Krebs solution showed that the selective PKC inhibitor Ro 32-0432 (an analogue of staurosporine) abolished established contractions to PMA, with an $IC_{50}$ value of about 200 nM. In $Ca^{2+}$-free Krebs solution, 3 $\mu$M Ro 32-0432 abolished established PMA contractions ($-2.8\pm5.4$% of control, $n=4$) and partially reduced contractions to 100 $\mu$M phenylephrine ($60\pm12$% of control, $n=3$) (Figure 3a). However, Ro 32-0432 had no effect on sulprostone-induced contraction ($106\pm8$% of control, $n=5$).

In contrast, staurosporine (a high potency, low-selectivity PKC inhibitor) inhibited established responses to both PMA and sulprostone. Staurosporine at 30 and 100 nM reduced PMA-induced tone to $75\pm10$ and $15\pm7$% of control after 60 min contact (both $n=3$), but tension was still falling at this time. Its inhibition of sulprostone responses was also slow, with minimal effect ($96\pm4$% of control) at 10 nM, a slow linear fall to $28\pm7$% of control at 100 nM, and reduction almost to the resting tension at 1000 nM (all $n=5$, Figure 3a, b). Staurosporine had similarly slow kinetics in normal Krebs solution (low-responder preparations). Further experiments using a longer exposure time to staurosporine are described later.

*Rho-kinase inhibitors* Established responses to 300 nM sulprostone were markedly inhibited by the cumulative addition of the Rho-kinase inhibitors H-1152, Y-27632 and HA-1077 (0.1–10 $\mu$M) (Figure 3b, Table 1). The slopes of the inhibition curves were $-1.06\pm0.10$, $-1.03\pm0.15$ and $-1.09\pm0.25$ (mean$\pm$s.e.m.), respectively. The inhibitory effects were quite rapid: half-times for mid-range effects were 2.7–3.3–4.3, 2.0–3.2–3.6 and 3.4–3.9–4.4 min, respectively (mean and range, $n=8$). H-89, an inhibitor of both PKA and Rho-kinase, gave more variable results; sulprostone responses were slightly enhanced by 10–300 nM H-89, inhibited or further enhanced at 1 and 3 $\mu$M, and consistently inhibited at 10 $\mu$M (Figure 3b). The Ro 32-0432-resistant component of phenylephrine-induced contraction was abolished by post-addition of 10 $\mu$M Y-27632 (Figure 3a).

*MAP kinase inhibitors* SB 202190, SB 203580 and PD 98059 had minimal effects on sulprostone contractions at 1 and 3 $\mu$M and produced small reductions at 10 and 30 $\mu$M (Figure 3b). SB 202190 and SB 203580 (1–30 $\mu$M) produced similar weak inhibitions of U-46619 (100 nM)-induced contractions, while PD 98059 was more effective: $93\pm2$, $82\pm2$, $67\pm5$ and $43\pm6$% of control at 1, 3, 10 and 30 $\mu$M, respectively ($n=3$).

### *Effects of H-1152 and Y-27632 on established responses to contractile agents in normal Krebs solution*

On aorta preparations (mostly high-responders) in normal Krebs solution, H-1152 had similar inhibitory effects against established responses ($\sim$0.5 g) to both sulprostone (300 nM) and phenylephrine (1 $\mu$M) (Figure 4a); $IC_{50}$ values are given in Table 1. H-1152 reduced larger responses ($\sim$1.5 g) to 10 $\mu$M phenylephrine and 50 mM $K^+$ by a maximum of about 60 and 20%, respectively. Y-27632 had a very similar profile to H-1152, except that it was two to three times less potent (Figure 4b).

**Table 1** Potencies of protein kinase inhibitors for inhibition of guinea-pig aorta contraction and Rho-kinase catalysis

| | *Inhibitory potency* | | | | |
|---|---|---|---|---|---|
| | *Guinea-pig aorta $IC_{50}$* (nM) | | | *Rho-kinase assay* | |
| *Inhibitor* | *Sulprostone $Ca^{2+}$-free* | *Sulprostone 2.5 mM $Ca^{2+}$* | *Phenylephrine 2.5 mM $Ca^{2+}$* | *$IC_{50}$* (nM) | $K_i$ (nM) |
| H-1152 | 190 | 140 | 130 | — | 1.6[a] |
| Y-27632 | 770 | 320 | 310 | 800, 260[b] | 140[a], 90[c], 350[d] |
| HA-1077 | 2030 | — | — | 1900 | 330[a] |
| H-89 | Mixed effect – see text | — | — | 270 | 100[c] |
| Ro 32-0432 | >3000 | >10,000 | — | — | — |
| Staurosporine | ~47 | ~4.5† | — | 1.2[b] | 22[c] |
| PD 98059 | >30,000 | — | — | >50,000 | — |
| SB 202190 | >30,000 | — | — | >10,000 | — |
| SB 203580 | >30,000 | — | — | >10,000 | — |

