Does Phospholipase C Mediate Muscarinic Receptor-Induced Rat Urinary Bladder Contraction?

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ABSTRACT

Muscarinic acetylcholine receptors, particularly M₃ receptors, are physiologically the most important mechanism to induce urinary bladder smooth muscle contraction. Their prototypical signaling response is a stimulation of phospholipase C (PLC), and this also has been shown in the urinary bladder. Nevertheless, it has remained controversial whether PLC signaling mediates bladder contraction induced by muscarinic receptor agonists. Studies in favor and against a role for PLC differed in their experimental protocol (single versus repeated concentration-response curves within a single preparation) and in the PLC inhibitors that have been used. We have now tested whether previous differential conclusions regarding a role for PLC are related to inhibitors and/or experimental protocols. In a single curve protocol, U-73,122, 1-[6-(((17β)-3-methoxyestra-1,3,5(10)-tien-17-yl)aminohexyl]-1H-pyrrole-2,5-dione did not attenuate carbachol responses. In a repeated curve protocol, ET-18-OCH₃ (1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine) lacked significant inhibition relative to vehicle time controls. In contrast, D609 (O-tricyclo[5.2.1.0²,6]dec-9-yl dithiocarbonate potassium salt) depressed maximal carbachol effects but also nonspecifically inhibited contraction induced by KCl. Neomycin did not affect the carbachol-induced rat urinary bladder contraction. We conclude that previously reported differences relate to the use of inhibitors rather than experimental protocols and that the overall data do not support a role for PLC in M₃ muscarinic receptor-mediated rat bladder contraction.

Muscarinic receptors are physiologically the most important mechanism mediating urinary bladder contraction (Anderson, 1993). The mammalian bladder (including human) mainly expresses M₂ and M₃ muscarinic receptors, which coexist in an approximate 3:1 ratio (Abrams et al., 2006; Hegde, 2006). Whereas M₂ receptors can contribute to bladder smooth muscle tone under certain conditions, the primary mediator of normal mammalian bladder contraction is the minor population of M₃ receptors (Abrams et al., 2006; Hegde, 2006). Muscarinic receptor subtypes can couple to a range of signal transduction pathways, and the primary signaling of M₃ receptors is thought to occur by stimulation of a phospholipase C (PLC) to generate inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (Caulfield, 1993). Muscarinic receptor coupling to PLC activation and IP₃ formation in the mammalian urinary bladder also has been reported (An et al., 2002; Kories et al., 2003; Schneider et al., 2004b; Kajioka et al., 2005).

Nevertheless, the role of PLC, specifically phosphatidylinositol-PLC (PI-PLC), in mediating muscarinic receptor-stimulated bladder contraction has remained controversial, particularly in rats. Some investigators have proposed PLC involvement based on the finding that the PI-PLC inhibitor ET-18-OCH₃ reduced carbachol potency and that the phosphatidylincholine-PLC (PC-PLC) inhibitor D609 attenuated both the carbachol potency and maximal contractile response (Braverman et al., 2006a,b). Other investigators proposed a lack of PLC involvement based on the finding that the PI-PLC inhibitor U-73,122 did not affect carbachol contractile responses in a concentration where it abolished carbachol-induced IP formation in the bladder (Schneider et al., 2004b). A comparison between those studies shows that they have applied not only different inhibitors but also different experimental protocols; whereas Braverman et al. (2006a,b) tested the inhibitor and its vehicle in parallel bladder strips (i.e., performing only one carbachol curve per strip, “Philadelphia protocol”), Schneider et al. (2004b) have tested increasing inhibitor concentrations in subsequent carbachol curves within the same bladder strip (“Amsterdam protocol”). To
resolve this “PLC-controversy”, we have now performed crossover experiments. Thus, each inhibitor was tested by the other group using its own protocol. Our joint efforts suggest that previously reported controversies relate to the use of inhibitor rather than protocol and do not support a role for PLC in muscarinic receptor-stimulated rat bladder contraction.

Materials and Methods

All experiments were in line with the National Institutes of Health (Institute of Laboratory Animal Resources, 1996) and had been approved by the respective animal care and use committees. Based upon our previous studies, experiments either followed the “Philadelphia protocol” as routinely used in the Ruggieri laboratory or the “Amsterdam protocol” as routinely used in the Michel laboratory.

