M₂ and M₄ Receptor Knockout Mice: Muscarinic Receptor Function in Cardiac and Smooth Muscle In Vitro

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ABSTRACT

Peripheral muscarinic receptors play key roles in the control of heart rate and smooth muscle activity. In this study, brady¬

cardic and smooth muscle contractile responses to the mus¬

carinic agonist carbachol were compared in isolated tis¬

sues from M₂ and M₄ muscarinic receptor knockout mice and

their wild-type littermates. Carbachol (1 × 10⁻⁸–3 × 10⁻⁵ M) pro¬

duced similar concentration-dependent bradycardia in sporo¬

taneously beating atria from M₂ receptor knockout mice

and wild-type control mice. In contrast, carbachol did not

produce bradycardia in atria derived from M₄ receptor

knockout mice, whereas such atria were responsive to ade¬

nosine-induced bradycardia. Carbachol-induced contractile

responses were similar in stomach fundus, urinary blad¬

der, and tracheal preparations from M₄ receptor knockout

mice and their wild-type littermates for each tissue (logEC₅₀

values ranging from 6.20 ± 0.10 to 6.76 ± 0.08), suggesting

that M₄ receptors do not participate in smooth muscle con¬

traction in these tissues. In contrast, ~2-fold higher carbachol con¬

centration was required for contraction of stomach fundus,

urinary bladder, and trachea from M₂ receptor knockout mice

(logEC₅₀ = 6.39 ± 0.05, 6.07 ± 0.06, and 6.27 ± 0.12,

respectively) than from wild-type littermates (logEC₅₀ = 6.68 ± 0.07, 6.27 ± 0.07, and 6.56 ± 0.06, respectively).

Furthermore, the affinity of the M₂ "selective" receptor antag¬

onist AF-DX-116 in inhibiting carbachol-induced smooth

muscle contraction was significantly reduced in M₄ receptor

knockout mice compared with tissues from wild-type litter¬

mates. Collectively, these results provide direct and unambig¬

uous evidence that M₂ receptors mediate muscarinic receptor¬

induced bradycardia and play a role in smooth muscle

contractility, whereas M₄ receptors are not involved in stomach

fundus, urinary bladder, or tracheal contractility.

Activation of peripheral postjunctional muscarinic recep¬

tors produces biological responses such as bradycardia and

smooth muscle contraction (Brown and Taylor, 1996). How¬

ever, the ability to attribute physiological roles to specific

native muscarinic receptor subtypes in different tissues has

become complicated by the important advances in molecular

biology identifying multiple muscarinic acetylcholine recep¬

tors (Levey, 1993). Difficulty associating a specific musca¬

rinic receptor subtype to a physiological or pathophysiology-

cal response may occur as a result of the overlapping

expression pattern of the different muscarinic receptors, lo¬

calization of multiple muscarinic receptors within a given

tissue, and/or the lack of ligands (agonists and antagonists)

with sufficient muscarinic receptor subtype selectivity or

specificity to permit conclusive assignment of receptor sub¬
type (Weas, 1996). To date, molecular cloning techniques

have led to the discovery and identification of gene products

for five distinct muscarinic receptor subtypes designated M₁

to M₅ (Kubo et al., 1986a,b; Bonner et al., 1987, 1988; Peralta

et al., 1987a,b). Four of these receptors (M₁ to M₄), corre¬

sponding to the transfected muscarinic gene products for M₁

to M₄, have been pharmacologically characterized (for re¬
eviews, see Hulme et al., 1990; Caulfield, 1993; Eglen et al.,

1996).

mRNA for the muscarinic receptor subtypes M₁ to M₄, are

expressed in peripheral tissues such as atria (Hassall et al.,

1993; Hoover et al., 1994), stomach fundus, and urinary

bladder (Eglen et al., 1996). Although mammalian heart is

thought to possess predominantly M₂ muscarinic receptors

(Caulfield, 1993), confirmed by the localization of M₂ mRNA

in rat heart by in situ hybridization (Hoover et al., 1994),

expression of M₁, M₃, and M₄ muscarinic receptor genes in

guinea pig and rat intrinsic intracardiac neurons by in situ

hybridization (Hassall et al., 1993) and in canine atrial tissue

by reverse transcription-polymerase chain reaction (Shi et al.,

1999) also has been reported. The role of these gene

products and/or their presence has not yet been associated

with any functional response in atria.

