Contractions of the Mouse Prostate Elicited by Acetylcholine Are Mediated by M₃ Muscarinic Receptors

Carl W. White, Jennifer L. Short, John M. Haynes, Minoru Matsui, and Sabatino Ventura

ABSTRACT
Increased smooth muscle tone in the human prostate contributes to the symptoms associated with benign prostatic hyperplasia. In the mouse prostate gland, cholinergic innervation is responsible for a component of the nerve-mediated contractile response. This study investigates the muscarinic receptor subtype responsible for the cholinergic contractile response in the mouse prostate gland. To characterize the muscarinic receptor subtype, mouse prostates taken from wild-type or M₃ muscarinic receptor knockout mice were mounted in organ baths. The isometric force that tissues developed in response to electrical-field stimulation or exogenously applied cholinergic agonists in the presence or absence of a range of muscarinic receptor antagonists was evaluated. Carbachol elicited reproducible concentration-dependent contractions of the isolated mouse prostate, which were antagonized by the presence of muscarinic receptor antagonists. Calculation of antagonist affinities (pA₂ values) indicated a rank order of antagonist potencies in the mouse prostate of: darifenacin (9.08) = atropine (9.07) > 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (9.02) > cyclohexyl-hydroxy-phenyl-(3-piperidin-1-ylpropyl)silane (7.85) > cyclohexyl-(4-fluorophenyl)-hydroxy-(3-piperidin-1-ylpropyl)silane (7.39) > himbacine (7.19) > pirenzepine (6.88) > methoctramine (6.20). Furthermore, genetic deletion of the M₃ muscarinic receptor inhibited prostatic contractions to electrical-field stimulation or exogenous administration of acetylcholine. In this study we identified that the cholinergic component of contraction in the mouse prostate is mediated by the M₃ muscarinic receptor subtype. Pharmacological antagonism of the M₃ muscarinic receptor may be a beneficial additional target for the treatment of benign prostatic hyperplasia in the human prostate gland.

Introduction
Muscarinic receptors are G protein-coupled receptors consisting of five subtypes denoted M₁–M₅, which are activated by the endogenous agonist acetylcholine. Muscarinic receptors can be divided into two groups depending on their G protein-coupling properties. M₁, M₂, and M₅ receptor subtypes couple to Gq/11 proteins, and signal through the inositol phosphate pathway, whereas the M₂ and M₄ receptor subtypes couple to Gi/o proteins and inhibit adenylyl cyclase activity. In the prostate gland, cholinergic innervation acting at muscarinic receptors in the glandular epithelium is thought to be responsible primarily for the secretion of prostatic fluids (Ventura et al., 2006), we have shown a significant cholinergic component of the nerve-mediated contractile response whereby acetylcholine acts at muscarinic receptors to mediate contraction (White et al., 2010). Currently, the muscarinic receptor subtype mediating the contractile response in the mouse prostate gland is unknown.

In the canine prostate, M₂ muscarinic receptors are responsible for mediatory contraction (Fernández et al., 1998). In contrast, M₁ muscarinic receptors mediate the facilitation of contraction in the guinea pig prostate (Lau et al., 2000). In the rat prostate, the M₃ muscarinic receptor subtype has been reported to mediate contractile responses to carbachol (Lau and Pennefather, 1998). Moreover, immunohistochemical studies of the rat prostate have shown colocalization of the M₃ muscarinic receptor subtype with the prostatic

ABBREVIATIONS: M₃R, M₃ muscarinic receptor; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; ANOVA, analysis of variance; HHSID, cyclohexyl-hydroxy-phenyl-(3-piperidin-1-ylpropyl)silane; p-F-HHSID, cyclohexyl-(4-fluorophenyl)-hydroxy-(3-piperidin-1-ylpropyl)silane.
smooth muscle (Nadelhaft, 2003). However, a number of studies have shown that subtype-specific muscarinic receptor antibodies are largely nonspecific (Jositsch et al., 2009; Michel et al., 2009; Pradidarcheep et al., 2009). Previous immunohistochemical studies of the prostate using muscarinic subtype-specific antibodies should be viewed cautiously without additional functional evidence for the role of a particular muscarinic receptor subtype in the prostate.