Philadelphia Protocol. Experiments were basically performed as described previously (Braverman et al., 2006a,b). In brief, urinary bladders were removed from female Sprague-Dawley rats (body weight, 200–250 g; bladder weight, ~100 mg; Ace, Boyertown, PA) that had been euthanized by N2 asphyxiation following anesthesia with 5% isoflurane in oxygen. The bladder was divided in the mid-sagittal plane and cut into longitudinal smooth muscle strips (approximately 3 × 8 mm). The muscle strips were then suspended with 9.8 mN tension in tissue baths containing 15 ml of modified Tyrode’s solution (125 mM NaCl, 2.7 mM KCl, 0.4 mM NaH2PO4, 1.8 mM CaCl2, 0.5 mM MgCl2, 23.8 mM NaHCO3, and 5.6 mM glucose) and equilibrated with 95% O2/5% CO2 at 37°C. After equilibration to the bath solution for 30 min, a maximal contraction induced by a 3-min exposure to 120 mM KCl was recorded. The strips were ranked based on their contractile response to KCl and sorted so that the average response in each treatment group was equal. The strips were incubated for 30 min in the presence or absence of a PLC inhibitor. Concentration-response curves were derived from the peak tension developed after cumulative addition of carbachol (10 nM to 300 μM final bath concentration) and normalized to the response to 120 mM KCl. Only one concentration of enzyme inhibitor was used for each muscle strip, and each muscle strip was only exposed to a single carbachol concentration-response curve.

Amsterdam Protocol. Experiments were basically performed as described previously (Schneider et al., 2004b). In brief, urinary bladders were removed from male Wistar rats (body weight, 298 ± 5 g; Charles River, Maastricht, The Netherlands) that had been euthanized by decapitation under pentobarbital anesthesia. Urinary bladder strips were cut longitudinally into four strips (1 mm in diameter, 16 ± 0.4 mm in length, 8.4 ± 0.3 mg in weight). Bladder strips were mounted under the tension of 10 mN in 7 ml of Krebs-Henseleit buffer of the following composition: 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 0.025 mM Na4EDTA, 2.5 mM CaCl2, 1.2 mM KH2PO4, 25 mM NaHCO3, and 5.5 mM glucose at 37°C, yielding a total potassium concentration of 5.9 mM. The organ baths were continually gassed with 95% O2/5% CO2 to maintain a pH of 7.4.

After 75 min of equilibration, including washes with fresh buffer every 15 min, the bladder strips were challenged twice with 50 mM KCl, with a 60-min rest and washes between each challenge. After washout and an additional 45-min equilibration, cumulative concentration-response curves were constructed for carbachol in the absence of vehicle or inhibitor. Using a 10-min washout and then 30-min equilibration periods in between, up to three additional curves were then generated in the presence of increasing concentrations of the indicated inhibitors or their vehicles.

Data Analysis. Carbachol concentration-response curves were analyzed by fitting sigmoidal curves to the experimental data. The force of contraction was expressed as the percentage of the effect of 120 mM KCl observed in the same bladder strip (Philadelphia protocol) or in the same bladder strip in the first concentration-response curve, i.e., before addition of any inhibitor or vehicle (Amsterdam protocol). Alterations in Ec50 or pEC50 in its presence relative to the vehicle or first curve were compared within curves in the same experiment using one-way analysis of variance. If the effect of individual inhibitor concentration relative to the first curve was significant, Dunnett’s post-test was performed. All curve fitting and statistical calculations were performed with the Prism program (GraphPad Software Inc., San Diego, CA), and a p < 0.05 was considered to be significant.

Chemicals. Carbachol HCl, U-73,122 (1-[6-[[17β]-3-methoxy-estra-1,3,5(10)-ti-en-17-yl)-amino]hexyl]-1H-pyrrrole-2,5-dione), and neomycin were purchased from Sigma-Aldrich (St. Louis, MO). ET-18-OCH3 (1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine) and D609 (O-tricyclo[5.2.1.02,6]dec-9-yI dithiocarbonate potassium salt) were obtained from Biomol Research Laboratory (Plymouth Meeting, PA). Et-18-CHO3 (at 10 μM) was dissolved in ethanol. D609 and neomycin (at 10 μM) were dissolved in distilled water. U-73,122 (at 1 mM) was dissolved in dimethyl sulfoxide.

