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Dependency of detrusor contractions on calcium sensitization and calcium entry through LOE-908-sensitive channels

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1 The subcellular mechanisms regulating stimulus-contraction coupling in detrusor remain to be determined. We used Ca^{2+}-free solutions, Ca^{2+} channel blockers, cyclopiazonic acid (CPA), and RhoA kinase (ROK) inhibitors to test the hypothesis that Ca^{2+} influx and Ca^{2+} sensitization play primary roles.

2 In rabbit detrusor, peak bethanechol (BE)-induced force was inhibited 90% by incubation for 3 min in a Ca^{2+}-free solution. By comparison, a 20 min incubation of rabbit femoral artery in a Ca^{2+}-free solution reduced receptor-induced force by only 5%.

3 In detrusor, inhibition of sarcoplasmic reticular (SR) Ca^{2+} release by 2APB, or depletion of SR Ca^{2+} by CPA, inhibited BE-induced force by only 27%. The CPA-insensitive force was abolished by $LaCl_3$. By comparison, 2APB inhibited receptor-induced force in rabbit femoral artery by 71%.

4 In the presence of the non-selective cation channel (NSCC) inhibitor, LOE-908, BE did not produce an increase in $[Ca^{2+}]_i$ but did produce weak increases in myosin phosphorylation and force.

5 Inhibitors of ROK-induced Ca^{2+} sensitization, HA-1077 and Y-27632, inhibited BE-induced force by $\sim$50%, and in combination with LOE-908, nearly abolished force.

6 These data suggest that two principal muscarinic receptor-stimulated detrusor contractile mechanisms include NSCC activation, that elevates $[Ca^{2+}]_i$ and ROK activation, that sensitizes cross bridges to Ca^{2+}.

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Abbreviations: 2APB, 2-aminoethoxydiphenyl borate

Introduction

A key regulatory step in the development of smooth muscle contractile force is Ca^{2+} mobilization (Williams *et al*., 1987). In vascular smooth muscle, the primary Ca^{2+} mobilization mechanisms responsible for initiation and maintenance of contractions induced by stimulation of G protein-coupled receptors are, respectively, intracellular Ca^{2+} release and Ca^{2+} influx from the extracellular space (Deth & van Breemen, 1974; Iino, 1990; Ratz & Murphy, 1987). Although muscarinic receptor stimulation is known to cause release of stored intracellular Ca^{2+} and influx of extracellular Ca^{2+} (Anderson, 1993), whether the scenario describing Ca^{2+} regulation of vascular smooth muscle contraction applies to detrusor smooth muscle remains debatable (Batra *et al*., 1987; Iacovou *et al*., 1990; Maggi *et al*., 1989; Mostwin, 1985; Zhoa *et al*., 1993). Most studies exploring the link between Ca^{2+} mobilization and contraction of detrusor smooth muscle have not examined parameters of contractile protein activation other than force, and changes in Ca^{2+} do not necessarily correspond with changes in force (Ratz, 1999; Rembold & Murphy, 1986; Somlyo & Somlyo, 1994). Thus, one reason for the controversy over the relationship between Ca^{2+} regulation and contraction may be that, to regulate force, detrusor utilizes mechanisms such as sensitization of cross bridges to Ca^{2+} (Ca^{2+} sensitization; (Somlyo *et al*., 1999)). Moreover, agents that selectively inhibit L-type voltage-operated Ca^{2+} channels (VOCCs) produce only moderate inhibition of muscarinic receptor-stimulated detrusor contractions, whereas Ca^{2+}-free solutions have been shown to nearly abolish detrusor contractions (Batra *et al*., 1987; Fovaeus *et al*., 1987; Ratz *et al*., 1999). Thus, in addition to activation of VOCCs, muscarinic receptor stimulation of detrusor may activate other Ca^{2+} channels to elevate Ca^{2+} entry.

The present study examined the abilities of the non-selective cation channel (NSCC) inhibitor, LOE-908, and inhibitors of RhoA kinase (ROK)-induced Ca^{2+} sensitization, HA-1077 and Y-27632, to reduce BE-induced detrusor contractions. Involuntary detrusor contractions are characteristic of overactive bladder, a major chronic disorder that produces symptoms such as urge incontinence (Abrams & Wein, 2000; Payne, 1998). Overactive bladder may result from alterations in the regulation of smooth muscle contraction (Brading, 1997; de Groat, 1997; Fry & Wu, 1998). Current therapy of urge incontinence due to overactive bladder relies almost exclusively on anti-cholinergic drugs,

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but the utility of these drugs is limited by adverse effects (Chapple, 2000). By identification of alternate regulatory sites such as NSCCs and ROK-induced Ca^{2+} sensitization that, if selectively blocked, will limit detrusor contractile activity, the present study provides data that may assist in the development of novel therapeutic tools to more specifically modulate overactive detrusor contractions.

Methods

Tissue preparation

Tissues were prepared as described previously (Ratz, 1993; Shenfeld *et al*., 1998). Whole bladders and femoral arteries from adult female New Zealand white rabbits were removed immediately after sacrifice with pentobarbitone. Arteries were cleaned of adhering fat and connective tissue and the endothelium was mechanically removed by passing a stainless steel rod through the lumen. Arteries and bladders were washed several times and stored in cold (0–4°C) physiologic salt solution (PSS), composed of NaCl, 140 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 1.6 mM; Na_2HPO_4 1.2 mM; morpholinopropanesulphonic acid, 2.0 mM (adjusted to pH 7.4 at either 0 or 37°C, as appropriate); Na_2 ethylenediamine tetraacetic acid (EDTA; to chelate trace heavy metals), 0.02; and dextrose, 5.6 mM. High purity water (17 MΩ) was used throughout. For clarity in the results section, PSS will be referred to as 'Ca^{2+}-containing solution' while PSS with no $CaCl_2$ and the addition of 1 mM EGTA to chelate Ca^{2+} as 'Ca^{2+}-free solution'. Longitudinal muscle strips were cut from the the wall of the detrusor body, and rings were cut from the femoral artery. Each muscle strip and ring was incubated in aerated PSS at 37°C in a water-jacketed tissue bath (Radnotti Glass Technology, Monrovia, CA, U.S.A.) and secured by small clips to a micrometer for length adjustments and an isometric force transducer (model 52, Apparatus, South Natick, MA, U.S.A.) to measure muscle contraction.