Both the M₂ and M₄ muscarinic receptor subtypes have been observed in binding studies of whole human prostate homogenates (Aniszuzzaman et al., 2008). However, Ruggieri et al. (1995) observed only the M₁ muscarinic subtype, which was found to be localized immunohistochemically in the epithelium. In cultures of human prostatic smooth muscle cells only M₃, M₅, and M₄ muscarinic receptor subtype mRNA expression is observed (Obara et al., 2000), with the M₁ muscarinic receptor being the most abundant. In a separate study of human prostatic smooth muscle cell cultures, M₂ muscarinic receptors were observed by radioligand binding experiments and mediated a decrease in cAMP accumulation (Yazawa et al., 1994).

In the mouse prostate both the clinically selective M₃ muscarinic receptor antagonists darifenacin and solifenacin inhibited [³H]N-methylscopolamine binding after oral administration, indicating a population of muscarinic receptors (Oki et al., 2005; Yamada et al., 2006). With the availability of muscarinic receptor subtype knockout mice the M₁ and M₃, but not the M₂, M₄, or M₅ muscarinic receptor subtypes were shown to be present in mouse prostate by [³H]N-methylscopolamine binding studies (Ito et al., 2009). However, the location and function of either the M₁ or M₃ muscarinic receptor subtypes present in the mouse prostate were not elucidated. The muscarinic receptor subtype mediating contraction in the mouse prostate is of interest because an increase in prostatic smooth muscle tone contributes to the lower urinary tract symptoms associated with benign prostatic hyperplasia in humans. The aim of this study was to pharmacologically characterize the muscarinic receptor subtype mediating the cholinergic contractile response in the mouse prostate gland and to confirm the muscarinic receptor subtype by using muscarinic receptor knockout mice.

Materials and Methods

Animals. Heterozygous [M₃R+/−] breeding pairs of adult M₃ muscarinic receptor knockout [M₃R−/−] mice were purchased from the Centre for Animal Resources and Development (Kumamoto University, Kumamoto, Japan), and a colony was established at the Monash Animal Services facility (Clayton, Australia). Age-matched littersmates were used at 10 weeks for experimentation and obtained by matings of M₃R+/− breeding pairs maintained on a B6.D2 background. The resulting litters of M₃R+/−, M₃R+/+, and wild-type [M₃R+/+] mice were routinely genotyped by polymerase chain reaction using genomic DNA from tail samples obtained at weaning (21 days) as described previously (Matsui et al., 2000). For pharmacological characterization experiments wild-type adult male mice (≥ 8 weeks) from a C57BL/6 background were used. All mice were bred and housed at the Monash Animal Services facility, were exposed to a 12-h light/dark photoperiod, and had free access to food and water. Mice were weighed before being sacrificed by cervical dislocation. Prior approval for animal experimentation was granted by the Monash University Standing Committee on Animal Ethics, ethics numbers VCPA 2009/14 and VCPA 2009/15 for the use of genetically modified and wild-type mice, respectively. All studies conformed to the National Health and Medical Research Council, Australian code of practice for the care and use of animals for scientific purposes.

Isolated Organ Bath Studies. The whole mouse ventral prostate was obtained by an incision along the midline of the lower abdomen that exposed the urogenital tract. To reveal the prostate, the penile muscles, excess fat, and connective tissue were cut away. The prostate was then carefully dissected out and placed in a specimen jar containing Krebs-Henseleit solution (118.1 mM NaCl, 25.0 mM NaHCO₃, 11.7 mM glucose, 4.69 mM KCl, 1.2 mM KH₂PO₄, 0.5 mM MgSO₄, and 2.5 mM CaCl₂, pH 7.4). The prostate was mounted in a 10-ml water-jacketed organ bath containing Krebs-Henseleit solution maintained at 37°C and bubbled with 95% O₂/5% CO₂. One end of the tissue was attached to a perspex tissue holder and the other to a Grass FT03 force displacement transducer (Grass Instruments, Quincy, MA) for the measurement of isometric contractions. The force of contraction was recorded using a PowerLab 4/SP data acquisition system (ADInstruments Pty Ltd., Castle Hill, Australia) and LabChart software version 5 (ADInstruments Pty Ltd.). Tissues were maintained under a resting force of approximately 0.7g. Before experimentation tissue preparations were equilibrated for 1 h, during which time prostates were stimulated with electrical pulses of 0.5-ms duration, 60 V at 0.01 Hz. Electrical stimulation occurred via two parallel platinum electrodes incorporated into the tissue holder, connected to a Grass S88 stimulator (Grass Instruments). After experimentation, prostates were removed from the organ baths, blotted dry, and weighed.

