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Potential of carbachol-induced detrusor smooth muscle contractions by β -adrenoceptor activation

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Abstract

In strips of rabbit bladder free of urothelium, the β -adrenoceptor agonist, isoproterenol, significantly reduced basal detrusor smooth muscle tone and inhibited contractions produced by low concentrations of the muscarinic receptor agonist, carbachol. During a carbachol concentration-response curve, instead of inhibiting, isoproterenol strengthened contractions produced by high carbachol concentrations. Thus, the carbachol concentration-response curve was shifted by isoproterenol from a shallow, graded relationship, to a steep, switch-like relationship. The tyrosine kinase inhibitor, genistein, inhibited carbachol-induced contractions only in the presence of isoproterenol. Contraction produced by a single high carbachol concentration (1 μ M) displayed 1 fast and 1 slow peak. In the presence of isoproterenol, the slow peak was not strengthened, but was delayed, and U-0126 (mitogen-activated protein kinase kinase inhibitor) selectively inhibited this delay concomitantly with inhibition of extracellular signal-regulated kinase (ERK) phosphorylation. Isoproterenol reduced ERK phosphorylation only in the absence of carbachol. These data support the concept that, by inhibiting weak contractions, potentiating strong contractions, and producing a more switch-like concentration-response curve, β -adrenoceptor stimulation enhanced the effectiveness of muscarinic receptor-induced detrusor smooth muscle contraction. Moreover, β -adrenoceptor stimulation changed the cellular mechanism by which carbachol produced contraction. The potential significance of multi-receptor and multi-cell crosstalk is discussed.

Index Words

rabbit urinary bladder; smooth muscle; isometric contraction; signal transduction; muscarinic receptors; β -adrenergic receptor; ERK; genistein

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1. Introduction

Overactive bladder is a common disease characterized by involuntary detrusor smooth muscle contractions during bladder filling (Abrams and Wein, 2000). The etiology of overactive bladder is unknown, in part, because subcellular mechanisms regulating detrusor smooth muscle contraction are poorly understood (Gillespie, 2004). In particular, the roles played by sympathetic efferent nerve activation alone and in the presence of cholinergic nerve activity in the normal detrusor remain unresolved.

During bladder filling, tonic activation of the sympathetic nervous system may ensure bladder relaxation (de Groat, 1975; de Groat and Saum, 1972). The net effect of the adrenergic inhibitory system appears to be to prolong bladder filling time by reducing the level of bladder wall tension transduced by local mechanoreceptors (Vaughan and Satchell, 1995). Recent evidence shows that, in addition to expressing M_3 receptors that elevate Ca^{2+} and cause contraction when stimulated by muscarinic receptor agonists, detrusor smooth muscle expresses M_2 muscarinic receptors that can inhibit β -adrenoceptor-induced relaxation (Hegde and Eglen, 1999; Ostrom and Ehlert, 1998). Thus, in theory, detrusor smooth muscle may participate in regulation of voiding by “turning off” the smooth muscle relaxant effect of β -adrenoceptor activation.

It is well-known that β -adrenoceptor activation elevates Ca^{2+} entry and the strength of contraction in myocardial cells (reviewed by (Sperelakis *et al.*, 1994)). Less well appreciated is the fact that Ca^{2+} entry can also be elevated in smooth muscle cells by β -adrenoceptor activation (Fukumitsu *et al.*, 1990; Ishikawa *et al.*, 1993; Xiong *et al.*, 1994a; b) via subcellular mechanisms analogous to those occurring in myocardial cells (Zhong *et al.*, 1999). It is therefore possible that one function of β -adrenoceptor stimulation of detrusor smooth muscle is to enhance contractions produced by muscarinic receptor stimulation.

We tested the hypothesis that detrusor smooth muscle contraction is more effective when both β -adrenoceptor and muscarinic receptors are stimulated. Moreover, we examined the possibility that tyrosine phosphorylation plays a role in crosstalk between β -adrenoceptor and muscarinic signaling systems during regulation of detrusor smooth muscle contraction. To reduce the complexity inherent in bladder in which multiple cell-types reside, these studies were performed using isolated strips of rabbit detrusor devoid of underlying urothelium and overlying serosa.