Contraction of isolated detrusor strips and artery rings

Isometric contractions were measured as described previously (Ratz, 1995; Shenfeld *et al*., 1998). Voltage signals were digitized (model DIO-DAS16, ComputerBoards, Mansfield, MA, U.S.A.), visualized on a computer screen as force (g), and stored for analyses. All data analyses were performed using a multi-channel data integration program (DASYLab, TasyTec USA, Amherst, NH, U.S.A.). Tissues were equilibrated for a minimum of 30 min suspended without tension between micrometer and isometric force transducer. Tissues were then stretched to their optimum length for muscle contraction (L_O) using an abbreviated length-force determination (Herlihy & Murphy, 1973; Ratz & Murphy, 1987; Uvelius, 1976). The optimum force for muscle contraction (F_O) produced by 110 mM KCl at L_O was obtained for each tissue. To reduce tissue-to-tissue variability, subsequent contractions were reported as normalized to F_O (F/F_O). In all studies on detrusor using KCl as a stimulus, 0.1 μM atropine was added to prevent potential activation of muscarinic receptors resulting from depolarization of parasympathetic nerves. In all experiments except that shown in Figure 8b, peak contractile responses were analysed. In Figure 8b the effects of HA-1077 on peak and tonic bethanechol (BE)-induced contractions were assessed.

$[Ca^{2+}]_i$

Ratiometric fluorimetry was performed using an emission wavelength of 500 nm and alternating excitation wavelengths of 340 and 380 nm, as previously described (Ratz, 1993; Ratz & Lattanzio, 1992) with minor alterations. Tissues were stretched to L_O and incubated with 7.5 μM Fura-2/AM and 0.01% Pluronic acid for 2 h at room temperature, and then heated to 37°C for an additional hour. After extensive washing to remove extracellular fura, the tissue was incubated for an additional 30 min prior to performing the ratiometric analysis. For each tissue, the maximum 340-to-380 nm ratio after an initial stimulation with bethanechol (R_{peak}) and nadir ratio after incubation in a Ca^{2+}-free solution (R_{nadir}) were determined. Data were reported as normalized to maximum (R_{peak}) and minimum (R_{nadir}) values using the following formula: Intracellular free $Ca^{2+} = (R - R_{nadir})/(R_{peak} - R_{nadir}) \times 100$. These values were reported as '% maximum'.

Myosin light chain phosphorylation

The degree of myosin light chain (MLC) phosphorylation was measured as described previously (Ratz, 1993; 1995; Ratz *et al*., 1989). In short, tissues were quick-frozen in an acetone/dry ice slurry, slowly warmed to room temperature, weighed and homogenized on ice in 8 M urea, 2% Triton X-100 and 20 mM dithiothreitol. Isoelectric variants of the 20 kDa MLCs were separated using 2-dimensional (IEF/SDS) PAGE, then identified and visualized by Western blotting and colloidal gold staining. The relative amounts of phosphorylated and non-phosphorylated MLCs were quantified by digital image analysis, and the degree of MLC phosphorylation was reported as the amount of phosphorylated MLC ($MLC_{20\text{-}P}$) divided by the total amount of MLC present (MCL_{20}).

Drugs

LOE-908 was a kind gift from Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT, U.S.A. Y-27632 was a kind gift from Welfide Corporation, Ozaka, Japan. Nifedipine, verapamil and bethanechol were from Sigma Corporation. Diphenylborinic acid ethanolamine ester (2-aminoethoxydiphenyl borate; 2APB) was from Aldrich. Cyclopiazonic acid (CPA) and SKF-96365 were from Calbiochem. HA-1077 was from Alexis Corporation. LOE-908 and CPA were dissolved in DMSO, nifedipine and 2APB were dissolved in ethanol, and all other drugs were dissolved in water. DMSO and ethanol were added at final concentrations no greater than 0.1%.

Statistics

Analysis of variance and the Student-Newman-Keuls test, or the *t*-test, was used where appropriate to determine significance, and the Null hypothesis was rejected at $P<0.05$. The population sample size (*n* value) refers to the number of animals, not the number of tissues.

Results

Contractions in a Ca^{2+}-free solution

To determine the dependency of muscarinic receptor-stimulated contraction on extracellular Ca^{2+}, strips of detrusor were incubated in a Ca^{2+}-free solution (see 'Methods') for various durations and stimulated with a maximum bethanechol (BE) concentration (100 μM). BE was used to stimulate muscarinic receptors because it is resistant to hydrolysis by cholinesterases and is selective for muscarinic over nicotinic receptors (Brown & Taylor, 1996). For a comparison, tissues were stimulated with 40 mM KCl, an agent known to produce contraction by causing membrane depolarizing leading to enhanced Ca^{2+} influx through VOCCs. The peak KCl-induced contraction produced in tissues incubated for 10 s in a Ca^{2+}-free solution was $\sim$0.60 fold the peak contraction produced in a Ca^{2+}-containing solution ($F/F_{control}$, Figure 1A, solid squares, where $F_{control}$ = force produced in a Ca^{2+}-containing solution). This value was reduced to zero when tissues were incubated for 90 s in a Ca^{2+}-free solution (Figure 1A, solid squares). In the presence of the VOCC blocker, verapamil (VP, 10 μM for 10 min), even a short 10 s incubation in a Ca^{2+}-free solution abolished KCl-induced force (Figure 1A, open squares). These data suggest that $\sim$90 s was required for diffusion of extracellular Ca^{2+} out of the extracellular space of the inner tissue layers to reduce Ca^{2+} entry sufficiently to eliminate the ability of a stimulus that exclusively utilizes extracellular Ca^{2+} entry to produce a contraction (Ratz & Murphy, 1987). Peak contractions produced by BE were not completely eliminated by incubation of detrusor for 90 s in a Ca^{2+}-free solution, but the degree of force produced was a weak 0.17 ± 0.05 fold that produced in a Ca^{2+}-containing solution (Figure 1A, solid circles). Longer durations of incubation in the Ca^{2+}-free solution further reduced the degree of force produced by BE, although force was never abolished, and even after a 60 min incubation period, force was $\sim$0.05 fold that produced in a Ca^{2+}-containing solution. In the presence of VP, BE-induced peak contractile force produced by incubation for 10 s in a Ca^{2+}-free solution was $\sim$0.2 fold that produced in a Ca^{2+}-containing solution. In a Ca^{2+}-containing solution in which cyclopiazonic acid (CPA, 10 μM) was added to inhibit the sarcoplasmic reticulum (SR) Ca^{2+}ATPase and eliminate the inositol 1,4,5-triphosphate-sensitive SR Ca^{2+} store (Golovina & Blaustein, 1997; Seidler *et al*., 1989), BE produced a strong contraction that was 0.73 fold control (Figure 1B). The BE-induced contraction produced in the presence of CPA was abolished by the general Ca^{2+} channel blocker, 1 mM $LaCl_3$ (Figure 1B).

