Increased Relaxant Action of Forskolin and Isoproterenol against Muscarinic Agonist-Induced Contractions in Smooth Muscle from M₂ Receptor Knockout Mice^a

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Ringer Bicarbonate; E_{max} , maximal response.

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The ability of forskolin and isoproterenol to inhibit the contractile action of the muscarinic agonist, oxotremorine-M, was investigated in smooth muscle from wild type and M_2 muscarinic receptor knockout mice. Forskolin (5.0 μ M) caused a significant reduction in the contractile activity of oxotremorine-M in ileum, trachea and urinary bladder from both wild type and M_2 muscarinic receptor knockout mice. This reduction in contractile activity was characterized by decreases in potency and maximal response, but not always both. Similar results were obtained with isoproterenol (1.0 μ M). The relaxant effects of forskolin in ileum, trachea and urinary bladder from M_2 receptor knockout mice were approximately three- to nine-fold greater than those observed in the same tissues from wild type mice. Similar results were obtained with isoproterenol in ileum and urinary bladder, although the differences between wild type and M_2 receptor knockout tissues were less than those observed with forskolin. In contrast, there was no significant difference between the relaxant effect of isoproterenol in trachea from wild type and M_2 receptor knockout mice. In contrast to the results observed with oxotremorine-M as the contractile agent, forskolin and isoproterenol did not exhibit greater relaxant activity against KCl-induced contractions in M_2 receptor knockout mice compared with wild type mice. These results suggest that a component of the contractile response to muscarinic agonists in smooth muscle involves an M_2 muscarinic receptor-mediated inhibition of the relaxant effects of agents that increase cAMP levels.

Muscarinic M_2 and M_3 receptors are abundantly expressed postjunctionally in smooth muscle where they mediate the contractile effects of acetylcholine (see reviews by Eglen et al. (1996) and Ehlert et al. (1997)). When measured in the absence of other heterologous agents, the contractile response to muscarinic agonists is inhibited by subtype selective antagonists in a manner consistent with an M_3 mechanism. This behavior is consistent with the known coupling of M_3 receptors to pertussis toxin-insensitive G_q , which mediates phosphoinositide hydrolysis and mobilization of Ca^{2+} (Candell et al., 1990; Noronha-Blob et al., 1989; Peralta et al., 1988; Roffel et al., 1990). The muscarinic M_2 receptor is known to signal through G_i in smooth muscle to mediate pertussis toxin-sensitive responses including the inhibition of both adenylyl cyclase (Candell et al., 1990; Noronha-Blob et al., 1989; Yang et al., 1991) and Ca^{2+} -activated potassium channels (Kotlikoff et al., 1992) as well as the stimulation of a nonselective cation conductance (Bolton and Zholos, 1997; Inoue and Isenberg, 1990). Ultimately, M_2 mediated effects on contraction through these latter mechanisms are conditional upon Ca^{2+} mobilization via another receptor. Consequently, the pharmacological paradigms required to demonstrate a contractile role of the M_2 receptor are more complicated than the standard assay used to establish a role for the M_3 receptor. Nevertheless, pharmacological studies employing pertussis toxin, irreversible M_3 selective muscarinic antagonists, and heterologous contractile and relaxant agents have demonstrated two roles for the M_2 receptor in contraction – an inhibition of the relaxation caused by agents that increase cAMP and a conditional potentiation of the M_3 receptor-mediated contractions (Hegde et al., 1997; Sawyer and Ehlert, 1998; Sawyer and Ehlert, 1999b; Shen and Mitchelson, 1998; Thomas et al., 1993; Thomas and Ehlert, 1994; Thomas and Ehlert, 1996).

Recent studies on M_2 and M_3 receptor knockout mice are generally consistent with the results of pharmacological experiments on wild type animals of other species showing that it is primarily the M_3 receptor that mediates a direct contraction in smooth muscle. In M_3 receptor knockout mice, the muscarinic contractile response of the ileum and urinary bladder are greatly reduced compared to wild type mice (Matsui et al., 2000; Stengel et al., 2002), whereas a much smaller decrement in contractile function was noted in M_2 receptor knockout mice (Stengel et al., 2000). In mutant mice lacking both M_2 and M_3 receptors, the potent contractile response to muscarinic agonists in ileum and urinary bladder is completely eliminated, demonstrating that only M_2 and M_3 muscarinic receptors contribute to the direct contractile response in these tissues (Matsui et al., in press). So far,

no studies have been reported on the role of M_2 receptors in mediating an inhibition of the relaxant effects of agents that increase cAMP in muscarinic receptor knockout mice.

In this report, we describe the relaxant effects of isoproterenol and forskolin on muscarinic agonist-induced contraction of smooth muscle in wild type and M_2 receptor knockout mice. Our results show that the relaxant effects of forskolin on muscarinic agonist-induced contractions, but not those elicited by KCl, are greatly increased in the ileum, bladder and trachea of M_2 receptor knockout mice as compared with wild type mice. Under similar conditions, the relaxant effect of isoproterenol was also enhanced in the ileum and urinary bladder, but not in the trachea. These results demonstrate that a component of the contractile mechanism of muscarinic agonists in smooth muscle involves an M_2 receptor-mediated inhibition of the relaxant effects of agents that increase cAMP levels.

