Signal Transduction Underlying Carbachol-Induced Contraction of Human Urinary Bladder

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ABSTRACT

The present study was designed to reexamine the muscarinic acetylcholine receptor subtype mediating carbachol-induced contraction of human urinary bladder and to investigate the underlying signal transduction. Based upon the nonselective tolterodine, the highly M₃-selective (R)-4-[(2-[3-(4-methoxy-benzoyl)amino]-benzyl]-piperidin-1-ylmethyl]-piperidine-1-carboxylic acid amide (Ro-320-6206), and the highly M₃-selective darifenacin and 3-(1-carbamoyl-1,1-diphenylmethyl)-1-(4-methoxyphenylethyl)pyrrolidine (APP), contraction occurs via M₃ receptors. The phospholipase C inhibitor 1-[(6-[(17)]r)-3-methoxyestra-1,3,5-(10)tri-en-17-y]amino]hexyl)-1H-pyrole-2,5-dione (U 73,122) (1–10 μM) did not significantly affect carbachol-stimulated bladder contraction. In contrast, trans-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide (Y 27,632) (1–10 μM), an inhibitor of rho-associated kinases, concentration dependently and effectively attenuated the carbachol responses. We conclude that carbachol-induced contraction of human urinary bladder via M₃ receptors largely depends on Ca²⁺ entry through nifedipine-sensitive channels and activation of a rho kinase, whereas phospholipase D and store-operated Ca²⁺ channels contribute only in a minor way. Surprisingly, phospholipase C or protein kinase C do not seem to be involved to a relevant extent.

MUSCARINIC ACETYLCHOLINE RECEPTORS

Muscarinic acetylcholine receptors are the physiologically most important mechanism to elicit contraction of the urinary bladder (Andersson, 1993). M₃ and M₄ muscarinic receptors coexist in the bladder of various mammalian species, including humans, but the expression of M₃ receptors is much greater than that of the M₄ receptors (Wang et al., 1995; Goepel et al., 1998; Yamanishi et al., 2000; 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; Tong et al., 1997; Braverman et al., 1998; Choppin et al., 1998; Longhurst and Levendusky, 2000; Kories et al., 2003; Schneider et al., 2004), mice (Choppin and Eglen, 2001b), pigs (Yamanishi et al., 2000), and dogs (Choppin and Eglen, 2001a). Moreover, M₄ (but not M₃) receptor knockout mice exhibit bladder distension and develop urinary retention (Birdsall et al., 2001). In humans, two studies have investigated the muscarinic receptor subtype mediating bladder contraction and also proposed involvement of M₃ receptors (Chess-Williams et al., 2001; Fetscher et al., 2002). On the other hand, this previous work was based on antagonists with only modest subtype selectivity and/or with a mechanism of action that is not purely competitive; hence these studies were not well suited for detecting a potential minor component of M₃ receptors in bladder contraction.

M₄ receptor-activated cellular signaling pathways have been studied in many cell types, and the activation of a phospholipase (PL) C to generate inositol phosphates and diacylglycerol is considered to be the prototypical response (Caulfield and Birdsall, 1998). However, muscarinic receptors can also activate a PLD in a variety of cell types (Felder, 1995). Additionally, M₄ receptor stimulation typically results in elevation of intracellular Ca²⁺ concentrations, which can involve both mobilization from intracellular stores and influx...
from the extracellular space (Caulfield and Birdsal, 1998). Muscarinic stimulation of PLC has also been demonstrated in rat bladder slices (Kories et al., 2003; Schneider et al., 2004) and in cultured smooth muscle cells from human bladder (Marsh et al., 1996). Although PLC stimulation has been implicated in the muscarinic receptor-mediated contraction of the feline bladder (An et al., 2002), we have recently demonstrated that it does not contribute to that of rat bladder (Schneider et al., 2004), indicating possible species differences. We have also excluded a contribution of PLAr and cyclooxygenase but have detected a small role for PLD in rat bladder (Schneider et al., 2004).

