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Involvement of Rho kinase and protein kinase C in carbachol-induced calcium sensitization in β-escin skinned rat and guinea-pig bladders

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1 The signal transduction pathways involved in carbachol (CCh)-induced calcium sensitization in β-escin permeabilized rat and guinea-pig bladder smooth muscles were investigated and the results were compared with guinea-pig taenia caecum.

2 Calcium contractions elicited cumulatively (*p*Ca 7.5–5) in the presence of calmodulin were significantly increased in all three tissues when CCh (50 μM) was added to the medium.

3 Under constant $[Ca^{2+}]_i$ conditions (*p*Ca 6), calmodulin (1 μM) and then GTP (100 μM) initiated significant contractions. CCh (50 μM) added to the bath caused a further contraction in all three tissues – calcium sensitization. This sensitization was significantly inhibited by atropine (50 μM).

4 The incubation of the tissues with the IP_3-receptor blocker 2-APB (30 μM) reduced the subsequent development of calcium sensitization by CCh in rat bladder but did not affect it in guinea-pig bladder and taenia ceacum.

5 The Rho kinase (ROK) inhibitor Y-27632 (5 μM) added in the presence of CCh reversed the calcium sensitization in rat bladder, whereas a transient contraction followed by a relaxation to a level not significantly different from the CCh contraction was seen in both guinea-pig bladder and taenia caecum. Y-27632 (1 μM) continuously present significantly inhibited the CCh-induced Ca^{2+} sensitization in rat bladder but not in guinea-pig bladder or taenia caecum.

6 In the presence of cyclopiazonic acid (CPA) (1 μM) and calmodulin (1 μM), Y-27632 (5 μM) did not change the calcium response curve (3×10^{-7}–10^{-5} M) in rat bladder but increased the contractile responses significantly in both guinea-pig bladder and taenia caecum.

7 The protein kinase C (PKC) inhibitor GF 109203X (5 μM) added in the presence of CCh inhibited the calcium sensitization induced by this muscarinic agonist in all three tissues in different ratios.

8 In conclusion, muscarinic receptor activation induces calcium sensitization in rat and guinea-pig detrusor smooth muscles but there are differences in their pathways.
British Journal of Pharmacology (2006) **148,** 376–384. doi:10.1038/sj.bjp.0706723;
published online 27 March 2006

Keywords: Calcium sensitization; bladder smooth muscle; skinned; Rho kinase; protein kinase C; rat; guinea-pig

Abbreviations: 2-APB, 2-aminoethoxydiphenylborate; CCh, carbachol; CPA, cyclopiazonic acid; CPI-17, protein kinase C-potentiated protein phosphatase-1 inhibitory protein; DMSO, dimethylsulphoxide; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluromethoxyphenylhydrazone; GF 109203X, bisindolylmaleimide I; GPCRs, G protein coupled receptors; GTP, guanosine-5′-triphosphate; IP_3, inositol triphosphate; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; *p*Ca, –[log Ca^{2+}]; PC-PLC, phosphatidyl choline specific phospholipase C; PI-PLC, phosphatidylinositol specific phospholipase C; PKA, protein kinase A; PKC, protein kinase C; ROK, Rho kinase; SR, sarcoplasmic reticulum; Y-27632, (R)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxyamide 2HCl

Introduction

Smooth muscle contraction is normally thought to be activated by an increase in cytosolic calcium produced by calcium entry through voltage sensitive calcium channels or by calcium release from the sarcoplasmic reticulum (SR). Agonists activating G protein coupled receptors (GPCRs) increase force in smooth muscle *via* calcium-dependent myosin light chain phosphorylation. The ratio of activities of calcium/calmodulin dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) determines the level of myosin light chain phosphorylation and the activation of the muscle. There are, however, mechanisms which can lead to muscle contraction without any necessary change in intracellular calcium ($[Ca^{2+}]_i$). When $[Ca^{2+}]$i and MLCK activity are constant, agonists activating GPCRs may cause a leftward shift of the calcium/force response curve, and this is called calcium sensitization. A major pathway that can cause this (Horowitz *et al*., 1996; Somlyo & Somlyo, 2000) operates through Rho kinase (ROK) and protein kinase C (PKC), and is thought to involve inhibition of myosin phosphatase activity

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at constant calcium, and therefore enhance myosin light chain phosphorylation and cause an increase in force (Somlyo & Somlyo, 2003).

β-escin is a saponin ester which forms pores in the plasma membrane permeable to molecules of up to 150 kDa (Iizuka *et al.*, 1994). It differs from other tools used to study intracellular signal cascades such as saponin and α-toxin. Saponin retains the G protein/phospholipase C/inositol triphosphate (IP_3) cascade but uncouples the receptors, and α-toxin (first used in arterial smooth muscles (Nishimura *et al.*, 1988)) does not affect receptor–effector coupling but only allows small molecules (such as ATP and EGTA) to penetrate the cell membrane. β-escin retains receptor–effector coupling but opens holes in the plasma membrane that are relatively bigger allowing access to some higher molecular weight compounds including heparin and calmodulin (Kobayashi *et al.*, 1989). Smooth muscle preparations treated with either β-escin or α-toxin have been shown to respond to excitatory agonists through IP_3-mediated release of Ca^{2+}, and also to show calcium sensitization (Kitazawa *et al.*, 1989; Todoroki-Ikeda *et al.*, 2000). However, the ability to introduce larger molecules into the cell makes β-escin a preferable tool for the study of the pathways mediating agonist-induced contraction in smooth muscles (Iizuka *et al.*, 1994).