METHODS

Animals: The generation of M_2 muscarinic receptor knockout mice has been described previously (Matsui et al., in press). A mutant mouse line was established in a mixed background between 129/SvJ and C57BL/6. This line was backcrossed with C57BL/6 mice to yield an N3 generation of $M_2^{-/-}$ muscarinic receptor knockout mice, which were used in the pharmacological studies described in this report. C57BL/6 mice were purchased from Harlan, Indianapolis, Indiana.

Isolated smooth muscle: Wild type ($M_2^{+/+}$) male C57BL/6 mice and male M_2 muscarinic receptor knockout ($M_2^{-/-}$) mice were used in these studies. Their body weights were approximately 25 – 30 g. The mice were euthanized by CO_2 asphyxiation and various smooth muscle preparations were immediately removed. Segments of whole ileum (1.5 – 2 cm in length) were excised starting at a point approximately 5 cm rostral from the ileocecal junction and mounted longitudinally in an organ bath with silk thread. The whole trachea was dissected free of adhering tissue and mounted as a single ring on stainless steel supports. The whole urinary bladder was removed and mounted longitudinally with silk thread. All tissues were bathed at 37°C within 50 ml organ baths containing Krebs Ringer Bicarbonate (KRB) buffer (NaCl, 124 mM; KCl, 5.0 mM; $MgSO_4$, 1.8 mM; $NaHCO_3$, 26 mM; KH_2PO_4 , 1.2; $CaCl_2$, 1.8 mM; glucose, 10 mM) gassed with O_2/CO_2 (19/1). Indomethacin (1.0 μM) was present in the KRB buffer at all times. Indomethacin is often included in experiments on tracheal smooth muscle to remove the influence of endogenous prostaglandins (Muccitelli et al., 1987), to dissipate spontaneous tracheal tone (Small et al., 1990), and to maintain receptor-stimulated tone (Mansour and Daniel, 1986), although the reasons for these uses are incompletely understood. To maintain consistency, all tissues were exposed to indomethacin. The tissues were connected to force-displacement transducers and isometric tension was recorded using either a Polygraph (Grass Instruments, Quincy, Massachusetts) or PowerLab (ADInstruments, Grand Junction, Colorado) recording systems. Resting tensions were adjusted to loads equivalent to those generated by masses of 0.3, 2 and 0.5 g in the ileum, trachea and urinary bladder, respectively. The tissues were first allowed to equilibrate for at least 60 min, and then three test doses of KCl (50 mM) were applied. After each test dose, the tissues were washed with fresh KRB buffer and allowed to rest for approximately 5 to 10 min. The contractile response to the third test dose of

KCl was used in calculations to normalize the response to the muscarinic agonist, oxotremorine-M, relative to that elicited by KCl. The tissues were allowed to rest for 15 min, and then a cumulative concentration-response curve to oxotremorine-M was measured, with the agonist concentrations being spaced three-fold. Only the tonic phase of contraction was used in the calculation of concentration-response curves. The tissues were washed with fresh KRB buffer and allowed to rest for 30 min. A second control concentration-response curve to oxotremorine-M was measured, and this second curve was used as the control to which the curves measured in the presence of relaxant agents were compared. Tissues were washed extensively with fresh KRB buffer after measurement of each concentration-response curve and were allowed to rest for 30 min before the next concentration-response curve was measured.

Calculations: The EC_{50} value (concentration of agonist eliciting half-maximal contraction) and the maximal response (E_{max}) to oxotremorine-M were estimated from the concentration-response data by nonlinear regression analysis using an increasing logistic equation as described previously (Candell et al., 1990). In most cases, the relaxant agents caused a decrease in both the maximal response and potency of oxotremorine-M for eliciting contraction. In such instances, the effect of the relaxant agent could be simulated quantitatively by assuming that the relaxant agent caused a decrease in the proportion of receptors or the intrinsic efficacy of the receptor-oxotremorine-M complex. We refer to this phenomenon as the decrease in the “observed coupling efficiency” of oxotremorine-M caused by the relaxant agent. This measure of relaxant action was calculated as described previously (Ostrom and Ehlert, 1997) using a method akin to Furchgott analysis (Furchgott, 1966). This estimate is simply an empirical measure of the relaxant effect, and no conclusion about the mechanism of relaxation is deduced with this estimate. We simply use the estimate of observed coupling efficiency as a means of summing the inhibitory effects of the relaxant agent on the two disparate parameters, EC_{50} and E_{max} . For each experiment, the estimates of EC_{50} were converted to negative logarithms (pEC_{50}), and the effect of the relaxant agent on the EC_{50} value was calculated as the difference between the control pEC_{50} value and that measured in the presence of the relaxant agent (i.e., $\text{Log } EC_{50} \text{ shift}$). To assess whether the relaxant agent had a significant effect on the EC_{50} value, a t-distribution was used to determine if the $\text{Log } EC_{50} \text{ shift}$ values were significantly different from zero. Similarly, a t-distribution was used to determine if the logarithm of the observed coupling efficiencies were significantly

different from a value of zero (i.e., no change in coupling efficiency). A paired t-test was used to determine if the relaxant agent had a significant effect on the E_{max} . To determine if these estimates of relaxant activity in M_2 receptor knockout mice were significantly different from those measured in wild type animals, an unpaired t-test was used.

