Signal transduction underlying carbachol-induced contraction of rat urinary bladder.

I. Phospholipases and Ca\(^{2+}\) sources*

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Running title: Phospholipases and Ca^{2+} in muscarinic bladder contraction

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Number of text pages: 22 (including references)
Number of tables: 0
Number of figures: 9
Number of references: 42
Words in Abstract: 213
Words in Introduction: 508
Words in Discussion: 1070

Non-standard abbreviations:
IP, inositol phosphate
PL, phospholipase
PEtOH, phosphatidylethanol

Recommended section assignment: other
ABSTRACT

We have reexamined the muscarinic receptor subtype mediating carbachol-induced contraction of rat urinary bladder and investigated the role of phospholipase (PL) C, D and A2 and of intra- and extracellular Ca\(^{2+}\) sources in this effect. Based upon the non-subtype-selective tolterodine, the highly M2 receptor-selective Ro-320-6206 and the highly M3 receptor-selective darifenacin and APP contraction occurs via M3 receptors. Carbachol stimulated inositol phosphate formation in rat bladder slices, and this was abolished by the phospholipase C inhibitor U 73,122 (10 \(\mu\)M). Nevertheless U 73,122 (1-10 \(\mu\)M) did not significantly affect carbachol-stimulated bladder contraction. Carbachol had only little effect on PLD activity in bladder slices, but the PLD inhibitor butan-1-ol relative to its negative control butan-2-ol (0.3% each) caused detectable inhibition of carbachol-induced bladder contraction. The cytosolic PLA2 inhibitor AACOCF3 weakly inhibited carbachol-induced contraction at a concentration of 300 \(\mu\)M, but the cyclooxygenase inhibitor indomethacin (1-10 \(\mu\)M) remained without effect. The Ca\(^{2+}\) entry blocker nifedipine (10-100 nM) almost completely inhibited carbachol-induced bladder contraction. In contrast, SK&F 96,365 (10 \(\mu\)M), an inhibitor of store-operated Ca\(^{2+}\) channels, caused little inhibition. We conclude that carbachol-induced contraction of rat bladder largely depends on Ca\(^{2+}\) entry through nifedipine-sensitive channels and, perhaps, PLD, PLA2 and store-operated Ca\(^{2+}\) channels, whereas cyclooxygenase and, surprisingly, also PLC are not involved to a relevant extent.
Muscarinic acetylcholine receptors are the physiologically most important mechanism to elicit contraction of the urinary bladder (Andersson, 1993). In the bladder of various mammalian species including humans M2 and M3 muscarinic receptors coexist, but the expression of M2 receptors is much greater than that of the M3 receptors (Goepel et al., 1998; Yamanishi et al., 2000; Wang et al., 1995; Kories et al., 2003). Nevertheless, the contractile response to the exogenous agonist carbachol and to endogenous agonist released by field stimulation have been attributed predominantly if not exclusively to M3 receptors in rats (Longhurst et al., 1995; Hegde et al., 1997; Choppin et al., 1998; Tong et al., 1997; Braverman et al., 1998; Longhurst and Levendusky, 2000; Kories et al., 2003), mice (Choppin and Eglen, 2001b), pigs (Yamanishi et al., 2000), dogs (Choppin and Eglen, 2001a) and humans (Chess-Williams et al., 2001; Fetscher et al., 2002). Moreover, at least male M3 (but not M2) receptor knock-out mice exhibit bladder distension and develop urinary retention (Matsui et al., 2000). On the other hand, it should be considered that hitherto available antagonists have only modest subtype-selectivity and/or do not act in a purely competitive manner; hence they were not well suited for detecting a potential minor component of M2 receptors in bladder contraction. The present study was primarily designed to determine the proximal signaling mechanisms underlying M3 receptor-mediated contraction of rat urinary bladder but we have also re-investigated the role of muscarinic receptor subtypes using two novel and highly M2- and M3-selective antagonists, i.e. Ro-320-6206 (Zhao et al., 2001) and APP (MacKenzie and Cross, 1991), respectively.

