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Stimulated calcium entry and constitutive RhoA kinase activity cause stretch-induced detrusor contraction

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Abstract

Urinary bladder wall muscle (i.e., detrusor smooth muscle; DSM) contracts in response to a quick-stretch, but this response is neither fully characterized, nor completely understood at the subcellular level. Strips of rabbit DSM were quick-stretched (5 ms) and held isometric for 10 s to measure the resulting peak quick-stretch contractile response (PQSR). The ability of selective Ca^{2+} channel blockers and kinase inhibitors to alter the PQSR was measured, and the phosphorylation levels of myosin light chain (MLC) and myosin phosphatase targeting regulatory subunit (MYPT1) were recorded. DSM responded to a quick-stretch with a biphasic response consisting of an initial contraction peaking at 0.24 ± 0.02 -fold the maximum KCl-induced contraction (F_0) by 1.48 ± 0.17 s (PQSR) before falling to a weaker tonic (10 s) level (0.12 ± 0.03 -fold F_0). The PQSR was dependent on the rate and degree of muscle stretch, displayed a refractory period, and was converted to a sustained response in the presence of muscarinic receptor stimulation. The PQSR was inhibited by nifedipine, 2-aminoethoxydiphenyl borate (2-APB), 100 μM gadolinium and Y-27632, but not by atropine, 10 μM gadolinium, LOE-908, cyclopiazonic acid, or GF-109203X. Y-27632 and nifedipine abolished the increase in MLC phosphorylation induced by a quick-stretch. Y-27632, but not nifedipine, inhibited basal MYPT1 phosphorylation, and a quick-stretch failed to increase phosphorylation of this rhoA kinase (ROCK) substrate above the basal level. These data support the hypothesis that constitutive ROCK activity is required for a quick-stretch to activate Ca^{2+} entry and cause a myogenic contraction of DSM.

Index Words

smooth muscle; urinary bladder; rabbit; myogenic contraction; signal transduction; ROCK

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1. Introduction

A transient detrusor smooth muscle (DSM) contraction in response to mechanical quick-stretch or a hypo-osmolar solution has been identified in rabbit (Burnstock and Prosser, 1960) and man (Masters et al., 1999). This transient contraction is also revealed as a transient increase in pressure when whole bladders are subjected to rapid, step increases in volume (Andersson et al., 1988). Stretch-induced smooth muscle contraction occurs independently of neural input, and the contractile response is therefore termed myogenic. A myogenic response is produced in many, but not all, smooth muscle types (Johnson, 1980), and has been most extensively studied in vascular smooth muscle, where the myogenic response plays a key role in regulation of blood flow (Davis and Hill, 1999). Membrane depolarization leading to elevations in Ca^{2+} entry and cytosolic Ca^{2+} levels can cause myogenic contraction, although the upstream mechanisms leading to depolarization remain to be fully elucidated (Davis and Hill, 1999). In DSM, activation of non-selective cation channels (Wellner and Isenberg, 1994) and stretch-dependent ryanodine receptors (Ji et al., 2002) have been shown to participate in stretch-mediated myogenic contraction. In addition to Ca^{2+} -dependent mechanisms, there is evidence that ROCK, protein kinase C (PKC), phosphatidylinositol 3-kinase and nitric oxide participate in myogenic contraction (Lagaud et al., 2002; Wei et al., 2008).

The precise role that stretch-activated myogenic contraction plays in the regulation of bladder filling or in micturition remains to be determined. However, not every DSM cell is innervated by a post-ganglionic parasympathetic fiber. For this reason Elbadawi (Elbadawi, 1995) suggested that rapid shortening (contraction) of innervated DSM cells following nerve activation leads through mechanical coupling to myogenic activation and contraction of many non-innervated muscle cells, and thus, to a complete and uniform detrusor contractile response. This concept was recently supported by Ji et. al. (Ji et al., 2002). Moreover, myogenic contraction may play a role in the spontaneous (autonomous) rhythmic contraction that occurs during the bladder filling phase (Gillespie, 2004).

The signaling events acting downstream from stretch-activated release of intracellular Ca^{2+} (Ji et al., 2002) that are responsible for causing DSM contraction remain to be determined. In particular, multiple Ca^{2+} entry channels potentially participate in smooth muscle Ca^{2+} homeostasis and contraction (Beech et al., 2004), and contraction may be regulated not only by increases in Ca^{2+} , but also by increases in the sensitivity of contractile proteins to Ca^{2+} (Ratz et al., 2005; Somlyo and Somlyo, 2003). The present study was designed to characterize biomechanically the myogenic contraction produced in rabbit DSM in response to an imposed rapid length-step (quick-stretch), and to identify subcellular mechanisms linking muscle-stretch with the myogenic contractile response.

Overactive bladder is a disorder involving involuntary detrusor contractions that occur during bladder filling (Hampel et al., 1997). Although overactive bladder is a complex disorder that may be associated with several different conditions, one unifying concept proposed by Coolsaet and Blaivas (Coolsaet and Blaivas, 1985) and shown recently to occur in intact bladder (Gillespie et al., 2003; Streng et al., 2006) is that detrusor is never entirely inactive (Artibani, 1997), and a defect causing accentuated micro-motion would propagate a chain of events that may trigger urinary urge incontinence and related symptoms (Gillespie, 2004). Indeed, detrusor from patients with overactive bladder display enhanced spontaneous rhythmic contractile tone (Kinder and Mundy, 1987). Moreover, partial bladder outlet obstruction enhances the autonomous activity of discrete bundles of DSM, and coordination of these contractions is enhanced by stretch (Drake et al., 2003). By understanding the subcellular events associated with myogenic contractions, a means to therapeutically

modulate detrusor overactivity may be discovered that would provide effective treatment of urge incontinence.

