Neuronally Released Acetylcholine Acts on the M2 Muscarinic Receptor to Oppose the Relaxant Effect of Isoproterenol on Cholinergic Contractions in Mouse Urinary Bladder

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Abbreviations: KRB, Krebs Ringer Bicarbonate; E\textsubscript{max}, maximal response; EC\textsubscript{50}, agonist concentration eliciting half-maximal response; KO, knockout; mATP, α,β-methylene ATP.

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ABSTRACT

We investigated whether M₂ muscarinic receptor activation opposes isoproterenol-induced relaxation in mouse urinary bladder and whether endogenous acetylcholine acts through a similar M₂ mechanism. When measured in urinary bladder from M₃ receptor knockout mice, the muscarinic agonist oxotremorine-M elicited only very weak contractions. In the presence of α,β-methylene ATP (10 µM) and isoproterenol (1 µM), however, oxotremorine-M elicited a robust contractile response. This response was completely absent in bladder from M₂/M₃ double knockout mice, indicating that activation of the M₂ receptor inhibits the relaxant effect of isoproterenol on the contraction to α,β-methylene ATP. Similar results were obtained when PGF₂α was used as the contractile agent, but not when serotonin was used. Electrical field stimulation of the urinary bladder from wild type mouse elicited contractions that were inhibited 20% by atropine and 40% by desensitization with α,β-methylene ATP. When measured in the presence of α,β-methylene ATP to desensitize the purinergic component of contraction, isoproterenol exhibited moderately greater relaxant activity in field stimulated bladder from the M₂ knockout mouse compared to that observed in wild type bladder. This differential relaxant effect of isoproterenol was greatly increased in the presence of physostigmine. In contrast, no differential effects were noted for isoproterenol in similar experiments on bladders from M₃ knockout and M₂/M₃ double knockout mice in the presence of physostigmine. Our results suggest that neuronally released acetylcholine acts on the M₂ muscarinic receptor to inhibit the relaxant effect of isoproterenol on the minor, cholinergic component of contraction in the field stimulated mouse urinary bladder.
INTRODUCTION

Muscarinic receptors play an important role in human urinary bladder function by eliciting contraction of the reservoir and relaxation of the outlet (de Groat and Yoshimura, 2001). With regard to the former, most investigators assume a major role for the M₃ receptor because the pharmacological profile of the contractile response of isolated bladder strips from various species exhibits an M₃ character (Noronha-Blob et al., 1989; Longhurst et al., 1995; Choppin and Eglen, 2001), and the response is almost completely lost in urinary bladder from M₃ KO mice (Matsui et al., 2000). These observations have led many to question whether the more abundant M₂ receptor has any substantial physiological function in contraction of the urinary bladder.

This issue seems enigmatic when one considers the abundance of signaling mechanisms for the M₂ receptor in smooth muscle. The M₂ receptor mediates an inhibition of adenylly cyclase and opposes the increase in cAMP elicited by the β-adrenoceptor (Noronha-Blob et al., 1989). This mechanism accounts for M₂ receptor mediated inhibition of the relaxant effect of isoproterenol (disinhibition of contraction) in gastrointestinal (Thomas et al., 1993; Thomas and Ehlert, 1994) and urinary bladder smooth muscle (Hegde et al., 1997; Matsui et al., 2003; Ehlert et al., 2005). Also, the M₂ receptor mediates a nonselective cation conductance (Bolton and Zholos, 1997) and an inhibition of Ca²⁺ activated K channels (Cole et al., 1989; Kume et al., 1992; Wade and Sims, 1993), and these effects would be expected to promote contraction. However, when the contractile response to muscarinic agonists is measured under conditions of a simultaneous activation of both M₂ and M₃ receptors, the profile for competitive antagonism always resembles that of the M₃ receptor with little hint of an M₂ contribution, even in the presence of isoproterenol when an M₂ receptor mediated disinhibition of contraction would be expected to occur (Choppin, 2002; Ehlert et al., 2005). The key to understanding this enigma lies in the conditional nature of M₂ signaling mechanisms. M₂ receptor mediated inhibition of relaxation requires a contraction elicited by a Ca²⁺ mobilizing receptor in the first place; otherwise, there is no contraction to be relaxed by the β-adrenoceptor, and no β-adrenoceptor
response for the M2 receptor to inhibit. Also, the nonselective cation conductance and the inhibition of Ca\(^{2+}\) activated K channels are Ca\(^{2+}\) dependent, illustrating that these M2 responses are also dependent on activation of a Ca\(^{2+}\) mobilizing receptor. It has been shown that a response mediated by a directly acting receptor, like the M3, and a receptor whose action is contingent upon activation of the former exhibits a pharmacological profile of the directly acting receptor in competitive antagonist studies (Sawyer and Ehlert, 1999b; Ehlert, 2003). Thus, the M3 profile of the muscarinic contractile response in urinary bladder is not only consistent with a pure M3 response, but also with an interaction between a conditionally acting M2 receptor and a directly acting M3. Moreover, the near complete loss of muscarinic contractile function in urinary bladder from M3 KO mouse is also consistent with both hypotheses.