Guinea-pig aorta data are from the current study: inhibitors were added cumulatively following establishment of contractions to 300 nM sulprostone and 1 $\mu$M phenylephrine, except for †staurosporine, which was preincubated for 2 h before addition of 300 nM sulprostone. Rho-kinase assay data are from [a]Ikenoya *et al.* (2002) (cow), [b]Turner *et al.* (2002) (human, [ATP] = 10 $\mu$M), [c]Feng *et al.* (1999) (chicken) and [d]Uehata *et al.* (1997) (human); other data are from Davies *et al.* (2000) (rat, [ATP] = 100 $\mu$M).

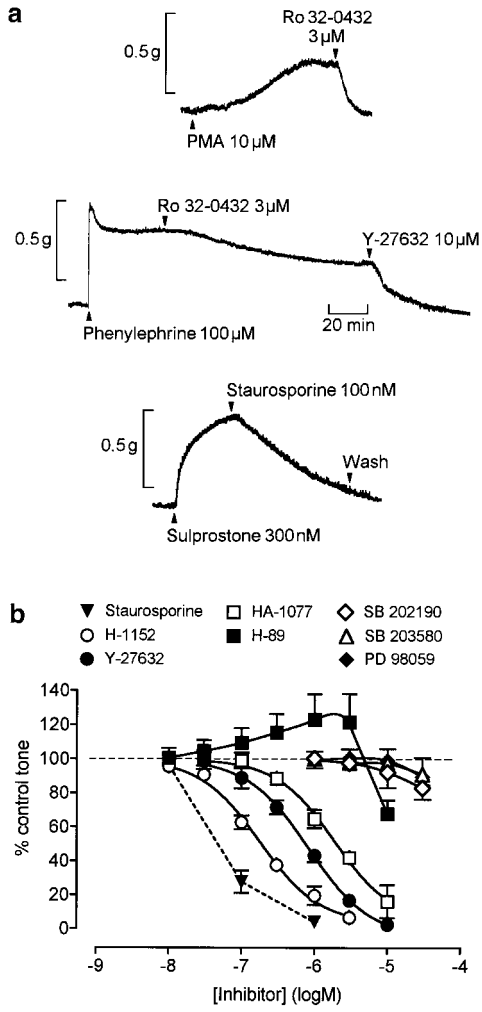


**Figure 3** Effects of protein kinase inhibitors on established contractile responses of guinea-pig aorta in $Ca^{2+}$-free Krebs solution (mixture of low- and high-responders). (a) Experimental tracings showing the effect of Ro 32-0432 on a PMA response, Ro 32-0432 followed by Y-27632 on a phenylephrine response, and staurosporine on a sulprostone response. (b) Log concentration–inhibition curves: tone induced by 300 nM sulprostone ($n=5$–7). For staurosporine, each preparation was exposed to a single concentration of staurosporine for 60 min; the broken line indicates that steady-state inhibition was not achieved. Values are means ± s.e.m.

