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Receptor-independent activation of Rho-kinase-mediated calcium sensitisation in smooth muscle

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1 The aim of this work was to determine whether Rho-kinase-mediated calcium sensitisation contributes to contractions of the mouse anococcygeus smooth muscle and, if so, whether the process was activated by receptor-dependent or receptor-independent mechanisms.

2 The Rho-kinase inhibitor Y27632 produced concentration-dependent decreases in tone raised by either the muscarinic receptor agonist carbachol (CCh), or the sarco-endoplasmic reticulum calcium ATPase inhibitor thapsigargin (Tg) (EC_{50} values against CCh and Tg of 8.4 ± 3.3 ($n=6$) and 6.1 ± 2.1 ($n=7$) μM, respectively). Pretreatment of tissues with Y27632 also inhibited contractions produced by 65 mM external potassium ($69 \pm 7\%$ ($n=4$) inhibition using 10 μM Y27632). Y27632 had no effect on contractions produced by the inhibitor of smooth muscle myosin light-chain phosphatase, calyculin-A.

3 In β-escin-permeabilised preparations, both CCh and Tg produced significant increases in tone over-and-above that produced by a combination of calcium (1 μM) and GTP (100 μM). These responses to CCh and Tg were inhibited by Y27632 (10 μM).

4 Western blot analysis of fractionated tissue samples probed for RhoA immunoreactivity, indicated that both CCh and Tg were able to induce translocation of RhoA from the cytosol to the membrane.

5 These findings suggest that Rho-kinase-mediated calcium sensitisation is activated by both receptor-dependent and receptor-independent mechanisms in the mouse anococcygeus.
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Abbreviations: BSA, bovine serum albumin; $[Ca^{2+}]_i$, concentration of free intracellular calcium; CCh, carbachol; GEF, GTP exchange factor; L-NOARG, L-N^G-nitroarginine; MLC_{20}, myosin light chain; MLCK, myosin light-chain kinase; PBS, phosphate-buffered saline; PKG, cGMP-dependent protein kinase; SERCA, sarco/endoplasmic reticulum CaATPase; SMPP-1M, smooth muscle myosin light-chain phosphatase; SNP, sodium nitroprusside; Tg, thapsigargin

Introduction

Calcium sensitisation in smooth muscle produces an increase in contractile force for a given concentration of free intracellular calcium ($[Ca^{2+}]_i$; reviews; Savineau & Marthan, 1997; Hori & Karaki, 1998; Somlyo & Somlyo, 1998). In addition to being involved in the maintenance of smooth muscle tone, the biochemical pathways that underlie calcium sensitisation offer targets through which inhibitory drugs or neurotransmitters might act to relax smooth muscle. The best-described sensitising mechanism involves inhibition of smooth muscle myosin light-chain phosphatase (SMPP-1 M) and is summarised in Figure 1. Contraction is initiated following phosphorylation of the regulatory myosin light chain (MLC_{20}) by the Ca^{2+}–calmodulin-dependent myosin light-chain kinase (MLCK). SMPP-1 M counteracts the effects of MLCK, dephosphorylating MLC_{20}, leading to relaxation. Inhibition of SMPP-1 M will increase the extent of MLC_{20} phosphorylation (and force) at a constant calcium concentration. This can occur following phosphorylation of SMPP-1 M by Rho-kinase, a Ser/Thr protein kinase, activated by the small monomeric G-protein RhoA. The precise mechanism by which RhoA is activated in smooth muscle remains to be elucidated, but it is thought to involve the exchange of GDP for GTP, mediated by guanine nucleotide exchange factors (GEFs), possibly initiated as a result of receptor activation of trimeric G-proteins (Somlyo & Somlyo, 1998).

Activation of Rho-kinase requires that RhoA-GTP becomes associated with the plasma membrane and consequently, calcium sensitisation is preceded by translocation of RhoA from the cell cytosol to the membrane (Gong *et al*., 1997).