To discriminate between these two possibilities, our lab developed a procedure for measuring the ability of the M2 receptor to disinhibit contractions elicited by other receptors, and we and others have used this technique to show that activation of the M2 receptor opposes the relaxant effects of isoproterenol and forskolin on contractions elicited by a variety of spasmogens in gastrointestinal and urinary bladder smooth muscle (Thomas et al., 1993; Thomas and Ehlert, 1994; Hegde et al., 1997; Ehlert et al., 2005). These experiments were carried out on isolated smooth muscle with exogenously applied muscarinic agonist, and hence, the results do not prove that endogenous acetylcholine released from parasympathetic nerves can mediate these M2 effects. To address this issue, we have used electrical field stimulation and P2X receptor-induced neuronal activation to demonstrate that acetylcholine released from nerves in gastrointestinal smooth muscle acts on the M2 receptor to oppose the relaxant effects of isoproterenol on neurogenic contractions mediated by the M3 receptor (Sawyer and Ehlert, 1999a; Sawyer et al., 2000).

In the present report, we have addressed these issues in mouse urinary bladder from wild type and muscarinic receptor knockout mice. We show that when applied exogenously, the muscarinic agonist oxotremorine-M acts on the M2 receptor to oppose the relaxant effects of isoproterenol on contractions elicited by exogenous PGF\(_{2\alpha}\) and the purinergic agonist \(\alpha,\beta\)
methylene ATP (mATP), but not those elicited by serotonin. We also show as others (Ekman et al., 2006) have that the contractile response of the field stimulated mouse urinary bladder is mediated by purinergic mechanisms and as well as a smaller cholinergic component. We found that the cholinergic component is involved in the interaction between the M₂ receptor and the β adrenoceptor.
MATERIALS AND METHODS

Mice: M2 muscarinic receptor knockout (M2–/–; M2 KO), M3 muscarinic receptor knockout (M3–/–; M3 KO), and M2/M3 muscarinic receptor double knockout (M2−/−, M3−/−; M2/M3 KO) mice were generated as described in Matsui et al. (2002) and Matsui et al. (2000), respectively. These hybrid lines were backcrossed with C57BL/6 mice to yield an N4 generation of M2 KO mice, an N8 generation of M3 KO mice and an N2 generation of M2/M3 KO mice. Except for the experiments on the field stimulated urinary bladder from M3 and M2/M3 KO mice, which were done on females, all other experiments were done on male knockout mice as well as male wild type (M2+/+, M3+/-) C57BL/6 mice.