2. Materials and methods

2.1. Tissue preparation

All experimental protocols involving animals were conducted within the appropriate animal welfare regulations and guidelines and were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Tissues were prepared as described previously (Speich et al., 2005). Whole bladders from adult female New Zealand white rabbits were removed immediately after sacrifice with pentobarbital. The bladders were washed several times, cleaned of adhering tissue, including fat and serosa, and stored in cold (0–4°C) physiologic salt solution (PSS), composed of NaCl, 140mM; KCl, 4.7 mM; MgSO₄, 1.2 mM; CaCl₂, 1.6 mM; Na₂HPO₄, 1.2 mM; morpholinopropanesulfonic acid, 2.0 mM (adjusted to pH 7.4 at either 0 or 37°C, as appropriate); Na₂ ethylenediamine tetraacetic acid (EDTA; to chelate trace heavy metals), 0.02; and dextrose, 5.6 mM. High purity (17MΩ) water was used throughout. For studies using gadolinium (Gd³⁺), phosphate and EDTA were not included in the PSS to avoid, respectively, precipitation of Gd³⁺ phosphate and Gd³⁺ chelation. For clarity in the results section, PSS will be referred to as a “Ca²⁺-containing solution” while PSS with no CaCl₂ and the addition of 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) to chelate Ca²⁺ as a “Ca²⁺-free solution.” Thin (~200 microns thick) longitudinal detrusor muscle strips ~3 mm wide and ~5 mm long at slack muscle length were separated from the underlying bladder urothelium using micro-dissection. Each muscle strip was incubated in aerated PSS at 37°C in a water-jacketed tissue bath (Radnotti Glass Technology, Monrovia, CA) and secured by small clips to a micrometer for length adjustments and an electronic lever (model 300H, Aurora Scientific, <http://www.aurorascientific.com/index.asp>). The lever was used to induce step (5 ms) and ramp (50 and 500 ms) muscle length changes (stretches), and to record isometric contractions.

2.2. Contraction of isolated detrusor strips

Isometric contractions were measured as described previously (Speich et al., 2005). Voltage signals were digitized (model DIO-DAS16, ComputerBoards, Mansfield, MA), 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). Tissues were equilibrated for a minimum of 30 minutes suspended without tension between micrometer and lever. Tissues were then stretched to their optimum length for muscle contraction (L_o) using an abbreviated length-force determination (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), or as the peak quick-stretch response (PQSR, Fig 1). To quantify peak and tonic forces produced by a quick-stretch in the presence and absence of a low concentration of carbachol (data presented in Fig 5), peak and tonic force values were obtained by subtracting the response produced in the Ca²⁺-free solution from that produced in the Ca²⁺-containing solution.

2.3. Applied length changes and resulting force responses

In most experiments, tissues secured to muscle clips were quickly (5 ms) stretched (quick-stretch) from L_o to a length 15% greater than L_o , held at this new length for 10 s, then released back to L_o . In one set of experiments, tissues were stretched more slowly (ramp-stretches of 50 and 500 ms), and in another set of experiments, the amplitude of the PQSR was measured in response to quick-stretches of three different lengths, 5%, 10% and 15% of

L_0 . Tissues that were quick-stretched responded with an immediate increase in force followed by a rapid decline in force characteristic of viscoelastic materials (stress-relaxation; (Fung, 1993)). In a Ca^{2+} -free solution, stress-relaxation continued towards a steady-state force (Fig 1, dotted line), but in a Ca^{2+} -containing solution, following a short latency period of 0.13 ± 0.01 s ($n=5$), tissues produced a strong, phasic contraction that peaked within 1.48 ± 0.17 s before declining to a weaker tonic contraction (Fig 1, solid line). Stress-relaxation is caused, in part, by frictional slippage due to breaking of non-covalent bonds. The rapid reversal of stress-relaxation by tissues exposed to a Ca^{2+} -containing solution suggests that rapid stretch activated crossbridges and force development to offset the viscous fall in force. The degree of force produced in the Ca^{2+} -containing solution minus that produced in the Ca^{2+} -free solution represented active muscle tone (Fig 1, cross-hatched area). To quantify the strong, phasic, contractile component, the PQSR was defined as the maximum myogenic tone produced above the minimum force attained immediately after the quick-stretch latency period (Fig 1). As such, the PQSR underestimated the actual strength of active force produced upon muscle-stretch, but provided a reliable method to measure and compare the transient myogenic responses under different experimental conditions.