For a number of years now, we have been using the mouse anococcygeus muscle as a relatively simple preparation in which to study smooth muscle excitation–contraction mechanisms. This tissue offers the advantage of producing strong, well-maintained contractions to a range of drugs acting through widely different mechanisms, including muscarinic receptor agonists, the sarco-endoplasmic reticulum calciumATPase (SERCA) inhibitor thapsigargin (Tg) and raised external potassium. An early observation that both carbachol (CCh) and Tg can produce strong, well-maintained contractions in the face of relatively small maintained increases in

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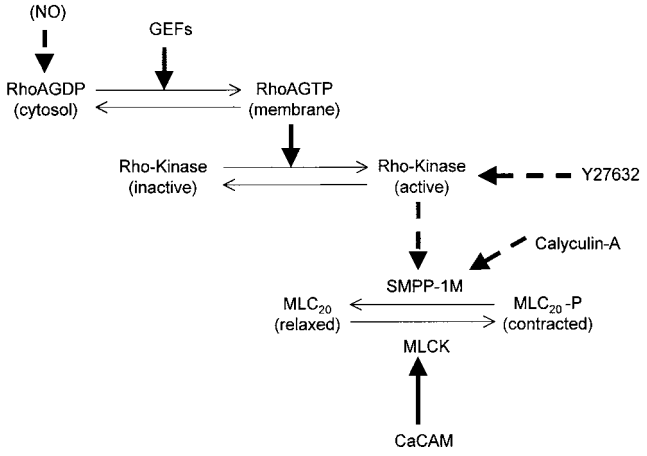

Figure 1 Diagram illustrating RhoA-mediated calcium sensitisation and sites at which Y27632 and calyculin act to interfere with the sensitisation process. Bold, solid arrows indicate stimulatory effects; bold broken arrows indicate inhibitory effects. See text for further details.

$[Ca^{2+}]_i$ (Wayman *et al*., 1999), suggested to us that not only might calcium sensitisation be important in this tissue, but that this process might be activated by both receptor-dependent and receptor-independent mechanisms (CCh and Tg, respectively). Recently, we presented preliminary data using β-escin-permeabilised tissues that suggested that calcium sensitisation does indeed occur in this muscle (Ayman *et al*., 2001). In the present study, we have extended these observations with the aim of determining first, whether RhoA/Rho-kinase-mediated inhibition of SMPP-1 M is involved in the calcium-sensitisation response; and second, whether calcium sensitisation can be initiated by receptor-independent pathways.

A preliminary report of these findings has been presented to the British Pharmacological Society (Ayman *et al*., 2002).

Methods

Tension studies using intact tissues

Male mice (LACA strain; 25–35 g) were killed by stunning and exsanguination. Anococcygeus muscles were dissected out and set up in 1 ml organ baths containing Krebs' bicarbonate buffer of the following composition (mM); NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.1, maintained at 37°C and gassed with 95% O_2/5% CO_2. An initial resting tension of 400 mg was applied to the tissue and changes in tension recorded using a force–displacement transducer (Grass FT03 or Biegestab K30) attached to a pen-recorder (Graphtec WR3101). Muscles were allowed to equilibrate for at least 30 min before beginning experimental procedures. In order to negate any effects that might be produced by noradrenaline released from sympathetic nerves within the tissue, the Krebs' solution contained phentolamine (1 μM) and the muscles were exposed to guanethidine (30 μM) for 10 min during the equilibration period. Similarly, the Krebs' solution also contained the nitric oxide (NO) synthase inhibitor L-N^G-nitroarginine (L-NOARG; 50 μM) to prevent the synthesis of NO within intrinsic inhibitory nerves. Where contractile responses to raised extracellular potassium were being studied, the concentration of KCl in the Krebs' solution was increased by the equimolar replacement of NaCl.

To record relaxations to drugs, the tone of the muscle was first raised using either CCh (50 μM) or the SERCA inhibitor Tg (100 nM). In previous studies, we have shown these concentrations to be equieffective at producing submaximal contractions (approximately EC_{90}) of the muscle. When Tg was used to raise tone, verapamil (10 μM) was included in the Krebs' solution to inhibit calcium entry through voltage-operated calcium channels. Relaxant drugs were applied only once a stable maintained increase in tone had been achieved. Increasing concentrations of relaxant drugs were applied cumulatively, each concentration being left in contact with the tissue until the response stabilised, before the addition of the next concentration. Relaxant responses were quantified as the per cent inhibition of the increase in tone produced by the contractile agent immediately prior to the first addition of the relaxant drug. In experiments where the contractile response to raised extracellular potassium was studied in the presence of Y27632, tissues were exposed to the inhibitor for 10 min prior to, and during, the exposure to the potassium-rich solution.