Isolated mouse urinary bladder: Whole mouse urinary bladder was removed, and silk thread was attached near the outlet and also at the dome of the bladder. The whole bladder was set up in a longitudinal orientation in an organ bath and connected to a force-displacement transducer using silk thread. The tissue was bathed in Krebs-Ringer Bicarbonate buffer (KRB buffer; 124 mM NaCl, 5 mM KCl, 1.3 mM MgSO4, 26 mM NaHCO3, 1.2 mM KH2PO4, 1.8 mM CaCl2, and 10 mM glucose) at 37°C and gassed with O2/CO2 (19/1) as described previously (Ehlert et al., 2005). The tissue was allowed to equilibrate for at least an hour, during which time the resting tension was adjusted to a load of 1 g. Next, the contractile response to three test doses to KCl (50 mM) were measured. After each test dose, the tissue was washed and allowed to rest for 10 min. The contractions to KCl were usually biphasic, consisting of an initial rapid peak that declined initially and then slowly rose to a stable plateau. The contractile response to KCl was calculated as the stable plateau value minus the initial resting tension. The contractile response to other agents were normalized relative to the largest response to KCl, which was usually occurred with the second or third test dose, both of which were usually similar.

The concentration-response curve to mATP was measured using a noncumulative technique. An aliquot of the agonist was added to the bath, and response was calculated as the
peak contraction that was achieved usually within a few sec minus the initial resting tension, which was adjusted to a mass equivalent of approximately 1 g. The bladder was washed two to three times, depending upon the agonist concentration, and allowed to rest for 10 min prior to the next measurement. The concentrations of agonist were added to the bath in order of increasing concentration to avoid the effect of desensitization on the response to low concentrations. In preliminary experiments, we found that the contraction elicited by mATP (30 µM) did not wane when measured repetitively (five times) using this schedule.

In some experiments on urinary bladder from M₃ KO mice, we measured the concentration-response curve to oxotremorine-M in the presence of isoproterenol (1 µM) and a non-muscarinic contractile agent (i.e., mATP, PGF₂α or serotonin) using a noncumulative technique. At the start of the experiment, two responses to the non-muscarinic contractile agonist were measured using the noncumulative method described above. The concentration of agonist was chosen so that it elicited a near maximal response (mATP, 30 µM; PGF₂α, 1 µM; and serotonin, 5 µM). Then the responses to increasing concentrations of oxotremorine-M were measured using the following strategy. First, isoproterenol was added to the bath at a final concentration of 1 µM. After two min, oxotremorine-M was added and the preparation was allowed to equilibrate for approximately 20–30 sec in the presence of both oxotremorine-M and isoproterenol. Then, the non-muscarinic contractile agent was added, and its peak (mATP) or stable response (PGF₂α and serotonin) was recorded. The preparation was washed two to three times and allowed to rest for approximately 10 min. This process was repeated on a 15 min cycle with increasing concentrations of oxotremorine-M and with the concentrations of isoproterenol and non-muscarinic contractile agent remaining constant. The response to oxotremorine-M was calculated as the measured response to the non-muscarinic contractile agent after isoproterenol and oxotremorine-M had been added to the bath minus the initial resting tension at the start of the experiment.
Field stimulated urinary bladder: The whole urinary bladder was set up as described above and mounted between two platinum ring electrodes spaced approximately 3.5 cm apart. The tissue was allowed to equilibrate for at least an hour, and three test doses to KCl were measured as described above. Three test trains of stimulation (0.2 Hz, 2 msec and 100 V), each for 5 min, were applied, and the tissue was washed and allowed to rest for five min after each stimulation. Under these conditions, the urinary bladder generated a train of twitch contractions, each twitch characterized by an initial rapid and transient contraction that decayed rapidly. The twitch response was calculated as the maximum peak tension minus the resting tension immediately before the pulse. These contractions were normalized relative to that elicited by KCl (50 mM).

Many, but not all, of the urinary bladders that we obtained from male M3 KO and M2/M3 KO mice did not respond to field stimulation, perhaps because of the prominent urinary bladder distension that occurs in male mice lacking the M3 receptor (Matsui et al., 2000), which could disrupt nerves innervating the bladder. Consequently, we used female mice for electrical field stimulation in M3 KO and M2/M3 KO mice. The relaxant response to isoproterenol was measured using a cumulative technique. Electrical field stimulation was applied to the bladder, and after one min, isoproterenol was added to the bath. Additional concentrations of isoproterenol were added at one-min intervals in order of increasing concentration. The contractile response in the presence of isoproterenol was calculated as the average of the heights of the last four twitches of the one-min cycle. Following electrical field stimulation, the urinary bladder was washed three times and allowed to equilibrate for 30 min prior to additional measurements. In experiments utilizing mATP or antagonists, these were applied to the tissue 30 min prior to field stimulation.