2.4. Myosin light chain (MLC) phosphorylation

The degree of MLC phosphorylation was measured as described previously (Jezior et al., 2001). Tissues were quick-frozen in an acetone-dry ice slurry, slowly warmed to room temperature, dried, weighed and homogenized on ice in 8M urea, 2% Triton X-100 and 20 mM dithiothreitol. Isoelectric variants of the 20 kDa MLCs were separated by 2-D (isoelectric focusing/sodium dodecyl sulfate) polyacrylamide gel electrophoresis followed by Western blot onto Immobilon-P membranes (Millipore; Bedford, MA), visualized by colloidal gold stain, and the relative amounts of phosphorylated and non-phosphorylated MLCs were quantified by digital image analysis.

2.5. Myosin phosphatase targeting subunit (MYPT1) phosphorylation

The degree of MYPT1 site-specific phosphorylation was measured as described previously (Porter et al., 2006). Detrusor strips were quick-frozen in an acetone-dry ice slurry, thawed, homogenized in 1% sodium dodecyl sulfate, 10% glycerol, 20 mM dithiothreitol, 25 mM Tris-HCl (pH 6.8), 5 mM EGTA, 1 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 20 mg/ml leupeptin, 2 mg/ml aprotinin, and 20 mg/ml (4-amidino-phenyl)-methanesulfonyl fluoride, heated 10 min at 100°C , clarified by centrifugation at 10,000 g for 10 min, and stored at -70°C . Thawed homogenates were assayed for protein concentration and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 12% polyacrylamide gels (12 mg of protein per well) followed by Western blotting onto Immobilon-P membranes (Millipore; Bedford, MA). Threonine-853 phosphorylated MYPT1 (MYPT1-p[Thr⁸⁵³]) was identified using a phospho-selective antibody (anti-MYPT-p[Thr⁸⁵³] primary antibody, BD Biosciences, Franklin Lakes, NJ) and detected using an horseradish peroxidase-labeled secondary antibody and enhanced chemiluminescence (Amersham). Quantification of visualized bands was obtained by digital image analysis software. To compensate for gel-to-gel variabilities in efficiencies of Western blotting, antibody labeling, enhanced chemiluminescence reaction, and film development, band intensities were reported as the degree of change from the Ca^{2+} -free basal value. Use of MYPT1 primary antibody (BD Biosciences, Franklin Lakes, NJ) revealed that protein-loading was consistently uniform across all lanes of the gel (see Fig 9B).

2.6. Drugs

Atropine, Gd^{3+} , HA-1077 (1-(5-Isoquinolinesulfonyl)-1H-hexahydro-1,4-diazepine dihydrochloride) and Y-27632 (trans-4-[(1R)-1-Aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride) were made as stock solutions in

distilled water. Nifedipine was dissolved in ethanol, which was added to tissue baths at a final concentration of 0.01%. LOE-908 (3,4-Dihydro-6,7-dimethoxy- α -phenyl-N,N-bis[2-(2,3,4-trimethoxyphenyl)ethyl]-1-isoquinolineacetamide hydrochloride), GF-109203X (2-[1-(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide), cyclopiazonic acid (CPA) and 2-APB (2,2-diphenyl-1,3,2-oxaza-borolidine) were dissolved in dimethyl sulfoxide, which was added at a final concentration of 0.1%. At these percentages, neither vehicle affected the myogenic response. Gd^{3+} was added to a phosphate- and EDTA-free physiological salt solution. LOE-908 was a kind gift from Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut. Y-27632 and CPA were from Calbiochem, 2-APB was from Cayman Chemical and GF-109203X was from Alexis Corporation. All other drugs were from Sigma Chemical Corp.

2.7. 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 bladders, not the number of tissues.

3. Results

3.1. Characterization of the myogenic response in rabbit detrusor

To determine whether the degree of myogenic contraction was dependent on the degree of stretch, tissues were subjected to quick-stretches of 5, 10 and 15% of L_o (Fig 2A), held at each new muscle length for 10 s to permit a myogenic contraction to occur, and the resulting PQSR (see “Methods” and Fig 1) values were recorded (Fig 2B). The PQSR produced by each length-step occurred ~ 1.5 s after application of the quick-stretch, and the degree of PQSR was dependent on the degree of length change (Fig 2C). Even the smallest length-step of 5% L_o produced a significant PQSR (Fig 2C, “5”) when compared to the level of force produced in tissues incubated in a Ca^{2+} -free solution (Fig 2C, “E”).

A 10-fold slower increase in length (50 ms ramp-stretch) still produced a strong PQSR that was not different than that produced by a 5 ms quick-stretch (Fig 3). However, a 100-fold slower ramp-stretch (500 ms) produced only a very weak PQSR (Fig 3). In all remaining studies, a 15%, 5 ms, quick-stretch was applied to produce a myogenic response.

Sequential quick-stretches were applied and the amplitude of each resulting PQSR was measured to determine whether a refractory period exists for development of myogenic tone. The amplitude of the PQSR was reduced by half when two length-steps lasting 10 s each were applied 10 s apart (Fig 4A, “1st” versus “2nd”). A 3rd quick-stretch applied 10 s after the 2nd quick-stretch further reduced the PQSR (Fig 4A, “2nd” versus “3rd”), but no further reduction in PQSR amplitude occurred during the 4th and 5th quick-stretches (Fig 4A). A small reduction in the mean strength of the PQSR occurred when quick-stretches lasting 10 s each were applied 30 s apart. This reduction was significant only after application of the 4th and 5th quick-stretch (Fig 4B). A significant reduction in PQSR amplitude was not induced when 3 quick-stretches lasting 10 s each were applied sequentially 90 s apart (Fig 4C). This was likewise true when 4 quick-stretches were applied sequentially 300 s apart (data not shown).

