M3-receptor knockout mice: muscarinic receptor function in atria, stomach fundus, urinary bladder, and trachea

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Stengel, Peter W., Masahisa Yamada, Jürgen Wess, and Marlene L. Cohen. M3-receptor knockout mice: muscarinic receptor function in atria, stomach fundus, urinary bladder, and trachea. Am J Physiol Regulatory Integrative Comp Physiol 282: R1443–R1449, 2002. First published January 17, 2002; 10.1152/ajpregu.00486.2001.—Negative chronotropic and smooth muscle contractile responses to the nonselective muscarinic agonist carbamylcholine were compared in isolated tissues from M3-muscarinic receptor knockout and wild-type mice. Carbamylcholine (10−8–3.0 × 10−5 M) induced a concentration-dependent decrease in atrial rate that was similar in atria from M3-receptor knockout and wild-type mice, indicating that M3 receptors were not involved in muscarinic receptor-mediated atrial rate decreases. In contrast, the M3 receptor was a major muscarinic receptor involved in smooth muscle contraction of stomach fundus, urinary bladder, and trachea, although differences existed in the extent of M3-receptor involvement among the tissues. Contraction to carbamylcholine was virtually abolished in urinary bladder from M3-receptor knockout mice, suggesting that contraction was predominantly due to M3-receptor activation. However, ~50–60% maximal contraction to carbamylcholine occurred in stomach fundus and trachea from M3-receptor knockout mice, indicating that contraction in these tissues was also due to M3-receptor activation. High concentrations of carbamylcholine relaxed the stomach fundus from M3-receptor knockout mice by M1-receptor activation. Thus M3-receptor knockout mice provided unambiguous evidence that M3 receptors 1) play no role in carbamylcholine-induced atrial rate reduction, 2) are the predominant receptor mediating carbamylcholine-induced urinary bladder contractility, and 3) share contractile responsibility with M2 receptors in mouse stomach fundus and trachea.

atrial rate; negative chronotropy; smooth muscle contraction; carbamylcholine-mediated responses

IN MANY SMOOTH MUSCLE PREPARATIONS where the muscarinic receptor population was resolved through antagonist radioligand-binding studies (6), M2 receptors comprised 70–80% and M3 receptors 20–30% of the receptor population. Although they are only a small proportion of the receptors, previous pharmacological studies indicated that muscarinic receptor-induced contractile responses in several smooth muscles were likely mediated by activation of M3 receptors (3, 6, 7). In addition, selective inactivation of M3 receptors by 4-DAMP mustard revealed a role for M3 receptors in smooth muscle contraction (7). Thus, with the availability of mutant mouse strains lacking functional M1, M2, M3, and M4 receptors (9, 10, 12, 24, 33), the physiological role of muscarinic receptors in native tissue can be unambiguously determined.

In previous studies comparing smooth muscle contractile responses in M2− and M4−receptor knockout mice with responses in wild-type littermates, carbamylcholine-induced contractile potency was reduced in stomach fundus, urinary bladder, and tracheal preparations only from M2-receptor knockout mice, indicating a role for M2, but not M4, receptors in smooth muscle contraction (33). However, in tissues from M2−receptor knockout mice, maximal contraction to carbamylcholine was not altered, suggesting a prominent contractile role for another cholinergic receptor. Recently, Matsui et al. (24) demonstrated marked impairment of the contractile response to carbamylcholine in ileum and urinary bladder from mutant mice lacking M3 receptors. Whether M3 receptors are also important in other smooth muscle preparations such as stomach fundus and trachea has not been examined in M3−receptor knockout mice. The fact that M3 receptors have been implicated in the regulation of intracellular calcium in stomach fundus (31) also raised the possibility of an important role for M3 receptors in smooth muscle contractility.

Mammalian heart is comprised predominantly of M2-muscarinic receptors (3), as determined by the localization of M2-receptor mRNA in rat heart by in situ hybridization (16) and, more recently, by the requirement of functional M2 receptors in muscarinic receptor-dependent atrial rate reduction (33). Nonetheless, expression of M3-muscarinic receptor genes in guinea pig and rat intrinsic intracardiac neurons by in situ hybridization (13) and in canine atria (30) and human heart (14, 36) by reverse transcription-polymerase chain reaction (30) has been described. In addition, on

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the basis of studies with isolated myocytes, Wang and colleagues (37) suggested that M₃ receptors mediated a potassium current involved in mammalian heart rate. However, no direct evidence exists for the role of these gene products in the regulation of atrial rate.