Results

Philadelphia Protocol. In the absence of any inhibitor, carbachol induced rat urinary bladder contraction with a pEC50 of 5.86 ± 0.10 and a maximal response of 16.7 ± 2.9 mN tension (n = 12). U-73,122 (10 μM) did not affect the potency or efficacy of carbachol to induce bladder contraction when expressed as percentage of the maximal carbachol response of each strip (Fig. 1), as absolute tension, or as percentage of KCl-induced contraction (data not shown). When darifenacin (30 nM) was added concomitantly with U-73,122 it induced a right shift of the carbachol concentration-response curve from which an apparent pA2 value of 8.5 was calculated (Fig. 1), indicating that the response in the presence of U-73,122 indeed occurs via a M3 receptor.

Amsterdam Protocol. In the first concentration-response curve within a bladder strip, i.e., in the absence of any inhibitor, carbachol increased force of contraction with a pEC50 of 5.94 ± 0.03 and maximal effects of 6.0 ± 0.3 mN/mg (n = 41 muscle strips). In confirmation of previous studies (Kories et al., 2003; Schneider et al., 2004b), three subsequent curves in the absence of any inhibitor yielded rather similar values. The PLC inhibitor vehicles (distilled water, ethanol, or dimethyl sulfoxide) caused minor if any right shifts of the curves.

Fig. 1. Effect of U-73,122 (10 μM) alone and in combination with darifenacin (30 nM) on carbachol-induced rat bladder contraction using the Philadelphia protocol (one curve per muscle strip). Data are means ± S.E.M. of 6 to 12 experiments. The maximal contractile tension responses (mean ± S.E.M.) are 17 ± 3 mN for control vehicle, 23 ± 5 mN for U-73,122, and 17 ± 2 mN for U-73,122 + darifenacin.
carbachol curves (up to 0.2 log units), but those failed to reach statistical significance (data not shown).

In confirmation of our previous findings (Schneider et al., 2004b), the addition of 1, 3, and 10 μM U 73,122 to a second, third, and fourth carbachol curve, respectively, did not have significant effects on the potency or efficacy of carbachol compared to vehicle time controls (Fig. 2). ET-18-OCH₃ (10–100 μM) only at the highest concentration caused a minor right shift of the carbachol curve (~0.4 log units; Fig. 3), which was not statistically significant compared to vehicle time controls. D609 had little effect against carbachol at 10 to 30 μM, but at a concentration of 100 μM, it almost abolished the carbachol response (Fig. 4). At 100 μM but not at 30 μM, D609 significantly attenuated contractile responses to 25, 50, 75, 100, and 120 mM KCl (n = 5 to 6; p < 0.01) compared to vehicle control using the Philadelphia protocol (Fig. 5), and 100 μM D609 similarly inhibited the response to 50 mM KCl in the Amsterdam protocol (data not shown). Neomycin (10–100 μM) did not affect the potency or efficacy of carbachol (Fig. 6).

Discussion

Although M₂ receptors can contribute to mammalian bladder contraction, most studies including data from M₂ and M₃ knock-out mice suggest a predominant role for the M₃ subtype under normal physiological conditions in most species, including humans (Abrams et al., 2006; Hegde, 2006). Activation of M₂ receptors prototypically leads to the hydrolysis of phosphatidylinositol 4,5-biphosphate to result in the generation of the second messengers IP₃ and diacylglycerol, which is then followed by elevation of intracellular calcium concentration and by activation of protein kinase C. It can also couple to the hydrolysis of phosphatidylcholine by a PC-PLC, which may contribute to sustaining smooth muscle contraction (Caulfield, 1993). However, the role of PLC, specifically PI-PLC, in mediating muscarinic receptor-stimulated bladder contraction has remained controversial, and the two laboratories contributing to the present study have taken somewhat different views on this role. Because we had previously used both of the different inhibitors and different experimental protocols to explore a role of PLC, we have now collaborated in a crossover study in which each inhibitor was tested in the opposite protocol.

Both protocols showed a very similar potency of carbachol to elicit rat bladder contraction under baseline conditions, thus providing cross-validation of our two techniques. The two laboratories also have used rats of different gender (females in Philadelphia protocol and males in Amsterdam protocol); however, previous work shows that gender does not affect carbachol-induced contraction or IP formation in rat bladder (Kories et al., 2003). The key difference between the two protocols had been the use of single versus repeated concentration-response curves within a single muscle strip. The reproducibility of pEC₅₀ and Eₘₐₓ across multiple curves (Kories et al., 2003; Schneider et al., 2004b) argues that no major desensitization occurred under these conditions in the Amsterdam protocol. However, it is possible that, during repeated curves, a switch from one underlying signaling mechanism to another could occur, which may not manifest in alterations of pEC₅₀ or Eₘₐₓ and may have led to missing a role for PLC in the Amsterdam protocol.