Muscarinic receptor stimulation has also been shown to elevate intracellular Ca2+ concentrations in rat and guinea pig bladder smooth muscle cells (Ikeda et al., 2002; Ma et al., 2002). Accordingly, L-type Ca2+ entry blockers can inhibit muscarinic receptor-mediated bladder contraction in guinea pigs (Ikeda et al., 2002), rats (Schneider et al., 2004), and humans (Sjögren et al., 1982; 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).

In other types of smooth muscle, a variety of protein kinases can link PL activation and/or Ca2+ elevation to contraction, including protein kinase (PK) C, tyrosine kinases, phosphatidylinositol-3-kinase, mitogen-activated PK, and rho kinase. In a recent study, we have demonstrated that muscarinic receptor-mediated contraction of rat bladder is markedly reduced by an inhibitor of rho kinase, whereas inhibitors of PKC, tyrosine kinases, phosphatidylinositol-3-kinase, or mitogen-activated PK were without effect (Fleichman et al., 2004).

Based on the above points, the present study was primarily designed to determine the signaling mechanisms underlying M3 receptor-mediated contraction of the human urinary bladder, specifically a possible involvement of PLC, PLD, L-type voltage-operated and receptor-operated Ca2+ channels, PKC, and rho kinase. Additionally, we have reinvestigated the role of muscarinic receptor subtypes using two novel and highly selective antagonists, i.e., Ro-320-6206 (Zhao et al., 2001) and APP (MacKenzie and Cross, 1991), respectively.

**Materials and Methods**

Human bladder specimens were obtained from patients undergoing cystectomy due to bladder cancer. All patients had given informed written consent in accordance with the approval by the local ethical committee at the University of Essen. The tissue samples were from tumor-free parts of the bladder. Contraction experiments were performed as described previously (Fetscher et al., 2002). Briefly, bladder strips of 10.4 ± 0.3-mm length and 1- to 2-mm diameter (mean weight 13.9 ± 0.7 mg, n = 111) were prepared and mounted 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. After washout and an additional 30 min of equilibration, cumulative concentration-response curves were constructed for carbachol. Using washout and 15-min equilibration periods in between, up to five consecutive curves were generated in the presence of increasing concentrations of the indicated antagonists or signaling inhibitors.

As in our previous studies with rat bladder (Fleichman et al., 2004; Schneider et al., 2004), carbachol concentration-response curves were analyzed by fitting sigmoidal curves to the experimental data, in which the bottom of the curve was fixed at zero. The force of contraction in the absence and presence of inhibitors were expressed as percentage of maximum carbachol effects observed within the same bladder strip in the first concentration-response curve, i.e., before addition of any inhibitor or vehicle. To assess inhibitor effects, alterations in Emax or pEC50 in its relative presence to the first curve were compared with those in the presence of a matching vehicle time control using two-way analysis of variance testing for main treatment effect and concentration dependence; if this indicated statistical significance, the effect of individual inhibitor concentrations relative to time-matched controls was assessed by Dunnett’s 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 Inc., San Diego, CA).

Carbachol HCl, nifedipine, 1-β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazol HCl (SK&F 96,365) and 1-β-[17β]-3-methoxyestradiol-1,3,5(10)-trien-17-yl)-aminohexyl]-1H-pprole-2,5-dione (U 73,122) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Bisindolylmaleimide I and calphostin C (from Cladosporium cladosprioides) were obtained from Calbiochem (Bad Soden, Germany). trans-4-{4-(4-methoxycyclohexyl)carboxamide (Y 27,632) was from Toeris Cookson Inc. (Bristol, UK). Darifenacin and tolterodine were provided by Pfizer (New York, NY), Ro-320-6206 (Ro 320-6206) and 3-(1-carbamoyl-1,1-diphenylmethyl)-1-(4-methoxyphenethyl)-pyrrolidine (APP) were synthesized as described previously (MacKenzie and Cross, 1991; Zhao et al., 2001).