On the basis of the possible role of M₃ receptors in atrial rate and smooth muscle contractility, the present study focused on comparing responses to carbamylcholine in isolated atria and three smooth muscle preparations (stomach fundus, urinary bladder, and trachea). Our results indicate, in a direct and unequivocal manner, that M₃ receptors are not involved in carbamylcholine-induced decreases in atrial rate but are essential for carbamylcholine-induced smooth muscle contraction, most prominently in the urinary bladder. Unanticipated findings from this study were 1) the substantial residual contraction to carbamylcholine in the stomach fundus and trachea from M₃-receptor knockout mice, indicative of pronounced M₂-receptor activation by carbamylcholine, and 2) the exposure of an M₁-receptor-mediated relaxation in the stomach fundus. These results emphasize the utility of muscarinic receptor knockout mice to evaluate the involvement of distinct muscarinic receptor subtypes in physiological functions.

METHODS

Animals. The generation of M₃-muscarinic receptor knockout mice has been described previously (38). Male M₃-receptor knockout and wild-type mice of the same genetic background used in the present study were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD) or Taconic (Germantown, NY). Animals were housed in polycarbonate ventilated cages. The animal room was maintained at 22°C and a relative humidity of 35–70% and daily light-dark cycle (lights on from 0600 to 1800). Dry pellet food (Laboratory Rodent Diet 5001, PMI Feed) and water were supplied ad libitum. Body weights of age-matched M₃-receptor knockout and wild-type mice were measured with a balance (model D1001 CA, Ohaus, Pine Brook, NJ). Mice were then killed by cervical dislocation, and the heart, stomach fundus, urinary bladder, and/or trachea were quickly excised and placed in modified Krebs bicarbonate buffer solution of the following composition (mM): 4.6 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 118.2 NaCl, 10.0 glucose, 1.6 CaCl₂·2H₂O, and 24.8 NaHCO₃. Experimental protocols and procedures were approved by the Eli Lilly and Company Animal Care and Use Committee. The investigation fully conformed with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals.

Atrial preparation. Spontaneously beating atria were dissected from ventricles, and the left atrium was attached with thread to a stationary glass rod, while the right atrium was tied with thread to a force displacement transducer. The atria were placed in organ baths containing 10 ml of bicarbonate buffer (see Animals for composition). The organ bath solution was maintained at 37°C and aerated with 95% O₂-5% CO₂. Smooth muscle preparations were placed under an initial optimal force of 2.0 g for tracheal rings and 4.0 g for stomach fundus and urinary bladder, as determined in length-tension optimizing studies with each preparation (33), and equilibrated for 1 h, during which time the tissues were washed at 15-min intervals. Isometric force in grams was measured with Sensotec transducers. Stomach fundus, urinary bladder, and trachea were initially challenged with 67 mM KCl to confirm viability of the preparation. Cumulative contractile concentration-response curves to 10⁻⁸–3.0 × 10⁻⁵ M carbamylcholine were generated and expressed as a percentage of the 67 mM KCl-induced contraction determined for each tissue. On each day, tissues from M₃-receptor knockout and wild-type mice were used to avoid the possibility of any daily systematic effect. Experiments were performed over multiple days.

In other experiments, stomach fundus from M₃-receptor knockout mice was incubated with 3.0 × 10⁻⁷ M pirenzepine dihydrochloride (Sigma Chemical) or vehicle for 20 min, and contractile concentration-response curves to 10⁻⁸–3.0 × 10⁻⁵ M carbamylcholine were generated. Peak contraction to carbamylcholine occurred at 10⁻⁶ M, and higher concentrations of carbamylcholine (3.0 × 10⁻⁶–3.0 × 10⁻⁵ M) relaxed the stomach fundus. The effect of pirenzepine on the relaxant response to carbamylcholine was examined in stomach fundus and measured as percent relaxation of the maximum contraction to 10⁻⁶ M carbamylcholine. Carbamylcholine-induced contraction and relaxation were similar before and after vehicle incubation in all tissues studied.