Muscarinic Receptor Characterization. After the equilibration period the prostates from wild-type C57BL/6 mice were exposed to a priming dose of 1 mM carbamylcholine chloride (carbachol) to ensure reproducible responses. Once a maximum contractile response was observed the tissue was washed four times with the volume of the organ bath. After a 30-min recovery period an initial discrete half-log concentration response curve to carbachol (10 nM-30 μM) was then constructed with 15-min intervals between drug additions, whereby for each concentration the response was allowed to reach a maximum and plateau before the tissue was washed out with four times the volume of the organ bath. To assess confounding effects on potency by α₁-adrenoceptors or cholinesterase activity, an appropriate time control curve to carbachol was constructed in the presence or absence of prazosin (0.3 μM) or physostigmine (10 μM). In subsequent experiments to prevent any confounding effects by α₁-adrenoceptors on characterization of the muscarinic receptor subtype, prazosin (0.3 μM) was added to the Krebs-Henseleit solution. To characterize the muscarinic subtype mediating contraction, after the initial concentration response curve, isolated prostates were exposed to a muscarinic receptor antagonist for 1 h before a second concentration response curve to carbachol (10 nM-1 mM) was constructed in the same manner. The muscarinic receptor antagonist was replaced after each wash.

Effect of M₃ Muscarinic Receptor Deletion on Nerve-Mediated Contraction. Frequency response curves were constructed in prostates taken from M₃R−/−, M₃R+/−, and M₃R+/+ mice after the equilibration period. Prostates were exposed to electrical-field stimulation at frequencies of 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 Hz (0.5-ms duration, 60 V) delivered at 10-min intervals in trains of pulses lasting 10 pulses or 10 s. An initial control frequency response curve was constructed to determine the contractile response of the tissue at each frequency. A second frequency response curve was then constructed after the prostate had been washed three times with the volume of the organ bath and exposed to atropine (1 μM) for 1 h. When a third frequency response curve was constructed, the prostate was washed as described previously and exposed to atropine (1 μM) plus prazosin (0.3 μM). After the final frequency response curve the prostate was washed again and allowed to rest for 1 h before the Krebs-Henseleit solution was replaced with a high K⁺ (80 mM) Krebs-Henseleit solution to measure the nonspecific contractile potential of the preparation.
Effect of M3 Muscarinic Receptor Deletion on Acetylcholine-Mediated Contraction. After the 1-h equilibration period, an initial discrete concentration response curve to acetylcholine (10 nM-1 mM), as described previously for carbachol, was constructed in prostates taken from M3R(−/−), M5R(+/−), and M3R(+/+) mice. After the initial concentration response curve, a second concentration response curve was constructed in the same manner; however, the tissue was exposed to atropine (1 μM) for 1 h before the first addition of acetylcholine and was replaced after each wash. In a separate experiment, using prostates taken from M3R(−/−) mice, the effect of mecamylamine (30 μM) on acetylcholine concentration response curves (control, 100 nM-1 mM) in the presence of mecamylamine, 100 nM-10 mM) was observed.

Data Analysis. The peak force (g) elicited by electrical-field stimulation or the exogenous application of muscarinic receptor agonists was measured at each frequency or concentration, in the presence and absence of antagonists. Mean frequency and concentration response curves were constructed using Prism version 5.00 for Windows (GraphPad Software, Inc., San Diego, CA). The mean curves were formed from the average of data from n experiments, where n is equal to the number of mice used. Results are expressed as mean ± S.E.M.

For characterization studies, the contractile responses to carbachol were expressed as a percentage of the maximum response of the initial concentration response curve. The nonlinear regression function of Prism version 5.00 was then used to fit an unconstrained sigmoidal concentration response curve to the data. For each antagonist, concentration ratios were determined by dividing the EC50 sigmoidal concentration response curve to the data. For each antagonism of Prism version 5.00 was then used to fit an unconstrained sigmoidal concentration response curve to the data. For each antagonist, concentration ratios were determined by dividing the EC50 value of carbachol in the presence of the antagonist by the EC50 value in the absence of the antagonist. After which, Arunlakshana-Schild plots were constructed whereby log (concentration ratio-1) was plotted versus log (antagonist concentration) (Arunlakshana and Schild, 1959). When the slope of the linear regression did not differ from unity, the slope was constrained to 1, and the pA2 value was calculated. Correlation and linear regression analysis was used to determine the muscarinic receptor subtype mediating contraction of the mouse prostate. pA2 values of the antagonists at the muscarinic receptors in the mouse prostate were compared against the mean published antagonist pK2 or pK3 values at each of the five muscarinic receptor subtypes (Table 1). Ionic strength of assay buffer is known to influence the binding affinity of muscarinic receptor antagonists (Loury et al., 1999). As such, pK2 or pK3 values were selected from Loury et al. (1999) and the International Union of Basic and Clinical Pharmacology G protein-coupled receptor database only from studies using buffers of comparable ionic strength to the Krebs-Henseleit solution used in this study. Where different k values reported for muscarinic receptor antagonists at the five muscarinic receptor subtypes (Table 1). Ionic strength of assay buffer is known to influence the binding affinity of muscarinic receptor antagonists (Loury et al., 1999). As such, pK2 or pK3 values were selected from Loury et al. (1999) and the International Union of Basic and Clinical Pharmacology G protein-coupled receptor database only from studies using buffers of comparable ionic strength to the Krebs-Henseleit solution used in this study. Where different antagonist affinities were given for the same subtype, the mean value was used for the correlation analysis. Pearson correlation coefficients (r) and associated p values, as well as the slope of linear regression, were calculated using Prism version 5.00.