In most smooth muscle preparations where the muscarinic

receptor population was determined through antagonist ra-

ABBREVIATIONS: M₁ to M₅, muscarinic acetylcholine receptors; AF-DX 116, 11-[[2-diethylamino-O-methyl]1-piperidinyl]acetyl]-5,11-dihydrol-
6H-pyridol[2,3-b][1,4]benzodiazepine-6-one.
dil ligand-binding studies (Eglen et al., 1996), the $M_2$ receptor subtype accounted for 70 to 80% of the receptor population, and the $M_3$ receptor subtype accounted for 20 to 30% of the receptor population. The contractile response of smooth muscle to muscarinic agonists is thought to be primarily mediated by activation of $M_3$ receptors (Ehler et al., 1997). Although controversial (Eglen et al., 1996), $M_4$ receptors have been implicated in contractile responses of guinea pig gall bladder (Ozkutlu et al., 1993; Oktay et al., 1998) and guinea pig uterus (Dörje et al., 1990), raising the possibility that $M_4$ receptors may play a role in the contractile response of other smooth muscle preparations. Thus, the availability of mutant mouse strains that lack functional $M_2$ and $M_4$ receptors (Gomez et al., 1999a,b) has provided the means to examine the physiological role of muscarinic receptors in native tissue in an unequivocal manner.

The present study focused on comparing muscarinic responses of isolated peripheral tissues derived from $M_2$ and $M_4$ receptor knockout mice and their wild-type littermates. Specifically, we examined carbamylcholine-induced bradycardia in isolated atria and contraction in three different smooth muscle preparations (stomach fundus, urinary bladder, and trachea). In addition, the antagonism of carbamylcholine-induced responses by the $M_2$ receptor “selective” antagonist AF-DX 116 (11-[[2-diethylamino-O-methyl]-1-piperidinyl]acetil-5,11-dihydro-6H-pyridol[2,3-b][1,4]benzodiazepine-6-one) (Hammer et al., 1986; Giachetti et al., 1986; Micheletti et al., 1987; Del Tacca et al., 1990), also was assessed in these tissues. Our results indicate, in a direct and unambiguous fashion, that $M_2$ receptors are essential for muscarinic receptor-dependent bradycardia and contribute to muscarinic agonist-induced smooth muscle contraction. These results demonstrate the usefulness of muscarinic receptor knockout mice as tools to assess the involvement of distinct muscarinic receptor subtypes in specific physiological functions.

Materials and Methods

Animals. The generation of $M_2$ and $M_4$ muscarinic receptor knockout mice has been described previously (Gomez et al., 1999a,b). Genetically, male mice used in the present study were 129B1/CF-1 hybrids ($M_2$ receptor knockout mice and their wild-type littermates, F2 generation) or 129Sv/Ei/CF-1 hybrids ($M_4$ receptor knockout mice and their wild-type littermates, F2 generation). Animals were housed in polycarbonate ventilated cages. The animal room was maintained at 22–24°C with a relative humidity of 35 to 70% and daily light/dark cycle (0600–1800 h light). Food (laboratory rodent diet 5001; PMI Feeds, St. Louis, MO) and water were supplied ad libitum. Mice (33–58 g) were sacrificed 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: 4.6 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 118.2 mM NaCl, 10.0 mM glucose, 1.6 mM CaCl$_2$(2H$_2$O), and 24.8 mM NaHCO$_3$. Experimental protocols and procedures were approved by the Eli Lilly and Company Animal Care and Use Committee.

Atrial Preparation. Spontaneously beating left and right atria were dissected from ventricles and the left atrium was attached with thread to a stationary glass rod, whereas the right atrium was tied with thread to a force displacement transducer. The atria were placed in organ baths containing 10 ml of Krebs-bicarbonate buffer (see above-mentioned composition). The organ bath solution was maintained at 37°C and aerated with a mixture of 95% O$_2$/5% CO$_2$.

The spontaneously beating left and right atria were placed under an initial force of 0.5 g and equilibrated for 20 min during which time the tissues were washed at 5-min intervals. Atrial rate in beats per minute was measured with Sensotec transducers (model MBL55140–02; Columbus, OH) that were coupled to a Compaq desktop-compatible data acquisition system (BIOPAC Systems, Inc., Goleta, CA). Cumulative concentration-response curves to carbamylcholine ($10^{-8}$–$10^{-4}$ M) or adenosine ($10^{-6}$–$10^{-7}$ M) were generated and expressed as a percentage of the initial atrial rate.