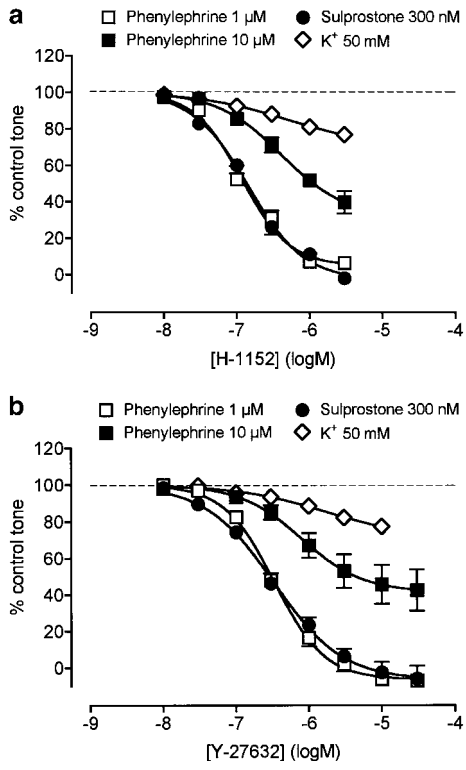


**Figure 4** Effects of Rho-kinase inhibitors on established responses of guinea-pig aorta preparations (mostly high-responders) in normal Krebs solution: (a) H-1152 and (b) Y-27632 vs phenylephrine ($n=4$ and 6), sulprostone ($n=4$ and 6) and $K^+$ ($n=10$ and 6). Values are means ± s.e.m.

In a separate series of experiments on mainly low-responder preparations, established responses to 50 mM $K^+$, which had been reduced to 0.25–0.45 g by 10 μM nifedipine, were further partially inhibited by H-1152 (0.03–3 μM) and Y-27632 (0.1–10 μM) as shown in Figure 5a,b; subsequent addition of 3 μM Ro 32-0432 had no significant effect. A similar profile was obtained when H-1152 and Ro 32-0432 were applied in reverse order, while vehicle controls had no significant effect (Figure 5c,d).

### *Effects of Y-27632 pretreatment on log concentration–response curves to contractile agents in normal Krebs solution*

When added alone, Y-27632 (10 μM) reduced the resting tension of the aorta preparations (range = −0.03 to −0.13 g). Figure 6a shows that 1 μM Y-27632 markedly suppressed sulprostone responses in low-responder preparations when added 20 min before the first agonist dose; the inhibition for 300 nM sulprostone was similar to the postaddition effect shown in Figure 4b. Pretreatment with 10 μM Y-27632 essentially abolished sulprostone responses (data not shown), and shifted the curves for both U-46619 and phenylephrine to the right with 15% reductions in maximum response (Figure 6a).

In a group of high-responder preparations, 100 nM CTX enhanced responses to sulprostone and postaddition nifedipine partially reduced control and CTX-treatment responses to the same tension (Figure 6b, cf Figure 1f). Pretreatment with 10 μM Y-27632 almost completely suppressed responses to 0.3–3 nM sulprostone enhanced by CTX; higher sulprostone concentrations overcame the Y-27632 inhibition, such that the 300 nM sulprostone response was about 50% of the CTX/sulprostone maximum. Postaddition nifedipine reduced the CTX/Y-27632 treatment response by about 80%. Y-27632 (10 μM) was also more effective against low than high phenylephrine responses enhanced by CTX (Figure 6c); postaddition of 1 μM nifedipine had no significant effect on the control phenylephrine response, whereas the corresponding CTX and CTX/Y27632 treatment responses were reduced by 22 and 26%, respectively. Y-27632 (10 μM) inhibited 50 mM $K^+$ responses by 27%, and again nifedipine only produced a further partial inhibition (Figure 6d).

In the same series of experiments, high-responder preparations were exposed to either staurosporine or vehicle for 2 h and then challenged with a single dose of PMA (10 μM, 60 min contact) or sulprostone (300 nM, 30 min contact) (data not