In mean frequency and concentration response curves, differences between the initial and subsequent drug-exposed curve were analyzed by Prism version 5.00 using a two-way, repeated-measure analysis of variance (ANOVA), followed by a Bonferroni post-test where required. The p values stated were used to evaluate the statistical significance of any difference between the two curves and represent the probability of the drug treatment causing a change. p < 0.05 was considered significant. All receptor nomenclature conforms to the International Union of Basic and Clinical Pharmacology nomenclature guidelines.

Reagents Used. (+)-Himbacine, acetylcholine chloride, atropine sulfate, carbamoylcholine chloride (carbachol), eserine hemisulfate salt (physostigmine), mecamylamine hydrochloride, methoctramine hemihydrate, and prazosin hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO), L.1-Dimethyl-4-diphenylacetoxypiperidin iodide (4-DAMP), cyclohexyl-hydroxy-phenyl-(3-piperidin-1-ylpropyl)silane (HHSilD), and cyclohexyl-(4-fluorophenyl)-hydroxy-(3-piperidin-1-yl propyl)silane (p-F-HHSilD) were obtained from Sigma/RBI (Natick, MA). Darifenacil hydrobromide was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and pirenzepine dihydrochloride was from Boehringer Ingelheim (Artamon, Australia). All drugs were dissolved and diluted in distilled water except HHSiD, which was dissolved in 10% dimethyl sulfoxide; all further dilutions were made in distilled water.

Results

Pharmacological Characterization. In the isolated wild-type mouse, prostate administration of exogenously applied carbachol (10 nM-1 mM) produced reproducible concentration-dependant contractile responses with a −log (EC50) of 5.99 ± 0.05 (n = 18; p = 0.95). The contractile response to carbachol was unaffected by physostigmine (10 μM; Fig. 1A; p = 0.20); however, prazosin (0.3 μM; Fig. 1B; p < 0.05) caused a 3-fold rightward shift in the concentration response curve to carbachol.

The muscarinic receptor antagonists used were: 4-DAMP (3, 10, and 30 nM), atropine (6, 20, and 60 nM), p-F-HHSiD (0.3, 1, and 3 μM), HHSiD (0.1, 0.3, and 1 μM), darifenacil (Fig. 2; 10, 30, and 300 nM), himbacine (0.1, 0.3, and 1 μM), methoctramine (1, 3, and 10 μM), and pirenzepine (0.6, 1, and 3 μM). All caused concentration-dependent parallel rightward shifts in the concentration response curve to carbachol. The slopes and pA2 values calculated by Arunlakshana-Schild analysis are shown in Table 2. None of the antagonists produced a slope that was significantly different from unity (Fig. 3; p > 0.05). The rank order of antagonist affinities (pA2 values) at the muscarinic receptor mediating contraction in the isolated mouse prostate (Table 2) was: darifenacil.[table]