In some experiments, AF-DX 116 was examined for its ability to antagonize the negative chronotrophic effect of cumulatively administered carbamylcholine. After initial responses were determined, atria were incubated with AF-DX 116 ($10^{-6}$ M) for 20 min. Responses to carbamylcholine ($10^{-7}$–$10^{-4}$ M) were then repeated in the presence of antagonist. Carbamylcholine-induced bradycardia was identical before and after vehicle.

Smooth Muscle Preparations. A longitudinal section of stomach fundus, the urinary bladder body (cut from the urethral opening to the dome), and a 5-mm length of trachea were prepared for in vitro examination. One end of the stomach fundus and urinary bladder was attached with thread to a stationary glass rod, whereas the other end was tied with thread to the transducer. Tracheal rings were mounted on hooks that were gently separated so that the lower hook was attached with thread to a stationary rod, and the upper hook was tied with thread to the transducer. All tissues were placed in organ baths containing 10 ml of Krebs-bicarbonate buffer (see above-mentioned composition). The organ bath solution was maintained at 37°C and aerated with a mixture of 95% O$_2$/5% CO$_2$. 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), 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. No significant differences in contractile responses to 67 mM KCl occurred among tissues from $M_2$ and $M_4$ receptor knockout mice and their wild-type littermates. Cumulative contracture concentration-response curves to carbamylcholine ($10^{-8}$–$10^{-5}$ M) were generated and expressed as a percentage of the 67 mM KCl-induced contraction determined for each tissue. On a given day, tissues from $M_2$ or $M_4$ receptor knockout mice and their wild-type littermates were used to avoid the possibility of any daily systematic effect. Experiments were performed over multiple days.

In some experiments, AF-DX 116 was examined for its ability to antagonize the contraction produced by the cumulative administration of carbamylcholine. After initial contractile responses were made, the smooth muscle preparations were incubated with AF-DX 116 ($10^{-4}$ M) for 60 min. Contractile responses to carbamylcholine ($3.0 \times 10^{-5}$-$3.0 \times 10^{-5}$ M) were then repeated in the presence of antagonist. Carbamylcholine-induced contractions were identical before and after vehicle incubation in all tissues studied.

The antagonist equilibrium dissociation constant ($K_B$) for AF-DX 116 versus carbamylcholine was determined according to the following equation (Furchgott, 1972): $K_B = [B]/(dose \times 1 - [B])$, where [B] is the concentration of the antagonist and the dose ratio is the EC$_{50}$ of the agonist in the presence of the antagonist divided by the control EC$_{50}$. EC$_{50}$ was the concentration of agonist required to elicit 50% of the maximal response. The antagonist equilibrium dissociation constant for AF-DX 116 was expressed as the negative logarithm of the $K_B$ (i.e., $-\log K_B$).

Statistical Analyses. Results were expressed as mean ± S.E. of 3 to 14 isolated tissues obtained from 3 to 14 animals. Agonist concentration-response curves were analyzed by a three-parameter logistic nonlinear model (De Lean et al., 1978). The three modeled parameters included the maximal response of the tissue, the EC$_{50}$, and the slope of the curves. Each curve was fitted with SAS (SAS...
Institute Inc., Cary, NC) on a Compaq (Deskpro 5133; Compaq, Houston, TX) personal computer. Unpaired Student’s t test was used to compare mean atrial rate, mean tissue KCl contractile response, and mean −logEC50 (EC50 was the agonist concentration for half-maximal response) values between two groups. One-way ANOVA was used to compare mean −logEC50 values among stomach fundus, urinary bladder, and trachea and Tukey test for all pairwise comparisons was performed when appropriate. Analyses were run with SigmaStat for Windows (version 2.03; SPSS Science Inc., Chicago, IL) on a Compaq personal computer (Deskpro 5133; Compaq). Comparisons were considered significant for P values of .05 or less.

**Drugs.** Carbamylcholine chloride and adenosine were purchased from Sigma Chemical Co. (St. Louis, MO). AF-DX 116 was provided by the Lilly Research Laboratories, Indianapolis, IN.

**Results**

**Basal Atrial Rate in Receptor Knockout Mice and Wild-Type Littermates.** Basal heart rates of spontaneously beating mouse atria derived from the M2 and M4 receptor knockout mice were not different from atrial rates derived from their wild-type littermates (Table 1). The similarity in basal atrial rate among the four groups suggests a lack of involvement of either M2 or M4 receptors in regulating basal sinoatrial nodal function in vitro.