Detrusor incubated for only 3 min in a Ca^{2+}-free solution and stimulated with a maximum concentration of BE (100 μM) produced a weak contraction of 0.11 fold F_O (Figure 2B). By comparison, arterial muscle incubated for

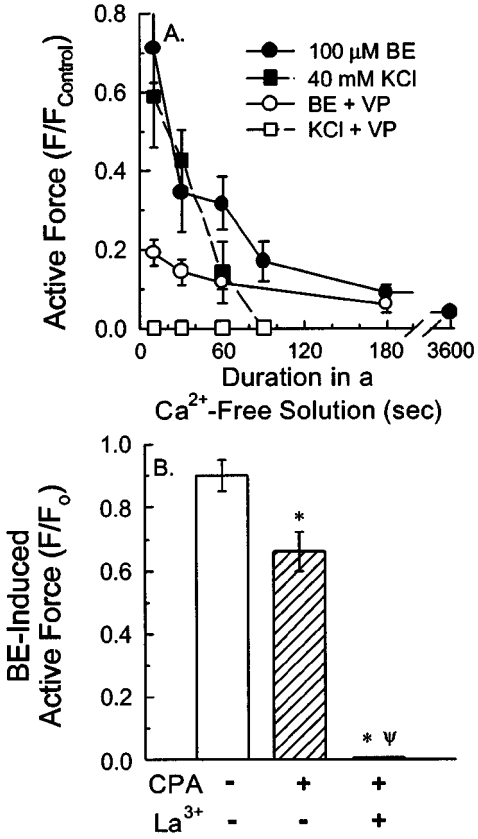

Figure 1 Effect of incubation in a Ca^{2+}-free solution (A) and treatment with 10 μM cyclopiazonic acid (CPA; B) on peak contractions produced by 100 μM bethanechol (BE). For a comparison, tissues incubated in a Ca^{2+}-free solution were also stimulated with KCl (A). Responses in (A) are reported as normalized to the peak force produced in a Ca^{2+}-containing solution ($F_{control}$). Data are means $\pm$ s.e.mean; $n = 3-8$; * = $P < 0.05$ compared to control; $\Psi = P < 0.05$ compared to +CPA. VP = 10 μM verapamil; La^{3+} = 1 mM $LaCl_3$.

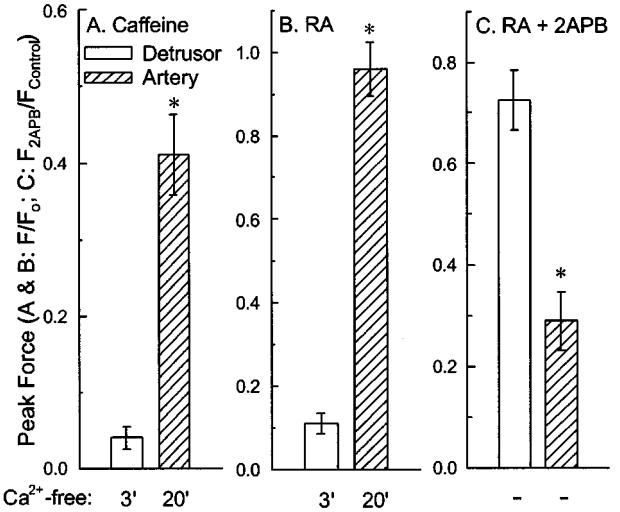

Figure 2 Comparison of the relative abilities of detrusor and artery to produce peak contractions when stimulated with 20 mM caffeine in a Ca^{2+}-free solution (A), and when stimulated with, respectively, the receptor agonists (RA) bethanechol (100 μM) and phenylephrine (10 μM) while tissues were incubated in a Ca^{2+}-free solution (B) or exposed to the inositol 1,4,5-trisphosphate receptor blocker, 2APB (C). The duration of incubation in a Ca^{2+}-free solution was 3 min (detrusor) and 20 min (artery; see x-axis labels, A and B). Peak PE contractions produced in (B) and (C) were recorded at 68 s. Responses in (A) and (B) are reported as normalized to F_O; responses in (C) are reported as normalized to the peak force produced in the absence of 2APB. Data are means $\pm$ s.e.mean; $n = 3-6$; * = $P < 0.05$ comparing artery to detrusor.

20 min in a Ca^{2+}-free solution and stimulated with a maximum concentration of the α_1-adrenergic receptor agonist, phenylephrine (PE; 10 μM), produced a strong peak contraction of 0.96 fold F_O (Figure 2B; RA refers to stimulation with a receptor agonist, either BE or PE). Moreover, 20 mM caffeine, an agent known to cause smooth muscle contraction by activation of sarcoplasmic reticular (SR) ryanodine receptors resulting in mobilization of intracellular Ca^{2+}, produced a strong contraction in arterial muscle incubated for 20 min in a Ca^{2+}-free solution, but produced a very weak contraction of 0.04 fold F_O in detrusor muscle incubated for only 3 min in a Ca^{2+}-free solution (Figure 2A). Lastly, an inhibitor of SR inositol 1,4,5-triphosphate receptors, 2APB (Ascher-Landsberg *et al*., 1999; Maruyama *et al*., 1997), strongly (71%) inhibited contractions produced by PE in arterial muscle, but only weakly (27%) inhibited contractions produced by BE in detrusor (Figure 2C). Interestingly, depletion of the inositol 1,4,5-trisphosphate-dependent SR Ca^{2+} pool by CPA, and inhibition of the inositol 1,4,5-trisphosphate receptor by 2APB, inhibited BE-induced peak detrusor contractions by the same degree, 27% (compare Figures 1B and 2C). These data taken together support the view that muscarinic receptor-induced detrusor contractions are more dependent on Ca^{2+} entry than on release of SR Ca^{2+}.