Tension studies with β-escin-permeabilised tissues

Mouse anococcygeus muscles were dissected and set up initially in Krebs' solution as described above, with the exception that the experiments were performed at 25°C to prolong tissue viability (Ayman *et al*., 2001). As before, the Krebs' solution contained phentolamine plus L-NOARG and the tissues were incubated with guanethidine for 10 min during a 30 min initial equilibration period. Following this, tissue viability was tested by the addition of CCh (50 μM) to the organ bath. A contraction of greater than 200 mg was considered obligatory before a tissue was taken on to the 'skinning' phase. The Krebs' solution was then replaced with a relaxing solution of the following composition (mM unless otherwise stated); PIPES 20, $MgCl_2$ 7.1, KCl 108, EGTA 2, Na_2ATP 5.9, creatine phosphate 2, creatine phosphokinase 4 U ml^{-1}, E-64 1 μg ml^{-1}, FCCP 1 μM; pH 6.8. The tissue was equilibrated in this solution for 20 min before being exposed to β-escin (50 μM for 10 min) that permeabilises the cell membrane to Ca^{2+}, while leaving G-protein-coupled receptors and their effectors relatively intact (Iizuka *et al*., 1994). The tissue was then returned to relaxing solution and changes in tension measured using a Grass FT03 force–displacement transducer attached to a MacLab® data-acquisition system. The final concentration of free calcium in the bathing solution was calculated (in the presence of 2 mM EGTA) using Chelator® for Windows software and adjusted to the required value by the addition of $CaCl_2$.

Western blot analysis of RhoA translocation

A minimum of 20 anococcygeus muscles (approximately 40 mg of tissue) were used in each assay to allow sufficient protein to be extracted into the cytosolic and membrane fractions. Following the dissection, the tissue was incubated at room temperature for 1 h in 1 ml of Krebs' containing either the drug under study or vehicle, prior to being 'snap frozen' in liquid nitrogen. Each sample was then thawed on ice, the Krebs' solution removed and replaced with 100 μl of ice-cold homogenisation buffer (composition; 'complete protease inhibitor cocktail' (Roche) with added ethylenediaminetetraacetic acid (EDTA) 1 mM, (N-[2-hydroxyethyl]piparazine-N'-[2-

ethanesulphonic acid]) HEPES 1 mM and sucrose 14 mM). The tissues were homogenised on dry ice using a pestle and mortar and the resulting homogenate centrifuged at $7000 \times g$ for 10 min at 4°C. The supernatant was collected and centrifuged further at $113{,}000 \times g$ (Optima TLX Ultracentrifuge, Beckman Instruments) for 1 h at 4°C and the resultant supernatant collected as the cytosolic fraction. The pellet, containing the membrane fraction, was resuspended over ice in 100 μl of homogenisation buffer containing 0.1% Triton X-100.

For Western blots, protein-matched samples were diluted with NuPage™ LDS sample buffer and subjected to electrophoresis (Excell II, Invitrogen) on NuPage™ 4–12% Bis-Tris gels (approximately 15 mg protein per well, depending on sample being assayed) at 150 V for 1 h in the presence of NuPage™ SDS running buffer. Proteins were then transferred to polyvinylidene difluoride membranes at 30 V for 1 hr in the presence of NuPage™ transfer buffer. Membranes were then washed in phosphate-buffered saline (PBS) before being blocked by treatment for 1 h at room temperature in PBS containing Tween-20 (0.05%), dried milk powder (5%) and bovine serum albumin (BSA; 1%). The membranes were then incubated with primary affinity purified polyclonal antibody (rabbit RhoA; 1 : 2000 diluted in blocking solution; Santa Cruz) for 1 h at room temperature before being washed in PBS and incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1 : 10,000 diluted in PBS; Perbio). Membranes were then washed in PBS containing Tween-20 (0.05%). Immunoreactive bands were detected using enhanced chemiluminescence (ECL *plus* kit, Amersham). The integrated optical density (OD) of bands corresponding to RhoA (approximately 21 kDa) in paired membrane and cytosolic fractions was quantified using Scion 'Image for Windows®' software. The OD of the band obtained from the membrane fraction was expressed as a percentage of the total OD of the bands in the membrane and cytosolic fractions to gain an estimate of the extent of RhoA translocation following various drug treatments.

Statistics

Statistical analysis was carried out using Student's *t*-test or ANOVA (with Student–Newman–Keuls *post hoc* test) as appropriate. A *P*-value less than 0.05 was considered significant. All results are expressed as mean ± s.e.m. for the stated number of observations.

Drugs used

All drugs were obtained from Sigma, Poole, U.K. unless otherwise stated. β-escin; calyculin-A (Tocris, Avonmouth, U.K.); CCh; creatine phosphate; creatine phosphokinase; *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64); EDTA; ethylene glycol-*bis*(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA); carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP); guanethidine monosulphate; HEPES; L-NOARG; phentolamine mesylate (Ciba Laboratories, Camberley, U.K.); piperazine-*N*,*N*′-*bis*[2-ethanesulphonic acid] (PIPES); Tg (Sigma and Calbiochem, Nottingham, U.K.); verapamil HCl; Y27632 (Yoshitomi Pharmaceutical Industries, Saitama, Japan). Drugs were prepared as stock solutions in deionised water with the exception of FCCP that was dissolved in 95% ethanol and Tg that was dissolved in dimethylsulphoxide.