The muscarinic receptor agonist, carbachol, at a low concentration ($3 \times 10^{-8}M$) that alone produced a weak contraction $\sim 10\%$ above basal tone (Fig 5, compare Carbachol open bar to Control open bar) converted the myogenic response from one characterized by a strong phasic and weak tonic contraction (Fig 5, Control hatched and cross-hatched bars) to one characterized by a strong, sustained response (Fig 5, Carbachol hatched and cross-hatched bars). Atropine (1 μM) abolished both pre-stretch weak contraction produced by carbachol

and the sustained phase of the myogenic response produced in response to stretch in the presence of carbachol (Fig 5, Atropine + Carbachol).

3.2. Effect on PQSR amplitude of atropine, CPA, inhibitors of Ca^{2+} channels, and inhibitors of ROCK and PKC

The effects of selective receptor, ion channel and enzyme inhibitors on PQSR were examined using the protocol shown diagrammatically in Fig 6A. Results from data shown in Fig 5 suggest that atropine did not affect the PQSR. To more definitively determine whether the PQSR was not the result of the action of acetylcholine released from cholinergic neurons on DSM, 1 μM atropine was added to tissues 10 and 15 minutes prior to quick-stretches to block DSM muscarinic receptors. Compared to control (Fig 6B, C1 and C2), atropine did not reduce the strength of the PQSR (Fig 6B, 10' (= 10 minutes) and 15' (= 15 minutes)). This concentration of atropine completely inhibited contractions produced by 10 μM bethanechol, a concentration of muscarinic receptor agonist that produces nearly a maximum rabbit DSM contraction (Shenfeld et al., 1998). To determine whether the PQSR could be abolished by sarcoplasmic/endoplasmic reticulum pump inhibition, tissues incubated with 10 μM CPA were subjected to the quick-stretch protocol shown in Fig 6A. CPA did not prevent the development of a strong PQSR (Fig 6C). In fact, the mean value of the PQSR in the presence of CPA was elevated compared to control, although this apparent increase was not significant. These data do not eliminate a role for the sarcoplasmic reticulum in causing a myogenic contraction, but suggest that Ca^{2+} entry, possibly in conjunction with sarcoplasmic reticulum Ca^{2+} -release, is a required signaling event linking the mechanical stimulus (quick-stretch) with the resulting contraction (PQSR).

L-type voltage-operated Ca^{2+} channels (VOCCs) and non-selective cation channels (NSCCs) may participate in stretch-activated Ca^{2+} influx in vascular smooth muscle cells (Davis and Hill, 1999). We therefore tested the hypothesis that these channel types also participate in quick-stretch-induced contraction of DSM by employing nifedipine and LOE-908, blockers of, respectively, VOCCs (McDonald et al., 1994) and NSCCs (Krautwurst et al., 1994). Nifedipine, at a concentration that inhibits KCl-induced contractions (1 μM), nearly abolished the PQSR (Fig 7A). Notably, 10 μM LOE-908, a concentration of NSCC blocker that strongly inhibits receptor-stimulated contractions in vascular smooth muscle (Krautwurst et al., 1994) and reduces by half the maximum contraction produced in detrusor by muscarinic receptor activation (Jezior et al., 2001), did not reduce the amplitude of the PQSR (Fig 7B). Gd^{3+} , a trivalent cation that concentration-dependently inhibits stretch-activated cation channels, certain transient receptor potential (TRP) channels, and VOCCs (Hamill and McBride, 1996; Lievremonet et al., 2005; Morita et al., 2007; Yang and Sachs, 1989; Zhu et al., 1998), had no effect at 10 μM , but greatly reduced the amplitude of the PQSR at 100 μM (Fig 7C). Because 10 μM Gd^{3+} inhibits TRP channels responsible for store-operated cation channel activity, whereas 100 μM Gd^{3+} is required to inhibit TRPC3/6/7 channels responsible for other forms of Ca^{2+} entry (Lievremont et al., 2005; Zhu et al., 1998), we also examined the effect of 2-APB at a concentration known to selectively inhibit TRPC3/6/7 channels (Lievremont et al., 2005), but to not inhibit VOCCs (Luo et al., 2001; Poteser et al., 2003). 2-APB at 10 μM , like nifedipine and 100 μM Gd^{3+} , strongly inhibited PQSR (Fig 7D).

Although G protein-coupled receptor stimulation and KCl are stimuli that cause activation of ROCK (Ratz et al., 2005; Somlyo and Somlyo, 2000), whether mechanical stretch of DSM also does so remains to be determined. In the present study, both Y-27632 (Fig 7E) and HA-1077 (Fig 7E), at concentrations that produce relatively selective inhibition of ROCK activity (Davies et al., 2000), caused a significant reduction in the amplitude of the PQSR.

Certain PKC isotypes may also participate in regulation of smooth muscle contraction through inhibition of MLC phosphatase (Eto et al., 2001). We used GF-109203X at a concentration that inhibits conventional and novel isotypes of PKC (PKC α , β), (Eto et al., 2001; Martiny-Baron et al., 1993), to test the hypothesis that PKC may play a role in regulation of stretch-activated detrusor contraction. At 1 μ M, GF-109203X did not reduce the amplitude of the PQSR (Fig 7F).