In this paper, we study the role of calcium sensitization in cholinergically induced contractions of rat and guinea-pig bladder smooth muscle using β-escin permeabilized preparations and carbachol (CCh) to activate the receptors. We compare the results with guinea-pig taenia caecum, a tissue that has been extensively studied by this method. CCh-induced contractions in the urinary bladder have been reported to be mediated by G protein coupled muscarinic M_3 receptors (Longhurst *et al.*, 1995; Tong *et al.*, 1997; Uchiyama & Chess-Williams, 2004). M_3 muscarinic receptors also mediate contraction in guinea-pig taenia caecum (Elnatan & Mitchelson, 1993). A recent study has demonstrated that calcium sensitization occurs in human bladder (Takahashi *et al.*, 2004), but to investigate the potential of drugs acting to modulate this pathway for future therapeutic use, the difficulty in obtaining suitable human material will necessitate development of a suitable animal model.

Methods

Tissue preparation

Male Wistar albino rats (150–200 g) and male guinea-pigs (350–400 g) were stunned and bled to death. The bladders from both species and taenia caecum from guinea-pigs were isolated and placed in HEPES buffered modified Krebs' solution. The mucosa and connective tissues were removed from the bladders under a dissecting microscope. Small strips (150–250 μm in diameter, 3–4 mm in length) of smooth muscle were dissected. Similar sized strips were dissected from the guinea-pig taenia caecum. A small hook was tied to one end of a strip to attach it to the transducer, and a snare of 5/0 surgical silk captured the other end and was used to mount the strip in a fixed position in a 500 μl chamber in one of a series of small chambers in a Perspex block. The chamber was filled with HEPES buffered modified Krebs' solution at room temperature and the strips were equilibrated for 30 min under a resting tension of 100 mg. Solution changes were made by moving the Perspex block. After stable responses had been achieved to 80 mM K^+ and 50 μM CCh in intact strips, they were moved into relaxing solutions, incubated for a few minutes and then permeabilized with 40 and 80 μM β-escin in relaxing solution for 30 min for rat and guinea-pig, respectively, at pH 6.8, followed by 2 min wash in relaxing solution before beginning an experiment. The contractile force was measured by a sensitive force transducer (AD Instruments) connected to an Apple Macintosh PowerBook 1400c computer using Chart software and MacLab 8 hardware.

Drugs and solutions

All drugs and solutions were prepared by using 18 MΩ-cm deionized water (Purelab UHQ, USF ELGA).

Normal tissues HEPES buffered modified Krebs' solution contained (mM) NaCl 126; KCl 6; $CaCl_2$ 2; $MgCl_2$ 1.2; glucose 14 and HEPES 10.5. The pH of this Krebs' solution was adjusted to 7.2 with NaOH. 80 mM K^+ Krebs' solution was prepared by replacing NaCl with an equivalent amount of KCl.

Permeabilized tissues Relaxing solution contained (mM) K propionate 130; $MgCl_2$ 4; Na_2ATP 4; tris-maleate 20; creatine phosphate 10; EGTA 4 and creatine phosphokinase 3.3 U ml^{-1}; protease inhibitor leupeptin (1 μM) and mitochondrial blocker FCCP (1 μM). The pH of this solution was adjusted to 6.8 with KOH. Activating solutions were the same as relaxing solution except that EGTA was lowered to 0.05 mM, free calcium concentration was adjusted to the desired value and 1 μM calmodulin added as specified. GTP (100 μM) was also added when CCh was used to activate the receptors. In experiments with cumulative calcium response curves, EGTA in the activating solution was kept at 0.05 mM. The free calcium concentration was calculated using a computer programme ('Bound and Determined', Brooks & Storey, 1992) and expressed as the negative logarithm (*p*Ca). When calcium response curves were elicited in the presence of an agonist or an antagonist, the substance was added to the calcium solution first and then the pH adjusted to 6.8. When drugs were added to an organ chamber, they were made up in relaxing solution containing 0.05 mM EGTA, and the concentration given is the estimated final concentration. In the expriments with 2-APB, the strips were first incubated with this inhibitor for 15 min in relaxing solution, and then contraction was elicited in the continuous presence of 2-APB. The experiments were carried out with the SR Ca^{2+}ATPase inhibitor cyclopiazonic acid (CPA; 1 μM) present in all solutions after permabilization. GF-109203X was disolved in DMSO and ryanodine was dissolved in ethanol but neither vehicle (0.5% of bathing solution) affected the contractions when tested alone.

Drugs used were β-escin (aescin), carbamylcholine chloride (CCh), creatine phosphokinase, leupeptin, ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), adenosine 5'-triphosphate (Na_2ATP), ryanodine, guanosine-5'-triphosphate (GTP), CPA, calmodulin, carbonyl cyanide *p*-trifluromethoxyphenylhydrazone (FCCP), dimethylsulphoxide (DMSO) from Sigma (St Louis, MO, U.S.A.) and creatine phosphate disodium salt, 2-aminoethoxydiphenylborate

(2-APB), (R)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxyamide 2HCl (Y-27632) ve bisindolylmaleimide I (GF-109203X) from Calbiochem (Nottingham, U.K.).