Effect of Ca^{2+} channel blockers

To determine whether Ca^{2+} entry through receptor-operated Ca^{2+} channels (Barritt, 1999) plays a significant role in peak detrusor contractions, we compared the inhibitory activities on peak BE-induced contractions of two receptor-operated Ca^{2+} entry blockers, LOE-908 and SKF-96365 (Clementi & Meldolesi, 1996; Leung & Kwan, 1999), to the selective VOCC blockers, verapamil and nifedipine (Figure 3). At the concentrations used, LOE-908 strongly inhibits non-selective cation channels (NSCCs), while SKF-96365 does not, and SKF-96365 abolishes store-operated Ca^{2+} channels (SOCCs) while LOE-908 does not (Iwamuro *et al*., 1999; Krautwurst *et al*., 1994). Moreover, the concentrations of nifedipine (2 μM) and verapamil (10 μM) used are known to abolish Ca^{2+} entry through VOCCs, but do not affect receptor-operated Ca^{2+} influx (Flaim *et al*., 1985). All agents inhibited peak BE-induced force by over 45% of control, although LOE-908 was the most effective, producing a 61% inhibition (Figure 3). In accordance with the fact that LOE-908 is a less potent inhibitor of VOCCs than of NSCCs (Krautwurst *et al*., 1994), 1 μM nifedipine inhibited 110 mM KCl-induced peak force by $93 \pm 2\%$ ($n=3$), while 10 μM LOE-908 produced a significantly weaker inhibition ($68 \pm 3\%$, $n=3$, $P<0.05$). Thus, these data suggest that BE activated NSCC as well as VOCCs. Tripling the concentration of LOE-908 to 30 μM further inhibited peak BE-induced force (10 μM LOE-908, 0.36 ± 0.08 F/F_O, $n=6$, compared to 30 μM LOE-908, 0.16 ± 0.01 F/F_O, $n=3$, $P<0.05$), presumably because this LOE-908 concentration more strongly inhibited VOCCs (IC_{50} of LOE-908 for VOCCS is 28 μM; (Krautwurst *et al*., 1994)). To avoid non-specific effects (Leung & Kwan, 1999), concentrations of SKF-96365 greater than 10 μM were not examined. By comparison, 10 μM LOE-908 had no effect on the strong peak contraction produced by 10 μM phenylephrine in femoral artery incubated in a Ca^{2+}-free solution (F/F_O values for control tissues and tissues incubated with LOE-908 both equaled 0.96, $n=3$).

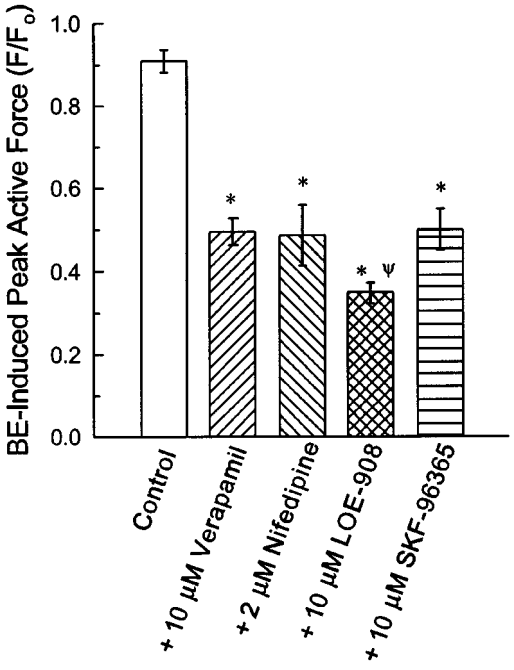

Figure 3 Effect of Ca^{2+} channel blockers on peak force produced by 100 μM bethanechol (BE). Data are means$\pm$s.e.mean; $n=4-12$; $*=P<0.05$ compared to control; $\Psi=P<0.05$ comparing LOE-908 to the other Ca^{2+} channel blockers.

Effect of verapamil and SKF-96365 on CPA-induced contractions

To determine whether detrusor smooth muscle contractions are dependent on activation of store-operated Ca^{2+} channels (SOCCs), tissues were contracted with 10 μM cyclopiazonic acid (CPA) alone and in the presence of the SOCC & VOCC blocker, SKF-96365, and the VOCC blocker, verapamil. In many smooth muscles, CPA causes depletion of SR Ca^{2+}, leading to activation of SOCCs and VOCCs, Ca^{2+} entry and contraction (Gibson *et al*., 1998; Parekh & Penner, 1997). In rabbit detrusor, CPA produced a slowly-developing but strong, phasic contraction that was not diminished by the muscarinic receptor antagonist, atropine, but was nearly abolished by 10 μM SKF-96365 (95% inhibition, Figure 4). These data suggest that SOCCs play a role in regulation of detrusor contractions. However, 10 μM verapamil was nearly as effective as SKF-96365 at inhibiting CPA-induced contractions (88% inhibition, Figure 4). Because SKF-96365 inhibits SOCCs and VOCCs with about the same potency (IC_{50} value $\cong 10$ μM (Krautwurst *et al*., 1994)), these data suggest that CPA-induced contractions are caused primarily ($\sim 90\%$) by activation of VOCCs, with a minimal contribution ($<10\%$) by SKF-96365-sensitive SOCCs.

Effect of LOE-908 on $[Ca^{2+}]_i$ and myosin light chain (MLC) phosphorylation

To determine whether the reduction in BE-induced peak force produced by LOE-908 correlates with a reduction in

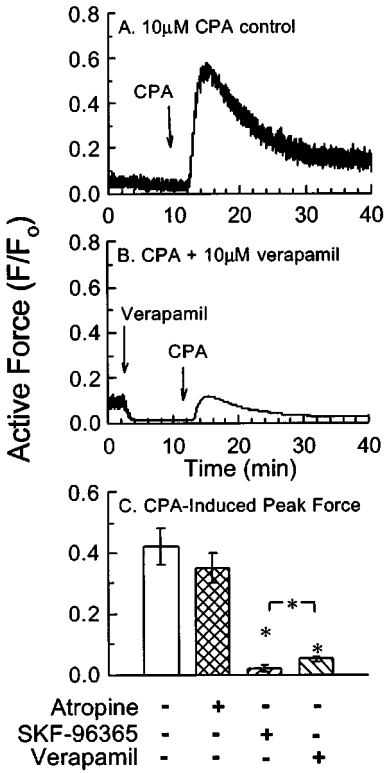

Figure 4 Effect of Ca^{2+} channel blockers and the muscarinic receptor antagonist, atropine (1 μM), on peak force (C) produced by 10 μM cyclopiazonic acid (CPA). Representative tracings of a CPA-induced contraction alone (A) and in the presence of the VOCC blocker, verapamil (B) are also shown. Data in C are means$\pm$ s.e.mean; $n=4-7$; $*=P<0.05$ compared to control; bracketed * indicates $P<0.05$ between identified groups.

