M₂ and M₃ Muscarinic Receptor Activation of Urinary Bladder Contractile Signal Transduction. II. Denervated Rat Bladder

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ABSTRACT

Normal rat bladder contractions are mediated by the M₃ muscarinic receptor subtype. The M₂ receptor subtype mediates contractions of the denervated, hypertrophied bladder. This study determined signal transduction mechanisms mediating contraction of the denervated rat bladder. Denervated bladder muscle strips were exposed to inhibitors of enzymes thought to be involved in signal transduction in vitro followed by a cumulative carbachol concentration-response curve. Outcome measures were the maximal contraction, the potency of carbachol, and the affinity of darifenacin for inhibition of contraction. Inhibition of phosphoinositide-specific phospholipase C (PI-PLC) with 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine (ET-18-OCH₃) has no effect on denervated bladder contractions, whereas inhibition of phosphatidyl choline-specific phospholipase C (PC-PLC) with O-tricyclo[5.2.1.0²,6]dec-9-yl dithiocarbonate potassium salt (D609) attenuates the carbachol maximum and potency. Inhibition of rho kinase with (R)-(+)trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride (Y-27632) reduces carbachol maximum, carbachol potency, and increases darifenacin affinity. Inhibition of rho kinase, protein kinase A (PKA), and protein kinase G (PKG) with 1-(5-isoquinolinesulfonfonyl)-homopiperazine-2HCl (HA-1077) reduces the carbachol maximum and potency. Inhibition of PKC with chelerythrine increases darifenacin affinity, whereas inhibition of rho kinase, PKA, PKG, and protein kinase C (PKC) with 1-(5-isoquinolinesulfonfonyl)-2-methylpiperazine-2HCl (H7) reduces the carbachol potency while increasing darifenacin affinity. Inhibition of rho kinase, PKA, and PKG with N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide-2HCl (H89) increases darifenacin affinity. This study demonstrates that different signal transduction mechanisms mediate the contractile response in the denervated rat bladder than in normal rat bladder. In normal rat bladder, PI-PLC and PC-PLC mediate the contraction, but in denervated bladder only PC-PLC is involved. In the denervated bladder, the rho kinase pathway is more dominant than in normal bladders. PKA seems to mediate a contractile response in normal bladders, whereas it seems to inhibit contraction in denervated bladders.

The affinity of subtype-selective muscarinic receptor antagonist drugs indicates that contraction is mediated by the M₃ receptor in most smooth muscles under normal conditions (Caulfield, 1993; Caulfield and Birdsal, 1998). However, direct M₂-mediated contractile response to muscarinic agonist has been shown for cat esophageal smooth muscle cells (Biancani et al., 1997). In contrast, cells isolated from the cat lower esophageal sphincter (LES) circular smooth muscle, like urinary bladder smooth muscle, are inhibited from contracting by subtype-selective antimuscarinic drugs with affinities typical of M₃ receptors. Induction of esophagitis by perfusing the esophagus with HCl switches the signal transduction mechanism in LES cells toward the pathway observed in esophageal cells. Thus, the affinity of antimuscarinic drugs becomes intermediate between their reported M₂ and M₃ affinities. In saponin-permeabilized LES cells from esophagitis cats, both anti-G₉₁₁ and Go₁₂,₁₅ antibodies inhibit contractions but not anti-G₁₅₁,₂ or Go₅ antibodies (Biancani et al., 1994). Acetylcholine fails to induce inositol 1,4,5-trisphosphate production and shortening is reduced by both the phosphatidylinositol-specific phospholipase C (PI-PLC) inhibitor U73122 as well as the phosphatidylcholine-specific phospholipase C (PC-PLC) inhibitor D609 and the

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ABBREVIATIONS:
LES, lower esophageal sphincter; PI-PLC, phosphoinositide-specific phospholipase C; PC-PLC, phosphatidyl choline-specific phospholipase C; ET-18-OCH₃, 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine; D609, O-tricyclo[5.2.1.0²,6]dec-9-yl dithiocarbonate potassium salt; Y-27632, (R)-(+)trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride; HA-1077, 1-(5-isoquinolinesulfonfonyl)homopiperazine-2HCl; H7, 1-(5-isoquinolinesulfonfonyl)-2-methylpiperazine-2HCl; H89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide-2HCl; PKA, protein kinase A; PKC, protein kinase; PKG, protein kinase G; ROCK, rho kinase; U73122, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrole-2,5-dione.