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 (Ratz, 1993; Shenfeld *et al.*, 1998). Whole bladders from adult female New Zealand white rabbits were removed immediately after sacrifice with pentobarbital. 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; $MgSO_4$, 1.2 mM; $CaCl_2$, 1.6 mM; Na_2HPO_4 , 1.2 mM; morpholinopropanesulfonic 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 (17M Ω) water was used throughout. Longitudinal detrusor muscle strips free of underlying urothelium were cut from the wall of the bladder above the trigone. Thin (~200 micron) muscle strips (~3 mm wide by ~5 mm long) were cut following the natural bundling that is clearly demarcated when bladders are in ice-cold

buffer, as described previously (Ratz and Miner, 2003). Muscle tissues were incubated in aerated PSS at 37°C in water-jacketed tissue baths (Radnotti Glass Technology, Monrovia, CA). Tissues that were to be stretched to their optimum length for muscle contraction (L_o) were secured by small clips to a micrometer for length adjustments and a force transducer (Harvard Bioscience, Holliston, MA and Radnotti Glass Technology, Inc, Monrovia, CA) for measurement of isometric contraction.

2.2. Contraction of isolated detrusor strips

Isometric contraction was measured as described previously (Ratz, 1995; Shenfeld *et al.*, 1998). 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 force transducer, then stretched to their optimum length for muscle contraction (L_o) using an abbreviated length-force determination in which the optimum force for muscle contraction (F_o) produced by 110 mM KCl at L_o was obtained (Herlihy and Murphy, 1973; Ratz and Murphy, 1987; Uvelius, 1976). Tissues were incubated in a Ca^{2+} -free solution and subjected to a quick-release protocol to obtain passive force values (Herlihy and Murphy, 1973). To reduce tissue-to-tissue variability, subsequent contractions were reported as normalized to F_o (F/F_o). Detrusor produces rhythmic contractions under basal conditions, and basal contractile tone is defined as the average rhythmic contractile force produced over a period of ~3 min minus passive force (Shenfeld *et al.*, 1999).

To construct a carbachol cumulative concentration-response curve, carbachol was added to tissues in half-log increments starting with 10^{-8} M carbachol and ending with 10^{-5} M carbachol (Fig 1A), and the peak force produced at each concentration was recorded. Tissues were washed several times by a complete buffer change to remove carbachol and cause complete relaxation. To examine the effect of isoproterenol and norepinephrine on carbachol-induced contractions, a control carbachol concentration-response curve was performed and, 60 min later, tissues were exposed to isoproterenol and norepinephrine and 15 min later a 2nd carbachol concentration-response curve was performed. The 2nd carbachol concentration-response curve was plotted with and compared to the 1st (Control) carbachol concentration-response curve. To examine the ability of genistein to alter the carbachol concentration-response curve, genistein was added 15 min before addition of isoproterenol.

To examine the effects of isoproterenol on single-dose carbachol contractions, tissues were contracted with carbachol for 3 min to produce a contraction designated as F_1 . Tissues were washed several times by a complete buffer change, and 60 min later, were exposed to isoproterenol for 15 min before carbachol was again added to produce a 2nd contraction. The 2nd contractions produced by carbachol were reported as F/F_1 .

2.3. Mitogen-activated protein kinase extracellular-signal regulated kinase 1 (ERK1) and vasodilator-stimulated phosphoprotein (VASP) phosphorylation