Results

Effects of the Rho kinase inhibitor Y27632 on intact tissues

The muscarinic acetylcholine receptor agonist CCh (50 μM) produced a strong (316 ± 63 mg; $n = 6$), well-maintained contraction of the mouse anococcygeus. The cumulative addition of Y27632 (0.1–200 μM) to tissues precontracted with CCh produced a concentration-dependent inhibition of tone (EC_{50} 8.4 ± 3.3 μM; $n = 6$; Figure 2). To determine whether this effect of Y27632 represented an inhibition of muscarinic receptor-mediated calcium sensitization, we carried out a similar series of experiments in tissues precontracted with Tg (in the presence of verapamil), which activates store-operated calcium entry in the mouse anococcygeus (Wallace *et al*., 1999) and produces contractions by a receptor-independent mechanism. Like CCh, Tg (100 nM) produced strong (247 ± 38 mg; $n = 7$) well-maintained contractions of the tissue and this effect was again inhibited by the cumulative addition of Y27632 with an EC_{50} of 6.1 ± 2.1 μM ($n = 7$; Figure 2), a value not significantly different to that seen when CCh was used as the contractile agent. This suggested that Y27632 did not rely on concomitant receptor activation by an agonist to produce its relaxant effect. To confirm this further, we studied the effect of the drug on contractions produced by increasing the extracellular concentration of potassium to 65 mM. As such contractions were not well maintained, we were unable to construct cumulative response curves to Y27632. However, the contractions to potassium were inhibited by 69 ± 7% ($n = 4$) following a 10 min pre-exposure to Y27632 (10 μM; Figure 3a). Using a similar protocol, contractions to CCh (50 μM) were reduced by 56 ± 7% ($n = 4$; Figure 3a). One possible interpretation of these findings is that Y27632 is producing a rather nonspecific inhibition of smooth muscle contractility. In an attempt to clarify this, we studied the effects of calyculin-A, a cell-permeable inhibitor of Type I and Type II phosphatases (Mitsui *et al*., 1994), used widely to inhibit SMPP-1 M in smooth muscle (Ishihara *et al*., 1989; Shiozaki *et al*., 2000; Burdyga & Wray, 2002; Gibson *et al*., 2002). We reasoned that if the relaxant effect of Y27632 was being produced as a result of enhanced SMPP-1 M activity due to removal of the inhibitory influence of Rho-kinase, then its effects should be blocked in tissues treated with calyculin-A. At a concentration of 1 μM, the phosphatase inhibitor produced a slowly developing contraction that reached a peak at around 10 min before diminishing to a stable plateau over a further 10–20 min. Addition of Y27632 (10 μM) during this plateau phase failed to relax the increase in tension produced by calyculin-A (Figure 3a). In contrast, the general smooth muscle relaxant papaverine was equally effective against tone raised by either CCh or calyculin-A (Figure 3b).

Effects of the Rho kinase inhibitor Y27632 on permeabilised tissues

β-escin-permeabilised, or 'skinned', smooth muscles have been used widely to study calcium sensitisation mechanisms,