The prototypical signal transduction mechanism of M3 receptors is stimulation of a phospholipase (PL) C to generate inositol phosphates and diacylglycerol (Caulfield and Birdsall, 1998). Muscarinic stimulation of PLC has also been demonstrated in cultured smooth muscle cells from human bladder (Marsh et al., 1996) and in rat bladder slices (Kories et al., 2003), and
the latter response was shown to be M₃ receptor-mediated. However, muscarinic receptors can also activate a PLD or PLA₂ in a variety of cell types (Felder, 1995), the latter possibly leading to cyclooxygenase activation (Nishimura et al., 1995). Several cyclooxygenase products can contract isolated detrusor muscle (Andersson, 2000), and cyclooxygenase activation was shown to at least partly mediate rat urinary bladder contraction induced by protease-activated receptor-2 (Nakahara et al., 2003). Therefore, we have determined the possible roles of PLC, PLD, PLA₂ and cyclooxygenase in muscarinic M₃ receptor-mediated contraction of rat urinary bladder.

Similar to all other types of smooth muscle, urinary bladder contraction evoked by muscarinic receptor stimulation involves elevations of intracellular Ca²⁺ concentrations in rat and guinea pig bladder smooth muscle cells (Ma et al., 2002; Ikeda et al., 2002). Accordingly, L-type Ca²⁺ entry blockers can inhibit muscarinic receptor-mediated bladder contraction in guinea pigs and humans (Sjögren et al., 1982; Ikeda et al., 2002; Masters et al., 1999). However, Ca²⁺ sources apart from L-type channels may also contribute in human bladder smooth muscle cells (Masters et al., 1999; Visser and van Mastrigt, 2000). Therefore, we have also determined the roles of nifedipine-sensitive and receptor-operated Ca²⁺ channels in M₃ receptor-mediated rat bladder contraction.
METHODS

Force of contraction: Urinary bladder strips were prepared from female Wistar rats (body weight 231 ± 9 g, bladder weight 65 ± 2 mg) obtained from the central animal breeding facility at the University of Essen. Experiments were performed as previously described (Kories et al., 2003). Briefly, longitudinal bladder strips (approximately 1 mm diameter, 18 ± 1 mm length, 9.6 ± 0.5 mg weight, n = 95) were mounted under a tension of 10 mN in 10 ml organ baths containing Krebs-Henseleit solution (119 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.18 mM KH₂PO₄, 1.17 mM MgSO₄, 2.5 mM CaCl₂, 0.027 mM EDTA, 5.5 mM glucose and 10 mM HEPES) which was aerated with 95% O₂ and 5% CO₂ to yield a pH of 7.4 at 37°C. After 60 min of equilibration including washes with fresh buffer every 15 min, the bladder strips were challenged three times with a combination of 50 mM KCl and 0.1 mM carbachol with 5 min rest and washes between each challenge. Following washout and an additional 30 min of equilibration, cumulative concentration-response curves were constructed for carbachol in the absence of any inhibitor or vehicle. Using 15 min washout and then 15 min equilibration periods in between, up to 4 additional curves were then generated in the presence of increasing concentrations of the indicated antagonists and inhibitors, their negative controls or their vehicles. Previous work had shown that carbachol-induced rat bladder contraction remains fairly stable under these conditions (Kories et al., 2003).

Carbachol concentration-response curves were analyzed by fitting sigmoidal curves to the experimental data, in which the bottom of the curve was fixed at 0. The force of contraction in the absence and presence of inhibitors were expressed as % of maximum carbachol effects observed within the same bladder strip in the first concentration-response curve, i.e. prior to addition of
any inhibitor or vehicle. To assess inhibitor effects, alterations in $E_{\text{max}}$ or pEC$_{50}$ in its presence relative to the first curve were compared to those in the presence of a matching vehicle time control using two-way analysis of variance testing for main treatment effect and concentration-dependency; if this indicated statistical significance, the effect of individual inhibitor concentrations relative to time-matched controls was assessed by Bonferroni post-tests. A $p < 0.05$ was considered to be significant in all statistical analyses. To assess antagonist effects, analysis according to Arunlakshana and Schild (1959) was performed. All curve fitting and statistical calculations were performed with the Prism program (version 4.0, Graphpad Software, San Diego, CA).