The degree of ERK1 and VASP phosphorylation was measured as described previously (Ratz, 2001). Briefly stated, detrusor strips were quick-frozen in an acetone-dry ice slurry, thawed, homogenized in 1% SDS, 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-amidinophenyl)-methanesulfonyl fluoride, heated 10 min at 100°C, clarified by centrifugation at 5,000 g for 10 min, and stored at -70°C. Thawed homogenates were assayed for protein concentration (NanoOrange, Molecular Probes; Eugene, OR), and proteins were separated (SDS-PAGE) on 12%

polyacrylamide gels (12 mg of protein per well) followed by Western blotting onto Immobilon-P membranes (Millipore; Bedford, MA). Active (i.e., doubly phosphorylated) ERK1 was identified using anti-active MAP kinase (ERK) antibody (Promega; Madison, WI) and detected using an horseradish peroxidase-labeled secondary antibody and enhanced chemiluminescence (ECL) and ECL film (Amersham). VASP and phosphorylated VASP (VASP-pS239) were identified using anti-VASP and anti-VASP-pS239 antibodies and detected using identical methods. 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, ECL reaction, and film development, a control sample (basal) was included in one lane of each gel, and band intensities from other lanes were reported as the degree of change from basal. Some samples were stripped and re-probed with ERK1 primary antibody (Santa Cruz Biotechnology; Santa Cruz, CA) to double-check that protein loading was consistently uniform across all lanes of the gel.

2.4. Drugs and Statistics

Genistein, daidzein, resveratrol and nifedipine were made as stock solutions in ethanol, which was added at a final concentration of 0.1%. 1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene (U-0126) was dissolved in distilled water. All drugs were from Calbiochem (EMD Chemicals Inc), Alexis Biochemicals Corporation or Sigma Chemical Corporation. 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.

3. Results

3.1. Effects of isoproterenol on the degree of contraction produced by a cumulative addition of carbachol

A cumulative carbachol concentration-response curve (Fig 1A, Control and Fig 1B, open symbols) was shallow (slope of the sigmoidal curve was ~ 1 , Fig 2D open bar) and the maximum contraction produced at 10 μM carbachol was $\sim 20\%$ weaker than the peak contraction produced by KCl (i.e., force was ~ 0.8 -fold F_0 , Figs 1A, 1B open symbol, and 2B, open bar). However, in the presence of isoproterenol, the carbachol concentration-response curve became steeper (slope of the sigmoidal curve was nearly twice that of control, Figs 1B and 2D), and although force produced at low carbachol concentrations was reduced by isoproterenol (Fig 1), that produced at high carbachol concentrations was strengthened (Figs 1 & 2B). Moreover, isoproterenol reduced the potency of carbachol (Fig 2C) and abolished basal contractile tone (Fig 2A).

3.2. Effect of isoproterenol on contraction produced by a single, low carbachol concentration

A single, low dose of carbachol (0.1 μM) produced a contraction peaking between 20% and 30% that produced by KCl (Fig 3). As expected, 100 nM isoproterenol reduced the strength of the carbachol-induced contraction (Fig 3). However, isoproterenol did not diminish the rapid rhythmic contractions produced during the low dose carbachol contractions (inset of Fig 3A).

3.3. Time-dependent control carbachol concentration-response curve and effect of norepinephrine on a carbachol concentration-response curve

The protocol used in this study was to construct two sequential carbachol concentration-response curves in the same tissue, the 1st concentration-response curve served as the

control, and the second was produced in the presence of isoproterenol and norepinephrine (see “Methods”). To determine whether the changes apparently produced by isoproterenol in the carbachol concentration-response curve characterized by a steepening of the curve and strengthening of the maximum contraction were simply due to time-and use-dependent changes in carbachol-induced signaling events, two control carbachol concentration-response curves were compared. A 2nd carbachol concentration-response curve was found to be identical to a 1st carbachol concentration-response curve (Fig 4A), suggesting that the changes induced by isoproterenol were not due to the protocol employed. The endogenous sympathetic neurotransmitter, norepinephrine, in the presence of the α -adrenoceptor blocker, phentolamine (1 μ M), like isoproterenol, steepened and strengthened the carbachol concentration-response curve (Fig 4B).

3.4. Effect of the general tyrosine kinase inhibitor, genistein, on potentiation by isoproterenol of the carbachol concentration-response curve

Genistein (10 μ M) had no effect on the carbachol concentration-response curve (Fig 5A). However, in the presence of isoproterenol, genistein inhibited the carbachol concentration-response curve at all carbachol concentrations greater than 0.03 μ M (Fig 5B).