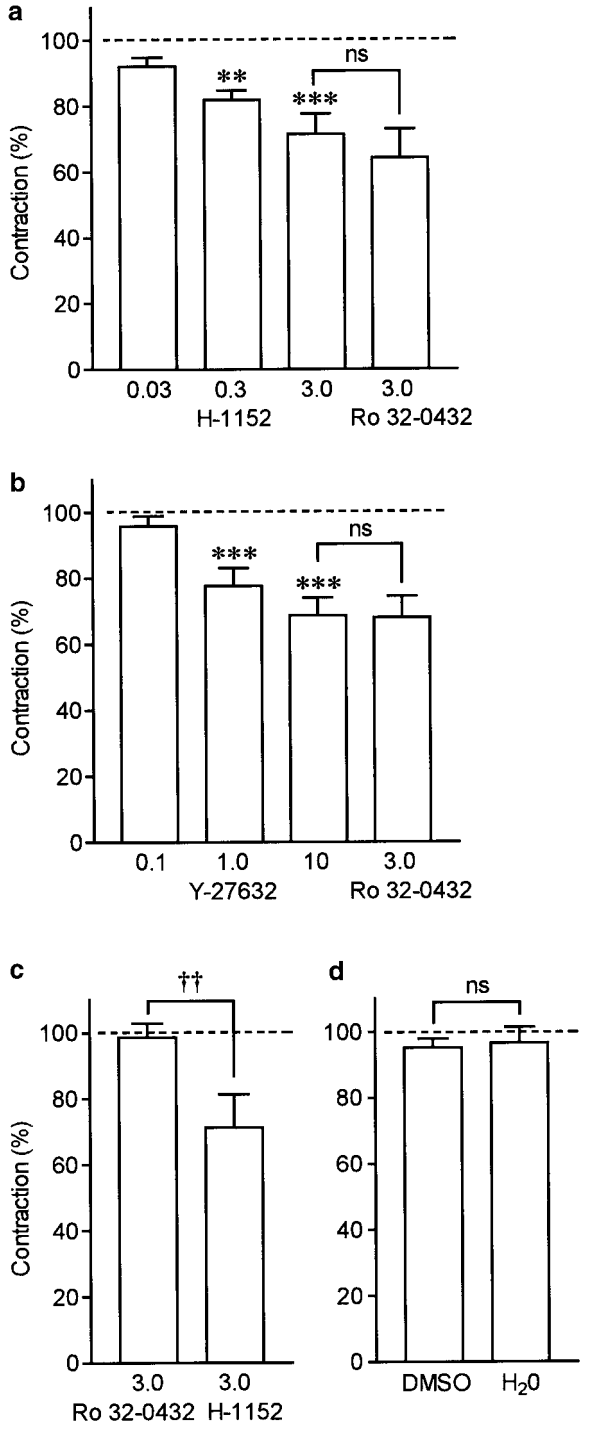


**Figure 5** Effects of cumulative addition of protein kinase inhibitors on $K^+$ (50 mM)-induced contraction of guinea-pig aorta preparations (mostly low responders) suppressed by addition of 10 $\mu$M nifedipine. (a) H-1152 followed by Ro 32-0432, (b) Y-27632 followed by Ro 32-0432, (c) Ro 32-0432 followed by H-1152, (d) vehicle controls for panel c (all $n=4$). $^{**}P<0.01$, $^{***}P<0.001$ compared to control value ($=100\%$); ns $P>0.05$, $^{\dagger\dagger}P<0.01$ for comparison indicated by parenthesis (repeated-measures one-factor ANOVA performed on raw data for each panel). Values are means$\pm$s.e.m.