3.3. Effect of Y-27632 on basal and quick-stretch-induced MLC phosphorylation

To determine whether quick-stretch causes increases in MLC phosphorylation, and whether inhibition of the PQSR by Y-27632 corresponded with reductions in the degree of MLC phosphorylation, tissues were quick-stretched, frozen ~1 s later, and processed to measure MLC phosphorylation. In agreement with our previous report (Ratz and Miner, 2003), basal MLC phosphorylation was ~15% and was inhibited by 1 μ M Y-27632 (Fig 8, “Basal”). Quick-stretch produced a strong increase in MLC phosphorylation (Fig 8, “Stretch”) that was also inhibited by Y-27632 (Fig 8, hatched bar). Like Y-27632, the Ca²⁺ channel blocker, nifedipine (1 μ M) inhibited both basal and quick-stretch-stimulated increases in the degree of MLC phosphorylation (Fig 8, cross-hatched bars).

3.4. Effects of Y-27632 and nifedipine on basal and quick-stretch-induced MYPT1 site-specific phosphorylation at threonine 853 (MYPT-p[Thr⁸⁵³])

ROCK can regulate myosin phosphatase activity by phosphorylating MYPT1 at threonine 853 (Kitazawa et al., 2003; Velasco et al., 2002). We therefore measured the relative degree of MYPT-p[Thr⁸⁵³] in resting (basal) detrusor and at ~1 s after tissues had been quick-stretched. Quick-stretch did not increase the degree of MYPT-p[Thr⁸⁵³] compared to the basal level (Fig 9A), and although the mean value was reduced, this apparent decrease was not significant. The amounts of MYPT-p[Thr⁸⁵³] produced both basally and during the 1st second of a quick-stretch were reduced by ~30% by 1 μ M Y-27632, but not by 1 μ M nifedipine (Fig 9A). No differences in levels of total MYPT were identified (Fig 9B).

In summary, the strong increase in MLC phosphorylation and transient contraction (PQSR) induced by quick-stretch were both inhibited by Y-27632, but quick-stretch did not cause phosphorylation of the ROCK substrate, MYPT1, suggesting that quick-stretch did not activate ROCK above the basal level. These data together support the hypothesis that ROCK was constitutively active, and that this constitutive activity was required for full development of the PQSR.

4. Discussion

This study provides data supporting the hypothesis that the contractile response produced upon quick-stretch of DSM strips (PQSR) was caused by activation of Ca²⁺ channels resulting in increases in cytosolic Ca²⁺ levels in the presence of constitutively active ROCK, an enzyme known to enhance the degree of MLC phosphorylation by inhibition of MLC phosphatase activity (Hartshorne et al., 1998). The significance of this finding is two-fold. First, in addition to playing a principal role in regulation of stimulus-induced Ca²⁺ sensitization of smooth muscle contractile protein activation (Ratz et al., 2005; Somlyo and Somlyo, 2000), results from the present study support the novel hypothesis that ROCK appears also to play a prominent permissive role in DSM contractions, a result supported by our previous finding that basal rhythmic tone and MLC phosphorylation are also strongly attenuated by ROCK inhibition in strips of rabbit detrusor (Ratz and Miner, 2003). Second, there is strong evidence that the most proximal, or upstream linkage between mechanical quick-stretch and initiation of cell signaling events in DSM is activation of ryanodine receptors and release of sarcoplasmic reticulum Ca²⁺ causing Ca²⁺ sparks (Ji et al., 2002).

Our data reveal that the downstream events must include stimulated Ca^{2+} entry along with constitutive ROCK activity for production of a full stretch-induced contraction. Thus, we propose that stimulated Ca^{2+} entry is necessary and links mechanical quick-stretch with contraction. In this working model (Fig 10), stimulated Ca^{2+} entry alone is necessary but insufficient to generate a full myogenic contraction, because constitutive ROCK activity is also required to maintain MLC phosphatase in an inactive conformation, permitting a strong increase in force. Moreover, based on a comparative pharmacological approach, TRPC3/6/7 and L-type VOCC channels appear to have played the primary role in the Ca^{2+} entry linking quick-stretch with contraction.