Data analysis

Contractions are expressed as % of the response to 80 mM K^+ elicited in intact tissues before skinning. Data were given as mean ± s.e.m. of *n* experiments. Statistical analyses were carried out by using one way analysis of variance (ANOVA) followed by Bonferroni test for comparing multiple groups. Student's *t*-test was used for comparing two groups. $P<0.05$ were accepted as statistically significant.

Results

The effect of CCh on cumulative calcium response curves

Cumulative calcium response curves (*p*Ca 7.5–5) were elicited in β-escin permeabilized smooth muscles in 0.05 mM EGTA solution in the presence of calmodulin in rat bladder, guinea-pig bladder and taenia caecum, in the presence and absence of CCh (Figure 1a–c). CCh (50 μM) significantly increased the calcium contractions compared to control curves in all three tissues ($P<0.05$).

CCh-induced calcium sensitization

The ability of CCh to sensitize the contractile apparatus at constant $[Ca^{2+}]_i$ was further studied in β-escin permeabilized smooth muscle strips exposed to calcium (*p*Ca 6) in 0.05 mM EGTA solution. After approximately 10 min and the development of a small contraction, subsequent addition of calmodulin (1 μM) and then GTP (100 μM) caused significant further contraction ($P<0.01$ and $P<0.001$, respectively). Under these constant $[Ca^{2+}]_i$ conditions, CCh (50 μM) added to the bath caused a further contraction in all three tissues used (Figure 2) that was significant compared to GTP contraction. When calcium sensitization is referred to in the rest of this paper, this procedure was used. CCh-induced calcium sensitization was significantly inhibited by the muscarinic receptor antagonist atropine (50 μM) added to the bath ($P<0.05$; Figure 2b–d).

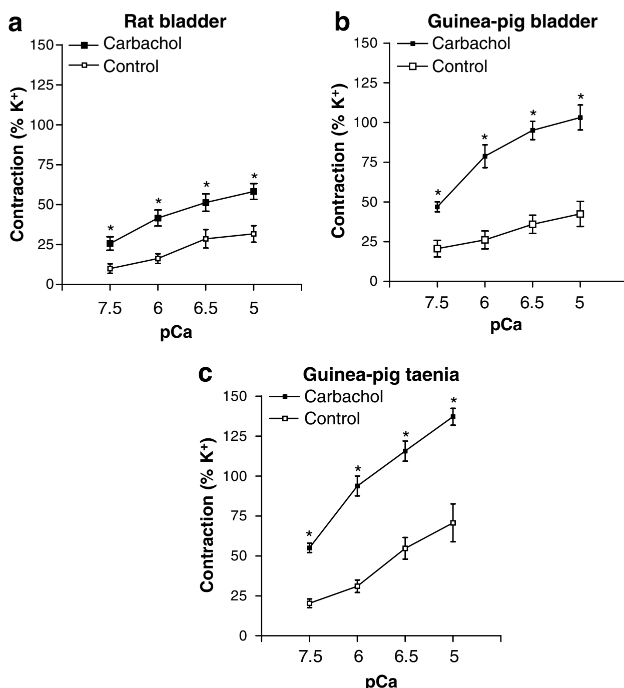

Figure 1 The effect of 50 μM CCh on cumulative calcium response curves. (a) Rat bladder, (b) guinea-pig bladder, and (c) guinea-pig taenia caecum. The *p*Ca was adjusted in activating solutions containing 0.05 mM EGTA, 1 μM CPA, 1 μM calmodulin and 100 μM GTP. In all three groups CCh increased the contraction responses significantly compared to their own controls ($n=4$–6, *$P<0.05$, ANOVA).

The effect of 2-APB on CCh-induced calcium sensitization in the presence of CPA

Since the receptor pathways are intact in these tissues, CCh will induce IP_3 formation and could potentially release Ca^{2+} from SR stores. Although we assume that during skinning in well-buffered solutions (4 mM EGTA, no added Ca^{2+}) the stores will empty, and that addition of CPA will prevent their filling in the activating solutions, to check that the ability of CCh to enhance force does not involve residual release of stored Ca^{2+}, the effects of the IP_3-receptor blocker 2-APB (membrane permeable inhibitor of IP_3 induced Ca^{2+} release) was investigated. Incubation (15 min) with 2-APB (30 μM) reduced the subsequent development of calcium sensitization by CCh in rat bladder but did not affect it in guinea-pig bladder and taenia ceacum. In the latter tissues, despite the presence of 2-APB, CCh induced further contraction (to a level significantly greater than with GTP, $P<0.05$; Table 1).

The effect of Y-27632 on CCh-induced calcium sensitization

The effect of the ROK inhibitor Y-27632 (5 μM) on CCh-induced calcium sensitization was observed to evaluate whether the ROK pathway has a role in CCh-induced calcium sensitization in β-escin skinned smooth muscle. In rat bladder, Y-27632 (5 μM) added in the presence of CCh reversed the calcium sensitization obtained by this muscarinic agonist ($P<0.05$) reducing the contraction to 55.2 ± 8.4% of its original height. In guinea-pig bladder and taenia caecum, Y-27632 (5 μM) added in the presence of CCh induced a transient contraction in both tissues, followed by relaxation to a level not significantly different from the CCh contraction in both groups ($P>0.05$). The results are given in Table 2. In a further set of experiments in the continuous presence of a lower concentration of Y-27632 (1 μM), CCh-induced Ca^{2+} sensitization was significantly inhibited in rat bladder but not in guinea-pig bladder or taenia caecum (Figure 3b–d).