Analysis of agonist concentration response curves: The maximal response (Emax), concentration of agonist eliciting half-maximal response (EC50) and Hill slope (n) were estimated from the agonist concentration-response curve by nonlinear regression analysis using Prism (GraphPad Software, Inc., San Diego). The significance of difference between the parameter estimates obtained on urinary bladders for wild type and M2 KO mice was determined using a Student’s T
test. For this analysis, EC$_{50}$ values were first converted to logarithms before doing the calculations.
RESULTS

Contraction of urinary bladder to exogenous agonists: We investigated the ability of the purinergic agonist mATP to elicit contractions in urinary bladder from wild type, M2 KO, M3 KO and M2/M3 KO mice. In wild type mice, mATP elicited powerful phasic contractions that usually reached a peak within a few seconds of application to the organ bath, and thereafter, the contraction slowly waned. A plot of the peak contraction, expressed relative to that elicited by KCl (50 mM), against the Log molar concentration of mATP is shown in Figure 1. The mean value ± SEM for the pEC50 and Emax values of mATP in the urinary bladder from wild type (6.09 ± .21 and 85 ± 13%), M2 KO (6.14 ± .11 and 87 ± 12%), M3 KO (5.85 ± .14 and 87 ± 22%) and M2/M3 KO mice (6.06 ± .12 and 79 ± 11%) were similar.

To investigate the role of the M2 receptor in opposing the relaxant effect of isoproterenol on contraction elicited to other contractile agents we used the following strategy, which is a modification of our prior method utilizing a cumulative technique for measuring the agonist concentration-response curve (Thomas et al., 1993; Ehlert et al., 2005). Here, we use a non-cumulative technique because the response to mATP is transient. Since there are no readily available muscarinic agonists that activate M2 receptors with high selectivity, we measured contraction to the muscarinic agonist oxotremorine–M in urinary bladder from M3 KO mice, which exhibits only very weak M2 receptor-mediated contractions to muscarinic agonists (Matsui et al., 2000; Ehlert et al., 2005). Thus, under these conditions, we can be assured that the response to muscarinic stimulation is due to activation of the M2 receptor only, and not both the M2 and M3 receptors. We measured contractions in the presence of isoproterenol (1.0 µM) and a non-muscarinic contractile agent (mATP, PGF2α or serotonin). In combination, the contractile agent and isoproterenol had little or no net effect because their actions oppose one another. However, if activation of the M2 receptor inhibits the relaxant effect of isoproterenol on the contractile agent, it should be possible to measure muscarinic agonist induced contractions through the M2 receptor that are characterized by an Emax equivalent to contraction elicited by the
non-muscarinic contractile agent (e.g., mATP) by itself. Figure 2B shows that oxotremorine-M elicits a potent contractile response in M3 KO mice when measured in the presence of mATP (30 µM) and isoproterenol (1 µM). This response was completely absent in urinary bladder from the M2/M3 KO mouse illustrating that the response is mediated by the M2 receptor. Moreover, the Emax is much greater than the weak M2 response measured in M3 KO mice in the absence of isoproterenol and mATP (Figure 2A), illustrating that the mechanism is consistent with an M2 receptor mediated disinhibition of the mATP induced contraction. Also, the Emax of the contraction is approximately the same as the contraction to mATP by itself, which is also consistent with the M2 disinhibitory mechanism. Similar results were obtained with PGF2α (Figure 2C), but little or no disinhibitory mechanism was detected when serotonin was used as the non-muscarinic contractile agent (data not shown).

To determine if the action of isoproterenol was altered in muscarinic receptor KO mice, we measured the relaxant effect of isoproterenol against mATP induced contractions in urinary bladder from wild type, M2 KO, M3 KO and M2/M3 KO mice (Figure 3A). The mean values ± SEM for the pEC50 and maximal inhibitory effect of isoproterenol in wild type (8.4 ± .2 and 61 ± 4%), M2 KO (8.4 ± .1 and 68 ± 2%), M3 KO (8.4 ± .1 and 71 ± 13%) and M2/M3 KO (8.4 ± .1 and 70 ± 2%) were approximately the same. We also measured the relaxant effect of isoproterenol against KCl induced contractions and found no difference in its pEC50 value or maximal inhibitory effect in wild type (7.68 ± .15 and 48 ± 4%) and M2 KO mice (7.62 ± .39 and 52 ± 11%), respectively (Figure 3B).