3.5. Effect of genistein, daidzein, resveratrol and nifedipine on changes produced by isoproterenol in a single-“dose” carbachol contraction

To investigate the mechanism by which isoproterenol potentiated carbachol contractions at high carbachol concentrations, single-dose contractions were examined. At 1 and 3 μ M, carbachol produced contractions consisting of 1 fast and 1 slow peak. The fast peak maximum contraction occurred at ~20–30 sec, which was followed by a stronger slow peak that produced a maximum contraction at ~60–80 sec (Fig 6). Although isoproterenol increased the strength of the maximum carbachol-induced contractions produced during a carbachol concentration-response curve (see Figs 1B and 2B), 100 nM isoproterenol reduced the strength of the fast, but not slow, peak during a single-dose carbachol contraction (Figs 6 & 7). Interestingly, isoproterenol caused a delay in the slow peak such that the maximum force occurred ~30 sec later (Figs 6 & 8). Isoproterenol did not produce a delay in the fast peak (Figs 6 & 8).

The inhibition by isoproterenol of the carbachol-induced fast peak contraction could not have caused a strengthening of the carbachol concentration-response curve at high carbachol concentrations. However, the delay in the slow peak could have caused a strengthening in the maximum contraction produced during the time-dependent carbachol concentration-response curve by prolonging the strongest part of the carbachol contraction. We therefore examined the effects of genistein and related compounds on the slow peak of a 1 μ M carbachol-induced contraction.

Genistein, daidzein and resveratrol are dietary phytoestrogens that can inhibit calcium channel activity (Dobrydneva *et al.*, 2002; Yokoshiki *et al.*, 1996). Genistein is also a general tyrosine kinase inhibitor (Jayatilake *et al.*, 1993), while daidzein is considered an inactive isomer of genistein (Akiyama *et al.*, 1987). Resveratrol has been shown to also inhibit conventional PKC isoforms (Slater *et al.*, 2003). U-0126 is a selective inhibitor of mitogen-activated protein kinase kinase (Duncia *et al.*, 1998), the tyr and ser/thr dual-specificity kinase responsible for ERK phosphorylation (Alessi *et al.*, 1994), and is reported to not block calcium channels (Pereira *et al.*, 2002). Genistein (10 μ M) did not inhibit the carbachol-induced slow peak force (Fig 9A, compare open bars for “No Drug” and “Genistein”). However, carbachol-induced slow peak force was reduced by genistein in the presence of isoproterenol (“Genistein”, Fig 9A, open compared to cross-hatched bar). Moreover, the delay in the carbachol-induced slow peak induced by isoproterenol (“No

Drug”, Fig 9B, peak force at ~120 sec compared to ~85 sec under control conditions) was eliminated by genistein (“Genistein”, Fig 9B, cross-hatched bar compared to open bar). Like genistein, daidzein and resveratrol (10 μ M) also reduced the force in the slow peak contraction only in the presence of isoproterenol (Fig 9A), but unlike genistein, daidzein and resveratrol did not reduce the delay in force produced by isoproterenol (Fig 9B). U-0126 (0.1 μ M), like genistein, reduced the delay in the slow peak contraction produced by isoproterenol (Fig 9B), but unlike genistein, did not produce a significant reduction in force produced in the presence of isoproterenol (Fig 9A). Genistein, daidzein, and U-0126, but not resveratrol, also produced reductions in the strength of the fast peak contraction produced by carbachol (control: 1.00 ± 0.02 F/F₁, $n=12$, vs. genistein: 0.86 ± 0.02 F/F₁, $n=4$, $P<0.05$; daidzein: 0.91 ± 0.03 F/F₁, $n=4$, $P<0.05$; U-0126: 0.92 ± 0.01 F/F₁, $n=4$, $P<0.05$; resveratrol: 1.04 ± 0.03 F/F₁, $n=4$, NS).