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>9.4, r</td>
<td>9.0, r</td>
<td>9.3, r</td>
<td>9.7, r</td>
<td>9.4, r</td>
</tr>
<tr>
<td>Darifenacil</td>
<td>7.8, h</td>
<td>7.0, h</td>
<td>8.8, h</td>
<td>7.7, h</td>
<td>8.0, h</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>8.9, r</td>
<td>8.2, r</td>
<td>9.2, r</td>
<td>9.1, r</td>
<td>8.9, r</td>
</tr>
<tr>
<td>Himbacine</td>
<td>6.7, h</td>
<td>7.9, h</td>
<td>6.9, h</td>
<td>7.8, h</td>
<td>6.1, h</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>6.7–7.0*, h (6.85)</td>
<td>7.3*, h</td>
<td>6.1–6.3*, h (6.2)</td>
<td>7.0*, h</td>
<td>6.3*, h</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>7.8, h</td>
<td>6.6–6.4, h (6.2)</td>
<td>6.6, h</td>
<td>7.3, h</td>
<td>6.6, h</td>
</tr>
<tr>
<td>p-F-HHSiD</td>
<td>7.5, h</td>
<td>6.4, h</td>
<td>7.6, h</td>
<td>7.3, h</td>
<td>6.3, h</td>
</tr>
<tr>
<td>HHSiD</td>
<td>7.7, h</td>
<td>6.7–6.8, h (6.75)</td>
<td>7.7, h</td>
<td>7.7, h</td>
<td>6.8, h</td>
</tr>
</tbody>
</table>

* and h indicate studies using tissues containing muscarinic receptors taken from rats or humans, respectively.

Values shown are selected pK2 or pK3 values at each of the five muscarinic receptor subtypes. Where a range is given the mean value in parentheses was used for the correlation analysis. Only estimates obtained from studies that used a physiological buffer are included here.
Deletion of the M₃ muscarinic receptor resulted in a reduction in mouse weight (Fig. 5A; \( p < 0.001 \)); however, no effect on prostate weight was observed (Fig. 5B). Furthermore, the contractile response to high K⁺ (80 mM) Krebs-Henseleit was reduced in prostates taken from M₃R(-/-) mice compared with prostates taken from M₃R(+/+) mice (Fig. 5C; \( p < 0.01 \)).

In prostates taken from M₃R(+/+) and M₃R(-/-) mice, electrical-field stimulation (0.1–20 Hz) elicited frequency-dependent contractions. Contractile responses were reduced in prostates taken from M₃R(-/-) mice but not M₃R(+/+) mice compared with prostates taken from M₃R(+/+) mice (Fig. 6). A maximum inhibition of contraction of 76% for M₃R(-/-) mice compared with M₃R(+/+) mice was observed at 2 Hz. Furthermore, atropine (1 \( \mu M \))

**TABLE 2**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Slope ± S.E.M.</th>
<th>( \mu A_2 ) ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>0.93 ± 0.04</td>
<td>9.07 ± 0.02</td>
</tr>
<tr>
<td>Darifenacin</td>
<td>1.02 ± 0.24</td>
<td>5.22 ± 0.57</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>1.01 ± 0.35</td>
<td>2.66 ± 0.11</td>
</tr>
<tr>
<td>Himbacine</td>
<td>0.98 ± 0.06</td>
<td>2.66 ± 0.11</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>0.65 ± 0.18</td>
<td>1.34 ± 0.08</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>0.15 ± 0.38</td>
<td>2.66 ± 0.11</td>
</tr>
<tr>
<td>p-F-HHSiD</td>
<td>1.16 ± 0.05</td>
<td>7.39 ± 0.05</td>
</tr>
<tr>
<td>HHSiD</td>
<td>0.91 ± 0.21</td>
<td>7.85 ± 0.07</td>
</tr>
</tbody>
</table>

**Fig. 1.** Left, mean log concentration response curves to carbachol (Cch) in the isolated mouse prostate expressed as a percentage of the maximum response in the initial curve before and after the administration of physostigmine (10 \( \mu M \)) (A) or prazosin (0.3 \( \mu M \)) (B). Points represent mean force ± S.E.M. (\( n = 6 \)), \( p \) values determined by a two-way, repeated-measures of ANOVA represent the probability of a significant interaction between concentration and treatment. Right, histograms represent the mean ± S.E.M. (\( n = 6 \) maximum contractile response in the absence (filled bars) or presence (open bars) of physostigmine (10 \( \mu M \)) (A) or prazosin (0.3 \( \mu M \)) (B).

(9.08) = atropine (9.07) > 4-DAMP (9.02) > HHSiD (7.85) > p-F-HHSiD (7.39) > himbacine (7.19) > pirenzepine (6.88) > methoctramine (6.20).

Correlation and linear regression analysis revealed significant correlation and linear regression for the M₃ (Fig. 4C; \( r = 0.98; p < 0.001 \); slope = 1.05 ± 0.08) and M₅ (Fig. 4E; \( r = 0.88; p < 0.01 \); slope = 1.02 ± 0.22) muscarinic receptor subtypes; whereas weaker linear regressions and correlations were observed for the M₁ (Fig. 4A; \( r = 0.77; p < 0.05 \); slope = 0.64 ± 0.21), M₂ (Fig. 4B; \( r = 0.51 \); slope = 0.44 ± 0.30), and M₄ (Fig. 4D; \( r = 0.78; p = 0.05 \); slope = 0.66 ± 0.22) subtypes.