**Carbamylcholine-Induced Bradycardia.** Carbamylcholine (10⁻⁸-10⁻⁵ M)-induced bradycardia was similar in spontaneously beating isolated atria from M2 (−logEC50 = 6.14 ± 0.06) and M4 (−logEC50 = 6.25 ± 0.11) wild-type mice (Fig. 1). Interestingly, carbamylcholine-induced bradycardia in atria from M4 receptor knockout mice was significantly reduced at lower carbamylcholine concentrations (3.0 × 10⁻⁸-3.0 × 10⁻⁷ M) compared with the bradycardia in atria from wild-type littermates (Fig. 2). The reduced efficacy of carbamylcholine in M4 receptor knockout mice was accompanied by a modest, and almost statistically significant reduction (P = .06) in the potency of carbamylcholine (−logEC50 = 6.02 ± 0.03) relative to the potency (−logEC50 = 6.25 ± 0.11) in atria from wild-type mice (Fig. 2). This trend toward a reduction in carbamylcholine-induced bradycardia in atria from M4 receptor knockout mice suggests that M4 receptors may be necessary to maximize the bradycardic effect produced by this agonist. Strikingly, the bradycardic activity of carbamylcholine (10⁻⁸-10⁻⁴ M) was abolished in atria derived from M2 receptor knockout mice (Fig. 3, top), even at the highest carbamylcholine concentration used (10⁻⁴ M).

**Adenosine-Induced Bradycardia.** Although heart rate of M4 receptor knockout mice was not reduced by carbamylcholine, adenosine produced a similar concentration-dependent bradycardia in atria from M4 wild-type and M4 receptor knockout mice (Fig. 3, bottom). The similar bradycardic effect of adenosine indicated that atria from M2 receptor knockout

**TABLE 1**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Mouse Genotype</th>
<th>Heart Rate (n)−beats/min</th>
<th>Knockout</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>372.0 ± 16.7 (14)</td>
<td>418.3 ± 26.9 (14)</td>
</tr>
<tr>
<td></td>
<td>M4</td>
<td>388.6 ± 17.7 (13)</td>
<td>396.0 ± 21.9 (7)</td>
</tr>
</tbody>
</table>

n, number of mice per group.

Values are mean ± S.E.
Bamylcholine concentration-response curves determined with smooth muscle derived from \( M_4 \) receptor knockout and \( M_2 \) receptor wild-type mice were virtually superimposable in all three tissues (Fig. 5). Accordingly, no differences occurred in the potency of carbachol in the stomach fundus, urinary bladder, and trachea (Table 3). These data suggest that the \( M_4 \) muscarinic receptor is not involved in postjunctional smooth muscle contraction in the stomach fundus, urinary bladder, and trachea.

Likewise, carbachol (\( 10^{-8}-10^{-5} \) M) contracted stomach fundus, urinary bladder, and trachea from \( M_2 \) receptor knockout mice and their wild-type littermates (Fig. 6). However, the potency of carbachol in all three tissues from the \( M_2 \) receptor knockout mice was reduced by a factor of \( \sim 2 \) compared with responses in \( M_2 \) wild-type mice. The differences in \( -\log EC_{50} \) values were statistically significant in the stomach fundus, urinary bladder, and trachea (Table 3). These studies suggest that \( M_2 \) receptors play a role in the contractile response of these smooth muscle preparations to muscarinic agonists.

Antagonism of Carbachol-Induced Contraction of Smooth Muscle Preparations by AF-DX 116. AF-DX 116 (\( 10^{-6} \) M) competitively inhibited carbachol-induced bradycardia in spontaneously beating atria from \( M_2 \) (top) and \( M_4 \) (middle) receptor wild-type mice and from \( M_4 \) (bottom) receptor knockout mice. Values are mean ± S.E. of the number of atria in parentheses. Absence of error bars indicates that the magnitude of error was less than the symbol size.

Fig. 3. Comparison of the negative chronotropic effect induced by carbachol (top) and adenosine (bottom) in spontaneously beating atria from \( M_2 \) receptor knockout and \( M_2 \) receptor wild-type mice. Values are mean ± S.E. of the number of atria in parentheses. Absence of error bars indicates that the magnitude of error was less than the symbol size.