**Field stimulated urinary bladder:** Electrical field stimulation (100 V, 2 msec, 0.2 Hz) of the isolated urinary bladder from male wild type mice elicited transient contractions with a frequency equivalent to the frequency of stimulation. These contractions were stable over 10 min periods of stimulation, and these 10-min trains of contractions were stable when measured at various times over a period of several hr. Tetrodotoxin (0.1 µM) caused an (85% inhibition) the contractions, indicating that most of the contraction was mediated by neuronal release and
approximately (15%) by direct activation of the muscle. When normalized relative to the contraction elicited by 50 mM KCl, the field stimulated contraction in urinary bladder from wild type mice (25.9 ± 1.4%) was not significantly different from that measured in M2 KO mice (27.6 ± 2.0) (P = 0.50, df = 38). In urinary bladder from wild type mice, prior treatment with mATP (0.1 mM) for 30 min caused a significant 38 ± 3.5% inhibition of field stimulated contractions (P = 3.5 x 10⁻⁵), whereas treatment with atropine 1 µM only caused a small but significant 15 ± 4.1% inhibition (P = 0.024). Treatment with both mATP (0.1 mM) and atropine (1.0 µM) caused a highly significant, near additive 69 ± 2.1% inhibition of the contraction (P = 9.5 x 10⁻⁵). We also found that methoxamine (1 µM), indomethacin (1 µM), or ketansarin (1 µM) caused no further inhibition of the residual contraction that persisted in the presence of atropine and mATP, indicating that the residual response is not mediated by prostanoids, norepinephrine acting on α-adrenoceptors or serotonin acting on 5-HT2 receptors. Atropine and mATP were a little less effective at inhibiting field stimulated contraction in urinary bladder from the M2 KO mouse, and this difference in mouse strains was significant with respect to mATP (P = 0.016), but not for atropine. The results described above are summarized in Figure 4.

Effects of isoproterenol on the field stimulated urinary bladder: We reasoned that if neuronally released acetylcholine acts on the M2 receptor to oppose β-adrenoceptor-mediated relaxation, then the relaxant effect of isoproterenol should be greater in urinary bladder from the M2 KO mouse relative to that from the wild type mouse when measured under conditions of field stimulation. To test this postulate, we measured the effects of various concentrations of isoproterenol on the field stimulated urinary bladder from wild type and M2 KO mice. As shown in Figure 5A, however, there was no difference in the mean ± SEM values for the pEC50 and maximal inhibitory effect of isoproterenol in urinary bladder from wild type (7.6 ± 0.2 and 90 ± 2%) and M2 KO (7.7 ± .1 and 87 ± 3%) mice, respectively. These results reveal no role for the M2 receptor in mediating a disinhibition of contraction via neuronally released acetylcholine.
Since there are three components to the contractile response under field stimulation (cholinergic, purinergic and that elicited by another unidentified mediator), we wondered if the M2 receptor might play a selective role in disinhibiting a minor component of the contraction. To test this postulate, we measured the relaxant effects of isoproterenol in urinary bladder pretreated with mATP (0.1 mM) to desensitize the purinergic component, and hence, to eliminate its contribution to contraction (Figure 5B). Under these conditions, isoproterenol exhibited a small, but statistically significant greater relaxant potency (P = .044) at inhibiting field stimulated contractions in the urinary bladder from M2 KO mice (pEC50 = 7.39 ± .18) compared to that from wild type mice (pEC50 = 6.95 ± .17). This difference corresponded to a 2.6-fold increase in potency. There was little difference in the maximal relaxant effect of isoproterenol in wild type (84.5 ± 5.4) and M2 KO mice (82.3 ± 4.3).