APP (10 mM), calphostin C (1 mM), darifenacin (10 mM), bisindolylmaleimide I (1 mM), Ro-320-6206 (10 mM), tolterodine (10 mM), and U 73122 (3 mM) were dissolved in dimethyl sulphoxide. Nifedipine was dissolved at 1 mM in ethanol. SK&F 96,365 (1 mM) and Y 27,632 (10 mM) were dissolved in distilled water. The experiments involving nifedipine were performed in light-shielded organ baths.

**Results**

Before 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 pEC50 of 6.11 ± 0.03 and maximum effects of 47.3 ± 2.6 mN (n = 111 muscle strips). All further contraction data are expressed as percentage of the maximum carbachol effect within the same preparation before addition of any inhibitor.
Similar to our previous observations in rats (Schneider et al., 2004) and humans (Fetscher et al., 2002), the contractile response of isolated human bladder strips slightly decreased with time. Thus, relative to the first curve in the absence of any antagonist, the second to fifth consecutive curve within a preparation exhibited a pEC50, which was $-0.07 \pm 0.03$, $0.36 \pm 0.09$, $0.49 \pm 0.15$, and $0.76 \pm 0.09$ log units smaller, respectively; concomitantly, maximum effects were reduced by $-1 \pm 2$, $13 \pm 7$, $18 \pm 5$, and $42 \pm 8\%$, respectively ($n = 12$). 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 that was significantly different from vehicle (data not shown), whereas 10, 30, 100, and 300 nM darifenacin caused reductions exceeding those in the time-matched vehicle group 15, 20, 27, and 17\% respectively ($p < 0.05$ versus vehicle for the 10 and 100 nM concentration). All four antagonists concentration dependently right-shifted the carbachol concentration-response curve (Fig. 1). The Schild-regression for the nonselective tolterodine (30–1000 nM), M3-selective darifenacin (10–300 nM), and the M3-selective APP (10–300 nM) did not differ significantly from unity, i.e., were 0.99 $\pm$ 0.10, 1.14 $\pm$ 0.10, and 1.14 $\pm$ 0.10, respectively. The corresponding calculated $pA2$ values were 7.99 (95% confidence interval 7.57–8.56), 8.41 (8.11–8.77), and 8.51 (8.20–8.90), respectively. 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 $\mu$M it right-shifted the carbachol concentration-response curve by only 0.22 $\pm$ 0.07, 0.26 $\pm$ 0.11, 0.61 $\pm$ 0.16, and 1.05 $\pm$ 0.10 log units, respectively; accordingly, a Schild-slope of only 0.56 $\pm$ 0.11 was obtained, and the x-axis intercept of this shallow regression line was 6.70 (95% confidence interval 6.07–7.66).

The PLC inhibitor U 73,122 (1–10 $\mu$M) did not significantly alter the potency or maximum effects of carbachol-induced bladder contraction relative to its vehicle (Fig. 2). The PLD inhibitor butan-1-ol (0.03–0.3%) 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 maximum effects of carbachol was obtained at a butan-1-ol concentration of 0.3% (Fig. 3).

The Ca2+ entry blocker nifedipine (10–100 nM) markedly inhibited carbachol-induced bladder contraction relative to its vehicle ethanol (Fig. 4). This inhibition was due to reductions of maximum carbachol responses reaching 40% at 100 nM, which were not accompanied by statistically significant alterations of the agonist potency for the remaining response. SK&F 96,365 (1–10 $\mu$M), an inhibitor of receptor-operated Ca2+ channels, did not significantly affect carbachol-induced bladder contractions (Fig. 5).

A potential role of PKC was assessed using the inhibitor bisindolylmaleimide I (1–10 $\mu$M), which did not significantly affect the potency nor maximum effects of carbachol-induced bladder contraction relative to paired time-control experiments in the presence of vehicle (Fig. 6). Similar data were obtained with another PKC inhibitor, calphostin C (0.1–1 $\mu$M), in a smaller number of experiments ($n = 4$, data not shown).