While not affecting the fast peak of a 1 μ M carbachol-induced contraction (0.98 ± 0.02 vs. 1.00 ± 0.1 , $n=4$), 30 nM nifedipine (L-type Ca²⁺ channel blocker) inhibited the strength of the slow peak in the absence and presence of isoproterenol (Fig 10A). Nifedipine did not reduce the delay in the carbachol-induced slow peak produced by isoproterenol (Fig 10B).

3.6. Effect of isoproterenol, U-0126 and carbachol on the degree of ERK phosphorylation

The selective mitogen-activated protein kinase kinase inhibitor, U-0126 (0.1 μ M), reduced the degree of basal ERK phosphorylation (Fig 11, U compared to the basal level of “1”), indicating that ERK was active under basal conditions. Likewise, 100 nM isoproterenol reduced the degree of basal ERK phosphorylation (Fig 11). In tissues stimulated with carbachol for 2 min, isoproterenol did not, but U-0126 and isoproterenol + U-0126 did reduce ERK phosphorylation in the presence of carbachol (Fig 11).

3.7. Effect of isoproterenol on VASP-pS239

VASP is a substrate of both cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase, and can serve as a reliable monitor of *in situ* activities of these enzymes (Butt et al., 1994; Lohmann and Walter, 2005; Oelze et al., 2000). As expected because these agents activate, respectively, cGMP-dependent protein kinase and PKA (Lohmann and Walter, 2005; Morita et al., 1986), 100 μ M 8-bromo-cGMP and forskolin produced strong increases in VASP-pS239 (Fig 12). At 0.1 μ M, isoproterenol produced a weak increase in VASP-pS239 (Fig 12), which is consistent with the hypothesis that at this low concentration, isoproterenol produced a modest increase in cAMP levels and PKA activity in detrusor smooth muscle. Thus, these data suggest that the isoproterenol-induced change in carbachol-induced force was likely mediated, at least in part, by isoproterenol-dependent increases in cAMP levels and PKA activity.

4. Discussion

In this investigation, β -adrenoceptor activation of detrusor smooth muscle with isoproterenol abolished basal detrusor tone and reduced the strength of carbachol-induced contractions produced at low muscarinic receptor agonist concentrations. These data support the concept that β -adrenoceptor activation reduces “contractile noise” to sustain a relatively quiescent detrusor during bladder filling (Khadra et al., 1995; Levin and Wein, 1979). The most significant aspect of the present study was the finding that, in addition to reducing contractile force, β -adrenoceptor activation of detrusor smooth muscle caused an *increase* in muscarinic receptor-induced contractile force at high muscarinic receptor occupancy. The result was to increase the steepness of the concentration-response curve to muscarinic receptor stimulation by 2-fold such that the contractile response was less graded and more “switch-like” (Ferrell, 1996). If the function of detrusor smooth muscle is to remain

quiescent in the presence of exposure to weak contractile stimuli, and to contract maximally when the stimulus is strong, then results from the present study indicate that the full function of detrusor smooth muscle is best preserved by concomitant activation of β adrenergic and muscarinic receptors.

The strengthened force of contraction produced at high muscarinic receptor occupancy during the carbachol concentration-response curve in the presence of β -adrenoceptor activation could not be ascribed simply to stronger maximum force production at each concentration of carbachol. Rather, the use of selective inhibitors supports the concept that certain mechanisms by which carbachol produced contraction were altered in the presence of β -adrenoceptor activation, and this alteration contributed to potentiation of maximum carbachol-induced force in a cumulative concentration-response curve. In particular, genistein, a general inhibitor of tyrosine kinases (Jayatilake *et al.*, 1993) and voltage-operated calcium channels (Dobrydneva *et al.*, 1999; Yokoshiki *et al.*, 1996), while totally ineffective at reducing carbachol-induced contractions in the absence of concomitant β -adrenoceptor stimulation, was a strong inhibitor of carbachol-induced force in the presence of β -adrenoceptor activation (compare Figs 5A and 5B, for example). These data suggest that signaling events involving tyrosine phosphorylation or calcium channel activation played a more significant role in contractions produced in the presence of β -adrenoceptor activation than in its absence.