The most significant recent advance coupling stimulus-dependent Ca^{2+} channel physiology such as store- and receptor-operated Ca^{2+} entry with Ca^{2+} channel type was the discovery of TRP channels, a family of over 50 structurally related proteins displaying a diverse stimulation profile (Beech, 2005). Thus far, the only organic channel blocker known to be selective for certain TRP channels is 2-APB (Lievremont et al., 2005). 2-APB displays an IC_{50} of 20 μM for TRPC5 channels (Xu et al., 2005), and at 10 μM , 2-APB inhibits TRPC3/6/7 by more than 50% while displaying no inhibition of inositol trisphosphate-dependent Ca^{2+} release (Lievremont et al., 2005). 2-APB does not inhibit VOCCs (Luo et al., 2001; Poteser et al., 2003) ryanodine receptors (Wu et al., 2000), arachidonic acid-activated cation channels (Luo et al., 2001), S-nitrosylation-activated channels (van Rossum et al., 2000), Ca^{2+} -activated Cl^- entry channels (Wu et al., 2000), or stretch-activated cation channels thought to be dependent on TRPM4-like non-selective cation channels (Sarkozi et al., 2005). Our data showed that the PQSR in DSM was nearly abolished by 10 μM 2-APB. Because TRPC3/6/7 channels are expressed by smooth muscle (Dietrich et al., 2005), these data suggest that TRP3/6/7 channels may have participated in the Ca^{2+} entry responsible for stretch-activated contraction in rabbit DSM. This conclusion supports the proposal that TRPC3/6/7 can serve not only as a receptor-operated and store-operated channel, but also as a mechano-sensitive channel (Beech, 2005). However, a recent study (Bai et al., 2006) reports that at the concentration used in the present study, 2-APB can effectively inhibit certain connexin isoforms and gap junction formation. Thus, our data cannot rule out the possibility that quick-stretch of rabbit DSM strips enhanced gap junction formation, permitting cell-to-cell coupling and a strong myogenic contraction.

TRPC3/6/7-dependent Ca^{2+} entry that is not dependent on sarcoplasmic reticulum store depletion is inhibited by 100 μM , but not 10 μM Gd^{3+} (Lievremont et al., 2005; Zhu et al., 1998). Thus, our data showing that 100 μM , but not 10 μM Gd^{3+} inhibited the PQSR supports our contention that a TRPC3/6/7 channel was activated downstream from mechanical stretch. Moreover, because Gd^{3+} inhibits ryanodine receptor Ca^{2+} channel activity with an IC_{50} of 5–6 μM (Sarkozi et al., 2005), these data suggest that the quick-stretch-induced myogenic contraction of rabbit DSM in the present study was not dependent on ryanodine receptor activation.

L-type VOCCs are sensitive not only to membrane depolarization, but also to mechanical stretch (Langton, 1993), and our finding that nifedipine strongly inhibited the PQSR suggests that VOCCs also played a role in stretch-induced contraction in DSM. However, several studies indicate that certain TRP-dependent channels may be as sensitive to inhibition by nifedipine and related compounds as are L-type VOCCs (Curtis and Scholfield, 2001; Krutetskaia et al., 1997; Willmott et al., 1996; Zhu et al., 1998), which is supported by evidence for sequence similarity between TRP channels and the α_1 subunit of L-type VOCCs (Phillips et al., 1992). For example, the VOCC blocker, verapamil, inhibits TRPC3 channels with an IC_{50} of 4 μM (Zhu et al., 1998). Also, in the present study, the PQSR was not inhibited by a low concentration (10 μM) of Gd^{3+} , which has been shown to effectively inhibit VOCCs (Hamill and McBride, 1996; Wu and Davis, 2001). Thus, an alternative

conclusion is that VOCCs did not contribute to the PQSR, and instead, that TRPC3/6/7 channels sensitive to nifedipine were responsible for stretch-activated Ca^{2+} entry leading to the PQSR in rabbit DSM. LOE-908 effectively inhibits not only basal tone but also muscarinic receptor-induced contraction in rabbit detrusor (Jezior et al., 2001), indicating that NSCCs play an essential role in these responses. Thus, stretch-activated myogenic contraction is distinct from basal tone and muscarinic receptor-induced contractions because LOE-908-sensitive NSCCs did not appear to play a role in myogenic contraction. These data, along with the finding that atropine did not suppress the PQSR, indicate that the myogenic response produced by quick-stretch in rabbit DSM did not involve muscarinic receptor activation.

The muscarinic receptor antagonist, atropine, did not inhibit the ability of quick-stretch to cause a contraction. These data can be taken as evidence that the isolated longitudinal bundles of DSM in the micro-dissected bladder strips in our preparation did not contain urothelium and suburothelial tissues, which respond to stretch by releasing acetylcholine causing bladder contraction (Yoshida et al., 2006).

Both ROCK and PKC have been implicated in regulation of vascular myogenic tone (Dubroca et al., 2005; Yeon et al., 2002). ROCK, PKC α and PKC δ can enhance Ca^{2+} -activated contraction by inhibition of MLC phosphatase, thus causing Ca^{2+} sensitization, an increase in the degree of force for a given increase in $[\text{Ca}^{2+}]_i$. Y-27632 is highly selective for inhibition of ROCK compared to MLC kinase, conventional PKC isoforms such as PKC α , and several other enzymes (Davies et al., 2000). However, Y-27632 also inhibits PKC δ , a novel PKC isoform (Eto et al., 2001). Thus, the use of Y-27632 alone is insufficient to distinguish between ROCK and PKC δ in causing smooth muscle contraction by enhancing Ca^{2+} sensitivity. For this reason, the use of GF-109203X and Y-27632 have been used to discriminate between ROCK and PKC δ activities (1 μM Y-27632 inhibits PKC δ and ROCK, but 1 μM GF-109203X inhibits PKC δ , not ROCK (Eto et al., 2001; Martiny-Baron et al., 1993)). In the present study, 1 μM GF-109203X did not reduce the amplitude of the PQSR, while 1 μM Y-27632 reduced the amplitude by more than 50%. These data suggest that ROCK, not PKC δ , was involved in the stretch-activated myogenic contraction. Moreover, 10 μM HA-1077 nearly abolished the PQSR, and HA-1077 is a potent inhibitor of ROCK compared to MLC kinase and PKC α (Sward et al., 2000). Because 1 μM GF-109203X inhibits PKC α (Martiny-Baron et al., 1993), this enzyme may also be ruled out as participating in the stretch-induced myogenic response in rabbit DSM.