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phospholipase D inhibitor propranolol. This switch induced by esophagitis has been suggested to be because of reduced resting levels of intracellular calcium since depletion of calcium stores in normal LES cells reproduces the changes induced by esophagitis (Biancarni et al., 1994).

Several studies have shown that under certain experimental conditions, the M₂ receptor subtype contributes to the contractile response. This includes selective alkylation of M₃ receptors in an environment of increased intracellular levels of cAMP in the rat urinary bladder (Hegde et al., 1997; Braverman and Ruggieri, 1999), guinea pig ileum (Ehlerdt and Thomas, 1995), and trachea (Thomas and Ehlerdt, 1996) or after alkylation without increasing intracellular cAMP levels in other tissues such as the guinea pig gallbladder (Braverman et al., 2000) and colon (Sawyer and Ehlerdt, 1998). Other studies of smooth muscle contraction after experimentally induced pathologies, for example, in a cat model of experimentally induced esophagitis (Sohn et al., 1997), in the denervated rat bladder (Braverman et al., 1998), and in a model of acute cholecystitis in the guinea pig gallbladder (Braverman et al., 2000), also suggest that the M₂ receptor participates in contraction. Additional evidence for an M₂ receptor-mediated contractile pathway was demonstrated by the synergistic effects of M₂- and M₃-selective antagonists for inhibition of bladder contraction in normal bladders treated with thapsigargin and denervated bladders (Braverman et al., 2002).

Our previous studies showed that both bilateral major pelvic ganglion electrocautery and spinal cord injury in the rat induce bladder hypertrophy and a change in muscarinic receptor subtype mediating bladder contraction from M₃ toward M₂ (Braverman et al., 1998; Braverman et al., 1999; Braverman and Ruggieri, 2003). These studies were conducted to determine whether these changes are also accompanied by alteration in the signal transduction mechanisms mediating contraction.

**Materials and Methods**

**Materials.** The following drugs or chemicals were obtained from the sources indicated: carbachol (Sigma-Aldrich, St. Louis, MO) and ET-18-OCH₃, D609, Y-27632, HA-1077, chelerythrine [1,2-dime-thoxy-N-methyl-1,3]benzodioxolo(5,6-c)phananthrindinium chloride], H₇, and H₈9 (BIOMOL Research Laboratories, Plymouth Meeting, PA). Darifenacin was a generous gift from Pfizer Central Research (Sandwich, Kent, UK).

**Surgery.** Rats (200- to 250-g female Sprague-Dawley rats; Ace Animals Inc., Boyertown, PA) were anesthetized with 25 μg/kg buprenorphine and 2% isoflurane in oxygen and a midline incision was made in the lower abdomen. The pelvic peritoneum was exposed. For bilateral denervation, both the left and right major pelvic ganglion were cauterized with a hand stitching pencil attached to a model SSE 2 solid state electrosurgery device (Valleylab, Boulder, CO). For sham-operated animals, the plexus was exposed but left intact. The urine was expressed by manual pressure on the abdomen twice daily.

**Muscle Strips.** Urinary bladders were removed from rats euthanized by CO₂ asphyxiation. The urinary bladder body (tissue above the ureteral orifices) was dissected free of the serosa and surrounding fat. The bladder was divided in the mid-sagittal plane, and then cut into longitudinal smooth muscle strips (approximately 4 mm × 10 mm). The muscle strips were then suspended with 1 g of 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.