Drugs and chemicals: The reagents used in this study were obtained from the following sources. Oxotremorine-M, Research Biochemicals International, Natick, MA; isoproterenol, indomethacin and tetrodotoxin, Sigma Chemical Company, St. Louis, MO; forskolin, Calbiochem, La Jolla, CA.

RESULTS

Oxotremorine-M elicited contractions in ileum, trachea and urinary bladder of wild type mice with mean pEC_{50} values $\pm$ SEM of 6.70 ± 0.037 , 6.94 ± 0.036 and 6.58 ± 0.044 and E_{max} values, expressed as mass equivalents, of 1.36 ± 0.083 , 2.60 ± 0.23 and 4.3 ± 0.47 g, respectively. When expressed relative to the contraction elicited by KCl (50 mM), the E_{max} values of oxotremorine-M in these tissues were 188 ± 20 , 218 ± 14 and $223 \pm 11\%$, respectively. In M_2 receptor knockout mice, the potency of oxotremorine-M was less. This decrease in potency corresponded to mean EC_{50} values that were 2.1-, 1.21- and 1.48-fold greater in the ileum, trachea and urinary bladder, respectively. These differences reached statistical significance in ileum ($P = 0.00013$), but not in trachea ($P = 0.124$) or urinary bladder ($P = 0.114$). There were no significant differences between wild type and M_2 receptor knockout mice with regard to the E_{max} values of oxotremorine-M expressed relative to the contraction elicited to KCl in the three tissues ($P = 0.93$, 0.12 and 0.11 in ileum, trachea and urinary bladder, respectively). A similar conclusion was reached when E_{max} was expressed in units of mass equivalents. These data are summarized in Figures 1 and 2 and Table 1. In a limited number of experiments, we found that tetrodotoxin (1 μ M) was without effect on the contractile response to oxotremorine-M in wild type and M_2 receptor knockout mice.

Figure 1 shows the relaxant effect of forskolin (5.0 μ M) on the contractile response to oxotremorine-M in ileum, trachea and urinary bladder. In wild type mice, forskolin caused a significant reduction in the potency of oxotremorine-M in each tissue (i.e., increase in EC_{50} or decrease in pEC_{50}), and a significant reduction in E_{max} in the trachea, but not in ileum or urinary bladder. These changes resulted in a significant reduction in the observed coupling efficiency of oxotremorine-M in the three tissues. Similar results were obtained with forskolin in smooth muscle from M_2 receptor knockout mice although the effects were greater. Forskolin caused 3.6-, 3.1- and 9.3-fold greater reductions in the coupling efficiency of oxotremorine-M in the ileum, trachea and urinary bladder of M_2 receptor knockout mice as compared with wild type mice. These results are summarized in Table 2.

Similar results were obtained with isoproterenol (1.0 μ M) except that there was a smaller difference between the relaxant effects of isoproterenol in wild type and M_2 receptor knockout mice (see Figure 2). The net effect of isoproterenol was to cause a significant reduction in the coupling efficiency of oxotremorine-M in the three tissues in both wild type and M_2 receptor knockout mice. Isoproterenol elicited 2.0- and 2.3-fold greater

reductions in the observed coupling efficiency of oxotremorine-M in ileum and urinary bladder from M_2 receptor knockout mice as compared to wild type mice (see Table 3). In contrast, the relaxant effects of isoproterenol in trachea were practically the same in wild type and M_2 receptor knockout mice (i.e., no significant differences between wild type and M_2 knockout with regard to the effect of isoproterenol on E_{max} , shift in EC_{50} or observed coupling efficiency, $P = 0.54, 0.39$ and 0.29 , respectively). The mean values for the effects of isoproterenol on E_{max} , EC_{50} and observed coupling efficiency in the trachea showed greater relaxant effects in wild type mice compared with M_2 receptor knockout mice, although these differences between groups were not statistically significant. These results are summarized in Table 3.