Fig. 4. Effect of the selective \( M_2 \) antagonist AF-DX 116 (\( 10^{-6} \) M) on carbachol-induced bradycardia in spontaneously beating atria from \( M_2 \) (top) and \( M_4 \) (middle) receptor wild-type mice and from \( M_4 \) (bottom) receptor knockout mice. Values are mean ± S.E. of the number of atria in parentheses. Absence of error bars indicates that the magnitude of error was less than the symbol size.
Similarly, AF-DX 116 (10^{-6} M) produced a rightward shift of carbamylcholine concentration-response curves in all three smooth muscle preparations from M2 wild-type mice (Fig. 8). The magnitude of this shift was similar to the one observed with the corresponding tissues from M4 receptor knockout mice and their wild-type littermates. However, the AF-DX 116-induced rightward shift of the carbamylcholine concentration-response curves was significantly less in smooth muscle preparations derived from M2 receptor knockout mice (Fig. 8). As shown in Table 2, −logK_B values for AF-DX 116 determined in stomach fundus, urinary bladder, and trachea tissues from M2 receptor knockout mice were significantly lower than the corresponding values determined with smooth muscle preparations from wild-type littermates.

**TABLE 2**
Antagonist dissociation constant (−logK_B) for inhibition of carbamylcholine-induced responses by AF-DX 116 in mouse atria and smooth muscle

<table>
<thead>
<tr>
<th>Tissue</th>
<th>M2 Mouse Genotype</th>
<th>M4 Mouse Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>Knockout</td>
</tr>
<tr>
<td></td>
<td>−logK_B ± S.E. (n)</td>
<td>−logK_B ± S.E. (n)</td>
</tr>
<tr>
<td>Atria</td>
<td>7.23 ± 0.06 (5)</td>
<td>7.28 ± 0.04 (5)</td>
</tr>
<tr>
<td>Stomach fundus</td>
<td>6.49 ± 0.17 (4)</td>
<td>6.55 ± 0.07 (4)</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>6.47 ± 0.06 (4)</td>
<td>6.64 ± 0.14 (4)</td>
</tr>
<tr>
<td>Trachea</td>
<td>6.54 ± 0.07 (3)</td>
<td>6.53 ± 0.07 (3)</td>
</tr>
<tr>
<td></td>
<td>7.45 ± 0.11 (5)</td>
<td>5.89 ± 0.18 (4)</td>
</tr>
<tr>
<td></td>
<td>6.48 ± 0.12 (4)</td>
<td>6.12 ± 0.06 (4)</td>
</tr>
<tr>
<td></td>
<td>6.72 ± 0.11 (4)</td>
<td>6.28 ± 0.10 (4)</td>
</tr>
</tbody>
</table>

ND, not determined.

**Fig. 5.** Cumulative contractile response to carbamylcholine in stomach fundus (top), urinary bladder (middle), and trachea (bottom) from M4 receptor knockout mice and their wild-type littermates. Values are mean ± S.E. of the number of tissues in parentheses. Absence of error bars indicates that the magnitude of error was less than the symbol size.

Peripheral muscarinic receptors are involved in the regulation of many important physiological functions, including the control of heart rate, the stimulation of glandular secretion, and smooth muscle contraction (Nathanson, 1987; Hulme et al., 1990; Caulfield, 1993; Brown and Taylor, 1996). In this study, we have used M2 and M4 receptor-deficient mice (Gomeza et al., 1999a,b) to study the potential role of these two muscarinic receptor subtypes in atrial bradycardia and smooth muscle contractile responses in stomach fundus, urinary bladder, and trachea. Previous studies with mouse stomach fundus (Mizuguchi et al., 1997; Pheng et al., 1997), urinary bladder (Durant et al., 1991; Lundbeck and Sjögren, 1992), and trachea (Henry et al., 1990; Garssen et al., 1993) have demonstrated muscarinic-induced contractile responses, but have not detailed the multiple receptor subtypes involved. Furthermore, although M1, M2, and M4 receptor knockout mice have been studied (Hamilton et al., 1997; Gomeza et al., 1999a,b), there are no reports of cardiac or smooth muscle responses in isolated tissues from these mice. Carbamylcholine was selected as a prototypic nonselective muscarinic agonist not subjected to degradation by acetylcholinesterase (Roberts and Konjovic, 1969).