**Carbachol Dose Response.** After equilibration to the bath solution for 30 min, a maximal contraction induced by a 3-min exposure to 120 mM potassium 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 one of the enzyme inhibitors and in the presence or absence of 30 nM darifenacin. Because higher doses of darifenacin seemed insurmountable and lower doses did not produce a significant shift in the concentration-response curve, a single dose of 30 nM darifenacin was used. Dose-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-induced contraction. Only one concentration of enzyme inhibitor and/or darifenacin was used for each muscle strip. Dose ratios were determined based on the average of the responses of antagonist free strips. Because some of the enzyme inhibitors decreased the maximal contraction to less than 50%, darifenacin affinity is based on the EC₅₀ values. The EC₅₀ and EC₅₀ values were determined for each strip using a sigmoidal curve fit of the data (Origin; OriginLab Corp., Northampton, MA.). The EC₅₀ values determined in the presence of darifenacin were used to estimate the pKᵦₐ values, and the dose ratios were determined using the same concentration of inhibitor with and without darifenacin. The estimated pKᵦₐ for darifenacin was calculated using the formula pKᵦₐ = -(log[darifenacin concentration]) – (log[dose ratio – 1]).

Because there were statistically significant differences in the variance between groups, statistical analysis of multiple-group comparisons was performed by nonparametric Kruskal-Wallis analysis of variance followed by a post hoc Mann-Whitney U test for pairwise comparisons (GB-STAT; Dynamic Microsystems, Silver Spring, MD). Because there was no statistical difference between the outcome measures for the different vehicle control groups, these data were pooled. For maximal contraction and carbachol potency, groups with enzyme inhibitors were compared with these pooled no-inhibitor-added vehicle controls.

**Results**

**Inhibition of Phospholipases.** Inhibition of PI-PLC with ET-18-OCH₃ had no effect on the carbachol-stimulated maximal contraction or on carbachol potency (Fig. 1). Inhibition of PC-PLC with 100 μM D609 reduced the maximal contraction, whereas both 30 and 100 μM D609 reduced carbachol potency (Fig. 2). Inhibition of either PI-PLC with ET-18-OCH₃ or PC-PLC with D609 had no effect on the affinity of darifenacin (Table 1). The nonspecific PLC inhibitor neomycin had no effect on either the carbachol maximal contraction, carbachol potency (Fig. 3), or darifenacin affinity (Table 1).

**Inhibition of Protein Kinases.** Inhibition of ROCK with 10 μM Y-27632 reduced the maximal contraction, whereas all concentrations reduced carbachol potency (Fig. 4). Moreover, 3 and 10 μM Y-27632 resulted in a significant increase in the affinity of darifenacin in the denervated tissue from values consistent with M₃-mediated contractions to values consistent with M₂-mediated contractions (Table 1). Inhibition of ROCK as well as PKG and PKA by 10 and 30 μM HA-1077 reduced the carbachol maximal contraction, whereas 10, 30, and 100 μM HA-1077 reduced carbachol potency (Fig. 5). No concentration of HA-1077 had any effect on darifenacin affinity. Inhibition of PKC with chelerythrine had no effect on the maximal contraction response or carba-
chol potency (Fig. 6). The lowest dose of chelerythrine (1 mM) significantly increased darifenacin affinity (Table 1). Inhibition of ROCK, PKA, PKC, and PKG by all concentrations of H7 reduces carbachol potency without effect on maximal response (Fig. 7), whereas only 30 μM H7 increased darifenacin affinity (Table 1). Inhibition of PKA, ROCK, and PKG with H89 had only marginal effects; the highest concentration of H89 increased darifenacin affinity (Fig. 8; Table 1).

Discussion

Our previous studies showed that within 3 days after denervation, there was a decrease in the affinity of M3-selective muscarinic antagonists that was not apparent at 24 h, and the decrease was maintained for at least 3 weeks. The response to electric field stimulation was slightly decreased 6 h after major pelvic ganglion electrocautery and absent by 24 h. Even though there was a large increase in bladder weight by 24 h, no differences in the carbachol-induced maximal contraction were seen at any time point (Braverman and Ruggieri, 2003). Therefore, the 3-day postsurgery time point was chosen for all of the current studies.