Figure 3 shows the relaxant effects of forskolin ($5.0 \mu\text{M}$) and isoproterenol ($1.0 \mu\text{M}$) on the contractile response elicited by KCl (50 mM). Forskolin caused a significant inhibition of KCl-induced contractions in ileum (55%), trachea (84%) and urinary bladder (39%) from wild type mice. However, in contrast to that observed with oxotremorine-M as the contractile agent, the relaxant effects of forskolin against KCl-induced contractions were not significantly different between wild type and M_2 receptor knockout mice in ileum ($P = 0.78$), trachea ($P = 0.073$) and urinary bladder ($P = 0.84$). There was a substantial, though statistically not significant, difference in the trachea; this change was in the direction of forskolin having a larger inhibitory effect in wild type trachea (84%) compared with that from M_2 receptor knockout trachea (76%). Similar results were obtained when isoproterenol was used as the relaxant agent against KCl-induced contractions. In wild type mice, isoproterenol caused a highly significant inhibition of KCl-induced contractions in ileum (39%), trachea (82%) and urinary bladder (54%). In ileum and urinary bladder, there were no significant differences in the effects of isoproterenol on KCl-induced contractions between wild type and M_2 receptor knockout mice ($P = 0.95$ and 0.99 in ileum and urinary bladder, respectively). In trachea, isoproterenol had similar relaxant effects against KCl-induced contractions in the two types of mice, however, the relaxant effect of isoproterenol was slightly yet significantly greater in wild type mice as compared with M_2 receptor knockout mice ($P = 0.039$). Thus, in contrast to the results of the experiments in which oxotremorine-M was used as the contractile agent, the relaxant effects of isoproterenol and forskolin against KCl-induced contractions in M_2 receptor knockout mice were equal to or less than those observed in wild type mice.

We also investigated the effects of the potassium channel activator, pinacidil, on the contractile response to oxotremorine-M in smooth muscle from wild type and M₂ receptor knockout mice (see Table 4). Pinacidil caused a decrease in the potency of oxotremorine-M in ileum, trachea and urinary bladder and a small increase in E_{max} in both wild type and M₂ receptor knockout mice. However, there were no significant differences between the effects of pinacidil in wild type and M₂ receptor knockout mice.

DISCUSSION

Our observations on the contractile action of the selective muscarinic agonist, oxotremorine-M, in trachea and urinary bladder of M_2 muscarinic receptor knockout mice are similar to those reported by Stengel et al. (2000) using the nonselective cholinergic agonist carbachol. We found that the potency of oxotremorine-M decreased 1.21- and 1.48-fold in trachea and urinary bladder from M_2 receptor knockout mice, respectively, compared with wild type, whereas Stengel and coworkers (2000) observed 1.9- and 1.6-fold reductions in potency in these tissues with carbachol. We also observed a significant 2.1-fold reduction in the potency of oxotremorine-M in ileum in M_2 receptor knockout mice compared with wild type. These results suggest that, in wild type mice, the M_2 receptor plays some modulatory roles although the M_3 receptor is capable of eliciting most of the direct contractile response to muscarinic agonists in these tissues. Moreover, Matsui and coworkers (in press) showed that the direct muscarinic contractile response is completely eliminated in ileum and urinary bladder from mutant mice lacking both M_2 and M_3 receptors. These results show that the postjunctional effects of muscarinic agonists can be attributed almost entirely to M_2 and M_3 muscarinic receptors.

Prior studies on gastrointestinal (Candell et al., 1990; Sawyer and Ehlert, 1998; Zhang and Buxton, 1991), tracheal (Yang et al., 1991) and urinary bladder smooth muscle (Noronha-Blob et al., 1989) have shown that the M_2 muscarinic receptor mediates an inhibition of adenylyl cyclase. In ileum and trachea, this effect has been shown to be prevented by pertussis toxin-treatment (Sankary et al., 1988; Thomas and Ehlert, 1994). In contrast, pertussis toxin-treatment has no inhibitory effect on M_3 receptor-mediated phosphoinositide hydrolysis in gastrointestinal or tracheal smooth muscle, or on the contractile response to muscarinic agonists in guinea pig ileum, colon or trachea (Ostrom and Ehlert, 1999; Sawyer and Ehlert, 1999a; Sawyer and Ehlert, 1999b; Thomas and Ehlert, 1994; Thomas and Ehlert, 1996). This result suggests that the M_2 receptor has little role in mediating the potent contractile response to muscarinic agonists in guinea pig smooth muscle, because uncoupling M_2 receptor signaling with pertussis toxin does not inhibit contraction. However, pertussis toxin-treatment has been shown to enhance the relaxant effects of forskolin on oxotremorine-M-mediated contractions in ileum and trachea (Ostrom and Ehlert, 1997; Ostrom and Ehlert, 1998). Presumably, in the presence of forskolin, part of the contractile mechanism of oxotremorine-M involves an M_2 receptor-mediated inhibition of the relaxant effect of

forskolin. This mechanism probably involves an M_2 receptor-mediated inhibition of adenylyl cyclase because forskolin is thought to elicit relaxation in smooth muscle through cAMP (see review, Berridge (1975)). By uncoupling this M_2 mechanism with pertussis toxin, the muscarinic contractile response is now more susceptible to inhibition by forskolin. This interpretation is consistent with the observation that pertussis toxin is without effect on forskolin-mediated inhibition of histamine-induced contractions (Ostrom and Ehlert, 1997; Ostrom and Ehlert, 1998). Since histamine elicits contraction in smooth muscle through activation of H_1 receptors (Black et al., 1972), which signal through G_q without activation of G_i (Arrang et al., 1995), one would not expect pertussis toxin to influence histamine-induced contractions or their inhibition by forskolin. Our results in M_2 KO mice with forskolin are consistent with our previous work in guinea pigs using pertussis toxin to inactivate M_2 receptor signaling.