Statistical analyses. Values are means ± SE of three to nine isolated tissues obtained from three to nine animals. Carbamylcholine concentration-response curves were analyzed by a three-parameter logistic nonlinear model (4). The three modeled parameters included the maximal response of the tissue, the EC₅₀, and the slope of the curves. Each curve was fitted using SAS software (SAS Institute, Cary, NC). Two-way repeated-measures ANOVA was used to compare carbamylcholine responses in stomach fundus, urinary bladder, and trachea between wild-type and M₃-receptor knockout mice and to examine the effect of 3.0 × 10⁻⁷ M pirenzepine on carbamylcholine-induced relaxation in stomach fundus from M₃-receptor knockout mice. Bonferroni’s correction was performed to control for multiple comparisons. Unpaired Student’s t-test was used to compare mean atrial rates, mean tissue KCl contractile responses, and mean pEC₅₀ (−log EC₅₀) values to carbamylcholine between M₃-receptor knockout and wild-type mice using SigmaStat for Windows (version 2.03, SPSS Science, Chicago, IL). Comparisons were considered significant for P ≤ 0.05.
RESULTS

Age and body weight comparisons between M3-receptor knockout and wild-type mice. The M3-receptor knockout mice did not significantly differ in age from wild-type mice: 23.5 ± 2.6 and 18.9 ± 3.7 wk, respectively (P = 0.30). In contrast, mean body weights of M3-receptor knockout mice were significantly lower than mean body weights of wild-type mice: 30.7 ± 1.5 and 35.7 ± 1.6 g, respectively (P < 0.05).

Basal atrial rate in M3-receptor knockout and wild-type mice. Basal rates of spontaneously beating mouse atria from the M3-receptor knockout mice were not different from atrial rates in wild-type mice: 472.9 ± 14.1 and 451.8 ± 12.0 beats/min, respectively. The similarity in basal atrial rate between the two groups indicates that M3 receptors do not modulate basal sinoatrial nodal function in vitro.

Carbamylcholine-induced negative chronotropy. The potency and maximal decrease in atrial rate to 10⁻⁸–10⁻⁵ M carbamylcholine were comparable in atria from M3-receptor knockout and wild-type mice (pEC₅₀ = 5.96 ± 0.07 and 6.02 ± 0.08, respectively; Fig. 1). This lack of difference in reduction in atrial rate to carbamylcholine between M3-receptor knockout and wild-type mice indicates that M3 receptors do not play a role in the atrial negative chronotropic response to carbamylcholine.

Carbamylcholine-induced contraction in smooth muscle. Carbamylcholine (10⁻⁸–3.0 × 10⁻⁵ M) contracted the stomach fundus, urinary bladder, and trachea from M3-receptor knockout and wild-type mice (Fig. 2). However, the characteristics of the contractile response to carbamylcholine differed among the tissues. In the stomach fundus and trachea, there was a marked reduction (~50–60%) in the maximal response to carbamylcholine in tissues from M3-receptor knockout mice compared with wild-type mice, although the potency of carbamylcholine was not altered (Table 1). In the stomach fundus and trachea, although maximal contraction was reduced, a residual maximal contraction to carbamylcholine (~50% of the contraction in tissue from wild-type mice) was apparent in tissues from the M3-receptor knockout mice.

Furthermore, in the stomach fundus but not the trachea, high concentrations of carbamylcholine (>10⁻⁶ M) relaxed the preparations from M3-receptor knockout mice (Fig. 2), an effect that did not occur in the trachea from M3-receptor knockout mice. In the presence of the M3-selective receptor antagonist pirenzepine (3.0 × 10⁻⁷ M), the relaxant response to high concentrations of carbamylcholine (>10⁻⁶ M) in stoma-
ach fundus from M₃-receptor knockout mice was abolished, indicating the involvement of M₁-receptor activation in carbamylcholine-induced relaxation (Fig. 3).

In contrast to the stomach fundus and trachea, maximal carbamylcholine-induced contraction of urinary bladder was markedly diminished. Little residual contractile response to carbamylcholine (<10% of the contraction in tissue from wild-type mice) remained in urinary bladder from the M₃-receptor knockout mice (Fig. 2, Table 1).

Although contractions to carbamylcholine were reduced in stomach fundus, urinary bladder, and trachea from M₃-receptor knockout mice, 67 mM KCl-induced contraction did not statistically differ in stomach fundus, urinary bladder, and trachea from M₃-receptor knockout and wild-type mice (Table 2). Thus stomach fundus, urinary bladder, and trachea were responsive to a noncholinergic smooth muscle contractile agent, indicating that the deletion of M₃ receptors did not alter contractile machinery in these tissues.