The effect of Y-27632 on cumulative calcium response curves

In order to examine further the unexpected contractile response to Y-27632 (5 μM), cumulative calcium response curves (*p*Ca 7.5–5) were elicited in the presence and absence of Y-27632. In these experiments, CPA (1 μM) and calmodulin (1 μM) in 0.05 mM EGTA were present with various calcium concentrations in the activating solution (Figure 4a–c). The

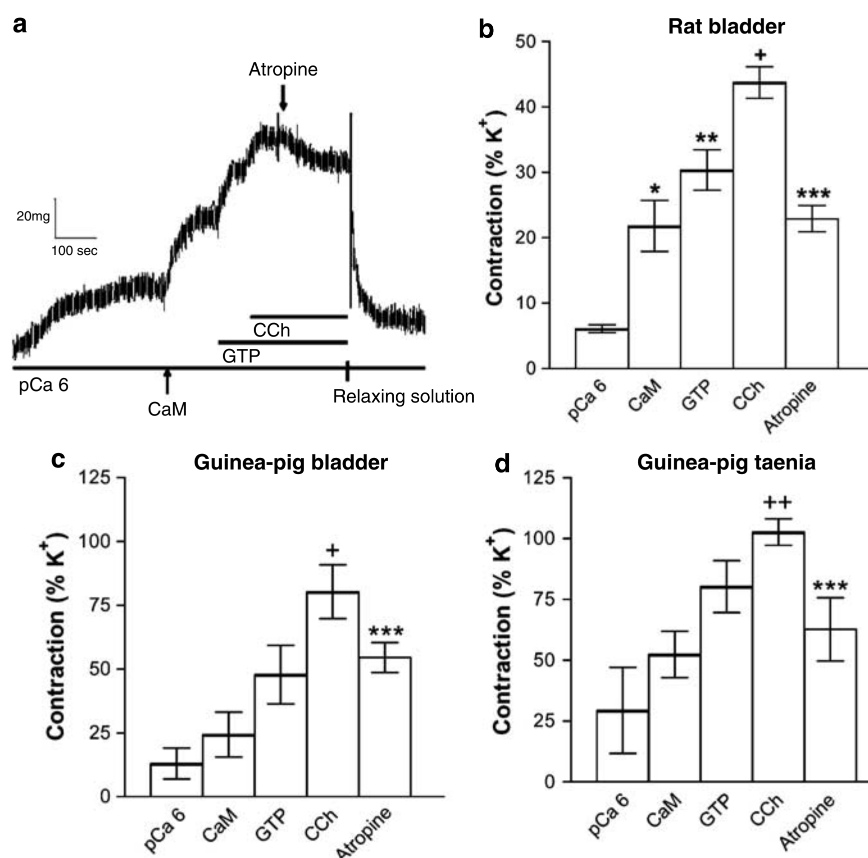

Figure 2 CCh-induced calcium sensitization in rat bladder and guinea-pig bladder and taenia caecum with calcium clamped at *p*Ca 6. (a) Consecutive increases in tension in rat bladder were induced by calmodulin (CaM; 1 μM), GTP (100 μM) and CCh (50 μM). Atropine (50 μM) added to the organ chamber in the presence of CCh reversed the contraction by this muscarinic receptor agonist. Summary of results from four tissues in (b) rat bladder, (c) guinea-pig bladder and (d) guinea-pig taenia caecum. ($n=4$–5, * significant compared to *p*Ca 6 ($P<0.01$); + significant compared to GTP ($P<0.05$), *** significant compared to CCh ($P<0.05$), ** significant compared to *p*Ca 6 ($P<0.001$), + + significant compared to CaM ($P<0.05$), Student's *t*-test).

Table 1 The effect of IP_3 inhibitor 2-APB (30 μM) on carbachol-induced calcium sensitization in the presence of CPA (1 μM) in (a) rat and (b) guinea-pig bladder and in (c) guinea-pig taenia caecum ($n=5$–6)

	Rat bladder		*Guinea-pig bladder*		*Guinea-pig taenia CECI*	
	Control	*2-APB*	*Control*	*2-APB*	*Control*	*2-APB*
*p*Ca 6	6.1±0.6	6.2±3.9	13.0±6.1	11.3±6.6	29.4±17.7	32.3±2.6
CaM	21.8±3.9	14.7±5.6	24.3±8.8	43.8±7.5	52.4±9.6	58.3±4.8
GTP	30.4±3.1	22.8±6.8	47.8±11.5	66.3±6.9	80.3±10.7	79.2±2.4
CCh	43.8±2.4*	29.8±8.5	80.3±10.6*	93.8±3.6*	102.7±5.5*	101.0±6.0*

*Significant compared to GTP ($P<0.05$), Student's *t*-test. Contractions are given as % of the initial contraction of the intact strip to 80 mM K^+.

ROK inhibitor Y-27632 (5 μM) added to the organ chambers did not change the calcium response curve in rat bladder compared to control (Figure 4a). However, in both guinea-pig bladder and taenia caecum, Y-27632 increased the contractile responses significantly compared to their own controls ($P<0.05$).