Since these data indicate that neuronally derived acetylcholine acts on the M2 receptor to oppose the relaxant effect of isoproterenol on a non-purinergic component of contraction, we tested whether we could amplify this action by carrying out experiments in the presence of a low concentration of the cholinesterase inhibitor physostigmine to inhibit the hydrolysis of acetylcholine. Physostigmine (70 nM) caused a significant increase in the contractile response to field stimulation in urinary bladder from both wild type and M2 KO mice by approximately 46% (Figure 6) (P = 7.2 x 10^{-4}). When measured in the presence of physostigmine, isoproterenol exhibited much greater relaxant potency in urinary bladder from the M2 KO mouse (pEC50 = 8.1 ± .1) compared to that observed in the wild type mouse (pEC50 = 7.5 ± .2) (see Figure 7A). This difference in pEC50 was significant (P = 0.037). Also, the maximal relaxant effect of isoproterenol in urinary bladder from M2 KO mice (90 ± 1.2%) was significantly greater than that observed in wild type mice (68 ± 4.3%) (P = 0.0082). We repeated these experiments in urinary bladder that had been treated with mATP (0.1 mM) to desensitize the purinergic component and to eliminate its contribution to contraction. In the presence of mATP, physostigmine (70 nM) increased contractions to field stimulation in wild type mice by approximately 36% (Figure 6) (P = 0.046). Under these conditions, isoproterenol also exhibited
significantly greater relaxant potency in urinary bladder from the M2 KO mouse (pEC$_{50}$ = 7.12 ± .29) compared to that measured in the wild type mouse (pEC$_{50}$ = 6.28 ± .18; P = 0.043) (Figure 7B). Finally, we repeated these experiments in urinary bladder from M3 KO and M2/M3 KO mice. Physostigmine (70 nM) caused little increase in contractions elicited to electrical field stimulation in urinary bladder from either M3 KO or M2/M3 KO mice (Figure 6), presumably because there is no M3 muscarinic component to the contraction. Under these conditions, we found little difference in the relaxant potency and maximal inhibitory effect of isoproterenol in urinary bladder from M3 KO (7.4 ± .2 and 65 ± 5.1) and M2/M3 KO mice (7.3 ± .1 and 68 ± 2.7).
DISCUSSION

Our results showing that bath applied muscarinic agonist can activate M₂ receptors to inhibit the relaxant effect of isoproterenol on contractions elicited to mATP are consistent with our prior studies showing the same role for the M₂ receptor in opposing the relaxant effect of isoproterenol and forskolin on contractions elicited to PGF₂α and M₃ receptor activation (Ehlert et al., 2005). In our prior studies with PGF₂α, we used a cumulative technique for measuring the concentration-response curve. Here we repeated our experiments with PGF₂α using a non-cumulative technique and observed an even greater M₂ response to oxotremorine-M.

Evidence from a variety of studies on the urinary bladder from the mouse (Vial and Evans, 2000) and other species indicates that mATP elicits contraction through the P₂X₁ subtype. Our observation that the contraction to mATP is transient and desensitizes quickly is consistent with a P₂X₁ mechanism since these ligand gated ion channels are known to desensitize rapidly to mATP. This rapid desensitization requires a non-cumulative method for measurement of the concentration-response curve to mATP and also for measurement of the role of the M₂ receptor in opposing the relaxant effect of isoproterenol on mATP induced contractions.

The results of our experiments on the components of the electrically induced contraction of the mouse urinary bladder differ from those of Ekman et al. (2006) and Lai et al. (2004) who found nearly equal contributions (50%) of purinergic and muscarinic cholinergic components to the field stimulated contractions of the mouse urinary bladder. In our experiments, the purinergic component was less (about 40%) and the atropine-sensitive, muscarinic component was only approximately 20%. In addition, we observed another non-purinergic, non-cholinergic component that represented approximately 30% of the contraction in wild type urinary bladder. This additional component could not be attributed to adrenergic, serotonergic or prostanoid mechanisms. The reason for the difference in the composition of the electrically induced contraction in our experiments compared to those of other investigators may be explained the
difference in stimulation parameters and by our use of the whole, intact urinary bladder rather than strips of it. In our experiments, we utilized a continuous 0.2 Hz stimulation for several minutes, with each stimulus at 2 msec, whereas Ekman et al. (2006) and Lai et al. (2004) used a brief 5 sec high frequency stimulation (20 - 60 hz) at 0.5 msec. In guinea pig urinary bladder, the contribution of muscarinic receptor component to neurogenic contractions is only 20 - 25% whereas the remainder can be attributed to purinergic contractions (Westfall et al., 1983; Kennedy et al., 2007). Similarly, in rat the cholinergic component of the electrogenic contraction in the urinary bladder is small (Carpenter, 1963).