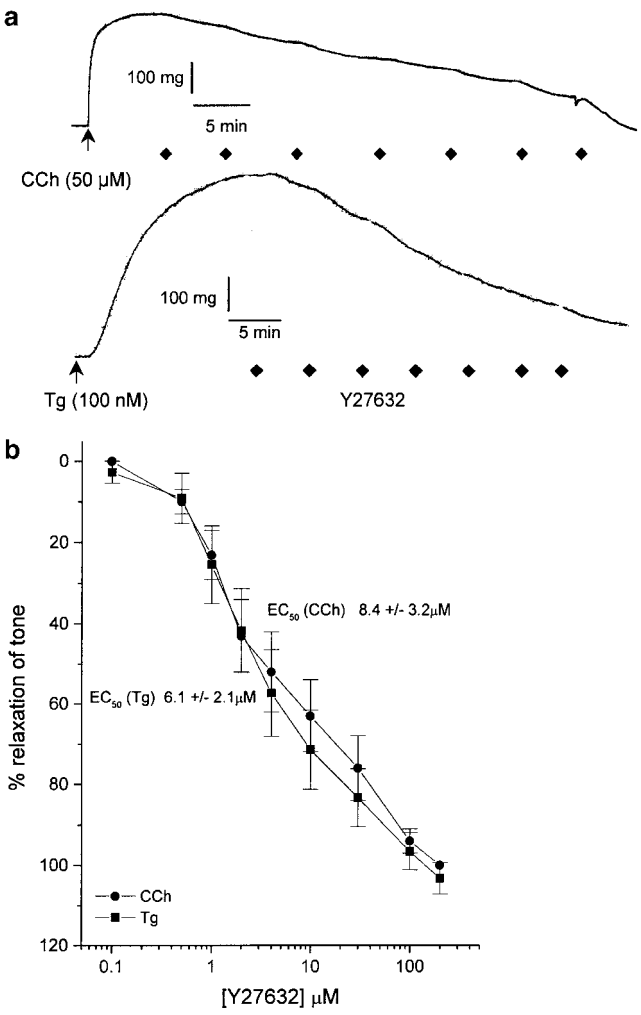

Figure 2 Effects of Y27632 on contractile responses in the mouse anococcygeus to CCh and Tg. Panel (a) shows example traces illustrating the inhibitory effect of Y27632 against CCh- and Tg-induced tone (upper and lower panels, respectively). Increasing concentrations of Y27632 (1, 2, 4, 10, 30, 100 and 200 μM) were applied cumulatively at the time points indicated by the diamond symbols. CCh and Tg were applied where indicated by the arrows. Panel (b) shows full, cumulative concentration–response curves for the inhibitory effects of Y27632 against CCh- and Tg-induced tone ($n=6$ and 7 tissues, respectively).

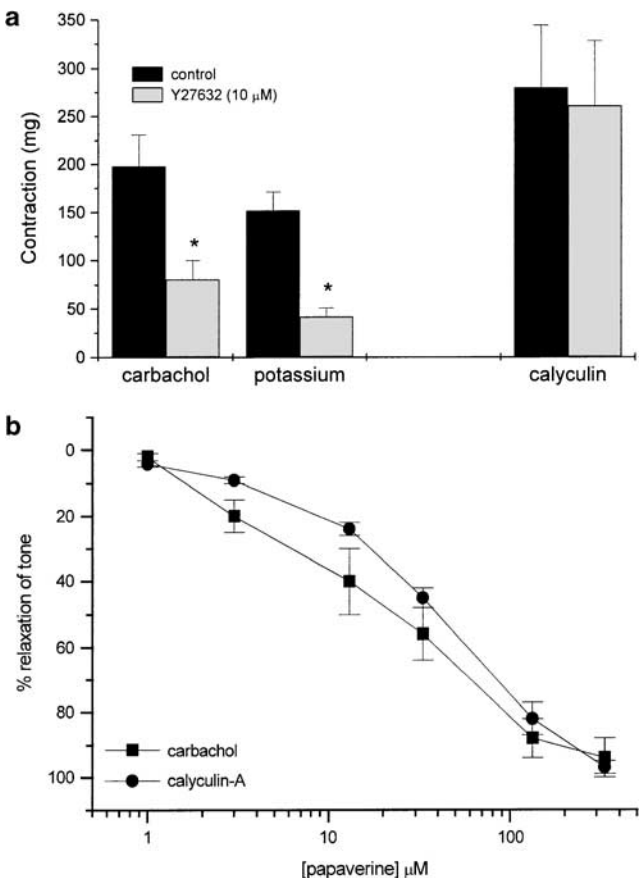

Figure 3 Y27632 is ineffective against contractions produced by the phosphatase inhibitor calyculin-A. Panel (a) shows the inhibitory effect of Y27632 (10 μM for 10 min) against contractions produced by either CCh (50 μM; $n=4$), potassium (65 mM; $n=4$) or calyculin-A (1 μM; $n=3$). *indicates significant difference ($P<0.05$) from matching control. As shown in panel (b), the differential effect of Y27632 against CCh and calyculin-A was in marked contrast to the general smooth muscle relaxant papaverine, which was equally effective at relaxing tone raised by either CCh (50 μM; $n=6$) or calyculin-A (1 μM; $n=6$).