To test a role of rho-associated kinase, its inhibitor Y 27,632 (1–10 $\mu$M; Fig. 7) was tested. Y 27,632 concentration dependently inhibited carbachol-induced bladder contraction, and this inhibition consisted mainly of a reduction of...
maximum responses (exceeding those in time-matched vehicle controls by 16, 23, and 27% at 1, 3, and 10 μM Y 27,632, respectively) without significant alterations of pEC$_{50}$.

**Discussion**

The present study was primarily designed to investigate proximal signaling mechanisms potentially involved in carbachol-induced muscarinic receptor-mediated contraction of human urinary bladder. Although M$_2$ receptors are more numerous in human bladder than M$_3$ receptors (Wang et al., 1995; Goepel et al., 1998), two studies have proposed that human bladder contraction is mediated predominantly if not exclusively by the minor population of M$_3$ receptors (Chess-Williams et al., 2001; Fetscher et al., 2002). However, these studies were based on antagonists with only moderate subtype selectivity or upon darifenacin, which has considerable selectivity for M$_3$ receptors but does not act purely competitively (Schneider et al., 2004); the not purely competitive antagonism by darifenacin was further confirmed in the present study. Therefore, we have reinvestigated the muscarinic receptor subtype mediating human bladder contraction using APP, a compound that similar to darifenacin is about 40-fold selective for M$_3$ receptors ($K_i$ of 2.6 versus 111 nM; S. Hegde, Theravance Inc., South San Francisco, CA, personal communication) but does not reduce maximum responses, and Ro-320-6206, an approximately 100-fold M$_2$-selective antagonist (5.0 versus 500 nM; Zhao et al., 2001); the nonselective 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 human bladder occurs via M$_3$ receptors. Moreover, the calculated apparent affinities of darifenacin and tolterodine in the present study were in good agreement with those previously reported for human bladder (Yono et al., 1999; Fetscher et al., 2002). Thus, humans seem to be similar to all previously studied animal species with regard to a primary involvement of the M$_3$ subtype of muscarinic receptors in bladder contraction.

The signal transduction underlying muscarinic receptor-mediated contraction of bladder smooth muscle has previously been studied in cats (An et al., 2002) and rats (Fleichman et al., 2004; Schneider et al., 2004). Because the results reported for the two species were not in agreement with each other, it seems important to specifically characterize those signaling pathways for human bladder. PLC activation is the prototypical signaling pathway of M$_3$ receptors (Caulfield and Birdsall, 1998), and it is also activated by muscarinic receptor stimulation in rat and human bladder (Marsh et al., 1996; Kories et al., 2003; Schneider et al., 2004). Thus, it was not surprising that an involvement of PLC in bladder contraction was reported in cats using neomycin as the PLC inhibitor (An et al., 2002). On the other hand, we have excluded a PLC involvement in rat bladder, because concentrations of the PLC inhibitor U 73,122, which fully suppressed inositol phosphate formation in rat bladder and also markedly inhibited α-adrenoceptor-mediated vasoconstriction, exhibited no inhibitory effects against carbachol-induced bladder contraction (Altman et al., 2003; Schneider et al., 2004). Similarly, U 73,122 did not significantly affect muscarinic receptor-mediated contraction of human bladder in the present study. These data do not support a role for PLC in contractile carbachol responses in human bladder and addi-

![Fig. 4. Effects of the Ca$^{2+}$ channel inhibitor nifedipine (10–100 nM) and its vehicle on carbachol-induced contraction. * and ***, $p < 0.05$ and <0.001, respectively, versus matching time controls in the presence of vehicle in a two-way analysis of variance followed by Dunnett’s post tests.](image)

![Fig. 5. Effects of SK&F 96,365 (1–10 μM), an inhibitor of receptor-operated Ca$^{2+}$ channels, and its vehicle on carbachol-induced contraction.](image)
tionally indicate that rats may be more similar to humans than cats in this regard.

Although muscarinic receptors can also activate PLD in several cell types (Felder, 1995), only very small if any PLD activation was seen in rat bladder upon carbachol stimulation (Schneider et al., 2004). Nevertheless, the PLD inhibitor butan-1-ol caused some inhibition of rat bladder contraction (Schneider et al., 2004), and similar findings were obtained in the present study with human bladder.