We found little difference in the relaxant effect of isoproterenol against field stimulated contractions in urinary bladder from wild type and M 2 KO mouse indicating that overall, M 2 receptor activation by neuronally release acetylcholine has little influence in opposing the relaxant action of isoproterenol on neurogenic contractions of the mouse urinary bladder. Nevertheless, we did observe a small but significant role for the M 2 receptor when the purinergic component of contraction was eliminated by desensitization with mATP. When the residual, non-purinergic component of the contraction was enhanced by treatment with physostigmine, the relaxant action of isoproterenol was much greater in urinary bladder from the M 2 KO mouse compared to that from the wild type mouse, indicating that neuronally released acetylcholine acts on the M 2 receptor to oppose the relaxant action of isoproterenol. The increased relaxant action of isoproterenol in urinary bladder from the M 2 KO mouse cannot be attributed to enhanced β-adrenoceptor-mediated relaxation in the M 2 KO mouse because there was no difference in the relaxant effects of isoproterenol against KCl- and mATP-induced contractions in these two mouse strains. It might be argued that in the presence of physostigmine, neuronally released acetylcholine accumulates and diffuses to M 2 receptors that oppose the relaxant effect of isoproterenol on purinergic contractions. This mechanism might explain the large increase in the relaxant effect of isoproterenol in M 2 KO mouse urinary bladder compared to wild type in the presence of physostigmine. However, this increase in the relaxant potency was not observed in the urinary bladder from the M 2/M 3 KO mouse compared to that from the M 3 KO mouse when
physostigmine was present. Thus, it appears that the enhanced effectiveness of the M₂ receptor in opposing the relaxant action of isoproterenol in the presence of physostigmine requires an M₃ component to the neurogenic contraction. This requirement suggests that neuronally released acetylcholine only acts on M₂ receptors that disinhibit M₃ receptor mediated contractions but not purinergic contractions. At the present time, we cannot rule out the possibility of a lack of acetylcholine release in parasympathetic nerves from M₃ KO and M₂/M₃ KO mice. It is known that urinary bladder from male, M₃ KO and M₂/M₃ KO mice undergoes prominent distention, which could damage nerves innervating the bladder (Matsui et al., 2000; Matsui et al., 2002). To avoid this potential problem, we used female M₃ KO and M₂/M₃ KO mice for our experiments on field stimulation. Previous studies on urinary bladder from wild type, M₂ KO, M₃ KO and M₂/M₃ KO mice have shown no differences between males and females with regard to the contractile action of carbachol in these four mouse strains (Matsui et al., 2000; Matsui et al., 2002).

Our hypothesis that neuronally released acetylcholine acts on the M₂ receptor to inhibit β-adrenoceptor-mediated relaxation of M₃ receptor-mediated contractions implies that it should be possible to demonstrate this mechanism with bath applied muscarinic agonist. We have previously shown that isoproterenol is more effective at inhibiting contractions of the urinary bladder from the M₂ KO mouse compared to those from wild type mouse when equiactive muscarinic stimuli are compared (Matsui et al., 2003). In contrast, there is no difference in the relaxant effect of isoproterenol against noncholinergic stimuli in urinary bladder from wild type and M₂ KO mice.