In denervated bladders, inhibition of PI-PLC with ET-18-OCH3 had no effect on any of the three outcome measures, indicating that PI-PLC is not involved in mediating the M3 contractile signal. The PC-PLC inhibitor D609 inhibits max-

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<td>10</td>
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* p < 0.05; ** p < 0.01 compared with vehicle controls.
imal contraction in both normal (Braverman et al., 2005) and denervated bladders with no effect on darifenacin affinity. This indicates that PC-PLC is activated via M₃ receptors in normal bladders and by the M₂ receptor in denervated bladders. Similar findings have been reported in the cat lower esophageal sphincter (Biancani et al., 1994). The results with the nonspecific PLC inhibitor neomycin (i.e., no effect on any of the parameters) is consistent with the effects of the specific PI-PLC inhibitor ET-18-OCH₃ and suggests that increased phosphoinositide turnover is not necessary for contraction of the denervated bladder. The reason neomycin does not mimic the effects of the PC-PLC inhibitor is unclear but could involve D609 inhibition of addition enzymes that may be involved in contraction other than PC-PLC such as sphingomyelin synthase (Luberto and Hannun, 1998). This is different from the normal rat bladder where ET-18-OCH₃ reduces both carbachol and darifenacin potency, and the lowest concentration of neomycin inhibits the maximal agonist-induced contraction (Braverman et al., 2005).

Y-27632, a specific inhibitor of rho kinase, reduced carbachol contraction in both normal (Braverman et al., 2005) and denervated bladders with no effect on darifenacin affinity.

Fig. 3. A, cumulative carbachol concentration-response curves of rat bladder contraction with and without the PLC inhibitor neomycin. B, effect of neomycin on the maximal carbachol response. C, effect of neomycin on carbachol EC₅₀. No inhibitor (n = 21), 10 μM (n = 4), 30 μM (n = 4), and 100 μM (n = 4).

Fig. 4. A, cumulative carbachol concentration-response curves of rat bladder contraction with and without the rho kinase inhibitor Y-27632. B, effect of neomycin on the maximal carbachol response. C, effect of neomycin on carbachol EC₅₀. No inhibitor (n = 21), 10 μM (n = 7), 30 μM (n = 9), and 100 μM (n = 4). *, p < 0.05; **, p < 0.01.

Fig. 5. A, cumulative carbachol concentration-response curves of rat bladder contraction with and without the rho kinase, PKA, and PKG inhibitor HA-1077. B, effect of HA-1077 on the maximal carbachol response. C, effect of HA-1077 on carbachol EC₅₀. No inhibitor (n = 21), 10 μM (n = 4), 30 μM (n = 4), and 100 μM (n = 3). *, p < 0.05; **, p < 0.01.
potency and darifenacin affinity in both normal (Braverman et al., 2005) and denervated rat bladders while also reducing the carbachol maximal contraction in the denervated bladders. In the presence of Y-27632, the affinity of darifenacin for inhibiting contraction is higher in both normal (Braverman et al., 2005) and denervated bladders, suggesting that the M3 receptor subtype is mediating contraction after inhibition of the rho pathway. This is consistent with the conclusion that the rho pathway is activated by stimulation of the M2 receptor subtype and may explain why ROCK inhibition has greater effects on the maximal agonist induced contraction in the predominantly M2-mediated contraction in denervated bladders. This suggests that the signal transduction pathway activated by the M2 receptor subtype may not be able to induce enough myosin light chain kinase activation and thus is dependent on inhibition of myosin light chain phosphatase to reach maximal contraction.

In denervated bladders, the affinity of darifenacin is in-

Fig. 6. A, cumulative carbachol concentration-response curves of rat bladder contraction with and without the PKC inhibitor chelerythrine. B, effect of chelerythrine on the maximal carbachol response. C, effect of chelerythrine on carbachol EC50. No inhibitor (n = 21), 10 μM (n = 6), 30 μM (n = 4), and 100 μM (n = 5).

Fig. 7. A, cumulative carbachol concentration-response curves of rat bladder contraction with and without the rho kinase, PKA, PKG, and PKC inhibitor H7. B, effect of H7 on the maximal carbachol response. C, effect of H7 on carbachol EC50. No inhibitor (n = 21), 10 μM (n = 6), 30 μM (n = 4), and 100 μM (n = 5). *p < 0.05; **p < 0.01.