The effect of GF 109203X on CCh-induced calcium sensitization

The effect of the PKC inhibitor GF 109203X (5 μM) on CCh-induced calcium sensitization was examined. In all three tissues GF 109203X added in the presence of CCh inhibited the calcium sensitization induced by this muscarinic agonist but by different ratios. In rat and guinea-pig bladders, the inhibition of CCh contractions by GF 109203X was to a level close to that seen after GTP (Figure 5a), whereas in taenia caecum, the reversal of calcium sensitization by GF 109203X was to a level close to that seen in CaM ($P<0.05$) (Figure 5b). These relaxations by GF 109203X, as a percantage of the cumulative contraction elicited in the presence of calcium, calmodulin, GTP and CCh, were by 38.4±5.8, 34.1±3.2, 41.0±1.6% in rat bladder, guinea-pig bladder and taenia caecum, respectively (Figure 5).

Table 2 The effect of Rho kinase inhibitor Y-27632 (5 μM) on carbachol-induced calcium sensitization in rat bladder and guinea-pig bladder and taenia caecum

	Rat bladder	*Guinea-pig bladder*	*Guinea-pig taenia*
*p*Ca 6	6.0±1.4	13.0±5.0	19.5±3.1
CaM	19.3±2.8	35.5±4.9	56.5±6.0
GTP	30.1±4.6	66.8±5.7	76.1±6.8
CCh	39.8±3.8*	82.9±5.9*	98.1±11.0*
Y-27632 contraction	—	104.6±10.5**	113.0±14.6
Y-27632 relaxation	18.5±4.4**	85.3±5.6	107.0±15.3

Calmodulin (CaM; 1 μM) and GTP (100 μM) added to the organ chamber increased the tension significantly compared to *p*Ca 6 contraction in all three tissues. Calcium sensitization obtained by added carbachol (CCh; 50 μM) further increased tension ($n = 4$ for rat and guinea-pig bladder, $n = 5$ for taenia caecum, *$P < 0.05$ in comparison with GTP contraction, Student's *t*-test). In rat bladder, Y-27632 (5 μM) added in the presence of carbachol relaxed the tissue significantly compared to carbachol contraction (**$P < 0.05$). In guinea-pig, Y-27632 added in the presence of carbachol first induced a contraction (Y-27632 contraction), significant compared to carbachol contraction in the bladder ($n = 4$, **$P < 0.05$, Student's *t*-test) and then a subsequent relaxation (Y-27632 relaxation) to a value not different from carbachol contraction in both groups ($n = 4$, $P > 0.05$, Student's *t*-test).

Discussion

β-escin permeabilization preserves the receptor–effector pathways in smooth muscle while allowing manipulation of the internal environment. In the present study, CCh was able to induce contraction in these chemically skinned tissues at constant calcium concentration-(*p*Ca 6) as shown in Figure 2 for rat bladder and for guinea-pig bladder and taenia caecum, and atropine reversed the CCh contraction demonstrating that CCh does induce calcium sensitization in these three tissues through muscarinic receptor activation. In the absence of calmodulin and GTP at *p*Ca 6 the tissues generated little tone, but sequential addition of these agents each caused contraction, implying that these two endogenous substances had been lost from the tissues. Similar loss during skinning has been reported in many studies including ileum, vascular and bronchial smooth muscle (Loirand *et al*., 1999; Todoroki-Ikeda *et al*., 2000; Chiba *et al*., 2001), and it is known that β-escin permeabilization opens pores in the cell membrane that allow high molecular weight compounds (up to 150 kDa) to penetrate (Iizuka *et al*., 1994). In the study by Kobayashi *et al*. (1989) addition of calmodulin increased the calcium induced contractions in β-escin skinned guinea-pig ileum longitudinal smooth muscle and CCh only induced contraction in the