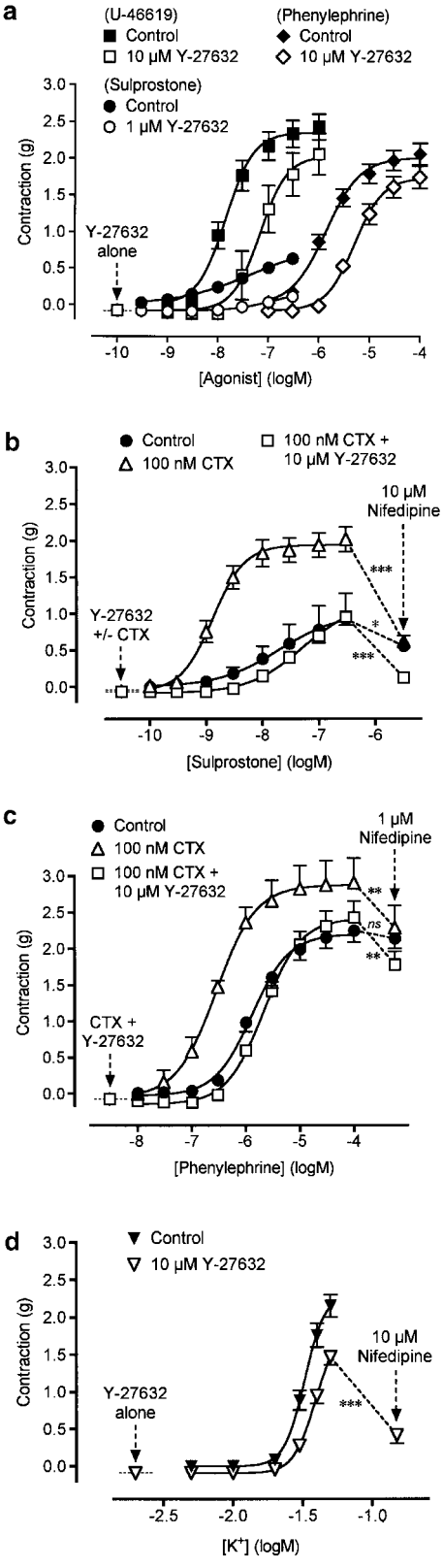


**Figure 6** Guinea-pig aorta in normal Krebs solution: effects of Y-27632 on log concentration–response curves for (a) U-46619, phenylephrine and sulprostone (low-responder preparations, $n=4$), (b) sulprostone in the presence of CTX, (c) phenylephrine in the presence of CTX, (d) $K^+$ (all high-responder preparations, $n=4$). Statistical analysis by repeated-measures two-factor ANOVA: ns $P>0.05$, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ for comparison of pre- and post-nifedipine data. Values are means$\pm$s.e.m.

shown). PMA responses were abolished by 30 and 100 nM staurosporine (both $n=4$), while sulprostone responses were reduced to $64\pm12$ ($P=0.074$), $37\pm6$ ($P<0.01$) and $20\pm8\%$ ($P<0.01$) of control by 2, 10 and 100 nM staurosporine, respectively ($n=4$, one-factor ANOVA); the $IC_{50}$ value of staurosporine was about 4.5 nM by interpolation (Table 1).

## Discussion

Contraction of guinea-pig thoracic aorta induced by the $EP_3$ receptor agonist sulprostone clearly has two components. The primary component is present in all preparations, is independent of the presence of external $Ca^{2+}$, and occurs at a threshold concentration of about 0.3 nM. The secondary component is only present in about 40% of preparations, involves $Ca^{2+}$ influx *via* L-type $Ca^{2+}$ channels, and occurs at sulprostone concentrations $\geqslant10$ nM. In both situations, the maximum response is lower than that achievable by either an $\alpha_1$-adrenoceptor agonist (phenylephrine) or a TP receptor agonist (U-46619). The guinea-pig aorta contains prostanoid $EP_2$ and IP (prostacyclin) receptors mediating relaxation, and we have previously shown that increasing concentrations of $PGE_2$ and its analogue misoprostol, which are potent agonists for both $EP_2$ and $EP_3$ receptors, exhibit reversal of contraction as their $EP_2$ effects gain the upper hand (Jones *et al*., 1998). Although sulprostone responses showed neither fade nor reversal of contraction, this does not exclude functional antagonism as a cause of its lower maximum. However, we believe that this is unlikely given the reported high selectivity of sulprostone for $EP_3$ receptors over $EP_2$ and IP receptors in both isolated tissue (Coleman *et al*., 1987) and membrane preparations (Boie *et al*., 1997; Kiriyama *et al*., 1997; Abramovitz *et al*., 2000) from several species.

### *Nature of the primary contractile component*

Repeated application of phenylephrine under $Ca^{2+}$-free conditions (Low *et al*., 1994) was used to deplete SR $Ca^{2+}$ stores involved in phasic contraction to $\alpha_1$-adrenoceptor activation (probably a $G_q$/PLC$\beta$/$InsP_3$-mediated event). This procedure did not affect the log concentration–response curve for sulprostone when compared to the $Ca^{2+}$-free condition. More extensive depletion of SR $Ca^{2+}$ stores occurs with CPA, a powerful inhibitor of the SR $Ca^{2+}$ pump (Deng & Kwan, 1991; Darby *et al*., 1993; Dettbarn & Palade, 1998). At high concentrations ($>1\,\mu$M) of CPA, $Ca^{2+}$ depletion becomes so effective that store-operated $Ca^{2+}$ channels (SOCC) are activated as a means of replenishing the SR stores; omission of $Ca^{2+}$ from the bathing solution prevents their replenishment (Putney, 1986; Putney & McKay, 1999; Ng & Gurney, 2001). Again, the sulprostone curve obtained following the CPA store-depletion procedure (or even both store-depletion procedures) did not differ from that under $Ca^{2+}$-free conditions. We therefore propose that the primary component of sulprostone action involves a contractile pathway that is largely independent of both the influx of $Ca^{2+}$ from the extracellular fluid and release of $Ca^{2+}$ from SR stores.