making use of the fact that because the internal milieu of the smooth muscle is a continuum of the extracellular medium in such preparations, the experimenter has some control over the intracellular concentration of calcium ions. As we have reported previously (Ayman *et al*., 2001), the addition of calcium to mouse anococcygeus permeabilised with β-escin produced concentration-dependent contractions, with 1 μM calcium producing approximately 60% of the maximal response. In the present series of experiments, we have used 1 μM calcium throughout and muscle tension is normalised with respect to that seen in the presence of this concentration of calcium. The addition of GTP (100 μM) to tissues exposed to calcium increased tension to $168\pm8.9\%$ ($n=22$). Tension was further increased by the addition of either CCh (50 μM; $304\pm44\%$; $n=6$) or Tg (100 μM; $250\pm31\%$; $n=5$). The response to GTP was not reversed by the addition of Y27632 (10 μM; $n=8$). However, the Rho-kinase inhibitor did reverse the effects produced by CCh ($n=6$) and Tg ($n=5$), tension returning to values (203 ± 42 and $172\pm8.1\%$, respectively) close to those recorded in the presence of calcium and GTP alone (Figure 4). These results suggest that Y27632 can inhibit the sensitising actions of CCh and Tg in β-escin-permeabilised mouse anococcygeus muscle.

Effects of CCh and Tg on RhoA translocation

The results obtained from contractile studies in intact and β-escin-permeabilised tissues point to Rho-kinase-mediated calcium sensitiation being activated by both receptor-dependent (CCh) and receptor-independent (Tg and raised potassium) mechanisms in the mouse anococcygeus. As mentioned above, activation of this sensitisation pathway is associated with translocation of RhoA from the cytoplasm to the plasma membrane and so, by way of confirming our results in the contractile studies, we looked to see whether CCh and Tg were able to initiate RhoA translocation.

As shown in Figure 5, in samples prepared from unstimulated muscles, the amount of Rho-A detected in the membrane fraction was $9.0\pm4.6\%$ ($n=4$) of the total amount detected.

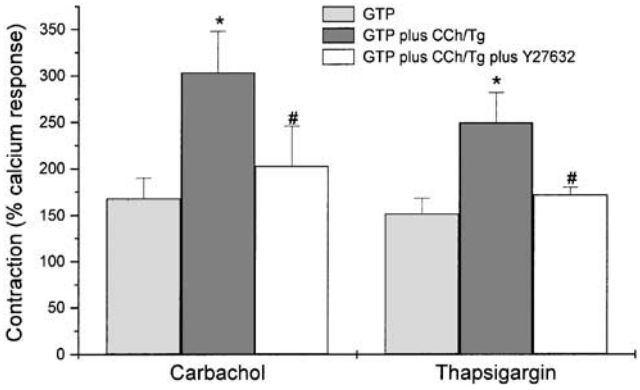

Figure 4 Y27632 inhibits CCh and Tg induced tone in β-escin-permeabilised muscles. Experiments were performed in the presence of 1 μM free calcium. Contractile responses to GTP, CCh and Tg are expressed as a percentage of those seen in the presence of calcium alone. Application of GTP (100 μM) produced a significant increase in muscle tone. This was further increased by the addition of either CCh (50 μM; $n=6$) or Tg (100 nM; $n=5$). The increases in tension produced by both CCh and Tg were inhibited by a subsequent addition of Y27632 (10 μM). *Indicates significantly different ($P<0.05$) from paired contraction to GTP alone. #Indicates significantly different ($P<0.05$) from paired contraction to GTP plus CCh or Tg.

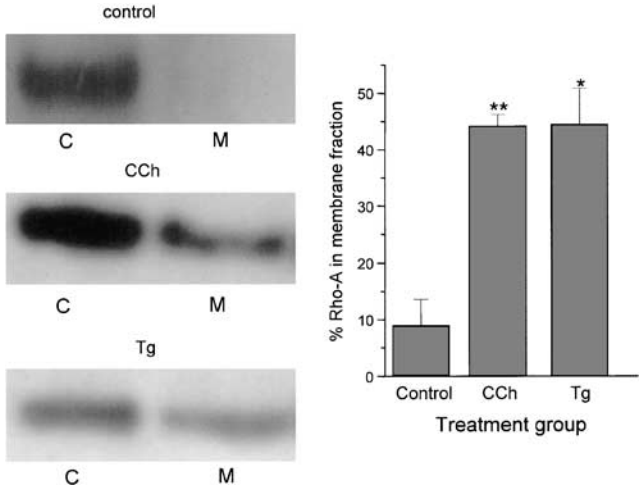

Figure 5 Both CCh (50 μM) and Tg (100 nM) cause RhoA translocation in mouse anococcygeus. The left-hand side of the figure shows representative Western blots of RhoA immunoreactivity in the cytosolic (C) and membrane (M) fractions obtained from tissues treated with either vehicle control, CCh or Tg. The histogram shows pooled data from four control tissues, four tissues treated with CCh and three treated with Tg. Data are presented as the percentage of the total RhoA immunoreactivity detected in the membrane fraction. *indicates $P<0.05$ and **$P<0.01$ when compared to control values.