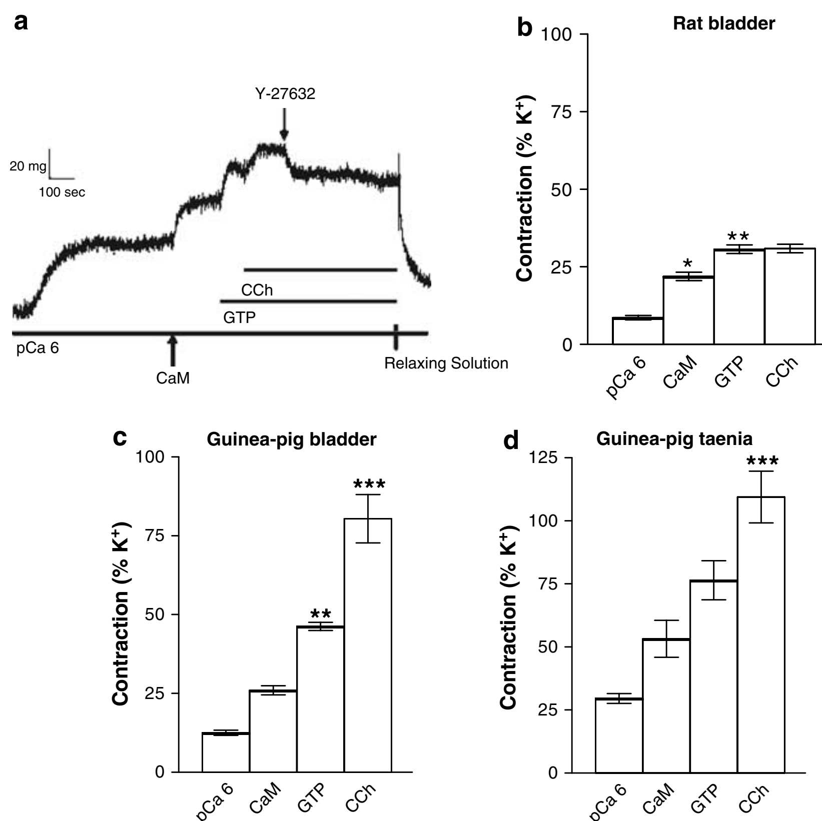

Figure 3 The effect of ROK inhibitor Y-27632 (1 μM) on CCh-induced calcium sensitization. (a) Rat bladder: original tracing showing acute effect of Y-27632 (1 μM) addition. (b–d) Summary histograms showing contractile responses in the continuous presence of Y-27632 (1 μM). Addition of calmodulin (CaM; 1 μM) and GTP (100 μM) increased the tension significantly ($n = 5$, *significant compared to *p*Ca 6 contraction ($P < 0.05$); ** significant compared to CaM($P < 0.05$), Student's *t*-test) in all tissues, whereas CCh (50 μM) no longer caused calcium sensitization in rat bladder in the presence of Y-27632 (1 μM) (b) but still caused calcium sensitization in guinea-pig bladder and (c) taenia caecum (d). ***Calcium sensitization significant compared to GTP ($n = 5$, $P < 0.05$, Student's *t*-test).

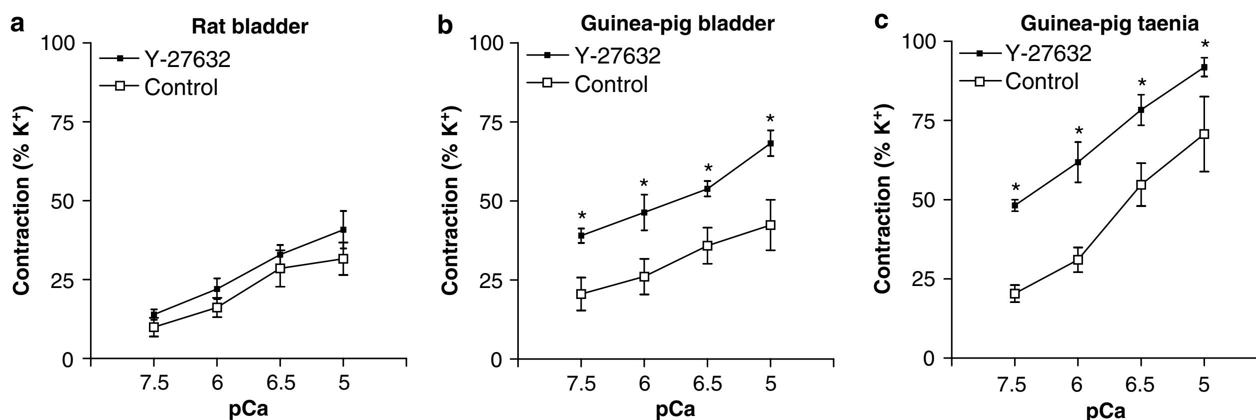

Figure 4 The effect of ROK inhibitor Y-27632 (5 μM) on cumulative calcium response curves (3×10^{-7} to 10^{-5} M) in 0.05 mM EGTA buffered activating solutions in (a) rat bladder, (b) guinea-pig bladder and (c) guinea-pig taenia caecum. In rat bladder, control calcium response curve obtained in the presence of Y-27632 was not significantly different from the control curve. In both guinea-pig bladder and taenia caecum, Y-27632 increased the contractile responses significantly compared to their own controls ($n=4$–6, $^*P<0.05$, ANOVA).