In total, four different PLC inhibitors have been tested in...
the present study. In this regard, U-73,122 and ET-18-OCH₃ are considered PI-PLC inhibitors, D609 is considered a PC-PLC inhibitor, and neomycin is considered a nonselective PLC inhibitor. U-73,122 had previously lacked inhibitory effects against carbachol-induced contraction in rat bladder in a concentration where it fully abolished carbachol-induced IP formation (Schneider et al., 2004b). This PI-PLC inhibitor had also failed to block carbachol-induced contraction of mouse (Wegener et al., 2004) or human bladder (Schneider et al., 2004a), all of which had used the Amsterdam or a similar protocol. In the present study, U-73,122 also did not affect the carbachol response in the Philadelphia protocol. It could be argued that PI-PLC is involved in this response, but its role can be concealed by a switch from M₂ to M₃ receptors in the presence of U-73,122. However, the high potency of darifenacin to antagonize contraction in the presence of U-73,122 indicates that this contraction is indeed M₃-mediated. Thus, the lack of U-73,122 to inhibit bladder contraction in a concentration where it inhibited IP formation does not support a role for PI-PLC in carbachol-induced bladder contraction.

Previously, ET-18-OCH₃ (30–100 μM), i.e., at 6 to 20 times its reported Kᵣ value for PI-PLC (Braverman et al., 2006b), was reported to decrease the apparent potency of carbachol without effect on Eₘ₃ₐₓₙ in the Philadelphia protocol (by ~1 log unit at 100 μM) (Braverman et al., 2006b). In the present studies using the Amsterdam protocol, such antagonism was less pronounced and did not reach statistical significance compared to vehicle time controls. The reason for this discrepancy remains unclear, but it should be noted that the final ethanol concentration in the assay in the original Philadelphia experiments had been only 0.35%, whereas it was 1.0% in the present experiments using the Amsterdam protocol. Moreover, in light of the unequivocal U-73,122 data, this minor discrepancy between the reported data has little effect on the overall conclusion regarding a relative lack of a PI-PLC component in the contractile carbachol response.

It has been proposed that a PC-PLC may be involved in the sustained phase of smooth muscle contraction (Somlyo and Somlyo, 1994; Makhlof and Murthy, 1997). Using the Philadelphia protocol, it had been reported previously that the PC-PLC inhibitor D609 at 100 μM, but not at lower concentrations, could reduce the Eₘ₃ₐₓₙ of carbachol in rat bladder (Braverman et al., 2006b). The present study using the Amsterdam protocol confirms this observation. However, this may not reflect a role for PC-PLC in carbachol-induced bladder contraction because the same concentration of D609 also suppressed the receptor-independent contraction induced by KCl in both the Philadelphia and the Amsterdam protocol. Moreover, D609 may have additional effects unrelated to PC-PLC (Kiss and Tomono, 1995; van Dijk et al., 1997). Which, if any, of those D609 effects is involved in the inhibition of the carbachol response in the bladder remains to be investigated. However, regardless of this consideration, these data do not support a specific role of PC-PLC in the carbachol response.

Finally, neomycin is a rather nonspecific inhibitor of PLC, which had been used to support a claim for an involvement of PLC in feline bladder smooth muscle contraction (Am et al., 2002). However, neither the previous data using the Philadelphia protocol (Braverman et al., 2006b) nor the present data using the Amsterdam protocol support such effects, at least in rats.

Taken together, the available data clearly show that muscarinic receptors in the bladder can couple to PLC activation (An et al., 2002; Kories et al., 2003; Schneider et al., 2004b; Kajioka et al., 2005) but do not support a major role for such activation in mediating bladder contraction induced by muscarinic receptor agonists. In contrast, undisputed roles exist for an influx of extracellular calcium through voltage-dependent channels (Ikeda et al., 1999; Schneider et al., 2004a,b) and for activation of a rho kinase (Peters et al., 2006) in mediating bladder contraction. Moreover, calcium release from intracellular stores mediated by a ryanodine receptor may also play a role (Kajioka et al., 2005).

References


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