**DISCUSSION**

The development of muscarinic-receptor knockout mice has permitted a detailed and unequivocal exploration of the role for each of the known muscarinic receptors to behavior (9, 10, 12, 26) and peripheral tissue responses (24, 33). Interestingly, elimination of the M₃ receptor resulted in a pronounced reduction in body weight transiently (24) or over longer time periods (38; this study), an effect not reported in animals lacking other muscarinic receptors (9, 10, 12). The reduction in body weight observed in M₃-receptor knockout mice was associated with a decrease in dry food intake in these animals (24, 38; this study). The weight loss was previously attributed to altered hypothalamic responsiveness (38). However, our study raises the possibility that delayed gastric emptying resulting from alterations in gastric motility in M₃-receptor knockout mice might also contribute to the reduction in body weight in M₃-receptor knockout mice. Support for this hypothesis comes from studies demonstrating that atropine delayed gastric emptying more in obese than in lean human subjects (35), suggesting reduced gastric vagal cholinergic activity in lean subjects. This observation is consistent with decreased M₃-receptor activation in gastrointestinal tissue contributing to decreased body weight. Thus, in addition to a central hypothalamic effect, reduced gastric contractility may also contribute to the reduction in body weight observed in M₃-receptor knockout mice.

Peripheral muscarinic receptors are implicated in the control of numerous fundamental physiological processes such as heart rate regulation, glandular secretion, and smooth muscle contraction (2, 3, 17, 27). In this study, we used M₃ receptor-deficient mice (24, 38) to study the potential role of this muscarinic receptor subtype in atrial rate and smooth muscle contractile responses in stomach fundus, urinary bladder, and trachea.

Initial studies documented a virtual loss of negative chronotropy to carbamylcholine in atria from M₂-receptor knockout mice (33), supporting a predominant role of the M₂ receptor in mediating carbamylcholine-
induced atrial rate reduction. However, the M3 receptor has also been strongly implicated in cardiac function on the basis of its localization (36) and reported role in altering potassium current in myocytes (37). On the basis of these observations, we further explored the effects of carbamylcholine in atria from M3-receptor knockout mice. The present studies unequivocally document that carbamylcholine-induced lowering of atrial rate was unaffected by the absence of the M3 receptor in mouse atria. It is possible that the M3 receptor proposed to alter heart rate (37) does not reside in the mouse atria or the alteration in potassium currents produced by pilocarpine in canine and guinea pig myocytes (37) may have resulted from activation of other muscarinic receptor(s). Although Wang et al. (37) attempted to utilize concentrations of pharmacological tools selective for specific muscarinic receptors, the compounds used were relatively nonselective. Nevertheless, the present studies with M3-receptor knockout mice add further support to the important and predominant role of M2 receptors in mediating atrial rate and convincingly document a lack of M3-receptor involvement. These studies do not, of course, address the role of M3 receptors in atrioventricular node conduction or Purkinje fiber transmission, which could affect cardiac conduction and heart rate in the intact cardiac system, but they do support the use of isolated mouse atria for the study of selective M2-receptor agonists and antagonists.

Prior pharmacological investigations using mouse stomach fundus (25, 29), urinary bladder (5, 23), and trachea (8, 15) have demonstrated muscarinic-induced contractile responses but have not detailed the multiple receptor subtypes involved. However, previous studies with muscarinic-receptor knockout mice demonstrated that M2, but not M4, receptors participated in smooth muscle contractility (33). Carbamylcholine was significantly less potent (by ~2-fold) in contracting stomach fundus, urinary bladder, and trachea from M2-receptor knockout mice than from M4-receptor knockout or wild-type mice (33). In addition, Matsui et al. (24) recently reported impaired contractility of urinary bladder and ileal smooth muscle preparations from M3-receptor knockout mice compared with wild-type mice. Taken in concert, these studies suggested an important role for M2 and M3 receptors in carbamylcholine-induced contraction in these tissues.

With the use of M3-receptor knockout mice, the current detailed examination of carbamylcholine-induced contraction revealed marked differences in responses among smooth muscle preparations from mutant mice. In the trachea from M3-receptor knockout mice, carbamylcholine produced a classical contractile response, albeit with significantly lower maximal contraction than in trachea from wild-type mice. Furthermore, maximal contraction was maintained as carbamylcholine concentration increased. A significant residual contraction to carbamylcholine (~60% of the response in tissues from wild-type mice) remained even in the absence of M3 receptors. This observation, coupled to the previous demonstration of decreased potency to carbamylcholine in trachea from M2-receptor knockout mice (33), suggests that M3-receptor activation is required for maximal tracheal contractility to carbamylcholine, whereas M2-receptor activation increases sensitivity or potency to carbamylcholine.

In contrast, carbamylcholine-induced contraction was virtually abolished in urinary bladder from M3-receptor knockout mice, confirming the previous observation of Matsui et al. (24). Thus, in urinary bladder, contraction to carbamylcholine is clearly and unequivocally mediated predominantly by activation of M3 receptors. M2 receptors play only a minor role in urinary bladder contractility to carbamylcholine (33). Thus the urinary bladder may serve as a useful preparation to study the effects of compounds and their signaling mechanisms at endogenous M3 receptors.