In support of our model (see Fig 10) that stretch stimulated Ca^{2+} entry to cause activation of MLC kinase (MLCK), we found that stretch increased the degree of MLC phosphorylation above the basal level of ~15% by ~3-fold to ~45%, and that nifedipine abolished this increase. Stretch did not cause an increase above the basal level in the ROCK substrate responsible for causing Ca^{2+} sensitization, MYPT-p[Thr⁸⁵³]. However, basal MYPT-p[Thr⁸⁵³] was reduced by ~40% by the ROCK inhibitor, Y-27632, and not by nifedipine. Y-27632 likewise reduced basal MLC phosphorylation to under 10%. Together, these data support the hypothesis that ROCK was constitutively active in DSM, and that the constitutive activity contributed to the contraction produced upon quick-stretch, but that quick-stretch did not further activate ROCK. Given the recent report that a threshold level of MLC phosphorylation of ~15% must be attained before contraction can occur in smooth muscle (Rembold et al., 2004), these data suggest that constitutive ROCK activity is essential to permit a rapid increase in MLC phosphorylation above the threshold level causing rapid contraction upon muscle stimulation.

While similar, myogenic tone of the detrusor and vasculature are distinct in at least two ways. First, both ROCK and PKC regulate myogenic tone of basilar artery (Yeon et al.,

2002), whereas the present study indicates that ROCK plays the principal role in detrusor. Second, myogenic tone in the vasculature is sustained (Davis and Hill, 1999), while in detrusor, it is transient. Interestingly, in the present study, weak stimulation of muscarinic receptors known to activate PKC converted the transient myogenic contraction to a sustained contraction.

The amplitude of the myogenic contraction was dependent on the amplitude of the length-step (see Fig 2). These data support the hypothesis that the role of the myogenic contraction is to provide high fidelity mechanical coupling of DSM cells acting as a unit. With such coupling, the degree of length change imposed on nearby DSM cells by, for example, rapidly contracting innervated DSM cells, would be proportional to the numbers of innervated cells activated, which would in turn translate to a proportional strength of myogenic contraction. Although the amplitude of the myogenic contraction was dependent on the amplitude of the length-step, only fast (5 or 50 ms), not slow (500 ms) stretches produced a myogenic contraction. Thus, the stimulus for induction of a myogenic contraction appeared to be muscle force rather than muscle length (Fung, 1993). One or more steps in the signaling sequence required less than 90 s, but more than 30 s for full recovery, because the amplitude of the peak myogenic response was significantly reduced when stretches were repeated every 10 or 30 s, but not when stretches were repeated every 90 s. Ion channel recovery from inactivation is usually measured on the ms time scale, suggesting that the refractory period for the myogenic response may not have been due to inactive ion channels. Interestingly, Ca^{2+} sensitivity involving ROCK can be down-regulated for long periods (Ratz, 1995), suggesting that inactivation of ROCK or ROCK signaling systems may have been responsible for the myogenic response refractory period. Alternatively, the refractory period may reflect mechanical accommodation of the stretch sensor.

In summary, the present study supports an active role for Ca^{2+} entry and a permissive role for ROCK activity in the DSM contraction induced by muscle quick-stretch. Our data ruled out LOE-908-sensitive NSCCs from participating in myogenic contraction, but showed that a 2-APB- and nifedipine-sensitive, but 10 μM Gd^{3+} -insensitive channel participates. The most likely channel consistent with this pharmacological profile is a TRPC3/6/7 cation channel. Moreover, this study showed that the amplitude of the detrusor myogenic response was dependent on the rate and degree of muscle stretch, displayed a relatively long (~30 s) refractory period, and was converted from a biphasic to a sustained contraction by weak stimulation of muscarinic receptors. Myogenic detrusor contraction may participate in the mechanical synchronization of contractions of multiple muscle cells permitting syncytial-like detrusor contraction (i.e., the rapid contraction of one cell would stretch an adjacent cell, causing membrane depolarization and contraction). As such, the detrusor myogenic response may represent a novel target for therapeutic agents designed to treat urological disorders such as overactive bladder.

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Figure 1.

Example of the force response produced upon a rapid (5 ms) imposed length-step equal to 15% of the optimum muscle length (L_0) in detrusor strips incubated in a Ca^{2+} -free solution (dotted line) and in a Ca^{2+} -containing solution (solid line). Myogenic tone is the force response produced by tissues in the Ca^{2+} -containing solution (hatched area). The peak quick-stretch response (PQSR) was defined as the difference between maximum myogenic force produced above the minimum force attained, on average, 0.13 ms after the stretch.

Figure 2.