A possible mechanism for the primary contractile component is enhancement of the $Ca^{2+}$ sensitivity of the MLC-contractile protein system. Historically, conventional and novel PKCs (cPKC, nPKC), activated by DAG derived from $G_q$/PLC$\beta$ activation, have been the main contenders for this mechanism (see Somlyo & Somlyo, 2000; Webb *et al*., 2000). Activated cPKC/nPKC phosphorylates CPI-17, which inhibits myosin phosphatase leading to a rise in the level of phosphorylated MLC (Eto *et al*., 1995; Senba *et al*., 1999). In our study, the staurosporine analogue Ro 32-0432 was used as a selective PKC inhibitor based on its $IC_{50}$ values for cPKC and nPKC of 9–37 and 108 nM, respectively, and its much weaker inhibition of MLC kinase ($IC_{50}=11.3\,\mu$M), PKA (22.4 $\mu$M), and various tyrosine kinases ($>100\,\mu$M) (Wilkinson *et al*., 1993; Birchall *et al*., 1994). Ro 32-0432's $IC_{50}$ value of 200 nM for reduction of PMA-induced contraction is in good agreement with the above values for PKC inhibition. Ro 32-0432 did not affect sulphostone responses (in either the presence or absence of external $Ca^{2+}$), indicating that PKC is unlikely to be involved in the primary component of $EP_3$ agonist action. PKC present in guinea-pig aorta smooth muscle cells is certainly activated by G-protein-coupled receptors as shown by the inhibitory effect of Ro 32-0432 on phenylephrine-induced contraction under $Ca^{2+}$-free conditions.

A search for other pathways involved in $Ca^{2+}$ sensitization in smooth muscle revealed that downregulation of PKC by prolonged incubation with a phorbol ester inhibited $Ca^{2+}$-sensitization due to the phorbol ester, but not that due to agonists coupling to G-proteins (Hori *et al*., 1993; Jensen *et al*., 1996). The Rho-kinase pathway emerged as a major candidate. Following activation by the small G-protein Rho-A in its GTP-bound form, Rho-kinase phosphorylates myosin phosphatase converting it to an inactive form, and thereby raising the level of phosphorylated MLC (Kimura *et al*., 1996). From experiments in which GTP-Rho levels were measured, it has been shown that contractions of rabbit isolated aorta by noradrenaline and U-46619 involve activation of Rho-kinase (Sakurada *et al*., 2001). In relation to the present study, the bovine $EP_{3B}$ receptor expressed in the PC12 cell line activates Rho-kinase to cause neurite retraction, and PKC is not involved (Katoh *et al*., 1996).

In our experiments, we used four agents with high to medium potency against Rho-kinase (Table 1), but little activity against PKC or MLC kinase: H-1152 (Ikenoya *et al*., 2002), HA-1077 (Nagumo *et al*., 2000) and H-89 (Chijiwa *et al*., 1990) are isoquinoline-sulphonamides, while Y-27632 (Uehata *et al*., 1997) is a 4-aminopyridine analogue. H-1152 and Y-27632, which show high selectivity for Rho-kinase over PKA ($K_i=0.0016$ vs 0.63 and 0.14 vs 25 $\mu$M, respectively), abolished the contractile action of sulprostone in the absence of external $Ca^{2+}$. HA-1077, which has a lower selectivity ($K_i=0.33$ vs 9.3 $\mu$M), also inhibited sulprostone contractions with a slope similar to H-1152 and Y-27632, but the inhibitions at the highest concentration tested (10 $\mu$M) were quite variable. H-89 discriminates poorly between Rho-kinase and PKA ($IC_{50}=0.27$ vs 0.14 $\mu$M; Davies *et al*., 2000). Leemhuis *et al*. (2002) have warned against using H-89 to investigate the role of PKA because of the interrelationship between PKA and Rho-kinase. PKA phosphorylates RhoA and consequently reduces the activation of Rho-kinase; H-89 inhibits PKA and consequently disinhibits RhoA; at the same time H-89 can directly inhibit Rho-kinase. By analogy, enhancement of the sulprostone response by H-89 at low concentrations (0.03–1 $\mu$M) may be due to inhibition of a tonically active adenylate cyclase/PKA system, while reduction of response at higher H-89 concentrations (1–10 $\mu$M) may be due to inhibition of downstream Rho-kinase. The well-documented low selectivity

of staurosporine as a protein kinase inhibitor has recently been extended to include Rho-kinase: the $K_i$ for the human enzyme is 22 nM (Turner *et al*., 2002) and the $IC_{50}$ for the chicken enzyme is 1.2 nM (Feng *et al*., 1999). It is possible therefore that staurosporine's potent suppression of sulprostone-induced contraction of the aorta is due to inhibition of Rho-kinase.