**Genetic Confirmation.** Deletion of the M₃ muscarinic receptor resulted in a reduction in mouse weight (Fig. 5A; \( p < 0.001 \)); however, no effect on prostate weight was observed (Fig. 5B). Furthermore, the contractile response to high K⁺ (80 mM) Krebs-Henseleit was reduced in prostates taken from both M₃R(+/-) and M₃R(-/-) mice (Fig. 5C; \( p < 0.01 \)).

In prostates taken from M₃R(+/-), M₃R(+/-), and M₃R(-/-) mice, electrical-field stimulation (0.1–20 Hz) elicited frequency-dependent contractions. Contractile responses were reduced in prostates taken from M₃R(-/-) mice but not M₃R(+/+) mice compared with prostates taken from M₃R(+/+) mice (Fig. 6). A maximum inhibition of contraction of 76% for M₃R(-/-) mice compared with M₃R(+/+) mice was observed at 2 Hz. Furthermore, atropine (1 \( \mu M \))...
attenuated electrical-field stimulation-mediated contraction in prostates taken from both M3R(H11001/H11001/H11001) (Fig. 7A; \(p < 0.001\)) and M3R(H11001/H11001/H11002) mice (Fig. 7B; \(p < 0.001\)) with a maximum inhibition of contraction observed at 5 Hz of 76 and 70%, respectively. However, atropine (Fig. 7C; \(p = 0.54; 1 \mu M\)) had no effect on the magnitude of contraction in prostates taken from M3R(H11002/H11002/H11002) mice, whereas prazosin (Fig. 7C; \(p < 0.001; 0.3 \mu M\)) abolished the residual noncholinergic contraction.

The administration of the endogenous agonist acetylcholine (10 nM-100 \(\mu M\)) elicited concentration-dependent contractile responses (Fig. 8A) that were inhibited by atropine (1 \(\mu M\)) in prostates taken from M3R(H11001/H11001/H11001) and M3R(H11001/H11001/H11002) mice. A slightly decreased potency for acetylcholine was observed in prostates taken from M3R(H11002/H11002/H11002) mice [Fig. 8A; \(-\log(EC_{50}) = 6.46; 95\%\) confidence intervals 6.31–6.68]. The maximum contractile response was unchanged (Fig. 8A; \(p = 0.83\)). In prostates taken from M3R(H11002/H11002/H11002) mice acetylcholine elicited a small contractile response at concentrations higher than 10 \(\mu M\) (Fig. 8A). This response was unaffected by the addition of atropine (Fig. 8B; \(p = 0.47; 1 \mu M\)), but was abolished by mecamylamine (Fig. 8C; \(p < 0.001; 30 \mu M\)).

**Discussion**

We have previously identified that in the mouse prostate gland acetylcholine acting at muscarinic receptors mediates a nonadrenergic contractile response; however, the muscarinic receptor subtype mediating this response was not elucidated. Furthermore, it has been reported that both the M1 and M3 muscarinic receptor subtypes are present in the mouse prostate (Ito et al., 2009); however, their locations and functions are unknown. This study used pharmacological and genetic techniques to examine the muscarinic receptor subtype responsible for the functional contractile response.

The contractile response to carbachol was unaffected by cholinesterase activity and was therefore seen as more suitable than acetylcholine for use in the pharmacological characterization study because it eliminated this confounding factor. However, the response to carbachol was inhibited by the \(\alpha_1\)-adrenoceptor antagonist prazosin, suggesting that carbachol facilitates the adrenergic response. This is potentially caused by the release of noradrenaline mediated by prejunctional muscarinic receptors on adrenergic nerves or nicotinic receptor activation of postganglionic adrenergic nerves. In the guinea pig prostate, cholinergic agonists facil-
itate nerve-mediated contraction hypothesized to be mediated by noradrenaline release by prejunctional muscarinic receptors (Lau et al., 2000). In contrast, in both the human (Hedlund et al., 1985) and canine (Arver and Sjöstrand, 1982) prostate, muscarinic receptors produce a slight reduction in noradrenaline release. Likewise, sympathetic neurotransmitter release is inhibited in the mouse atria, urinary bladder, and vas deferens by muscarinic receptors (Trendelenburg et al., 2005). In the present study, atropine had no detectable effect on the contractile response to high concentrations of acetylcholine in prostates taken from M3R+/+ mice, whereas the noncompetitive nicotinic antagonist mecamylamine abolished the contractile response. This suggests that postganglionic nicotinic receptors rather than prejunctional muscarinic receptors mediate the release of neuronal noradrenaline.