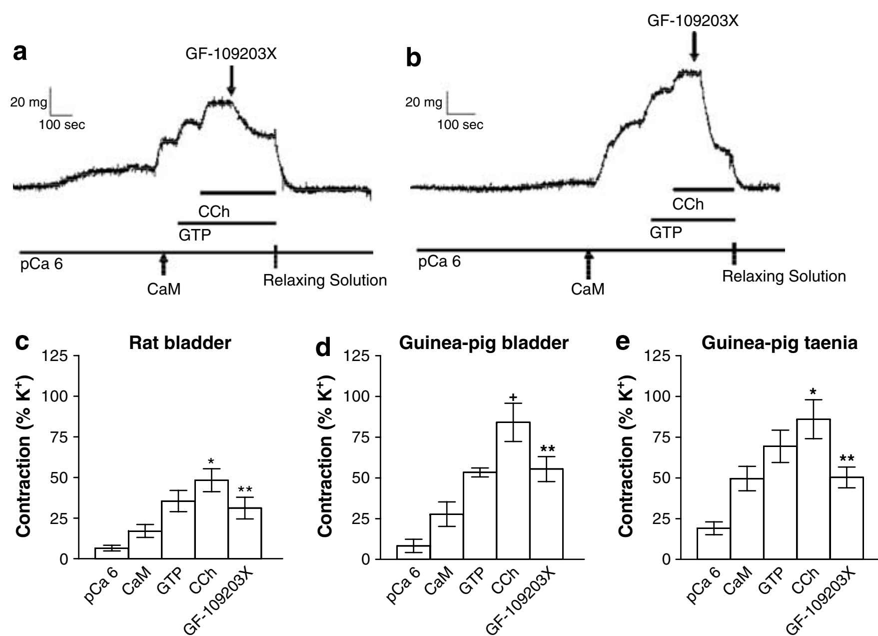

Figure 5 The effect of PKC inhibitor GF 109203X (5 μM) on CCh-induced calcium sensitization. (a and b) Original traces for rat bladder and guinea-pig taenia caecum. (c–e) Summary of results. Calmodulin (CaM; 1 μM) and GTP (100 μM) added after calcium was clamped at *p*Ca 6 significantly increased the tension compared to *p*Ca 6 contraction ($P<0.05$). Calcium sensitization obtained by added CCh (50 μM) was significant compared to CaM in rat bladder and taenia caecum, and was significant compared to GTP in guinea-pig bladder ($n=5$, $^*P<0.05$ compared to CaM, $^+P<0.05$ compared to GTP, Student's *t*-test). In all three tissues GF 109203X added in the presence of CCh inhibited the calcium sensitization induced by this muscarinic agonist but all in different ratios. In rat and guinea-pig bladders, the inhibition was close to the level of GTP contraction whereas in taenia caecum, it was close to the CaM contraction level. ($n=5$, $^{**}P<0.05$ compared to CCh contraction, Student's *t*-test).

presence of GTP. This confirms the requirement of GTP for receptor–effector coupling through G proteins (Litosch & Fain, 1986). Our findings support these studies since CCh without calmodulin and GTP did not cause a significant contraction (data not shown).

Muscarinic receptor activation is primarily responsible for the sustained contraction necessary for the emptying of the urinary bladder. M_2 and M_3 muscarinic receptor subtypes have been identified in rat and guinea-pig bladders and in taenia caecum and the M_3 receptor subtype has been shown to mediate the contraction in all three tissues (Elnatan & Mitchelson, 1993; Wang *et al*., 1995; Kories *et al*., 2003; Uchiyama & Chess-Williams, 2004). The role of the M_2 receptor is less certain. A recent study claimed that both M_2 and M_3 receptor subtypes are involved in normal rat bladder contractions with the M_3 subtype activating PI-PLC, PC-PLC, and PKA, and the M_2 subtype working through ROK, PKC, and an additional contractile signal transduction mechanism (Braverman *et al*., 2006).

In intact tissues, muscarinic receptor activation elevates $[Ca^{2+}]_i$ through release of intracellular calcium from IP_3 or ryanodine sensitive stores as well as from extracellular calcium influx (e.g. for bladder, Visser & van Mastrigt, 2000; Ma *et al*., 2002). It is thus possible that in skinned preparations, local release of calcium even in the presence of calcium buffers,

might have a role in the contractile responses seen. To check that this was not happening, we used 2-APB, an inhibitor of the IP_3 receptors, under conditions when the SR Ca^{2+}ATPase was blocked by CPA. This had no effect on contractions due to CCh-induced sensitization in guinea-pig bladder and taenia caecum, although it slightly reduced them in the rat bladder. All our experiments were carried out in the presence of CPA that should prevent filling of the store and allow contractile responses independent of SR. The fact that CCh still produced contraction in the presence of CPA and 2-APB did not alter this in the guinea-pig tissues reassured us that we were demonstrating sensitization. Therefore, we conclude that the CCh-induced calcium sensitization in guinea-pig tissues in this study is independent of calcium release from stores. Although the inhibitory effect of 2-APB in the rat under our conditions may suggest some involvement of store release, we think it is unlikely that there was any calcium in the stores, and that the reduction of the CCh response is more likely to be caused by nonselective effects of 2-APB.

In smooth muscles, the major mechanism for calcium sensitization is enhanced myosin light chain phosphorylation that is either independent of calcium-calmodulin (Weber *et al.*, 1999) or a result of MLCP inhibition (Kitazawa *et al.*, 1991a, b). There are two main pathways known to control independently the activity of MLCP; their determinants are Rho-associated protein kinase (ROK) and PKC, respectively (Somlyo & Somlyo, 2003).

ROK is activated through the monomeric GTP binding protein RhoA from Rho subfamily, and this pathway is known as the RhoA/ROK pathway. The involvement of RhoA in agonist-induced calcium sensitization has been reported in many tissues, for example, vas deferens, intestine, and airways (Fujita *et al.*, 1995; Otto *et al.*, 1996; Chiba *et al.*, 1999). RhoA also causes concentration-dependent calcium sensitization in β-escin permeabilized smooth muscles (Gong *et al.*, 1996). MLCP has three subunits: catalytic (PP1c), regulatory targeting (MYPT1), and one having an unknown function. The MYPT1 subunit binds MLCP to myosin, and enhances its ability to dephosphorylate the light chains. Phosphorylation of MYPT1 at Thr^{695} by ROK inhibits MLCP activity and induces calcium sensitization (Uehata *et al.*, 1997).

A specific ROK inhibitor Y-27632 has been shown to inhibit contractions in many smooth muscles such as vascular (Uehata *et al.*, 1997), airway (Iizuka *et al.*, 2000), genital (Chitaley *et al.*, 2001; Rees *et al.*, 2001) and ileal (Sward *et al.*, 2000). In rat bladder, the expression of two types of ROK (ROK I and ROK II) has been demonstrated (Wibberley *et al.*, 2003). In the present study, Y-27632 was used to test the involvement of ROK in CCh induced calcium sensitization. In rat bladder, Y-27632 abolished CCh-induced calcium sensitization suggesting a possible role of ROK as shown in Figure 3, and lowered the tone to 55.2% of the total contraction – similar to that before addition of GTP. However, in guinea-pig bladder and taneia caecum, Y-27632 added to bath in the presence of CCh first induced a contraction and then subsequently relaxed both tissues back to the level seen in CCh before Y-27632 was added, as given in Table 2. Thus, it did not abolish CCh-induced calcium sensitization. This lack of effect in the guinea-pig tissues was confirmed by carrying out experiments in the continuous presence of a lower concentration of Y-27632. Again calcium sensitization was abolished in the rat detrusor but not in the guinea-pig tissues.