The other three agents examined inhibit MAP kinase cascades: SB 202190 and SB 203580 are specific for p38 MAP kinase (SAPK2a) ($IC_{50} = 50$ nM for both, Davies *et al*., 2000), while PD 98059 prevents the activation of MAP kinase kinase (MKK1 or MEK1) (Alessi *et al*., 1995); it was however inactive in the MKKI assay of Davies *et al*. (2000). None showed any effect on sulprostone-induced contraction at concentrations less than 10 $\mu$M. The small reductions seen at 10–30 $\mu$M of the three agents may be due to inhibition of Rho-kinase, since Davies *et al*. (2000) have shown that 10 $\mu$M SB 202190 and 10 $\mu$M SB 203589 inhibit human Rho-kinase by $39 \pm 2$ and $23 \pm 1$%, respectively (mean $\pm$ s.e.m.); PD 98059 at 50 $\mu$M reduced Rho-kinase activity by $20 \pm 3$%; several bisindoylmaleimides related to Ro 32-0432 did not inhibit Rho-kinase.

Our finding of the same potency ranking for the more selective Rho-kinase inhibitors (H-1152 > Y-27632 > H-1077) in the functional and enzyme assay systems (Table 1) may imply a role of Rho-kinase in $EP_3$ agonist-induced contraction, and the results for the less-selective inhibitors add weight to this proposal. However, close agreement between the 'inhibition constants' would make the picture more convincing. Examination of Table 1 shows that, while agreement is generally good, H-1152 is much more potent in the Rho-kinase assay. The problems of using inhibition constants for protein kinase inhibitors obtained by an enzyme assay to infer second messenger mechanisms in a functional system have been discussed by Davies *et al*. (2000). The authors point out that inhibitory potency is often greater in the enzyme assay, and this may be due to two factors. Firstly, a low $IC_{50}$ value can result from inappropriate enzyme assay conditions, particularly the use of low ATP concentrations that do not match the high intracellular concentration of ATP. This is unlikely to be the explanation here since $K_i$ values are available for the important inhibitors. Secondly, the inhibitor may penetrate poorly into cells. Staurosporine showed slow kinetics on the guinea-pig aorta, and a similar profile has been reported previously on rabbit aorta against noradrenaline-induced contraction (Sasaki *et al*., 1991). Slow accumulation within the smooth muscle cells may be the cause. However, H-1152, Y-27632 and HA-1077 showed comparable and fairly fast time courses for inhibition of sulprostone responses. The question then arises: are the bathing fluid and intracellular concentrations of the inhibitor similar during the observed steady state? A third factor that may confound our comparison of functional and biochemical data is that the Rho-kinase inhibition constants shown in Table 1 derive from four species, none of which is the guinea-pig.

### *Tonic Rho-kinase activity*

H-1152 and Y-27632 showed similar profiles against established responses to sulprostone, phenylephrine and $K^+$, with greater inhibition of weak responses to phenylephrine and least inhibition of $K^+$ responses (which matched the strong response to phenylephrine). The simplest explanation for these data is that all of the agents directly activate the Rho-kinase pathway, while some also activate other pathways to produce contraction. Examination of data for rat intrapulmonary artery in Robertson *et al*. (2000) shows that 10 $\mu$M Y-27632 was less effective against matching responses to $K^+$ ($\sim$55% inhibition) compared to $PGF_{2\alpha}$ ($\sim$95% inhibition); $PGF_{2\alpha}$ was probably acting as a TP-receptor agonist in this preparation (our inference). It has also been reported that Y-27632 (10 $\mu$M) markedly reduced resting tone of human internal mammary artery (Batchelor *et al*., 2001); Y-27632 also reduced tension to below the resting level in our experiments. Batchelor *et al*. point out that, since Rho-kinase appears to be basally active (Gong *et al*., 1996; Fu *et al*., 1998), inhibition of Rho-kinase should still be effective in inhibiting contraction of agonists that exert their effects principally *via* $Ca^{2+}$ influx or $Ca^{2+}$ release from stores. The term *permissive* may be applied to this basal operation of the Rho-kinase pathway, which produces a limited degree of $Ca^{2+}$-sensitization and enhancement of agonist-induced contraction. Of possible relevance here is the finding by Hasegawa *et al*. (1997) that the $\beta$-isoform of the mouse $EP_3$ receptor expressed in MDCK cells activates $G_{13}$/Rho in an agonist-dependent mode, while the $\alpha$-isoform activates $G_{13}$/Rho constitutively. Determining the relative contributions of permissive and direct Rho-kinase mechanisms to $EP_3$ agonist action in vascular smooth muscle will in the first instance require a biochemical method capable of accurately measuring both the resting level of Rho-kinase activity and the reduction of this level by a Rho-kinase inhibitor. We are pursuing this goal, along with the identification of the $EP_3$ receptor isoforms present in guinea-pig aorta smooth muscle cells.