Correlation plots show that the antagonist affinities obtained in the pharmacological characterization studies correlate most noticeably with the binding affinities of the antagonists at the M3 muscarinic receptor subtype. Progressively poorer correlations were observed for the M5, M4, M1, and M2 muscarinic receptor subtypes. The slope of the regression analysis was also used to determine the muscarinic receptor subtype, with a slope of the line closest to unity \((x = y)\) considered to be the best correlation. The best regression analysis was observed for the M3 and M5 muscarinic receptor subtypes. Taken together, these results are indicative of the M3 muscarinic receptor subtype mediating contraction in the mouse prostate gland. These results are consistent with findings in the rat prostate, where the M3 muscarinic receptor subtype mediates contraction (Lau and Pennefather, 1998) and is localized in the stromal tissue (Nadelhaft, 2003). However, this is in contrast to findings in the human (Yazawa et al., 1994) and canine (Fernández et al., 1998) prostates where M2 muscarinic receptors are thought to mediate contraction and in the guinea pig prostate where M1 muscarinic receptors facilitate contraction (Lau et al., 2000).

It should be noted that a good correlation, with poor linear regression, was also observed for the M1 subtype. Given that a population of M1 receptors has previously been reported in the mouse prostate (Ito et al., 2009) this is intriguing. At the M1 muscarinic receptor the binding affinities for all of the antago-

Fig. 6. Comparison of mean contractile responses to electrical-field stimulation (0.5 ms, 60 V, 0.1–20 Hz, 10-s pulses) in prostates taken from wild-type mice [M3R+/+], open bars, M3R muscarinic receptor heterozygous mice [M3R+/−], gray bars, and M3R muscarinic receptor knockout mice [M3R−/−], black bars. Bars represent mean force \(±\) S.E.M. \((n = 6)\). Values determined by a two-way, repeated-measures of ANOVA represent the probability of the deletion causing a significant change in response.

Fig. 7. Mean contractile responses to electrical-field stimulation (0.5 ms, 60 V, 1–20 Hz, 10-s pulses) in prostates taken from wild-type mice [M3R+/+] (A), M3R muscarinic receptor heterozygous mice [M3R+/−] (B), and M3R muscarinic receptor knockout mice [M3R−/−] (C) in the absence (open bars) or presence of atropine (1 μM) (closed bars) (A–C) as well as atropine (1 μM) plus prazosin (0.3 μM) (gray bars) (C). Bars represent mean force \(±\) S.E.M. \((n = 6)\). Values determined by a two-way, repeated-measures of ANOVA represent the probability of the drug treatment causing a significant change.
nists used, except darifenacin and pirenzepine, are close to the binding affinities for the M₃ muscarinic receptor subtype (http://www.iuphar-db.org/DATABASE/FamilyMenuForward?familyId=2). Therefore darifenacin and pirenzepine impart the greatest strength when differentiating between M₁ and M₃ muscarinic receptors. The binding affinity for darifenacin (pA₂ = 9.08) exclusively suggests the M₃ subtype over the M₁. For pirenzepine, however, the binding affinity (pA₂ = 6.88) falls within the range for the M₃ subtype and somewhat within the range of the M₁ subtype. The Schild slope (Table 2) for pirenzepine is steeper than what is expected of a truly competitive antagonist, albeit not significantly. The lack of statistical significance in this difference may be the result of a broad S.E.M. (± 0.38) confounding the analysis and can be explained by the concentration range of antagonist used (0.6–3 μM) being insufficient large (Kenakin, 2009). When the binding affinity is calculated as a pKᵣ value, instead of a pA₂ value using the equation described by Furchgott (1972), the binding obtained is 5.89, which is well within the range of the M₃ but not the M₁ muscarinic receptor subtype.

Likewise, good linear regression analysis was observed for both the M₃ and M₅ subtypes. This is unsurprising given the dearth of muscarinic receptor antagonists capable of distinguishing between M₃ and M₅ muscarinic receptors. Given that Ito et al. (2009) failed to find a significant population of M₅ receptors in the mouse prostate gland, in addition to the results presented here using M₃ muscarinic receptor knockout mice, it seems unlikely that M₅ muscarinic receptors mediate contraction.