**Phospholipase C activation** was assessed as [$^3$H]inositol phosphate formation in 350 x 350 $\mu$m bladder slices as previously described (Kories et al., 2003). Briefly, slices were suspended in 10 ml Ringer solution (147.2 mM NaCl, 4.0 mM KCl, 2.25 mM CaCl$_2$, 10 mM glucose and 20 mM HEPES at pH 7.4) supplemented with 10 mM LiCl to block inositol phosphate degradation and 2 U/ml adenosine deaminase. They were incubated for 60 min at 37°C in the presence of 100 $\mu$Ci [$^3$H]myoinositol/12 ml. Thereafter, 300 $\mu$l of the slice suspension (corresponding to 6-8 mg slice wet weight) were pipetted into flat bottom polystyrene tubes under gentle swirling, and agonists and antagonists were added in the indicated concentrations to yield a final volume of 330 $\mu$l. After incubation for 45 min the reaction was stopped by addition of 400 $\mu$l ice-cold methanol and 700 $\mu$l chloroform. The mixture was vigorously vortexed twice and thereafter the phases were separated by centrifugation at 820 g for 10 min at 4°C. Aliquots (450 $\mu$l) of the upper phase were placed on Dowex AG1-X8 columns (200 mg per column). Free inositol was eluted twice each with 5 ml H$_2$O and 5 ml of 60 mM ammonium formate. Total inositol phosphates were eluted by addition of twice 1 ml 1 M ammonium formate dissolved in 100 mM
formic acid. Each data point was measured in quadruplicate within each experiment. Statistical significance of differences was determined by one-way analysis of variance; if this indicated significant differences among group means, individual groups were compared by Dunnett's multiple comparison tests.

**Phospholipase D** was assessed as \[^3\text{H}\]phosphatidylethanol (\[^3\text{H}\]PEtOH) formation. The bladder was quickly removed and stored in buffer containing 147.2 mM NaCl, 4 mM KCl, 2.25 mM CaCl\(_2\), 20 mM HEPES and 1 mg/ml glucose at 37°C and a pH of 7.4. Bladder slices of 200 x 200 \(\mu\)m were prepared as above. The suspension was resuspended and incubated twice with adenosine deaminase (2 U/ml) for 15 min each. Next, the slices were resuspended in 7 ml of fresh buffer containing 40 \(\mu\)l \[^3\text{H}\]oleic acid (specific activity 5 mCi/ml) and again incubated at 37°C for 60 min. After resuspending the slices in fresh buffer containing 5% (v/v) ethanol, they were incubated in a total volume of 400 \(\mu\)l with the indicated drugs for 45 min at 37°C. Afterwards, the incubation was stopped by adding 0.5 ml each of ice-cold methanol, trichloromethane and H\(_2\)O. The mixture was vortexed vigorously twice and centrifuged for 10 min at 2000 g and 4°C. Four hundred \(\mu\)l of the lower phase were put into small reaction tubes and the solvent was evacuated using a SpeedVac centrifuge. The pellet was then resuspended with 25 \(\mu\)l of a 1:1-mixture of chloroform and methanol, and 20 \(\mu\)l were placed on Silica Gel 60 thin layer chromatography plates (Whatman). The lipids were separated using the organic phase of a mixture of ethyl acetate/iso-octane/acetic acid/water (91:14:21:70 by vol.), migrated with authentic standards and were localized by iodine staining. Areas corresponding to the PEtOH standard were scraped into scintillation vials, as were the areas below containing other labeled phospholipids. The formation of \[^3\text{H}\]PEtOH was assessed as ratio of total labeled phospholipids and is given as percent of basal.
**Chemicals:**

Carbachol HCl, nifedipine, SK&F 96,365

(1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole HCl), U 73,122

(1-(6-[(17β)-3-methoxyestra-1,3,5[10]-trien-17-yl]-amino)hexyl)-1H-pyrrole-2,5-dione) and U 73,343

(1-(6-[(17β)-3-methoxyestra-1,3,5[10]-trien-17-yl]-amino)hexyl)-2,5-pyrrolidinedione) were obtained from Sigma-Aldrich (Taufkirchen, Germany). AACOCF₃ (arachidonyltrifluoromethyl ketone), indomethacin and phorbol myristyl acetate were from Calbiochem (Bad Soden, Germany). [³H]-myo-inositol (specific activity 115 Ci/mmol) was from Amersham (Braunschweig, Germany), and [³H]oleic acid (specific activity 23 Ci/mmol) was from PerkinElmer (Boston, MA, USA). Darifenacin and tolterodine were provided by Pfizer (New York, NY), Ro 320-6206

((R)-4-{2-[3-(4-methoxy-benzoylamino)-benzyl]-piperidin-1-ylmethyl}-piperidine-1-carboxylic acid amide) and APP

(3-(1-carbamoyl-1,1-diphenylmethyl)-1-(4-methoxyphenylethyl)pyrrolidine) were synthesized as previously described (MacKenzie et al., 1991; Zhao et al., 2001).