We have also observed that pertussis toxin-treatment enhances the relaxant effect of isoproterenol against oxotremorine-M-induced contraction of the guinea pig ileum, but not those elicited in the trachea (Ostrom and Ehlert, 1997; Ostrom and Ehlert, 1998; Thomas and Ehlert, 1994). As observed with forskolin, pertussis toxin-treatment has no effect on the ability of isoproterenol to inhibit histamine-induced contraction in guinea pig ileum and trachea. Our results on M_2 receptor knockout mice are consistent with these observations. We observed a significant increase in the relaxant action of isoproterenol against oxotremorine-M-induced contraction in ileum and urinary bladder from M_2 receptor knockout mice, but not in trachea. Collectively, our results suggest that pertussis toxin is a useful tool for exploring the role of M_2 receptors in smooth muscle.

It seems unlikely that the increased relaxant effectiveness of forskolin and isoproterenol in M_2 muscarinic receptor knockout mice is due to an increase in the sensitivity of smooth muscle to relaxant agents. Our experiments utilizing KCl as the contractile stimulus showed that there was no increase in the sensitivity of ileum, trachea or urinary bladder to the relaxant effects of forskolin and isoproterenol in M_2 receptor knockout mice compared with wild type. Moreover, we found that there was no increase in the relaxant action of the potassium channel activator, pinacidil, against oxotremorine-M-induced contraction in M_2 receptor knockout mice. Since the relaxant mechanism of pinacidil does not involve cAMP, one would not expect to observe a change in the ability of pinacidil to inhibit oxotremorine-M-induced contractions in M_2 receptor knockout mice. Thus, our studies with KCl and pinacidil in M_2 receptor knockout mice are consistent with the postulate that the increased relaxant action

of forskolin and isoproterenol against oxotremorine-M-induced contractions is due to the loss of M_2 receptors and not to an increase in the relaxant effectiveness of forskolin and isoproterenol.

The pattern of changes in M_2 muscarinic receptor knockout mice observed in this study show close agreement with prior pharmacological studies in wild type guinea pigs. One powerful method for investigating the function of M_2 receptors in wild type smooth muscle involves inactivating all non- M_2 muscarinic receptors with the aziridinium ion of 4-DAMP mustard (N-(2-chloroethyl)-4-piperidinyll diphenylacetic acid). Following this treatment, the muscarinic contractile response is measured in the presence of histamine and forskolin. Under these conditions, the contractile response to oxotremorine exhibits an M_2 profile for pharmacological antagonism in the ileum and trachea (Thomas et al., 1993; Thomas and Ehlert, 1996). Presumably, the contractile mechanism of oxotremorine-M under these conditions involves an M_2 receptor-mediated inhibition of the relaxant effect of forskolin on histamine-induced contractions. When, isoproterenol is used in the paradigm in place of forskolin in the ileum, the potency of oxotremorine-M is less, and the pharmacological antagonism exhibits a profile midway between M_2 - and M_3 -like (Thomas et al., 1993). Analogous types of experiments with isoproterenol in guinea pig urinary bladder (Hegde et al., 1997) and with isoproterenol and forskolin in guinea pig taenia caeci (Shen and Mitchelson, 1998) have yielded similar results. These data suggest that the M_2 receptor has a smaller role in inhibiting the relaxant effects of isoproterenol as compared to those of forskolin. Moreover, in the trachea, this type of experimental paradigm shows no role for the M_2 receptor in opposing the relaxant effects of isoproterenol on histamine-induced contractions (Ostrom and Ehlert, 1998; Ostrom and Ehlert, 1999). These prior pharmacological studies are consistent with the present data on M_2 receptor knockout mice. As described above, we found a greater increase in the relaxant effectiveness of forskolin compared with isoproterenol against oxotremorine-M-induced contractions in ileum from M_2 receptor knockout mice as compared with wild type. Also, we found no difference between trachea from wild type and M_2 receptor knockout mice with regard to the relaxant effectiveness of isoproterenol against oxotremorine-M-induced contractions. These results indicate that the M_2 receptor does not oppose isoproterenol-induced relaxation in the trachea.