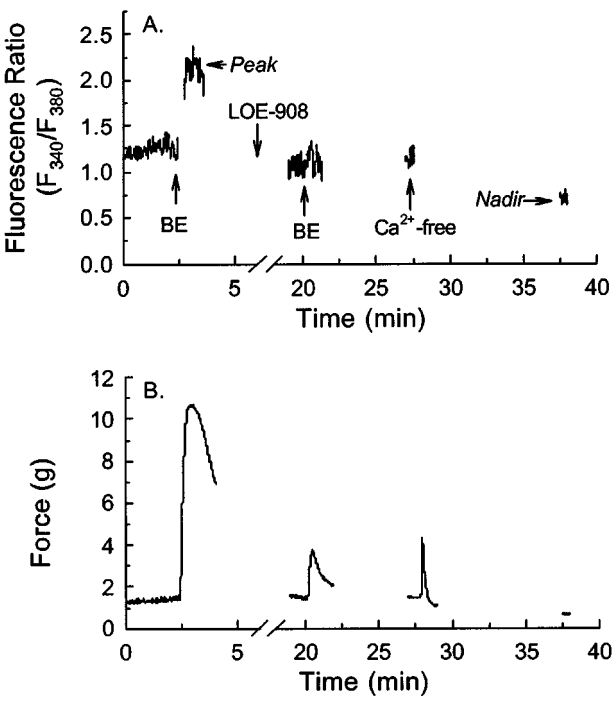

Figure 5 Typical tracing of fluorescence ratio (A), recording changes in $[Ca^{2+}]_i$, and corresponding force tracing (B). The effect of 10 μM LOE-908 is shown. Control tissues (not shown) were not exposed to LOE-908. Maximum (response to a 1st bethanechol (BE) stimulus, R_{peak}) and minimum (response to a Ca^{2+}-free solution, R_{nadir}) ratio values were obtained to calculate and report changes in basal and a bethanechol (BE)-induced $[Ca^{2+}]_i$ as per cent maximum in Figure 7A (see 'Methods'). Tissues were not continuously exposed to the excitation light to avoid photo-bleaching, and the force record was linked to the light record, thus creating the gaps in both tracings. The 'spike' in force upon addition of the Ca^{2+}-free solution was an artifact caused by buffer washout.

$[Ca^{2+}]_i$ tissues loaded with the Ca^{2+}-indicator, fura-2, were incubated with 10 μM LOE-908 for 30 min and stimulated with 100 μM BE. In Figure 7A, $[Ca^{2+}]_i$ is reported as a per cent of the maximum response produced by an initial stimulation with BE (see 'Peak' in Figure 5A). These $[Ca^{2+}]_i$ values reflect $[Ca^{2+}]_i$ above that produced in a Ca^{2+}-free solution (zero $[Ca^{2+}]_i$ represents the value measured after a 30 min incubation in a Ca^{2+}-free solution; see 'Methods' and 'Nadir' in Figure 5A). Detrusor displays basal tone (Shenfeld *et al*., 1999). In the present study, basal tone was 0.10 fold F_O (Figure 7B), and basal $[Ca^{2+}]_i$ was $\sim$50% of that produced by an initial BE stimulation (Figure 7A). Stimulation with BE produced an increase in $[Ca^{2+}]_i$ approximately equivalent to the initial BE response (i.e., $\sim$100%, Figure 7A), and an increase in force of 0.91 fold F_O (Figure 7B). LOE-908 (10 μM) significantly reduced basal $[Ca^{2+}]_i$ from $\sim$50 to $\sim$10% (Figure 7A), and reduced basal tone from 0.10 fold F_O to 0.03 fold F_O (Figure 7B). Interestingly, BE failed to produce an increase in $[Ca^{2+}]_i$ in the presence of 10 μM LOE-908 (Figure 7A). However, as reported in Figure 3, BE-induced peak force was $\sim$0.35 fold F_O in the presence of LOE-908 (Figure 7B).

A 30 s stimulation of detrusor with BE produced an increase in the degree of MLC phosphorylation from a basal level of 16% to nearly 35% (Figure 6 and 7C). LOE-908 produced a reduction in the average value of MLC phosphorylation from 16 to 12%, but this apparent reduction was not statistically significant (Figure 7C). In the presence of 10 μM LOE-908, BE still produced an increase in the degree of MLC phosphorylation above the basal level (from 12 to 21%), but this increase was greatly reduced compared to that produced in the absence of LOE-908 (Figure 7C). In summary, in the presence of 10 μM LOE-908, 100 μM BE produced an increase in MLC phosphorylation and force, but not $[Ca^{2+}]_i$. However, the level of $[Ca^{2+}]_i$ in the presence of BE and LOE-908 was elevated well above that produced by a Ca^{2+}-free solution (see Figure 7A).

Effect of the RhoA kinase (ROK) inhibitors, HA-1077 and Y-27632, and effect of LOE-908 plus ROK inhibitors, on BE-induced contractions

It is generally accepted that increases in $[Ca^{2+}]_i$ produce increases in smooth muscle force. However, because G protein-coupled receptor (GPCR) stimulation may induce an increase in the Ca^{2+} sensitivity of smooth muscle contractions by activation of ROK, increases in force produced upon GPCR stimulation may occur despite weak increases, or no increase in $[Ca^{2+}]_i$ (Karaki, 1989; Somlyo & Somlyo, 2000). Thus, we tested the hypothesis that, in detrusor smooth muscle, BE-induced increases in force were due, in part, to activation of a ROK-induced increase in Ca^{2+}

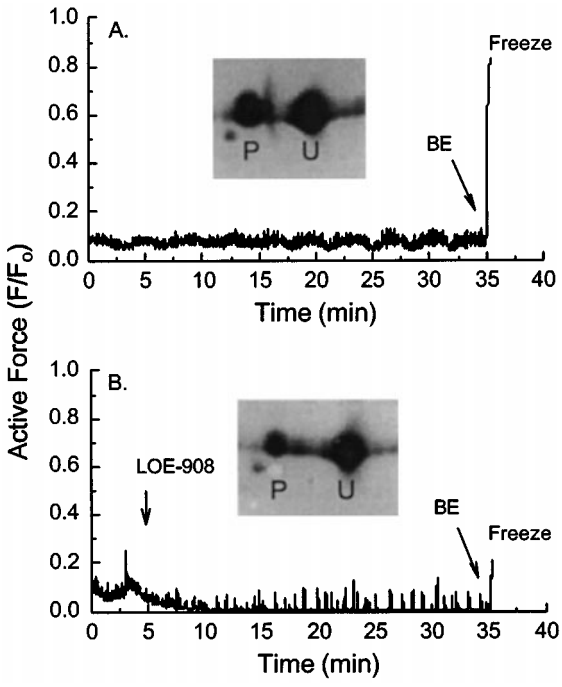

Figure 6 Typical tracings of basal and 100 μM bethanechol (BE)-induced increases in force produced by a control tissue (A) and a tissue exposed to 10 μM LOE-908 (B). Tissues were frozen at 30 s of BE stimulation and changes in the degree of 20 kDa myosin light chain (MLC) phosphorylation (inserts; P = phosphorylated MLC, U = unphosphorylated MLC) were measured using 2-D (IEF/SDS) PAGE, trans-blotting and colloidal gold staining (see 'Methods').