This percentage was significantly increased in tissues treated with either CCh (50 μM; 44.2$\pm$2%; $n=4$) or Tg (100 nM; 44.5$\pm$6.4%; $n=3$).

Discussion

There are two major findings of this study. First that RhoA/Rho-kinase-mediated calcium sensitisation is essential in the maintenance of tone in the mouse anococcygeus muscle; and second that this process is activated both receptor-dependent and receptor-independent mechanisms.

The Rho-kinase inhibitor, Y27632, produced powerful relaxations of both intact and β-escin-permeabilised preparations of the mouse anococcygeus. This effect relies on a functional SMPP-1 M enzyme, being absent in tissues treated with the SMPP-1 M inhibitor, calyculin-A. RhoA/Rho-kinase-mediated calcium sensitisation is generally thought to be a receptor-mediated phenomenon (Savineau & Marthan, 1997; Hori & Karaki, 1998; Somlyo & Somlyo, 1998). However, several observations point to receptor-independent activation of calcium sensitisation in the mouse anococcygeus: first, the inhibition of Tg- and potassium-mediated contractions by Y27632; second, the contractions produced by Tg in β-escin-permeabilised preparations; third, the enhanced translocation of RhoA from the cytosol to the membrane in muscles treated with Tg.

Y27632 was equally effective at inhibiting tone raised by CCh, Tg and high extracellular potassium. While CCh, acting through M3 muscarinic receptors, would be expected to initiate calcium sensitisation, Tg and potassium produce contractions by receptor-independent mechanisms; the SERCA inhibitor by activating store-operated calcium entry; and potassium by depolarising the muscle membrane and opening voltage-operated calcium channels (Gibson & McFadzean, 2001). Neither mechanism would be expected to activate calcium sensitisation. Broadly speaking there are two possible explanations for these findings. First, the relaxation produced by Y27632 might not result from Rho-kinase inhibition, but from some nonspecific action. The EC_{50} of around 7 μM obtained for Y27632 against both CCh- and Tg-induced tone in the mouse anococcygeus is similar to values reported in other smooth muscles including rabbit aorta (IC_{50} 0.7 μM; Uehata *et al*., 1997), rat aorta (IC_{50} approximately 0.7 μM; Sauzeau *et al*., 2000), human bronchus (IC_{50} 2 μM; Yamagata *et al*., 2000), human pulmonary artery (IC_{50} 1.6 μM; Ito *et al*., 2001), rat bronchus (IC_{50} approximately 10 μM; Chiba *et al*., 2001) and rat mesenteric artery (IC_{50} approximately 1 μM; VanBavel *et al*., 2001). It is however some 30-fold higher than the K_i reported for inhibition of purified Rho-kinase enzyme (Uehata *et al*., 1997). In that study, the drug was reported to inhibit purified protein kinase C and cAMP-dependent kinase, with K_i values around 25 μM. More recently, Davies *et al*. (2000), while generally confirming the selectivity of Y27632, reported that the drug is equieffective at inhibiting Rho-kinase and a protein kinase C-related protein kinase (PKCRK), with an IC_{50} of around 700 nM. Thus, although some nonselective action might underlie the effects of Y27632 in the present study, this would seem unlikely. Our findings that Y27632 did not relax tone raised by calyculin-A in intact muscles or calcium plus GTP in β-escin-permeabilised preparations also argue against a nonspecific inhibitory effect of Y27632 on muscle contractility.

In rabbit aorta, 10 μM Y27632 produced approximately 30% inhibition of the contraction produced by potassium and inhibited receptor-mediated contractions by around 90% (Uehata *et al*., 1997). In the present study, the same concentration of Y27632 inhibited the potassium-evoked contraction by approximately 70% and receptor-mediated contractions by around 55%. Thus, there is a clear distinction between the two tissues in the ability of Y27632 to inhibit tone raised by receptor agonists when compared to that raised by

potassium. Conceivably, raising external potassium may cause the release of excitatory neurotransmitters from intrinsic nerves within the mouse anococcygeus muscle preparation, such that the contraction of the muscle will include a receptor-mediated component produced by these neurotransmitters. However, this is unlikely for two reasons. First, our experiments are carried out on tissues treated with guanethidine and phentolamine, both of which act to abolish the noradrenergic contractile responses seen on electrical field stimulation (Gibson *et al*., 1990). Second, the contractions to potassium are abolished following blockade of voltage-operated calcium channels using nifedipine, while those to CCh and noradrenaline are only inhibited by around 20% (Gibson *et al*., 1994). We are therefore confident that the contractile response to raised potassium is due to a direct effect on the smooth muscle.