Effect of the degree of muscle stretch on the peak quick-stretch response (PQSR). Each tissue was quick (5 ms)-stretched by amounts equaling 5, 10 and 15% of L_0 and held at the new muscle length for 10 s, permitting the development of myogenic contraction. An example of the length perturbation for three muscle strips is shown in panel A (upstroke of length-step lasted 5 ms, duration of length-step lasted 10 s), and the corresponding force response for each length-step is shown in panel B. Summary data is shown in panel C. PQSR measured for stretches in a Ca^{2+} -containing solution (hatched bars) were compared to the force response produced in a Ca^{2+} -free solution (open bar). Data in (C) are mean $\pm$ S.E.M., $n = 5$, * $P < 0.05$ compared to "E".

Figure 3.

Effect of the rate of muscle stretch on peak quick-stretch response (PQSR) amplitude. Tissues were subjected to stretches equaling 15% L_0 at 5, 50 and 500 ms (insert shows time on the x-axis and muscle length on the y-axis), and the resulting PQSR was recorded. Data are mean $\pm$ S.E.M., $n = 3-5$, * $P < 0.05$ compared to 5 and 50 ms.

Figure 4.

Identification of a refractory period for the peak quick-stretch response (PQSR). Tissues were quick (5 ms)-stretched by amounts equaling 15% of L_0 and maintained at the new length for 10 s to permit development of a myogenic contraction before each muscle was returned to L_0 . This sequence was repeated 5 times every 10 (A) or 30 (B) s, or 3 times every 90 (C) s. Data are mean $\pm$ S.E.M., $n = 8$, * $P < 0.05$ compared to the 1st response.

Figure 5.

Effect of weak stimulation of muscarinic receptors on the peak and tonic myogenic force. At a concentration that produced a weak (~10%) contraction, carbachol converted myogenic contraction induced by a 5 ms quick-stretch of 15% L_0 from a transient response to a sustained response (tonic force was not different than peak force). Atropine (1 μ M) abolished the ability of quick-stretch to induce a strong tonic myogenic response. Data are mean $\pm$ S.E.M., $n = 3$. * $P < 0.05$ comparing peak and tonic forces, Ψ $P < 0.05$ compared to pre-stretch tone.

A. Protocol

a. Length-Steps (Stretches)

b. Force Responses

B. Atropine

C. CPA

Figure 6.

Protocol for the effect of selective drugs on the peak quick-stretch response (PQSR) is shown in panel A (a. imposed length change, b. resulting force change), and the effects of the cholinergic antagonist, atropine (1 μ M), and the sarcoplasmic/endoplasmic reticulum pump inhibitor, cyclopiazonic acid (CPA, 10 μ M), on PQSR are shown in, respectively, panels B and C. Tissues were quick (5 ms)-stretched by amounts equaling 15% of L_0 and maintained at the new length for 10 s to permit development of a myogenic contraction before each muscle was returned to L_0 . This was repeated again 5 min later and the PQSRs to these 1st and 2nd quick-stretches were labeled control 1 (C1) and control 2 (C2), respectively. Atropine was added and 10 min (10') and 15 min (15') later tissues were subjected to 3rd and 4th quick-stretches, respectively. Data are mean $\pm$ S.E.M., $n = 3-6$.

Figure 7.

Effects on the PQSR of the selective L-type voltage-operated Ca^{2+} channel (VOCC) blocker, nifedipine (A), the non-selective cation channel blocker, LOE-908 (B), the cation channel blocker, Gd^{3+} (C), the TRP channel blocker, 2-APB (D), the ROCK blockers Y-27632 (1 μ M) and HA-1077 (10 μ M, E), and the inhibitor of conventional and novel isoforms of PKC, GF-109203X (F). Drugs were added immediately after recovery from the 2nd quick-stretch, as shown in panel A of Fig 6. Responses to the 1st and 2nd quick-stretches were labeled control 1 (C1) and control 2 (C2), respectively, and to the 3rd and 4th quick-stretches after addition of drug were labeled 10 min (10') and 15 min (15'), respectively. Data are mean $\pm$ S.E.M., $n = 3-6$. * $P < 0.05$ compared to C1.

Figure 8.

Quick-stretch induced a large increase above the basal level in myosin light chain (MLC) phosphorylation at ~ 1 s ($\Psi P < 0.05$ compared to control basal). Y-27632 and nifedipine (1 μM) inhibited the degree of MLC phosphorylation produced basally and at 1 s of muscle quick-stretch. Data are mean $\pm$ S.E.M., $n = 3-8$. * $P < 0.05$ compared to respective control values.

Figure 9.

Effect of Y-27632 and nifedipine (1 μ M) on the degree of myosin phosphatase targeting subunit (MYPT) phosphorylation at threonine 853 (Thr⁸⁵³) under basal conditions and ~1sec after initiation of a quick-stretch. Data are mean $\pm$ S.E.M., n = 3–6. * P<0.05 compared to respective control values.

Figure 10.

Working model of cell signaling systems operating during quick-stretch-induced myogenic contraction. Development of a full quick-stretch-induced contraction required constitutive, not stimulated ROCK activity, and quick-stretch-stimulated activation of Ca^{2+} entry that was sensitive to blockade by nifedipine, low concentrations of 2-APB, and high concentrations of Gd^{3+} . Extracellular = extracellular space; PM = plasma membrane.; MLC = myosin light chain; MLCK = MLC kinase; MLCP = MLC phosphatase; MYPT1 = myosin phosphatase targeting subunit 1; TRPC3/6/7 = transient receptor potential canonical channel isoform 3, 6 or 7.