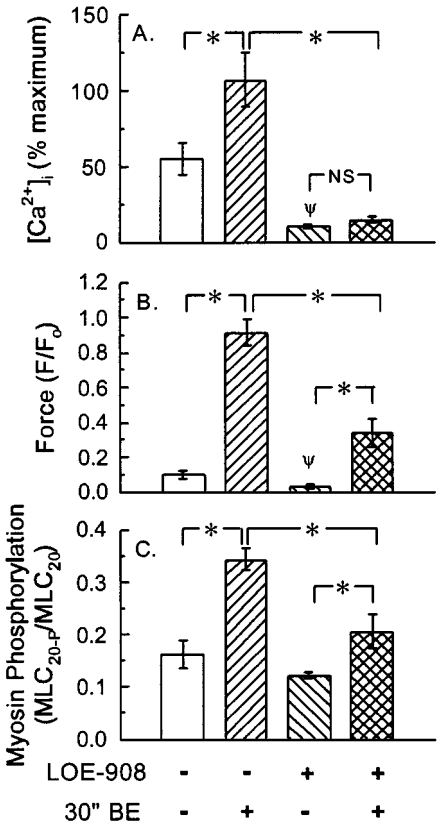

Figure 7 Effect of 10 μM LOE-908 on $[Ca^{2+}]_i$ (A) and force (B) produced under basal conditions and at 30 s (30″) of stimulation with 100 μM bethanechol (BE). Data are means ± s.e.mean; $n = 3-4$; bracketed * indicates $P < 0.05$ between identified groups; $\Psi = P < 0.05$ compared to basal (without (−) LOE-908 or BE).

sensitivity of contractions. HA-1077, an inhibitor of ROK (Nagumo *et al*., 2000; Sward *et al*., 2000; Uehata *et al*., 1997) reduced both peak (Figure 8A) and tonic (10 min; Figure 8B) BE-induced contractions. At 30 μM, HA-1077 did not abolish contractions, but inhibited peak and tonic force by, respectively, 65 ± 6.8% and 79 ± 5.3% (Figure 8). Another highly selective ROK inhibitor, Y-27632 (Feng *et al*., 1999a, b; Uehata *et al*., 1997), when added at a concentration <2 fold greater than the IC_{50} value for inhibition of Ca^{2+}-sensitization (Sward *et al*., 2000), also produced a strong inhibition of peak BE-induced force (Figure 9B). Most importantly, when added alone, the ROK inhibitors and the NSCC blocker, LOE-908, inhibited peak BE-induced force by only ~50% (Figure 9B). However, the combination of a ROK blocker plus LOE-908 nearly abolished contractions (Figure 9A), inhibiting peak BE-induced force by 90–93% (Figure 9B). Moreover, the degree of MLC phosphorylation produced by a 30 s stimulation with 100 μM BE in the presence of 10 μM LOE-908 plus 3 μM Y-27632 was lower than that produced in the presence of either agent alone (Figure 10).

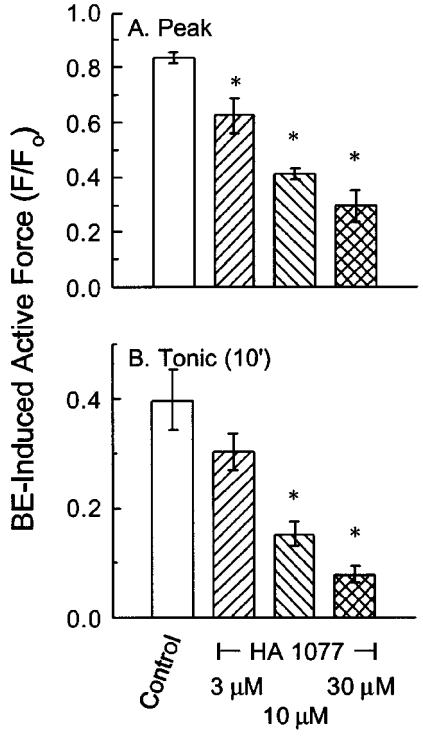

Figure 8 Effect of HA-1077 on peak (A) and tonic (B) contractions produced by 100 μM bethanechol (BE). Data are means ± s.e.mean; $n = 4$; * = $P < 0.05$ compared to control.

Discussion

Data from the present study support the hypothesis that maximum stimulation of detrusor smooth muscle muscarinic receptors produces a contraction that is highly dependent on both Ca^{2+} entry and an increase in the sensitivity of force to increases in $[Ca^{2+}]_i$. Our data using a Ca^{2+}-free solution,

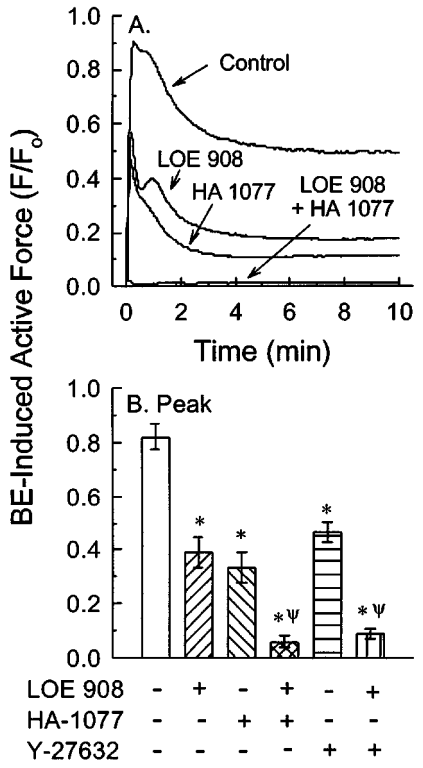

Figure 9 Effect of 10 μM LOE-908, 30 μM HA-1077 and 3 μM Y-27632 alone, and LOE-908 plus HA-1077 and LOE-908 plus Y-27632, on peak force (B) produced by 100 μM bethanechol (BE). LOE-908 selectively inhibits non-selective cation channels (NSCCs). HA-1077 and Y-27632 are inhibitors of RhoA kinase (ROK)-induced Ca^{2+} sensitization. Representative tracings of contractions produced by BE alone, in the presence of LOE-908, HA-1077, and LOE-908 plus HA-1077 are also shown (A). Note the dramatic inhibition of contraction produced by LOE-908 plus HA-1077 compared to that produced by each agent alone (A). LOE-908 plus Y-27632 also nearly abolished the ability of BE to cause contraction. Data in (B) are means $\pm$ s.e.mean; $n=4-7$; $*=P<0.05$ compared to control; $\Psi=P<0.05$ comparing the responses produced by LOE-908 plus a ROK inhibitor to that produced by each agent alone.