Y27632 inhibits Tg-induced contractions in guinea-pig tracheal smooth muscle (Ito *et al*., 2002) albeit at concentrations approximately 10-fold higher than those used in the present study. In trachea, high concentrations of Y27632 ($>10\,\mu$M) also inhibited the mobilisation of calcium in response to receptor agonists (but not Tg; Ito *et al*., 2001; 2002) that may point to a role for the RhoA/Rho-kinase system in intracellular calcium signalling in smooth muscle. Y27632 also inhibits (IC_{50}, $0.4\,\mu$M) the sustained phase of potassium-induced contractions in rat caudal arterial smooth muscle while having no effect on the calcium response (Mita *et al*., 2002).

β-escin-permeabilised smooth muscles are used widely to study calcium-sensitisation mechanisms. β-escin permeabilises the plasma membrane to small molecules, but leaves intact G-protein-coupled receptor signalling mechanisms and the sarcoplasmic reticular membrane (Iizuka *et al*., 1994). Thus, the ability of receptor agonists to cause contractions at a constant 'intracellular' calcium concentration can be taken as a measure of their ability to activate calcium sensitisation. As expected, raising the 'intracellular' calcium concentration from zero to $1\,\mu$M contracted β-escin-permeabilised muscles. This contraction was further enhanced by the addition of GTP, a substrate for RhoA, which might suggest that there is some tonic activation of the RhoA/Rho-kinase system in the tissue. Arguing against this was the finding that Y27632 had no effect on this GTP-mediated contraction. In itself, this was a surprising finding since GTP would be expected to activate RhoA-mediated calcium sensitisation and therefore any related contraction should be sensitive to block by Y27632. At present, we have no explanation for this discrepancy and further work–including measurement of RhoA translocation in response to GTP–is required to clarify this issue. Y27632 did however inhibit the additional contractions produced by both CCh and Tg in the presence of GTP plus calcium, again pointing to Rho-kinase-mediated calcium sensitisation being activated by receptor-independent mechanisms. Activation of RhoA is associated with translocation of the G-protein from the cytoplasm to the plasma membrane (Gong *et al*., 1997). Both CCh and Tg were able to stimulate RhoA translocation in the mouse anococcygeus, supporting our findings in contractile studies.

Calyculin A is a cell-permeable inhibitor of Type I and Type II phosphatases (Mitsui *et al*., 1994) used to study the role of SMPP-1 M in smooth muscle excitation–contraction coupling (Ishihara *et al*., 1989; Shiozaki *et al*., 2000; Burdyga & Wray, 2002; Gibson *et al*., 2002). Although we have not measured SMPP-1 M activity directly, calyculin-A caused a slow contraction of the mouse anococcygeus, as it does in other smooth muscles (Ishihara *et al*., 1989; Uehata *et al*., 1997; Shiozaki *et al*., 2000; Gibson *et al*., 2002), consistent with inhibition of the enzyme. This also suggests that under resting conditions, SMPP-1 M is responsible for maintaining the muscle in a relaxed state, presumably by counteracting tonic activity of MLCK. The finding that Y27632 was unable to relax the increase in tone produced by calyculin-A is consistent with a mechanism of action for Y27632 involving potentiation of SMPP-1 M, and is in marked contrast to papaverine that was equally effective against tone raised by CCh and calyculin-A.

In conclusion, our results suggest that RhoA/Rho-kinase-mediated calcium sensitisation potentiates contractions produced by both receptor-dependent and receptor-independent mechanisms in the mouse anococcygeus, although unequivocal evidence in support of this hypothesis will only come from direct biochemical measurement of the effects of contractile agents on SMPP-1 M activity. How Tg and high potassium might activate the RhoA/Rho-kinase pathway is unclear. One possibility is that activation of the RhoA/Rho-kinase-mediated calcium-sensitisation pathway is intimately linked to depletion of internal calcium stores and activation of store-operated calcium entry. Potassium (by calcium-induced calcium release), Tg (by inhibiting SERCA) and CCh (following liberation of inositol trisphosphate) might each be expected to deplete the sarcoplasmic reticulum of calcium. How this leads to activation of store-operated calcium entry remains a matter of debate in smooth muscle as in other cell types, but one intriguing possibility is that RhoA/Rho-kinase-mediated calcium sensitisation is activated following store depletion and serves to potentiate the effects of the small amounts of calcium that enter through store-operated channels. This could explain the powerful contractile effects produced by Tg in the mouse anococcygeus and some other tonic smooth muscles.

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