M₃R(−/−) mice were used to confirm the muscarinic receptor subtype mediating the cholinergic contractile response in the mouse prostate gland. However, a significant difference in the response to 80 mM K⁺ Krebs-Henseleit solution between genotypes was also observed. This result is interesting and unexplained; however, it may suggest that endogenously released acetylcholine acting at M₃ muscarinic receptors contributes to the contractile response mediated by 80 mM K⁺ Krebs-Henseleit solution. Equally, it may suggest that the deletion of the M₃ muscarinic receptor affects the contractile machinery in the mouse prostate gland. Nevertheless, to allow fair comparison between genotypes, results to electrical-field stimulation were plotted as a percentage of the contractile response to 80 mM K⁺ Krebs-Henseleit solution.

In prostates taken from M₃R(−/−) mice, we observed that the nerve-mediated contractile response was inhibited. Furthermore, atropine was without effect in M₃R(−/−) mice. These observations suggest that the M₃ muscarinic receptor subtype is solely responsible for the cholinergic component of nerve-mediated contraction and that compensatory changes by up-regulation of other muscarinic receptor subtypes do not occur in prostates taken from M₃R(−/−) mice. This does not exclude the possibility of up-regulation of noncholinergic mechanisms such as purinergic mechanisms, as observed in the bladders of M₃R(−/−) mice (Igawa et al., 2004). However, this seems unlikely in the prostate because the residual contractile response to electrical-field stimulation in prostates taken from M₃R(−/−) mice is abolished by the α₁-adrenoceptor antagonist prazosin. Furthermore this confirms that the nerve-mediated contractile response in the mouse prostate is mediated by both muscarinic receptors and α₁-adrenoceptors (White et al., 2010).

The decrease in the potency of acetylcholine observed in prostates taken from M₃ muscarinic receptor heterozygous mice may be a product of fewer M₃ muscarinic receptors being available, whereas the response to acetylcholine in prostates taken from M₃R(+/+) mice was comparable with our previous results (White et al., 2010). The absence of a contractile response to acetylcholine, except at high concentrations (as discussed previously) in M₃R(−/−) mice, further confirms the M₃ muscarinic receptor as the subtype mediating contraction in the mouse prostate gland.

The weight of the prostate was unaltered by the deletion of the M₃ muscarinic receptor, indicating the M₃ muscarinic receptor and/or gene does not play a role in growth of the mouse prostate gland. In primary human cell cultures and prostate cancer cell lines (Witte et al., 2008), as well as in the rat prostate (McVary et al., 1998), growth can be regulated by cholinergic agonists or parasympathetic innervation, respectively. Although the results of the present study exclude a role for M₃ muscarinic receptors in regulating growth, they
can not exclude the possibility of additional muscarinic receptor subtype regulating mouse prostate growth. It should be noted that the reduction in mouse weight at this age in M3R(–/–) mice, caused by an impairment of saliva production (Matsui et al., 2000) or appetite (Yamada et al., 2001), may have influenced this result.

Unlike in the mouse prostate, the cholinergic component of nerve-mediated contraction in the human prostate is small compared with the adrenergic response (Caine et al., 1975; Hedlund et al., 1985; Gup et al., 1989; Kester et al., 2003). However, another study has observed an adreno-muscarinic synergy in contraction of the human prostate (Roosen et al., 2009). Although the M3 muscarinic receptor subtype has not been demonstrated in the human prostate, recent reviews of clinical trials using muscarinic receptor antagonists (which included the M3 muscarinic receptor antagonists solifenacin and darifenacin) for the treatment of lower urinary tract symptoms, usually in combination with an adrenoceptor antagonist, show varying improvement in storage symptoms without worsening of voiding symptoms or significant development of acute urinary retention (Athanasopoulos, 2010; Chapple, 2010). Although these drugs may act by inhibition of detrusor instability in the bladder, prostate smooth muscle tone can not be ruled out as an additional site of action for the treatment of lower urinary tract symptoms by muscarinic receptor antagonists.

In conclusion, the results of the present study have shown that the M3 muscarinic receptor subtype is solely responsible for the nerve-mediated cholinergic component of contraction in the mouse prostate gland.

Authorship Contributions

Participated in research design: White, Short, and Ventura.

Conducted experiments: White.

Contributed new reagents or analytic tools: Matsui.

Performed data analysis: White and Ventura.

Wrote or contributed to the writing of the manuscript: White, Short, Haynes, and Ventura.

References


Address correspondence to: Dr. Sabatino Ventura, Medicinal Chemistry and Drug Action, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia. E-mail: sah.ventura@monash.edu.