M2 and M4 muscarinic receptors are difficult to discriminate by classical pharmacological techniques because these receptor subtypes share similar ligand-binding and G protein-coupling properties (Hulme et al., 1990; Caulfield, 1993; Wess, 1996). The use of tissues derived from M2 and M4 receptor knockout mice therefore offers the advantage that the potential involvement of these receptors in muscarinic agonist-dependent responses can be assessed in a straightforward and unambiguous fashion (Gomeza et al., 1999a,b). Such an approach combined with the use of pharmacological tools provides a powerful means to dissect and understand the receptor mechanisms governing muscarinic tissue responses. Furthermore, in muscarinic receptor knockout mice generated to date, immunoprecipitation studies in brain and heart have not revealed alteration or compensatory changes
because heart rate is known to be cholinergically regulated (Loffelholz and Pappano, 1985), the heart rate in animals whose atria do not possess either the M₂ or M₄ receptor can shed light on receptor mechanisms controlling this important function. Interestingly, basal heart rate was unaltered in isolated atria from mice lacking the M₂ or M₄ receptor, suggesting that these receptors do not play an important role in sino-atrial function in vitro. These data are consistent with the lack of effect of atropine on spontaneously beating canine right atria in vitro (Vicenzi et al., 1995). However, the fact that heart rate also was unaltered in $\beta_1/\beta_2$-adrenergic receptor double knockout mice (Rohrer et al., 1999) may suggest that in vitro studies will not detect changes in basal heart rates.

Strikingly, atria derived from M₂ receptor knockout mice were unresponsive to the bradycardic effects of carbamylcholine. However, the negative chronotropic effects of adenosine were fully retained in atria from M₂ receptor knockout mice, indicating that the biochemical pathway that links receptor activation to reduction in atrial rate remains intact in these animals. These observations provide direct evidence that the presence of the M₂ receptor subtype is essential for carbamylcholine-induced bradycardia. Our results are consistent with previous observations indicating that the muscarinic receptor population expressed by mammalian heart almost exclusively consists of M₂ receptors (Hulme et al., 1990; Caulfield, 1993). In agreement with these findings, $[^3H]$quinuclidinyl benzilate (a nonselective muscarinic antagonist)-binding activity was shown to be almost completely abolished in hearts derived from M₂ receptor knockout mice (Gomeza et al., 1999a). Moreover, functional studies with subtype-“selective” muscarinic antagonists suggested that muscarinic control of cardiac pacemaker activity is mediated by the M₂ receptor subtype (Hulme et al., 1990; Caulfield, 1993). Consistent with these observations, we showed in the present study that AF-DX 116 antagonized carbamylcholine-induced bradycardia in atria derived from wild-type mice with an inhibitory antagonist dissociation constant consistent with M₂ receptor blockade (Table 2).

Interestingly, in low concentrations, bradycardic effects of carbamylcholine ($3.0 \times 10^{-8}$-$3.0 \times 10^{-7}$ M) were significantly reduced in atria derived from M₄ receptor knockout mice compared with atrial preparations derived from wild-type littermates. This observation raises the possibility that M₄ receptors may play a modulatory role in muscarinic regulation of cardiac pacemaker activity. However, this modulatory activity appears to require the presence of functional M₂ receptors because carbamylcholine-induced bradycardia was completely abolished in M₂ receptor knockout mice. Although mammalian heart predominantly expresses M₂ receptors, M₄ receptor mRNA is present in guinea pig and rat intracardiac neurons (Hassall et al., 1993; Hoover et al., 1994) as well as in canine atrial myocytes (Shi et al., 1999). Clearly,
however, the potential involvement of the M₄ receptor subtype in modulating atrial rate to M₂ receptor agonists (at least in the mouse) will require more rigorous studies, including the demonstration that M₄ receptors are indeed expressed in mouse heart. This issue will be addressed in future studies.

Another major physiological function mediated by peripheral muscarinic receptors is the contraction of smooth muscle. Most smooth muscle preparations express multiple muscarinic receptor subtypes (Caulfield, 1993; Eglen et al., 1996; Ehlert et al., 1997). In most cases, smooth muscle expressed a major population of M₂ receptors with a clearly lower density of M₃ receptors (Caulfield, 1993; Eglen et al., 1996; Ehlert et al., 1997). However, although controversial, other muscarinic receptor subtypes, including the M₄ receptor (Dörje et al., 1990, 1991a; Ozkutlu et al., 1993; Oktay et al., 1998), also have been detected in some smooth muscle preparations by pharmacological and molecular techniques (for reviews, see Levey, 1993; Eglen et al., 1996).