Species differences were also seen in the effect of Y-27632 on the cumulative calcium response curves. In rat the curve was not affected by Y-27632, but in the guinea-pig tissues the drug increased the contractions at all levels of *p*Ca (Figure 5). It is not clear why Y-27632 enhanced contractions in the skinned guinea-pig tissues, and it is probable that this effect is independent of the ROK pathway. In intact guinea-pig bladder, it seems that ROK may have endogenous activity since Hashitani *et al.* (2004) showed that blocking its activity with Y-27632 reduced the contractile response initiated by action potentials, without altering the associated increase in intracellular calcium concentration.

Another pathway that results in MLCP inhibition involves the phosphatase inhibitor protein protein kinase C-potentiated protein phosphatase-1 inhibitory protein (CPI-17). Phosphorylation of CPI-17 by PKC enhances its ability to inhibit the catalytic subunit MLCP (Eto *et al.*, 1995; 1997). In tonic smooth muscles, the PKC-CPI-17 pathway is the dominant contributor to calcium sensitization caused by MLC phosphorylation. However, in phasic smooth muscles like vas deferens, the contribution of this pathway is minor (Woodsome *et al.*, 2001). In intact strips from rabbit detrusor, a PKC inhibitor GF 109203X inhibited CCh-induced contractions whereas in strips dissected from the fundus it did not (Ratz *et al.*, 2002). In guinea-pig bladder there is evidence that stimulation of muscarinic receptors activates PKC, leading to K_{ATP} channel inhibition. GF 109203X and calphostin C block these effects (Bonev & Nelson, 1993). In guinea-pig taenia caecum the response to M_2 receptor activation involves PKC (Shen & Mitchelson, 1998). In the present study, GF 109203X reversed CCh-induced calcium sensitization in all three tissues but in different ratios. The relaxation by GF 109203X as a percentage of total contraction is 38.4 % in rat bladder, 34.1 % in guinea-pig bladder and 41.0% in taenia caecum.

In recent studies in intact rat bladder, Fleichman *et al.* (2004) showed that the PKC inhibitors chelerythrine, calphostin C and bisindolylmaleimide I failed to affect CCh concentration–response curves although tested in high concentrations. In the same study, the ROK inhibitor Y-27632 concentration dependently inhibited CCh-induced contractions consistent with our present study (Fleichman *et al.*, 2004). Similarly, in human bladder, it has been shown that Y-27632, but not calphostin C and bisindolylmaleimide I, significantly reduced CCh-induced bladder contractions (Schneider *et al.*, 2004). In a study investigating calcium sensitization in α-toxin permeabilized human urinary bladder smooth muscle, it has been shown that CCh contracts human bladder by increasing intracellular calcium and causing calcium sensitization. According to these authors, CCh-induced calcium sensitization is regulated by ROK and PKC in human bladder and this was confirmed by the relaxation obtained by Y-27632 and GF 109203X when the tissue was contracted by GTP and CCh (Takahashi *et al.*, 2004). According to our results, ROK plays a major role in CCh-induced calcium sensitization in rat bladder whereas PKC is also involved in the signalling cascade, but is less important. In guinea-pig, ROK did not contribute to calcium sensitization in either tissue under our conditions whereas PKC did.

In conclusion, we have elucidated the mechanisms involved in calcium sensitization in detrusor by using β-escin permeabilized smooth muscle, a useful tool to study signal

Table 3 The effect of inhibitors of signal transduction on carbachol-induced calcium sensitization in rat bladder, guinea-pig bladder and taenia caecum

	Rat bladder	*Guinea-pig bladder*	*Guinea-pig taenia caecum*
Inhibitors			
2-APB (30 μM)	+	−	−
Y-27632 (1 μM)	+	−	−
Y-27632 (5 μM)	+	−	−
GF 109203X (5 μM)	+	+	+

(+) Compound caused an inhibitory effect on carbachol induced calcium sensitization, (−) inhibitor had no effect on calcium sensitization.

transduction if care is taken with interpreting the effects of added agonists and antagonists. We have shown that muscarinic receptor activation can induce calcium sensitization in rat and guinea-pig detrusor smooth muscle, and is likely to play a significant role in activation of bladder contraction under normal conditions. It is interesting, however, to note that the mechanisms activated differ between the species, and it will be necessary to compare the relative importance of the pathways used between human detrusor and any potential animal models if calcium sensitization is to become a target for drug development to treat the over-active bladder. According to our results, rat is the more appropriate model to study these mechanisms when compared with the published human bladder data given above. The data showing the similarities and differences in the contribution of the various signal transduction pathways to calcium sensitization for rat detrusor, guinea-pig detrusor and taenia caecum are summarized in Table 3.

N.T. Durlu-Kandilci was funded by The Scientific and Technological Research Council of Turkey.

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(Received September 27, 2005
Revised November 28, 2005
Accepted February 15, 2006
Published online 27 March 2006)