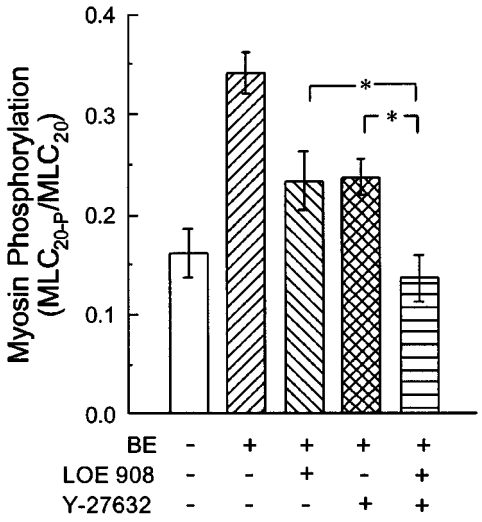

Figure 10 Effect of 10 μM LOE-908, 3 μM Y-27632, and LOE-908 plus Y-27632 on MLC phosphorylation produced at 30 s by 100 μM bethanechol (BE). LOE-908 selectively inhibits non-selective cation channels (NSCCs), and Y-27632 is an inhibitor of RhoA kinase (ROK)-induced Ca^{2+} sensitization. Data are means $\pm$ s.e.mean; $n=3-5$; bracketed * indicates $P<0.05$ between identified groups. Basal (open symbol) and BE-stimulated (right cross-hatched) MLC phosphorylation values are reproduced from Figure 7C for a comparison.

cyclopiazonic acid, 2APB, caffeine and Ca^{2+} channel blockers support the model developed by Batra *et al.* (1987) and Fovaeus *et al.* (1987) that describes detrusor contractions as depending primarily on Ca^{2+} entry rather than on release of Ca^{2+} from SR pools. A novel finding in the present study was that the NSCC blocker, LOE-908, completely inhibited the increase in $[Ca^{2+}]_i$ produced by BE, and reduced peak BE-induced force significantly more than did the selective VOCC blockers, nifedipine and verapamil. These data suggest that NSCCs play a principal role in regulation of detrusor contractions. Perhaps the most important finding of this study was that, in the presence of 10 μM LOE-908, BE produced a moderate increase in force ($\sim$35%) and myosin light chain (MLC) phosphorylation without producing a significant increase in $[Ca^{2+}]_i$ above the basal level. This receptor-stimulated increase in force that was retained in the presence of LOE-908 was nearly abolished by the combination of LOE-908 and an inhibitor of ROK-induced Ca^{2+} sensitization (HA-1077 or Y-27632). The significance of these data is that they support the hypothesis that muscarinic receptor stimulation produces a large increase in the Ca^{2+}-sensitivity of contractions. Thus, the measurement of force alone can not reliably be used to predict changes in stimulus-induced Ca^{2+} mobilization in detrusor smooth muscle. In summary, our data support the view that a significant component of force produced in the presence of a maximally effective concentration of a Ca^{2+} channel blocker may reflect stimulus-induced Ca^{2+} sensitization rather than simply mobilization of intracellular Ca^{2+}.

LOE-908 does not attenuate store-operated Ca^{2+} entry when used at 10 μM (Iwamuro *et al.*, 1999; Zhang *et al.*, 1999), minimally affects VOCCs ($IC_{50}=28$ μM (Krautwurst *et al.*, 1994)), but strongly inhibits NSCC current ($IC_{50}=0.56$ μM (Krautwurst *et al.*, 1994)). For example, in A7r5 smooth muscle cells, 10 μM LOE-908 inhibits NSCC current by $\sim$80% and L-type VOCC current by $\sim$13%. Moreover, LOE-908 does not appear to inhibit release of intracellular Ca^{2+} (Encabo *et al.*, 1996), and our data showed that 10 μM LOE-908 had no effect on contractions produced by arterial muscle incubated in a Ca^{2+}-free solution. Thus, at 10 μM, LOE-908 is considered a selective NSCC blocker. In the present study, 10 μM LOE-908 reduced basal Ca^{2+} entry and basal tone in detrusor. However, it is important to note that LOE-908 did not reduce $[Ca^{2+}]_i$ as much as did incubation in a Ca^{2+}-free solution, indicating that basal Ca^{2+} influx in detrusor is not entirely dependent on NSCCs. This concentration of LOE-908 not only reduced basal $[Ca^{2+}]_i$, but also completely inhibited the ability of BE to increase $[Ca^{2+}]_i$ above that produced in the presence of LOE-908 alone. Thus, these data together support the hypothesis that NSCCs play prominent roles in both establishment of basal tone and BE-stimulated Ca^{2+} entry in detrusor smooth muscle. Moreover, the finding that LOE-908 strongly inhibited BE-induced peak contraction in detrusor while having little effect on phenylephrine-induced peak contraction in artery indicates that NSCC inhibitors may target detrusor

smooth muscle over arterial smooth muscle. Such information may prove useful in the development of therapeutic agents designed to treat overactive bladder. Interestingly, although LOE-908 reduced basal tone, intermittent twitches often remained (see Figure 6B). We speculate that this may have been due to the ability of LOE-908 to inhibit K^+ channels (Krause *et al*., 1998) while incompletely inhibiting VOCCs.