AACOCF₃ (at 10 mM), APP (at 10 mM), darifenacin (at 10 mM), tolterodine (at 10 mM), Ro-320-6206 (at 10 mM), U 73122 (at 3 mM), U 73343 (at 3 mM) and phorbol myristate acetate (at 1 mM) were dissolved in dimethylsulfoxide. Indomethacin (at 10 mM) and nifedipine (at 1 mM) were dissolved in ethanol. SK&F 96,365 was dissolved at 1 mM in distilled water. The experiments involving nifedipine were performed in light-shielded organ baths.
RESULTS

Prior to addition of antagonist or inhibitor, i.e. in the first curve generated within each bladder strip, carbachol concentration-dependently increased force of contraction with a pEC$_{50}$ of 5.65 ± 0.03 and maximum effects of 35.4 ± 1.4 mN (n = 127 muscle strips). All further contraction data are expressed as % of the maximum carbachol effect within the same preparation prior to addition of any inhibitor.

Antagonist experiments

Relative to the first curve in the absence of any antagonist, the second to fifth consecutive curve within a preparation exhibited a pEC$_{50}$ which was -0.06, 0.05, 0.16 and 0.23 log units smaller, respectively; concomitantly, maximum effects were reduced by -2 ± 5%, 8 ± 9%, 18 ± 11% and 26 ± 13%, respectively (n = 6). These alterations were taken into account when analyzing the effects of the antagonists. Within the tested concentration range, tolterodine, Ro-320-6206 and APP did not affect maximum carbachol responses in a manner which was significantly different from vehicle (data not shown), whereas 10, 30, 100 and 300 nM darifenacin reduced it by 21 ± 5%, 38 ± 9%, 52 ± 11% and 60 ± 10%, respectively (except for highest concentration all p < 0.05 vs. vehicle). All four antagonists concentration-dependently right-shifted the carbachol concentration-response curve (Fig. 1). The Schild-regression for the non-selective tolterodine (30-1000 nM) had a slope of slightly less than unity (0.80 ± 0.06) and an x-axis intercept (apparent pA$_2$ value) of 8.93 (95% confidence interval: 8.57-9.42). The Schild-regression for the M$_3$-selective darifenacin (10-300 nM) had a slope close to unity (1.11 ± 0.15), and its x-axis intercept (apparent pA$_2$ value) of 8.67 (95% confidence interval: 8.20-9.38). The
Schild-regression for the M₁-selective APP (10-300 nM) had a slope close to unity (1.08 ± 0.15) and an x-axis intercept (apparent pA₂ value) of 8.73 (95% confidence interval: 8.24-9.49). The M₂-selective Ro-320-6206 had only little effect on the carbachol concentration-response curve. Thus, at concentrations of 0.3, 1, 3 and 10 µM it right-shifted the carbachol concentration-response curve by only 0.14 ± 0.10, 0.25 ± 0.11, 0.42 ± 0.14 and 0.75 ± 0.16 log units, respectively; accordingly, a Schild-slope of only 0.40 ± 0.11 was obtained, and the x-axis intercept of this shallow regression line was 6.72 (95% confidence interval 5.92-8.53).

Role of phospholipase C

In confirmation of previous findings from our laboratory (Kories et al., 2003), 1 mM carbachol enhanced IP formation by approximately 60% over basal (Fig. 2). This concentration of carbachol had been chosen based upon our previously published concentration-response curves in order to obtain a good signal/noise ratio. While 10 µM U 73,122 (10 µM) alone had no effect on basal IP formation, it abolished carbachol-stimulated IP formation (Fig. 2).