Fig. 8. A, cumulative carbachol concentration-response curves of rat bladder contraction with and without the rho kinase, PKA, and PKG inhibitor H89. B, effect of H89 on the maximal carbachol response. C, effect of H89 on carbachol EC50. No inhibitor (n = 21), 10 μM (n = 5), 30 μM (n = 5), and 100 μM (n = 4).
creased after partial inhibition of PKC with 1 μM chelerythrine, but it is not changed by 3 or 10 μM. This suggests that when PKC is only slightly inhibited, contraction occurs via an M₃-mediated pathway, but when PKC is further inhibited, contraction occurs via an M₂-mediated pathway independent of PKC, which is capable of inducing a maximal contraction with no change in agonist potency. These results are consistent with the idea that the level of PKC activity may determine whether the M₂ or the M₃ contractile pathway mediates contraction.

HA-1077 has approximately a 5-fold higher affinity for inhibition of ROCK than PKA and PKG. In denervated bladders, inhibition of PKA and PKG along with ROCK by HA-1077 reduces both carbachol potency and the carbachol maximum, the same effect as inhibition of ROCK alone. However, after ROCK inhibition, the affinity of darifenacin is increased. When PKA, PKG, and ROCK are inhibited by HA-1077, there is no change in darifenacin affinity, suggesting that contraction occurs via an M₂-mediated pathway independently of ROCK, PKA, and PKG.

H7 has a approximately a 6-fold higher affinity for inhibition of ROCK than PKA and approximately a 10-fold higher affinity for inhibiting ROCK than PKC and PKG. In denervated bladders, H7 reduced carbachol potency while increasing darifenacin affinity, suggesting that these enzymes are involved in mediating the M₂ receptor contractile signal; however, in this case, the remaining M₃ contractile signal was sufficient to stimulate maximum force. HA-1077, which inhibits ROCK, PKA, and PKG, reduced carbachol maximum along with carbachol potency with no effect on darifenacin affinity. These differences may be because of the additional inhibition of PKC by H7. As described above, partial inhibition of PKC resulted in an increase in darifenacin affinity, suggesting M₃ receptor-mediated contractions, whereas greater inhibition of PKC has no effect on darifenacin affinity.

H89 has approximately a 5-fold higher affinity for inhibiting PKA than ROCK and approximately a 10-fold higher affinity for inhibiting PKC than ROCK and PKG. In denervated bladders, inhibition of ROCK alone reduced the carbachol maximum, carbachol potency, and darifenacin affinity, whereas inhibition of ROCK predominately with some inhibition of PKA and PKG by HA-1077 also reduced the carbachol maximum and carbachol potency with no effect on darifenacin affinity. Inhibition of predominatey PKC with some ROCK inhibition with H89 had no effect on the maximum contraction or carbachol potency, only increasing darifenacin affinity. Thus, in denervated bladders, PKA probably mediates a pathway that relaxes the muscle, and inhibition of this pathway allowed for maximum contraction without any alteration in carbachol potency.

In control bladders, where the M₃ receptor subtype predominantly mediates contraction, PI-PLC, PC-PLC, ROCK, PKC, PKA, and/or PKG are all involved in transducing the contractile signal (Braverman et al., 2005). The M₃ receptor activated ROCK in the normal rat bladder because darifenacin affinity was increased after ROCK inhibition. This demonstrated that although the M₃ receptor seemed to predominate in mediating contraction in control bladders, the M₂ receptor did participate in the contractile signal. In denervated bladders, where the M₂ receptor subtype predominantly mediated contraction, PI-PLC did not seem to be involved in contraction, whereas PC-PLC was involved. In addition, ROCK played a more important role in mediating contraction in the denervated bladder than in normal bladders. In addition, PKA and/or PKG seemed to mediate a different effect in denervated than in normal bladders. Inhibition of rho kinase, PKA, and PKG with H89 attenuated the carbachol maximum and carbachol potency in normal bladders (Braverman et al., 2005), whereas it had no effect on the carbachol maximum or carbachol potency in the denervated rat bladder.

In conclusion, pathophysiological conditions such as denervation-induced hypertrophy altered the muscarinic receptor subtype mediating contraction. This alteration was accompanied by changes in the signal transduction mechanisms that mediate contraction. These findings may allow for the development of therapeutics that may be more effective in inhibiting abnormal bladder contractions while minimizing the side effects associated with inhibition of normal muscarinic receptor function.

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References


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