The finding that LOE-908, nifedipine and verapamil strongly inhibited BE-induced contractions indicates that NSCCs and VOCCs participate in regulation of contraction. Another receptor-activated channel type that participates in regulation of smooth muscle contractions in ileum (Ohta *et al*., 1995), pulmonary artery (De La Fuente *et al*., 1995) and spleen (Burt *et al*., 1995) is the store-operated Ca^{2+} channel (SOCC), which is opened by CPA-induced depletion of the inositol 1,4,5-trisphosphate-dependent SR Ca^{2+} store. Our data showed that CPA produced strong contractions, suggesting that SOCCs may participate in regulation of detrusor contractions. However, nearly 90% of the CPA-induced contraction was inhibited by verapamil, suggesting that, in rabbit detrusor, SR store-depletion activates VOCCs, and that 'classical' SOCCs contribute little. An alternate explanation is that one class of SOCCs is sensitive to VOCC blockers. This system also operates in the smooth muscle of some vascular beds, especially those that display intrinsic (myogenic) tone (Asano *et al*., 1998; Nomura *et al*., 1996). However, 10 μM SKF-96365 produced a significantly greater inhibition of CPA-induced peak force than did verapamil, suggesting that a very small fraction ($<10\%$) of the CPA-induced detrusor contraction was dependent on SOCC activity. This conclusion is based on the fact that, at 10 μM, SKF-96365 is more selective for SOCCs than for VOCCs or NSCCs. For example, when used at 10 μM in A7r5 smooth muscle cells, SKF-96365 abolishes SOCC activity (Iwamuro *et al*., 1999), and the potencies of SKF-96365 for VOCCs and NSCCs are 8 and 13 μM, respectively (Krautwurst *et al*., 1994). Lastly, SKF-96365 does not inhibit release of intracellular Ca^{2+} at 10 μM (Merritt *et al*., 1990). Thus, our data suggest that, in detrusor, store-depletion primarily activates VOCCs, although SOCCs may play a minor role.

Ca^{2+} channel blockers reduce BE-induced contractions by more than 50%, but even the most effective agent, LOE-908, did not abolish peak force. BE-induced contractions that occur in the presence of Ca^{2+} channel blockade may be due to mobilization of SR Ca^{2+}. Our data using 2APB (an inhibitor of SR Ca^{2+} release through inositol 1,4,5-trisphosphate receptors; (Ascher-Landsberg *et al*., 1999; Maruyama *et al*., 1997)) demonstrated that the early phase of receptor-stimulated contraction of arterial muscle was highly dependent on mobilization of SR Ca^{2+}, but that mobilization of SR Ca^{2+} contributed no more than 27% to the peak force produced by BE in detrusor. Moreover, BE produced only a very weak contraction of $\sim$0.2 fold F_O when detrusor was incubated for 90 s in a Ca^{2+}-free solution. BE produced stronger contractions when tissues were incubated for shorter durations, but KCl could also produce significant contractions in tissues incubated for $<$90 s in a Ca^{2+}-free solution. Thus, in tissues incubated in a Ca^{2+}-free solution for $<$90 s, contractions produced by BE that were stronger than $\sim$0.2 fold F_O were likely produced because sufficient time had not yet elapsed for Ca^{2+} to diffuse out of the inner tissue layers, and Ca^{2+}entry contributed to force production (Ratz & Murphy, 1987). This hypothesis is supported by our data showing that verapamil eliminated the ability of KCl to produce contractions, even when tissues were incubated for only 10 s in a Ca^{2+}-free solution. Verapamil blocks VOCC-induced Ca^{2+} influx, and does not gain access to intracellular sites (Mras & Sperelakis, 1981; 1982). Thus, the fact that verapamil reduced but did not eliminate the ability of BE to produce contractions in tissues incubated in a Ca^{2+}-free solution suggests that mobilization of intracellular Ca^{2+} may have contributed to force production.

An alternative hypothesis that could explain how BE produced a weak contraction when Ca^{2+} influx was eliminated by a Ca^{2+}-free solution or by Ca^{2+} channel blockers is that BE-activated a mechanism that sensitized cross bridges to the existing level of $[Ca^{2+}]_i$ (Karaki, 1989; Somlyo *et al*., 1999). Although Ca^{2+}-dependent increases in MLC phosphorylation have long been known to play a key role in activation of cross bridges generating increases in smooth muscle contractile force (Driska *et al*., 1981), force is not necessarily a reliable measure of changes in $[Ca^{2+}]_i$. For example, a decrease in $[Ca^{2+}]_i$ may not result in a reduction in force (Rembold & Murphy, 1986), and recent studies indicate that an increase in force may not require an increase in $[Ca^{2+}]_i$ (Ratz, 1999; Uehata *et al*., 1997; VanBavel *et al*., 1998).

An increase in force without a concomitant or proportional increase in $[Ca^{2+}]_i$ is termed an increase in Ca^{2+} sensitivity, and several different models have been proposed to explain the phenomenon (Hori & Karaki, 1998; Somlyo & Somlyo, 1994). One model involves Ca^{2+}-calmodulin independent MLC phosphorylation brought about by activation of RhoA kinase (ROK), which inhibits MLC phosphatase activity, thereby elevating the degree of MLC phosphorylation independently of increases in $[Ca^{2+}]_i$. Three experiments suggest that our data fit this model. First, the ROK inhibitors, Y-27632 and HA-1077, strongly inhibited BE-induced contractions at inhibitor concentrations recently used by others to identify the participation of ROK in regulation of contractions of ileal, vascular and respiratory smooth muscles (Chiba *et al*., 1999; Sward *et al*., 2000; Uehata *et al*., 1997). Second, 10 μM LOE-908 did not abolish BE-induced increases in MLC phosphorylation and force despite its ability to abolish a BE-induced increase in $[Ca^{2+}]_i$. And third, although Ca^{2+} channel blockade by 10 μM LOE-908, or ROK inhibition by either HA-1077 or Y-27632 alone did not abolish BE-induced MLC phosphorylation and force, inhibition of both NSCCs and ROK did. It is interesting to note that 30 μM HA-1077 abolishes the tonic contraction produced by muscarinic receptor stimulation of guinea-pig ileal smooth muscle, but leaves the peak contraction nearly intact (Sward *et al*., 2000). Moreover, activation of muscarinic receptors does not cause ROK-induced Ca^{2+} sensitization in chicken gizzard smooth muscle, despite the presence of ROK and of GTPγS-induced Ca^{2+} sensitization in this muscle-type (Anabuki *et al*., 2000). We found that 30 μM HA-1077 inhibited both peak and tonic contractions in rabbit detrusor, and that $\sim$20% of the tonic contraction remained (see Figures 8 and 9). Additional studies focusing on differences in the dependency of contractions of various smooth muscle systems on ROK may permit the design of

new drugs targeting disorders involving detrusor smooth muscle, such as overactive bladder.

In summary, our data support the hypothesis that muscarinic receptor-stimulated contractions of detrusor smooth muscle are regulated almost exclusively by ROK-induced Ca^{2+} sensitization and Ca^{2+} entry through NSCCs and VOCCs. Blockade of Ca^{2+}-sensitization or NSCCs may therefore provide novel targets for therapeutic drug discovery directed towards reducing detrusor overactivity that causes urgency or incontinence.

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