Nevertheless, U 73,122 (1-10 µM) did not significantly alter the potency or maximum effects of carbachol-induced bladder contraction relative to its vehicle (Fig. 3). In light of the unexpectedness of this finding, it was confirmed for 10 µM U 73,122 in a second series of experiments performed by a different investigator; in that series U 73,122 failed to significantly affect carbachol-induced bladder contraction not only relative to vehicle but also relative to 10 µM of its negative control U 73,343 (n = 11-12, data not shown). Moreover, we confirmed the effectiveness of U 73,122 in organ bath experiments by demonstrating that it markedly inhibited α₁-adrenoceptor-induced contraction of rat mesenteric microvessels (Altmann et al., 2003).
Role of phospholipase D

PLD activity was markedly stimulated by 1 µM of the positive control phorbol myristyl acetate (210 ± 19% over basal, n = 13). In contrast, 1 mM carbachol had only little effect on PLD activity, i.e. enhanced [³H]PEtOH accumulation non-significantly by only 13 ± 10% over basal (Fig. 4).

The PLD inhibitor butan-1-ol did not significantly alter carbachol-induced contraction relative to its negative control butan-2-ol when tested at concentrations of 0.03% or 0.1%, but a statistically significant reduction of potency and maximum effects of carbachol was obtained at a butan-1-ol concentration of 0.3% (Fig. 5).

Role of phospholipase A₂ and cyclooxygenase

The PLA₂ inhibitor AACOCF₃ did not significantly alter carbachol-induced contraction relative to its vehicle when tested at concentrations of 30 and 100 µM whereas a statistically significant reduction of maximum effects of carbachol (but not of its potency) was observed at an inhibitor concentration of 300 µM (Fig. 6).

The cyclooxygenase inhibitor indomethacin (1-10 µM) did not significantly alter the potency or maximum effects of carbachol-induced bladder contraction relative to its vehicle (Fig. 7).

Role of Ca²⁺ sources
The Ca\textsuperscript{2+} entry blocker nifedipine (10-100 nM) markedly inhibited carbachol-induced bladder contraction relative to its vehicle ethanol (Fig. 8). This inhibition was due to reductions of maximum carbachol responses reaching up to 90% at 100 nM, which were not accompanied by statistically significant alterations of the agonist potency for the remaining response.

SK&F 96,365, an inhibitor of receptor-operated Ca\textsuperscript{2+} channels, did not significantly affect carbachol-induced bladder contractions at concentrations of 1 or 3 µM, whereas a significant reduction of maximum responses (-47%) but not of carbachol potency was seen with 10 µM SK&F 96,365 (Fig. 9).
DISCUSSION

The present study was primarily designed to investigate proximal signaling mechanisms potentially involved in carbachol-induced muscarinic receptor-mediated contraction of rat urinary bladder. Although \( M_2 \) receptors are more numerous in rat bladder than \( M_3 \) receptors (Wang et al., 1995; Kories et al., 2003), numerous studies have proposed that rat bladder contraction is mediated predominantly if not exclusively by the minor population of \( M_3 \) receptors (Longhurst et al., 1995; Hegde et al., 1997; Choppin et al., 1998; Tong et al., 1997; Braverman et al., 1998; Longhurst et al., 2000; Kories et al., 2003). However, all of these studies were based on antagonist with only moderate subtype-selectivity or upon darifenacin, which has considerable selectivity for \( M_3 \) receptors but does not act purely competitively (as confirmed in the present study). Therefore, we have reinvestigated the muscarinic receptor subtype mediating rat bladder contraction using APP (MacKenzie et al., 1991), a compound which similar to darifenacin is about 40-fold selective for \( M_3 \) receptors (Ki 2.6 vs. 111 nM, S. Hegde Theravance Inc., personal communication) but does not reduce maximum responses, and R-320-6206, an approximately 100-fold \( M_2 \) selective antagonist APP (5.0 vs. 500 nM (Zhao et al., 2001)); the non-selective tolterodine and the \( M_3 \) selective darifenacin were studied in comparison. Using more selective and apparently purely competitive tools, our present experiments confirm that carbachol-induced contraction of rat bladder occurs via \( M_3 \) receptors.