An involvement of L-type Ca2+/H11001 channels in muscarinic receptor-mediated bladder contraction has previously been shown in rats (Schneider et al., 2004), guinea pigs (Ikeda et al., 2002), and humans (Sjögren et al., 1982; Masters et al., 1999). The present study confirms these findings in human bladder using nifedipine as an inhibitor. Interestingly, the potency of nifedipine to cause such inhibition was considerably higher than what we had previously observed in blood vessels under similar conditions (Chen et al., 1996; Bischoff et al., 2001), indicating that bladder contraction may rely to a greater extent on L-type Ca2+/H11001 channels than that of blood vessels. In this regard, it is interesting to note that human bladder smooth muscle exhibits spontaneous nifedipine-sensitive action potentials (Hashitani and Brading, 2003). Moreover, knockout mice lacking the Cα1,2 gene, which encodes for a subunit of voltage-operated Ca2+/H11001 channels, exhibit a markedly reduced bladder contraction in response to muscarinic stimulation (Wegener et al., 2003).

Previous studies have suggested that Ca2+/H11001 sources apart from L-type channels, e.g., thapsigargin- or ryanodine-sensitive intracellular stores, may also contribute to human bladder smooth muscle cells (Masters et al., 1999; Visser and van Mastrigt, 2000). In our previous studies, we have detected only little if any role for receptor-operated Ca2+/H11001 channels in rat bladder (Schneider et al., 2004). In the present study, SK&F 96,365, an inhibitor of these channels, also caused only minor inhibition of the carbachol response. Thus, L-type Ca2+/H11001 channels and thapsigargin- and/or ryanodine-sensitive mechanisms rather than receptor-operated channels seem to be important for M3 receptor-mediated contraction of human bladder.

Using three chemically distinct PKC inhibitors, we have previously demonstrated that PKC does not play a relevant role in carbachol-induced rat bladder contraction (Fleichman et al., 2004). Given the fact that PKC is typically activated after PLC stimulation and that the lack of PLC involvement was rather surprising, we have also tested the role of PKC in human bladder contraction with two of the previously used inhibitors. However, similar to our findings in the rat, neither inhibitor significantly affected human bladder contraction, demonstrating that PKC does not contribute to this effect. Because PKC activation frequently occurs secondary to PLC activation and because our present experiments in human as well as our previous ones in rat bladder (Schneider et al., 2004) did not detect a role for PLC in urinary bladder contraction, these data demonstrate that the PLC/PKC cascade may be activated by muscarinic receptors in rat and human urinary bladder (Marsh et al., 1996; Kories et al., 2003) but is not crucial for induction of its contraction.

Rho-associated kinase is gaining attention as a universal regulator of smooth muscle tone and is also expressed at high levels in rat urinary bladder (Wallner, 2003). Previous work has established its role in muscarinic receptor-
mediated rat bladder contraction (Ribble et al., 2003; Fleichman et al., 2004). This can involve several different mechanisms, including a direct effect on myosin light chains; effects on CPI-17, a phosphorylation-dependent inhibitory protein of myosin phosphatase; and, perhaps most importantly, a direct inhibition of myosin phosphatase (Fukata et al., 2001). The present data demonstrate that rho kinase is also involved in muscarinic receptor-mediated contraction of the human bladder.

In conclusion, carbachol-induced, M3 muscarinic receptor-mediated contraction of human bladder is largely mediated by Ca2+ influx through L-type, voltage-dependent channels and activation of a rho kinase. Surprisingly, PLC and PKC activation are not involved. Moreover, PLD and receptor-operated Ca2+ channels also play only a minor if any role in muscarinic receptor-mediated contraction. The similarity of these findings with those previously reported for rat urinary bladder (Wibberley et al., 2003; Fleichman et al., 2004; Schneider et al., 2004) indicates that rats may be a suitable model species to study signal transduction in the urinary bladder.

References
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