It has been suggested that the lack of the role of the M_2 receptor in opposing isoproterenol-induced relaxation in the trachea implies that the relaxant mechanism of isoproterenol in the trachea is through a non-cAMP mechanism (Ostrom and Ehlert, 1998; Ostrom and Ehlert, 1999). Torphy (1994) has previously proposed a

non-cAMP mechanism, which could involve stimulation of Ca^{2+} activated K^+ channels. In bovine trachea, it has been shown that muscarinic receptor activation inhibits the increase in cAMP levels elicited by both forskolin and isoproterenol (Ostrom and Ehlert, 1998). Also, when cAMP levels and relaxation of muscarinic agonist-induced contractions are measured under identical conditions, forskolin-induced relaxation obeys a saturable, increasing function of the cytosolic concentration of cAMP, suggesting a functional relationship between the two (Ostrom and Ehlert, 1998). In contrast, no functional relationship between relaxation and the cytosolic concentration of cAMP was observed for isoproterenol (Ostrom and Ehlert, 1998). An increase in relaxation from 30 to 100% occurred with no change in the cAMP concentration. The smaller role of the M_2 receptor in opposing isoproterenol-induced relaxation in the ileum compared with that caused by forskolin suggests that some, but not all, of the relaxation caused by isoproterenol in the ileum is mediated through cAMP. This could be explained if the G_s activated by β -adrenergic receptors acts through at least two pathways - adenylyl cyclase as well as another pathway that is unopposed by M_2 receptor activation. Forskolin, which acts downstream at the level of adenylyl cyclase, would be unable to elicit such a non-cAMP dependent mechanism for relaxation.

Prior studies in guinea pigs have shown that, in addition to opposing cAMP-mediated relaxation, M_2 receptor activation also causes a conditional potentiation of M_3 muscarinic receptor-mediated contractions (Sawyer and Ehlert, 1999b). This conclusion was based on the unique pattern of sensitivity of the muscarinic contractile response in the colon to pertussis toxin and both competitive and irreversible muscarinic antagonists. The data were consistent with the postulate that M_2 receptors have no direct contractile action by themselves, but cause a conditional potentiation of M_3 receptor-mediated contractions. As suggested previously (Ehlert, in press; Ehlert et al., 1999; Ehlert et al., 1997; Sawyer and Ehlert, 1999b), a possible mechanism for this action could be M_2 receptor-mediated stimulation of the non-selective cation conductance or inhibition of Ca^{2+} -activated K^+ channels. Both of these mechanisms are Ca^{2+} -dependent; hence, they fulfill the necessary criterion of being conditional upon Ca^{2+} mobilization by the M_3 receptor. At first, it might seem that a loss of these M_2 responses could account for the increased susceptibility of the muscarinic contractile response to relaxant agents in M_2 receptor knockout mice. However, our method of analysis makes this possibility unlikely. Our null method approach compares equivalent contractile stimuli in the presence and absence of the relaxant agents. There is no inherent reason to assume that a direct contractile stimulus mediated through an interaction between M_2 and M_3 receptors in wild type mice would

be more resistant to relaxant agents than an equivalent stimulus activated only by M_3 receptors in M_2 receptor knockout mice. Moreover, if loss of the latter mechanism were responsible for the increased relaxant action of forskolin and isoproterenol in M_2 receptor knockout mice, then, for a given tissue, we would have expected to observe the same increase in the relaxant effectiveness of both forskolin and isoproterenol. However, this was not observed as described above. In contrast, if the M_2 receptor mediates an attenuation of the relaxant stimulus directly (e.g., through inhibition of adenylyl cyclase), this mechanism could explain why the relaxant activity of forskolin and isoproterenol increased differentially in tissues from M_2 receptor knockout mice. Finally, it is important to mention that our null method also controls for compensatory changes in the activity of M_3 receptors in M_2 receptor knockout mice. Future studies on M_2 receptor knockout mice may help to sort out the mechanism of the M_2 receptor-mediated potentiation of M_3 receptor mediated contractions.

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FOOTNOTES

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Table 1: Contractile activity of oxotremorine-M in smooth muscle from wild type and M₂ muscarinic receptor knockout (KO) mice. ^a

	<i>Ileum</i>	<i>Trachea</i>	<i>Urinary Bladder</i>
<u>Wild Type</u> (n = 5)			
<i>E</i> _{max} (% KCl)	188 ± 20	218 ± 14	223 ± 11
<i>pEC</i> ₅₀	6.70 ± 0.037	6.94 ± 0.036	6.58 ± 0.044
<u>M₂ Receptor KO</u> (n = 5)			
<i>E</i> _{max} (% KCl)	185 ± 22	191 ± 6.1	188 ± 17
<i>pEC</i> ₅₀	6.38 ± 0.028 ^b	6.86 ± 0.037	6.41 ± 0.055

^a The data were calculated from the experiments shown in Figures 1 and 2. Mean values ± SEM from five wild type and five M₂ receptor knockout mice are shown.

^b Significantly different from that measured in wild type mice, P = 0.00013.