Co-immunoprecipitation studies demonstrate that the \( M_3 \) receptors in rat bladder couple predominantly to G-proteins of the \( G_{q/11} \) and, surprisingly, also the \( G_{i1} \) type (Wang et al., 1995). Activation of a PLC is the prototypical signaling response of \( G_q \)-coupled receptors in general and of \( M_3 \) muscarinic receptors in particular (Caulfield et al., 1998). Muscarinic receptor stimulation
also activates PLC in cultured smooth muscle cells from human bladder (Marsh et al., 1996) and in rat bladder slices (Kories et al., 2003), and at least the latter response is mediated by M₃ receptors, i.e. the same subtype mediating the contraction. Studies in feline isolated bladder smooth muscle cells using neomycin as the PLC inhibitor have proposed that PLC activation is important for carbachol-induced bladder contraction (An et al., 2002). The present data based on rat bladder strips and U 73,122 as the PLC inhibitor surprisingly do not support this proposal. While we do not know whether these discrepancies are due to differences in species (rat vs. cat), type of preparation (strip vs. cultured cell) or PLC inhibitor (U 73,122 vs. neomycin), it should be noted that the present study did not detect inhibition of contraction under conditions where PLC activation was clearly abolished within the same study. Thus, at least in rats, M₃ receptor mediated PLC activation and contraction occur concomitantly but contraction is not dependent on PLC activation.

Activation of PLD is another potential signaling mechanism of M₃ muscarinic receptors (Zhou et al., 1994; Schmidt et al., 1995). In the present study the PLD inhibitor butan-1-ol, relative to its negative control butan-2-ol, caused some inhibition of carbachol-induced bladder contraction, but the effect was weak and reached statistical significance only at the highest inhibitor concentration. However, butan-1-ol concentrations up to 0.5% can still be considered to be selectively inhibiting PLD (Bechoua and Daniel, 2001; Banno et al., 2001), and hence the inhibition by 0.3% butan-1-ol in our study is unlikely to be non-specific. The small extent of the inhibition is not surprising since carbachol caused only little if any PLD activation in rat bladder slices, i.e. an effect of less than 10% of the positive control phorbol myristate acetate. Thus, PLD activation appears to play only a minor role in M₃ receptor-mediated rat bladder contraction.
Activation of a cytosolic PLA₂, possibly followed by that of a cyclooxygenase, is another potential signaling mechanism of muscarinic receptors (Hunt et al., 1994; Felder, 1995; Nishimura et al., 1995). This could potentially also be involved in muscarinic receptor-mediated bladder contraction since several cyclooxygenase products are known to contract the bladder (Andersson, 2000) and since it has recently been shown that rat bladder contraction elicited by protease-activated receptor-2 involves activation of a cyclooxygenase (Nakahara et al., 2003; Kubota et al., 2003). In the present study AACOCF₃, an inhibitor of cytosolic PLA₂, caused only minor if any inhibition of rat bladder contraction. Moreover, the cyclooxygenase inhibitor indomethacin, when applied in concentrations inhibiting protease-activated receptor-2-mediated rat bladder contraction (Nakahara et al., 2003), was completely ineffective. Thus, cytosolic PLA₂ and cyclooxygenase do not appear to play a role for M₃ receptor-mediated rat bladder contraction.

Elevations of intracellular Ca²⁺ concentrations play a central role in smooth muscle contraction. Muscarinic receptor-induced Ca²⁺ elevations have been demonstrated in rat and guinea pig bladder smooth muscle cells (Ma et al., 2002; Ikeda et al., 2002). They could come from intracellular stores, e.g. inositol phosphate or ryanodine receptor-sensitive stores, or from the extracellular space via a variety of ion channels. In light of our negative data regarding an involvement of PLC we have not further investigated a possible role of inositol phosphate-sensitive Ca²⁺ stores. A role for ryanodine-sensitive Ca²⁺ stores has previously been demonstrated in muscarinic receptor-stimulated contraction of human detrusor isolated smooth muscle cells (Visser et al., 2000). In guinea pig and human bladder smooth muscle cells L-type Ca²⁺ channels also appear to contribute to the muscarinic receptor-mediated bladder contractions (Sjögren et al., 1982; Masters et al., 1999; Ikeda et al., 2002; Visser et al., 2000). In the present
study the L-type Ca\(^{2+}\) channel inhibitor nifedipine potently and effectively inhibited carbachol-induced bladder contraction. Indeed this response was much more sensitive to nifedipine than noradrenaline or sphingosylphosphorylcholine induced blood vessel contraction (Chen et al., 1996; Bischoff et al., 2001). Moreover, knock-out mice lacking the Ca\(_{1.2}\) gene, which encodes for a subunit of voltage-operated Ca\(^{2+}\) channels; exhibit a markedly reduced bladder contraction in response to muscarinic stimulation (Wegener et al., 2003). In contrast SK&F 96,355, an inhibitor of receptor-operated Ca\(^{2+}\) channels caused only minor if any inhibition of carbachol-induced bladder contraction in the present study. Thus, influx of extracellular Ca\(^{2+}\) through L-type voltage-dependent channels but not through receptor-operated channels appears to play a pivotal role for rat bladder contraction.