The temporal profile of a single high-dose (1 μ M) carbachol contraction revealed that β -adrenoceptor stimulation did not strengthen carbachol-induced force. Rather, β -adrenoceptor stimulation reduced the fast peak contraction, but delayed the slow peak contraction. Because carbachol-induced contractions are transient in nature, this delay in the slow peak contraction likely prolonged the duration of the strong force response, and the resulting summation may have produced stronger contractions at the highest carbachol concentrations in our cumulative concentration-response curve. An alternate possibility is that an increase in force could result from inhibition of desensitization. Our working hypothesis is that this delay reflected a change in the basic subcellular mechanisms controlling contraction. Whereas genistein and U-0126, a selective inhibitor of ERK phosphorylation (Duncia *et al.*, 1998), prevented this delay, daidzein and resveratrol, analogs of genistein displaying reduced tyrosine kinase inhibitory activity (Akiyama *et al.*, 1987), did not. Moreover, none of these inhibitors affected the slow peak contraction in the absence of β -adrenoceptor stimulation, whereas 30 nM nifedipine, a voltage-operated calcium channel antagonist, did. These data support the concept that stronger contractions occurred during a cumulative carbachol concentration-response curve in the presence of β -adrenoceptor stimulation because tyrosine phosphorylation, specifically via the ERK pathway, permitted potentiation of cumulative contractions by a mechanism not dependent on voltage-operated calcium channel activity.

U-0126 inhibited ERK phosphorylation in non-stimulated detrusor, indicating that ERK was active in the basal state. β -adrenoceptor activation stimulates ERK activity in a variety of cell-types by a PKA-dependent mechanism (Berkeley and Levey, 2003; Enserink *et al.*, 2002; Friedman *et al.*, 2002; Vossler *et al.*, 1997). However, PKA can also phosphorylate raf-1, the first upstream kinase of the ERK cascade, suppressing its activity and leading to reduced ERK phosphorylation (Dhillon *et al.*, 2002; Dumaz *et al.*, 2002). The latter mechanism may have operated in the present study because β -adrenoceptor stimulation with isoproterenol, like the effect of U-0126, reduced basal ERK activity. In the presence of maximum muscarinic receptor stimulation, U-0126 still inhibited ERK phosphorylation, but β -adrenoceptor stimulation was no longer an effective inhibitor of ERK activation. Thus, high muscarinic receptor occupancy may act as a “switch” in detrusor smooth muscle,

permitting ERK activity to remain “on” despite β -adrenoceptor stimulation which, in the absence of muscarinic receptor stimulation, would reduce ERK activity.

The precise mechanism by which ERK may modulate contraction in detrusor smooth muscle was not determined, and there are several possibilities. Prolongation of the slow peak contraction may have been accomplished by kinetic alterations resulting in a delayed enhancement of crossbridge activity through caldesmon phosphorylation and thin filament regulation (Hedges et al., 2000; Li et al., 2003), or through increases in myosin light chain kinase activity (D'Angelo and Adam, 2002). Alternatively, ERK may have initiated a change in Ca^{2+} kinetics (Xiao *et al.*, 2004). Slow peak contractions produced by carbachol were prolonged in the presence of isoproterenol, and this prolongation was blocked by U-0126. However, ERK activation was not greater in tissues stimulated by carbachol in the presence of isoproterenol compared to those stimulated in the absence of isoproterenol. This may mean that a temporal or spatial change occurred in ERK activity that we did not measure, and provides the rationale for future studies directed towards understanding the complex subcellular crosstalk between muscarinic and adrenergic signaling systems in detrusor smooth muscle.