Table 2: Effect of forskolin on the contractile response to oxotremorine-M in smooth muscle from wild type and M₂ muscarinic receptor knockout (KO) mice. ^a

	<i>Ileum</i>	<i>Trachea</i>	<i>Urinary Bladder</i>
<u>Wild Type (n = 5)</u>			
<i>Forskolin</i>			
E_{max} (% control)	90 ± 3.9	63 ± 5.2 ^d	103 ± 2.7
EC_{50} Shift (fold) ^b	1.6 ^d (0.20 ± 0.032)	6.7 ^d (0.83 ± 0.057)	2.2 ^d (0.35 ± 0.053)
$CE_{observed}$ ^c	0.57 ^d (-0.25 ± 0.033)	0.098 ^d (-1.01 ± 0.026)	0.46 ^d (-0.34 ± 0.058)
<u>M₂ Receptor KO (n = 5)</u>			
<i>Forskolin</i>			
E_{max} (% control)	70 ± 13	63 ± 3.1 ^d	74 ± 13
EC_{50} Shift (fold) ^b	4.9 ^{d,e} (0.69 ± 0.055)	20 ^{d,e} (1.30 ± 0.018)	16 ^{d,e} (1.20 ± 0.16)
$CE_{observed}$ ^c	0.16 ^{d,e} (-0.80 ± 0.14)	0.032 ^{d,e} (-1.50 ± 0.029)	0.049 ^{d,e} (-1.31 ± 0.11)

^a The data were calculated from the experiments shown in Figure 1. Mean values ± SEM from five wild type and five M₂ receptor knockout mice are shown.

^b Denotes the EC_{50} value of oxotremorine-M measured in the presence of the relaxant agent divided by that measured in its absence. The values in parentheses beneath each estimate represent the mean Log EC_{50} shift values ± SEM.

^c Denotes the observed coupling efficiency of oxotremorine-M in the presence of the relaxant agent relative to that in the absence of the relaxant agent. The values in parentheses beneath each estimate represent the mean Log $CE_{observed}$ values ± SEM.

^d A significant effect of the relaxant agent relative to control. The probability values for these differences are as follows. Wild type mice, effects of forskolin: E_{max} in trachea, P = 0.0020; EC_{50} shift in ileum, trachea and urinary bladder, P = 0.0079, 0.00013 and 0.0028; $CE_{observed}$ in ileum, trachea and urinary bladder, P = 0.0023,

7.0×10^{-9} and 0.0012. M_2 muscarinic receptor knockout mice, effects of forskolin: E_{max} in trachea, $P = 0.00030$; EC_{50} *shift* in ileum, trachea and urinary bladder, $P = 0.0011$, 2.1×10^{-7} and 0.0016; $CE_{observed}$ in ileum, trachea and urinary bladder, $P = 0.00046$, 1.8×10^{-10} and 1.3×10^{-7} .

- ^e Significantly different from the relaxant effect observed in wild type mice. The probability values for these differences are as follows: Effects of forskolin on EC_{50} *shift* in ileum, trachea and urinary bladder, $P = 0.00027$, 0.000047, and 0.0021; effects of forskolin on $CE_{observed}$ in ileum, trachea and urinary bladder, $P = 0.00090$, 2.9×10^{-6} and 0.00086.

Table 3: Effect of isoproterenol on the contractile response to oxotremorine-M in smooth muscle from wild type and M₂ muscarinic receptor knockout (KO) mice. ^a

	<i>Ileum</i>	<i>Trachea</i>	<i>Urinary Bladder</i>
<u>Wild Type</u> (n = 5)			
<i>Isoproterenol</i>			
E_{max} (% control)	83 ± 5.0 ^d	74 ± 4.4 ^d	107 ± 4.9
EC_{50} Shift (fold) ^b	3.1 ^d (0.49 ± 0.061)	14.1 ^d (1.15 ± 0.098)	1.8 ^d (0.26 ± 0.059)
$CE_{observed}$ ^c	0.28 ^d (-0.55 ± 0.060)	0.055 ^d (-1.26 ± 0.082)	0.62 ^d (-0.21 ± 0.065)
<u>M₂ Receptor KO</u> (n = 5)			
<i>Isoproterenol</i>			
E_{max} (% control)	63 ± 2.8 ^{d,e}	78 ± 5.9 ^d	106 ± 3.5
EC_{50} Shift (fold) ^b	4.5 ^d (0.66 ± 0.065)	11.4 ^d (1.06 ± 0.023)	4.0 ^{d,e} (0.61 ± 0.063)
$CE_{observed}$ ^c	0.14 ^{d,e} (-0.85 ± 0.066)	0.070 ^d (-1.15 ± 0.052)	0.27 ^{d,e} (-0.56 ± 0.057)

^a The data were calculated from the experiments shown in Figure 2. Mean values ± SEM from five wild type and five M₂ receptor knockout mice are shown.

^b Denotes the EC_{50} value of oxotremorine-M measured in the presence of the relaxant agent divided by that measured in its absence. The values in parentheses beneath each estimate represent the mean Log EC_{50} shift values ± SEM.

^c Denotes the observed coupling efficiency of oxotremorine-M in the presence of the relaxant agent relative to that in the absence of the relaxant agent. The values in parentheses beneath each estimate represent the mean Log $CE_{observed}$ values ± SEM.