To understand the roles of M₂ and M₄ muscarinic receptors in smooth muscle contraction, we examined carbamylcholine-induced contractile responses in stomach fundus, urinary bladder, and tracheal smooth muscle preparations derived from M₄ receptor knockout mice and their wild-type littermates. Carbamylcholine concentration-response curves in tissues from M₄ receptor-deficient mice were virtually superimposable with those obtained with the corresponding preparations derived from wild-type littermates. This finding clearly indicates that M₄ receptors do not play a role in modulating carbamylcholine-dependent contraction in the three tissues studied.

In contrast, carbamylcholine was significantly less potent (by a factor of ∼2) in contracting stomach fundus, urinary bladder, and trachea from M₂ receptor knockout mice compared with the corresponding preparations from wild-type littermates. These data clearly indicate that M₂ receptors participate in smooth muscle contraction to carbamylcholine. Note that contractile responses to 67 mM KCl were similar in these smooth muscle tissues from M₂ and M₄ receptor knockout mice and their wild-type littermates. However, in spite of the lack of functional M₂ receptors in smooth muscle from M₂ receptor knockout mice, carbamylcholine, although less potent, was still capable of eliciting maximal contractile responses in all three preparations. This observation is consistent with previous pharmacological studies suggesting that muscarinic receptor-induced contraction is primarily mediated by M₃ muscarinic receptors (Caulfield, 1993; Eglen et al., 1996; Ehlert et al., 1997).

In agreement with these findings, studies with AF-DX 116 resulted in a negative logarithm antagonist dissociation constant in smooth muscle from M₂ receptor knockout mice that was significantly lower than the negative logarithm antago-
nist dissociation constant in tissues from wild-type control animals. The lower affinity of AF-DX 116 \((-\log K_d)\) values of 5.89–6.28) in smooth muscle from M2 receptor knockout mice was consistent with the affinity of AF-DX 116 at M3 receptors (Table 4) and with the notion that smooth muscle contraction in these mice was mediated by the M3 subtype (Hulme et al., 1990; Caulfield, 1993; Eglen et al., 1996; Ehlert et al., 1997). Furthermore, if the antagonist dissociation constant for AF-DX 116 at cloned M2 and M3 receptors approximates 100 and 1000 nM, respectively (Table 4), the intermediate value \((\sim 320 \text{ nM})\) for the antagonist dissociation constant of AF-DX 116 in tissues from the M2 wild-type mice supports the contention that both M2 and M3 receptors participate in the contractile response to muscarinic agonists in the stomach fundus, urinary bladder, and trachea. Thus, use of smooth muscle from the M2 knockout mice provides a useful model for the study of physiological responses at M3 receptors.

In conclusion, this study highlights the usefulness of receptor knockout mice in combination with pharmacological tools to study the role of specific muscarinic receptor subtypes present in peripheral tissues. We demonstrated, in a direct and unequivocal manner, that muscarinic receptor-dependent bradycardia requires the presence of functional M2 receptors. Our data also suggest that M4 receptors may play a minor, facilitatory role in M2 receptor-mediated bradycardia, although this observation needs to be confirmed by

![Graphs showing effects of AF-DX 116 on smooth muscle contraction](image)

**Table 4**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Muscarinic Receptor Subtype</th>
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<tr>
<td></td>
<td>M1</td>
<td>M2</td>
</tr>
<tr>
<td>[^{3}H]NMS</td>
<td>5.89</td>
<td>6.73</td>
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<tr>
<td>[^{3}H]NMS</td>
<td>6.37</td>
<td>7.26</td>
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<td>[^{3}H]MQNB</td>
<td>ND</td>
<td>7.17</td>
</tr>
<tr>
<td>Mean</td>
<td>6.96</td>
<td>7.07</td>
</tr>
</tbody>
</table>

NMS, N-methylscopolamine; QNB, quinuclidinybenzilate; MQNB, N-methyl-3-QNB. ND, not determined.
more detailed studies. Finally, the present study demonstrates that although M₂ receptors are the predominant muscarinic subtype mediating contraction in stomach fundus, urinary bladder, and trachea, M₄ but not M₁ muscarinic receptors also play a role in carbachol-induced contraction.

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References