We have previously noted that the effectiveness of isoproterenol for relaxing electrical field stimulated contractions of the guinea pig ileum is greatly enhanced in ileum treated with pertussis toxin to interrupt M₂ receptor signaling (Sawyer and Ehlert, 1999a). Pertussis toxin treatment has no inhibitory effect on muscarinic agonist induced contractions of the guinea pig ileum, nor on field stimulated contractions. Pertussis toxin treatment does, however, increase the relaxant effect of isoproterenol against muscarinic agonist induced contraction of the guinea pig
ileum. Thus, these prior data strongly indicate that neuronally released acetylcholine acts on the M$_2$ receptor to inhibit the relaxant effect of isoproterenol on M$_3$ mediated contractions of the guinea pig ileum. In the guinea pig ileum, electrical field stimulated contractions are mediated entirely by acetylcholine acting on M$_3$ receptors (Kilbinger et al., 1984). As suggested above, it may be that in the mouse urinary bladder, the disinhibitory role of the M$_2$ receptor is also selective for M$_3$ receptor mediated contractions.

Although the disinhibitory role of the M$_2$ receptor in mouse urinary bladder is rather small, cholinergic contractions only make up a small component of the neurogenic contraction in the mouse under our conditions of electrical field stimulation. In human urinary bladder, the contribution of acetylcholine to neurogenic contractions is much greater (Sibley, 1984). Thus, it is possible that the disinhibitory role that we describe here for the M$_2$ receptor in mouse bladder is much greater in the human urinary bladder where cholinergic contractions predominate.
REFERENCES


FOOTNOTES

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**Figure 1:** The contractile response to mATP in urinary bladder from muscarinic receptor knockout mice. The contractile response to mATP was measured in urinary bladder from wild type, M2 KO, M3 KO and M2/M3 KO mice using a non-cumulative technique. Mean values ± SEM from 3 - 4 experiments are shown, each done on a different urinary bladder.

**Figure 2:** The contractile response to oxotremorine-M in mouse urinary bladder lacking the M3 muscarinic receptor in the absence (A) and presence (B and C) of isoproterenol (1 µM) and either mATP (30 µM) (B) or PGF2α (1 µM) (C). Mean values ± SEM from 3 - 6 experiments are shown, each done on a different urinary bladder.

**Figure 3:** Relaxant effect of isoproterenol against contractions elicited to mATP (A) and KCl (B) in wild type and muscarinic receptor knockout mice. Mean values ± SEM from 4 - 8 experiments are shown, each done on a different urinary bladder.

**Figure 4:** Effects of tetrodotoxin, mATP and atropine on electrical field stimulated contractions of urinary bladder from wild type and M2 KO mouse. A: A representative trace of field stimulated contractions of urinary bladder from a wild type mouse in the absence and presence of tetrodotoxin (1.0 µM). B: The effects of mATP (0.1 mM) and atropine (1.0 µM) and their combination on field stimulated contractions of urinary bladder from wild type and M2 KO mice. Mean values ± SEM from 3 - 7 experiments are shown, each done on a separate bladder.

**Figure 5:** Effects of isoproterenol on electrical field-stimulated contractions of urinary bladder from wild type and M2 KO mice in the absence (A) and presence (B) of mATP (0.1 mM). The data represent the mean values ± SEM from 7 experiments, each done on a different bladder.

**Figure 6:** Effect of physostigmine (70 nM), mATP (0.1 mM) and their combination on field stimulated contractions in urinary bladder from wild type and muscarinic receptor KO mice. The data represent the mean values ± SEM from 3 - 6 experiments, each done on a different bladder.

**Figure 7:** Effect of isoproterenol on field stimulated contractions of mouse urinary bladder in the presence of physostigmine (70 nM). A: Field stimulated contractions of urinary bladder from
wild type and M2 KO mice were measured in the presence of physostigmine (70 nM) and various concentrations of isoproterenol. B: The experiment in A was repeated in the presence of mATP (0.1 mM). C: The experiment in A was repeated in urinary bladder from M3 KO and M2/M3 KO mice. The data represent the mean values of 3 - 7 experiments, each done on a different urinary bladder.
Figure 1
Figure 3
Figure 4
Figure 5

A

B

Contraction (% Control)

Log [Isoproterenol]

WT

M₂ KO

Contraction (% Control)

Log [Isoproterenol]
Figure 6
Figure 7