^d A significant effect of the relaxant agent relative to control. The probability values for these differences are as follows. Wild type mice, effects of isoproterenol: E_{max} in ileum and trachea, P = 0.030 and 0.0038; EC_{50} shift in ileum, trachea and urinary bladder, P = 0.0014, 0.000030 and 0.0068; $CE_{observed}$ in ileum, trachea and urinary

bladder, $P = 0.000061$, 5.7×10^{-8} and 0.0099 . M_2 muscarinic receptor knockout mice, effects of isoproterenol: E_{max} in ileum and trachea, $P = 0.00093$ and 0.022 ; $EC_{50} shift$ in ileum, trachea and urinary bladder, $P = 0.0021$, 9.7×10^{-8} and 0.00022 ; $CE_{observed}$ in ileum, trachea and urinary bladder, $P = 0.000023$, 5.0×10^{-8} and 3.4×10^{-6} .

^e Significantly different from the relaxant effect observed in wild type mice. The probability values for these differences are as follows: Effects of isoproterenol on E_{max} in ileum, $P = 0.0089$; effects of isoproterenol on $EC_{50} shift$ in urinary bladder, $P = 0.0025$; effects of isoproterenol on $CE_{observed}$ in ileum and urinary bladder, $P = 0.018$ and 0.0059 .

Table 4: Effect of pinacidil (5.0 μ M) on the contractile response to oxotremorine-M in smooth muscle from wild type and M₂ muscarinic receptor knockout (KO) mice. ^a

	<i>Ileum</i>	<i>Trachea</i>	<i>Urinary Bladder</i>
<u>Wild Type</u> (<i>n</i> = 4)			
E_{max} (% control) ^b	125 $\pm$ 11	80 $\pm$ 4.1 ^d	107 $\pm$ 6.4
EC_{50} Shift (fold) ^c	3.2 ^d (0.50 $\pm$ 0.059)	4.1 ^d (0.62 $\pm$ 0.084)	1.7 (0.22 $\pm$ 0.070)
<u>M₂ Receptor KO</u> (<i>n</i> = 4)			
E_{max} (% control) ^b	137 $\pm$ 3.8 ^d	87 $\pm$ 4.4	111 $\pm$ 9.5
EC_{50} Shift (fold) ^c	2.2 ^d (0.35 $\pm$ 0.055)	3.9 ^d (0.59 $\pm$ 0.070)	1.5 (0.19 $\pm$ 0.070)

^a Mean values $\pm$ SEM from four wild type and four M₂ receptor knockout mice are shown.

^b Denotes the E_{max} value of oxotremorine-M measured in the presence of pinacidil divided by that measured in its absence, expressed as a percent.

^c Denotes the EC_{50} value of oxotremorine-M measured in the presence of pinacidil divided by that measured in its absence. The values in parentheses beneath each estimate represent the mean *Log shift* values $\pm$ SEM.

^d A significant effect of pinacidil relative to control. The probability values for these differences are as follows. Wild type mice, effects of pinacidil on E_{max} in trachea, P = 0.016; EC_{50} shift in ileum and trachea, P = 0.0035 and 0.0052. M₂ muscarinic receptor knockout mice, effects of pinacidil on E_{max} in ileum, P = 0.0022; EC_{50} shift in ileum and trachea, P = 0.0079 and 0.0035.

Legends to Figures

Figure 1: *Effects of forskolin on oxotremorine-M-mediated contractions in ileum (A,D), trachea (B,E) and urinary bladder (C,F) from wild type (A-C) and M_2 muscarinic receptor knockout mice (D-F). The contractile response to oxotremorine-M was measured in the absence (○) and presence (●) of forskolin (5.0 μ M). The magnitudes of the contractions are expressed relative to the contraction elicited by KCl (50 mM). The data represent the mean values $\pm$ SEM from five wild type and five knockout mice.*

Figure 2: *Effects of isoproterenol on oxotremorine-M-mediated contractions in ileum (A,D), trachea (B,E) and urinary bladder (C,F) from wild type (A-C) and M_2 muscarinic receptor knockout mice (D-F). The contractile response to oxotremorine-M was measured in the absence (○) and presence (●) of isoproterenol (1.0 μ M). The magnitudes of the contractions are expressed relative to the contraction elicited by KCl (50 mM). The data represent the mean values $\pm$ SEM from five wild type and five knockout mice.*

Figure 3: *Effects of forskolin (5.0 μ M) (A) and isoproterenol (1.0 μ M) (B) on contractions elicited to KCl (50 mM) in ileum, trachea and urinary bladder from wild type and M_2 muscarinic receptor knockout mice. The contractions are expressed as a percentage of the contraction elicited to KCl in the absence of the relaxant agents. The data represent the mean values $\pm$ SEM from four wild type and four knockout mice. *, significantly different from the relaxant effect observed in wild type mice, $P = 0.039$.*

Figure 1

Figure 2

Figure 3