The phytoestrogens genistein, daidzein and resveratrol reduced the strength of the carbachol-induced slow peak force in the presence but not absence of β -adrenoceptor stimulation, while U-0126 had no effect on the strength of the carbachol-induced slow peak force either in the presence or absence of β -adrenoceptor stimulation. At 10 μM , the concentration used in the present study, genistein, daidzein and resveratrol have been shown to strongly inhibit store-operated calcium channels in platelets (Dobrydneva *et al.*, 1999), and it is tempting to speculate that β -adrenoceptor stimulation increased the ability of muscarinic receptor stimulation to activate these calcium channels in detrusor smooth muscle. A recent study revealed that PKA causes detrusor smooth muscle relaxation by elevating Ca^{2+} entry, which in turn, elevates Ca^{2+} -dependent K^{+} efflux leading to plasma membrane hyperpolarization (Petkov and Nelson, 2005). Thus, it is clear that PKA in detrusor smooth muscle can, as in cardiomyocytes, elevate Ca^{2+} channel activity. Our data showed that, while not affecting the strength of the carbachol-induced fast peak contraction, 30 nM nifedipine inhibited the strength of the slow peak contraction by ~50%, both in the presence and absence of β -adrenoceptor stimulation, and β -adrenoceptor stimulation did not potentiate this inhibition (see Fig 10A). Thus, it is unlikely that β -adrenoceptor stimulation increased the degree of L-type calcium channel activation by carbachol. Rather, the data support the hypothesis that concomitant β -adrenoceptor and muscarinic receptor stimulation caused the down-stream activation of a nifedipine-insensitive calcium channel, such as phytoestrogen-sensitive store-operated calcium channels.

Significance, limitations and future studies

It is important to recognize that the urothelium was removed from tissues in this study in order to reveal potential synergistic regulatory mechanisms existing at the level of the detrusor smooth muscle cell. Thus, this preparation does not fully represent the signaling complexity inherent in the *in vivo* bladder (Birder *et al.*, 2002). Moreover, recent studies have revealed that interstitial cells in the detrusor wall and in other smooth muscle-dependent organs may be similar or identical to interstitial cells of Cajal that play an important role in the regulation of gastrointestinal smooth muscle contraction, in part, by acting as integrators of neuronal stimuli (Daniel, 2001; Exintaris et al., 2002; Hashitani, 2006; McCloskey and Gurney, 2002; Pucovsky et al., 2003; Sanders, 1996; van der et al., 2004). For this reason, one very important question that remains to be determined is whether the β -adrenoceptor and muscarinic-receptor signaling systems responsible for the crosstalk identified in this study were due to effects solely within detrusor smooth muscle cells, or also to interactions between interstitial cells of Cajal and detrusor smooth muscle cells.

In conclusion, our data support a speculative model of the bladder fill-void cycle in which β -adrenoceptor activity would eliminate “contractile noise” caused by spontaneous tone or by weak stimulation of muscarinic receptors during the bladder filling phase, and would be expected to enhance the strength of contraction produced during muscarinic receptor activation during voiding. We envision that based on our work, β -adrenoceptor agonists would reduce basal rhythmic contraction during the filling phase (which is increased in patients with overactive bladder) and would help strengthen the voiding contraction and thereby more completely empty the bladder and reduce residual urine volume post-void. Moreover, the finding that phytoestrogens such as genistein effectively and selectively inhibited the β -adrenoceptor-induced potentiation of muscarinic receptor-induced contractions suggests that new therapeutic agents specifically targeting treatment of bladder disorders may be revealed by further studies directed towards elucidating mechanisms of cholinergic and adrenergic cell signaling crosstalk.

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Figure 1.

Cumulative carbachol (CC) concentration-response curves (CRC) produced in the absence (Control) and presence of 100 nM isoproterenol (+ 100 nM ISO). Time-dependent force tracings for one muscle strip are shown in panel “A”, and summary data are shown in panel “B”. To construct the carbachol concentration-response curves, tissues were contracted with final concentrations of carbachol ranging from 10⁻⁸ to 10⁻⁵ M, washed to remove carbachol for 60 min, then incubated with isoproterenol for 15 min before a 2nd carbachol concentration-response curve was constructed. Data in panel “B” are mean ± S.E.M. *n* = 4.

Figure 2.

Effect of 10 nM and 100 nM isoproterenol (ISO) on basal contractile tone (A) and on the three parameters describing a cabachol (CC) sigmoidal concentration-response curve (CRC): maximum contractile response (B), potency (EC_{50} ; C), and steepness (slope of the curve; D). Data are mean $\pm$ S.E.M. $n = 4$. * $P < 0.05$ compared to control.