In conclusion, carbachol-induced, M3 muscarinic receptor-mediated contraction of rat bladder is largely mediated by Ca\(^{2+}\) influx through L-type, voltage-dependent channels. Surprisingly, PLC activation is not involved although it is concomitantly activated. Moreover, PLD, PLA\(_2\), cyclooxygenase and receptor-operated Ca\(^{2+}\) channels also play only a minor if any role in muscarinic receptor-mediated contraction of rat bladder. The role of various protein kinases, which may be activated secondary to these proximal signaling mechanisms, was determined in the accompanying manuscript (Fleichman et al., 2003).
Acknowledgements: The skilful technical assistance of Ms. Charlotte Fetscher and the help of Dr. Martina Schmidt in setting up the PLD assay are gratefully acknowledged.
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Footnote page:

* This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (Mi 294/7-1) and Theravance Inc. TS was recipient of a training fellowship from the intramural grant program of the University of Essen Medical School (IFORES).

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LEGENDS TO THE FIGURES

Figure 1: Schild plots for antagonism of carbachol-induced rat bladder contraction. Data are means ± SEM of 6 experiments each.

Figure 2: Effect of carbachol (1 mM), U 73,122 (10 µM) and their combination of inositol phosphate accumulation in rat bladder slices. Data are expressed as % of accumulation in the absence of either drug (basal) within the same experiment. *: p < 0.05 vs. basal in a one-way ANOVA followed by Dunnett’s multiple comparison test.

Figure 3: Effects of the phospholipase C inhibitor U 73,122 (1-10 µM) and its vehicle on carbachol-induced contraction of rat bladder (n = 7).

Figure 4: Effects of carbachol (1 mM) and atropine (1 µM) on [3H]PEtOH formation in rat bladder slices. Data are expressed as % of accumulation in the absence of either drug (basal) within the same experiment.

Figure 5: Effects of the phospholipase D inhibitor butan-1-ol (0.03-0.3%) and its negative control butan-2-ol on carbachol-induced contraction of rat bladder (n = 8).*: p < 0.05 vs. matching time controls in the presence of negative control in a two-way analysis of variance followed by Bonferroni post-tests.

Figure 6: Effects of the cytosolic phospholipase A2 inhibitor AACOCF3 (30-300 µM) and its vehicle on carbachol-induced contraction of rat bladder (n = 8). *: p < 0.05 vs. matching time
controls in the presence of vehicle in a two-way analysis of variance followed by Bonferroni post-tests.

**Figure 7:** Effects of the cyclooxygenase inhibitor indomethacin (1-10 µM) and its vehicle on carbachol-induced contraction of rat bladder (n = 8).

**Figure 8:** Effects of the Ca^{2+} channel inhibitor nifedipine (10-100 nM) and its vehicle on carbachol-induced contraction of rat bladder (n = 6). * and ***: p < 0.05 and < 0.001, respectively, vs. matching time controls in the presence of vehicle in a two-way analysis of variance followed by Bonferroni post-tests.

**Figure 9:** Effects of SK&F 96,365 (1-10 µM), an inhibitor of receptor-operated Ca^{2+} channels, and its vehicle on carbachol-induced contraction of rat bladder (n = 6). **: p < 0.01 vs. matching time controls in the presence of vehicle in a two-way analysis of variance followed by Bonferroni post-tests.
The graph shows the contraction response to carbachol at different concentrations. The y-axis represents the contraction as a percentage of the control maximum, while the x-axis represents the concentration of carbachol in log M.

There are four curves corresponding to different concentrations of carbachol: control, 1 μM, 3 μM, and 10 μM. Each curve shows the contraction response at n = 7.

Additionally, there are plots for E_max and pEC_{50} values for vehicle and U73,122 inhibitors. The E_max plots show the force of contraction as a percentage of control, while the pEC_{50} plots show the alteration of pEC_{50} values.
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