### *$Ca^{2+}$ influx component*

The $Ca^{2+}$ influx component of sulprostone-induced contraction was only discernible at the higher concentrations of sulprostone. It is possible that the $Ca^{2+}$ influx is a consequence of increasing activation of the Rho-kinase pathway by sulprostone. Alternatively, from our knowledge of recombinant $EP_3$ receptors (see Breyer *et al*., 2001), the higher sulprostone concentrations may activate an $EP_3$ isoform that couples to a G-protein regulating $Ca^{2+}$ influx (with no direct involvement of Rho-kinase). In this situation, it would still be possible for a Rho-kinase inhibitor to abolish contraction deriving from both mechanisms (e.g. in high-responders) if the $Ca^{2+}$ influx was modest and depended mainly on Rho-kinase activity for its expression. The same mechanism could explain how Y-27632 abolishes responses to low concentrations of sulprostone (e.g. 1 nM, Figure 6b) in the presence of CTX, which enhances $Ca^{2+}$ influx through L-type channels by reducing the hyperpolarizing influence of $K^+$ efflux through plasma membrane $BK_{Ca}$ channels. When $Ca^{2+}$ influx is larger (e.g. 300 nM sulprostone + CTX), the partial inhibition of contraction by the Rho-kinase inhibitor may be explained by a limited ability of noninhibited myosin phosphatase to oppose the drive from $Ca^{2+}$-MLC kinase. The same arguments could also be applied to the effect of Y-27632 on low and high phenylephrine responses in the absence and presence of CTX.

On guinea-pig aorta, Low *et al*. (1994) showed that the PKC inhibitor calphostin C and the 'nonspecific kinase inhibitor' H-7 reduced 100 mM $K^+$ responses by about 25 and 12%, respectively, while calphostin C reduced 100 mM $K^+$ responses by about 50% in the presence of nicardipine or the absence of

external $Ca^{2+}$. They suggested that high $K^+$ may activate PKC in addition to opening L-type $Ca^{2+}$ channels. In our experiments, the nifedipine-resistant response to 50 mM $K^+$ was unaffected by Ro 32-0432, but partially inhibited by H-1152 and Y-27632. Surprisingly, we could not show any inhibitory activity of calphostin C against either PMA- or sulprostone-induced contractions, either with or without photo-activation (see Bruns *et al*., 1991) of the calphostin C in the bathing fluid (unreported observations). Uehata *et al*. (1997) have reported a $K_i$ value of 0.45 $\mu$M for H-7 against Rho-kinase, and lesser activity against PKC (7.7 $\mu$M) and PKA (5.7 $\mu$M). It is possible therefore that the inhibitory effects of H-7 on $K^+$-induced contraction may be due to inhibition of Rho-kinase rather than PKC.

## Concluding remarks

In guinea-pig aorta, a major component of $EP_3$ receptor agonist-induced contraction is independent of $Ca^{2+}$ influx and $Ca^{2+}$ release from internal stores. Activation of Rho-kinase appears to be involved based on the inhibitory effects of both selective and nonselective Rho-kinase inhibitors, and this may lead to $Ca^{2+}$-sensitization. In separate studies using the patch-clamp technique, we have shown that sulprostone opens nonspecific cation channels in isolated smooth muscle cells from guinea-pig aorta (Shum *et al*., 2002). The ensuing inward current may produce sufficient membrane depolarization on its own or *via* the opening of $Ca^{2+}$-activated $Cl^-$ channels to open L-type $Ca^{2+}$ channels (Hirakawa *et al*., 1999), thereby causing further contraction (see Large, 2002). Further studies are in progress to determine the factors that might lead to variability in these currents in the isolated vessel and thereby explain the low/high-responder situation that we have described.

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