The carbamylcholine contractile response profile in the stomach fundus differed from that in the trachea or urinary bladder. In the absence of M3 receptors, maximal contraction to carbamylcholine was markedly reduced (<50% of the maximal response in tissues from wild-type mice). Thus M3 receptors play a predominant role in carbamylcholine-induced contraction in the stomach fundus. This observation was consistent with the previous demonstration that the M3-selective receptor antagonist zamifenacine suppressed gastric emptying in rats at a dose that did not alter heart rate (21). These results support our contention that blockade of the M3 receptor will decrease gastric contractility, stomach emptying, and body weight, as observed in the M3-receptor knockout mice.

As the concentration of carbamylcholine was increased in stomach fundus from M3-receptor knockout mice, a relaxant effect was observed, an effect that was not as apparent in stomach fundus from wild-type mice or in trachea from M3-receptor knockout mice. Previous studies using stomach fundus from pig (20) and rat (19) documented neurally induced nitric oxide-mediated relaxant responses. Such relaxant responses may be mediated by activation of M1 (1, 18, 28) or M4 (11) receptors, as documented with other smooth muscle preparations. Our results with the M1-selective receptor antagonist pirenzepine demonstrate that M1-receptor activation is likely the mechanism mediating carbamylcholine-induced relaxation in stomach fundus from M3-receptor knockout mice. These data document the presence of a relaxant cholinergic receptor in the stomach fundus that is most apparent in the absence of M3 receptors.

Clearly, M3-receptor deletion affected contraction differentially in the three smooth muscle preparations. The fact that M3-receptor deletion reduced contraction to carbamylcholine in stomach fundus and trachea by only 50% may be related to activation of other muscarinic receptor contractile mechanisms. For example, activation of the M2 receptor can inhibit adenylyl cyclase (3, 6, 7), and inhibition of adenylyl cyclase has been associated with contractile effects for other receptor agonists in vascular smooth muscle (34). Alternatively, activation of presynaptic M2 receptors by acetylcholine has been proposed to inhibit nitric oxide
release in cerebral blood vessels and thus reduce vaso-
dilation (22), which could translate into enhanced con-
tractility. If a similar action were to occur in stomach
fundus or trachea, enhanced contraction would like-
wise occur when carbachol activated presynap-
tic M2 receptors.

Thus, in stomach fundus, trachea, and urinary blad-
der, the maximal contractile response to carbachol-
line was diminished in tissues from M3-receptor knock-
out mice. The possibility can be raised that the marked
reduction in contraction to carbachol in smooth
muscles from the M3-receptor knockout mice was re-
lated to the significantly reduced body weight of the
M3-receptor knockout mice. However, this is unlikely,
since 1) no change in basal atrial rate occurred between
knockout and wild-type mice, 2) the contractile re-
sponses to carbachol did not change in a uni-
form fashion among the smooth muscle preparations,
and, lastly and most convincingly, 3) contraction to KCl
was not significantly altered in tissues from the M3-
receptor knockout mice. KCl has been used as a non-
selective depolarizing agonist to standardize function
in smooth muscle. Thus, although M3-receptor knock-
ut mice were significantly smaller in body weight and
tissue responsiveness to carbachol was reduced,
contraction to nonspecific agonists such as KCl was not
similarly affected.

In conclusion, this study illustrates the usefulness of
receptor knockout mice to examine the role of specific
muscarinic receptor subtypes present in peripheral
tissues. We showed, in a direct and unequivocal man-
er, that functional M3 receptors are not involved in
muscarinic receptor-dependent atrial rate reduction.
The present study also demonstrated that M3 receptors
are the primary muscarinic receptor subtype mediat-
ing contraction in urinary bladder. In contrast, a con-
siderable smooth muscle contractile response to car-
bachol remained in stomach fundus and trachea
from M3-receptor knockout mice, suggesting that, in
these tissues, M3 and M2 receptors importantly con-
tributed to muscarinic-induced contraction. Thus
marked differences exist in the relative role of M2 and
M3 receptors in the contraction produced by carbachol-
line in peripheral smooth muscle.

Perspectives

These results with smooth muscle from M3-receptor
knockout mice coupled with previous studies using
M2-receptor knockout mice (33) suggest that M2- and
M3-receptor activation contributes to smooth muscle
contractility, although the contribution of M2-receptor
activation varies widely among tissues. The develop-
ment of M23-double receptor knockout mice and the
study of smooth muscle contractility from such animals
would be required to test this hypothesis and defini-
tively rule out a contractile role for other muscarinic
receptors.

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