Figure 3.

Effect of 100 nM isoproterenol (ISO) on contraction produced by a single, low concentration (0.1 μM) of carbachol (CC). Raw data for one tissue is shown in panel “A”. Although the average force was reduced by isoproterenol, in general, rhythmic activity was not diminished by isoproterenol (insert, panel “A”). Data in panel “B” represents the average of 4 tissues ($n = 4$), and the insert shows that isoproterenol significantly reduced force produced at 2 min. Data in panel “B” are mean $\pm$ S.E.M. $n = 4$.

Figure 4.

Two control, sequential carbachol (CC) concentration-response curves (CRC) were not different from one another (A). Norepinephrine (NE; 1 μ M), like isoproterenol, inhibited contraction at low carbachol concentrations, and strengthened contraction at high carbachol concentrations (B). Data are mean $\pm$ S.E.M. $n = 4$. * $P < 0.05$ compared to control.

Figure 5.

Effect of the general tyrosine kinase inhibitor, genistein, on the carbachol (CC) concentration-response curves (CRCs) produced in the absence (A) and presence of 100 nM isoproterenol (+ ISO; B). Data are mean $\pm$ S.E.M. $n = 4$. * $P < 0.05$ compared to control.

Figure 6.

Example of time-dependent force tracings produced by a single “dose” (1 μ M) of carbachol (CC) in the absence (Control) and presence of 100 nM isoproterenol (+ ISO). Note that contractions consisted of two peak responses, labeled “fast peaks” and “slow peaks”. In the presence of isoproterenol, the fast peak was diminished in strength and the slow peak was delayed in time.

Figure 7.

Maximum strength of fast (A & C) and slow (B & D) peak contractile responses produced by 1 μM carbachol (CC) in the absence (CC Control) and presence of 100 nM isoproterenol (+ ISO) relative to a previous carbachol contraction produced in the absence of isoproterenol (F₁). Data are mean ± S.E.M. *n* = 4. * *P* < 0.05 compared to control.

Figure 8.

Time to fast (A & C) and slow (B & D) peak contractile responses produced by 1 μ M carbachol (CC) in the absence (CC Control) and presence of 100 nM isoproterenol (+ ISO). Data are mean $\pm$ S.E.M. $n = 4$. * $P < 0.05$ compared to control.

Figure 9.

Strength of (A) and time to (B) slow peak contractile responses produced by 1 μ M carbachol (CC) in the absence (CC Control) and presence of 100 nM isoproterenol (+ ISO), and in the absence of another drug (No Drug) and presence of 10 μ M genistein (general tyrosine kinase inhibitor), daidzein and resveratrol (related phytoestrogens), and 0.1 μ M U-0126 (inhibitor of ERK phosphorylation). Data are mean $\pm$ S.E.M. $n = 4$. * $P < 0.05$ compared to control.

Figure 10.

Strength of (A) and time to (B) slow peak contractile responses produced by 1 μ M carbachol (CC) in the absence (CC Control) and presence of 100 nM isoproterenol (+ ISO), and in the absence of another drug (No Drug) and presence of nifedipine (calcium channel blocker). Data are mean $\pm$ S.E.M. $n = 4$. * $P < 0.05$ compared to control.

Figure 11.

Effect of 100 nM isoproterenol (I), 0.1 μ M U-0126 (U), 1 μ M carbachol (CC), carbachol plus each agent alone and carbachol plus I and U (I+U) on the level of ERK phosphorylation. Data are mean $\pm$ S.E.M. *n* values are in parentheses. * $P < 0.05$ compared to 1 (the basal level).

Figure 12.

One hundred nM isoproterenol (ISO), 100 μ M 8-bromo-cGMP (8b-cGMP), and 10 μ M forskolin (FSK) increased the degree of phosphorylated VASP (VASP-pS239) and not total VASP compared to control in strips of rabbit detrusor smooth muscle (representative Western blot of $n=4$).