ABSTRACT—Muscarinic cholinoceptor subtypes in the rat prostatic membrane were characterized by using $[^3H]$-methyl-quinuclidinyl benzilate (QNB) in ligand binding studies. $[^3H]$-Methyl-QNB saturation experiments showed the existence of a homogeneous population of binding sites with a high affinity ($K_D$ value) of $0.24 \pm 0.04 \text{ nM}$ and a maximum binding site number ($B_{\text{max}}$) of $219 \pm 65 \text{ fmol/mg protein}$. Inhibition of $[^3H]$-methyl-QNB binding by nonlabelled compounds was in the following order of effectiveness in rat prostate: atropine $>$ 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) $>$ hexahydro-sila-difenidol hydrochloride, p-fluoroanalog (p-F-HHSiD) $>$ pirenzepine $>$ methoctramine $>$ [1]-[[2-((dimethylamino)methyl)-1-piperidinyl)acetyl]-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one] (AF-DX 116). This ranking order was similar to that for the salivary gland (M3 subtype), but not for the brain (M1 subtype) or the heart (M2 subtype). These results indicate that the muscarinic cholinoceptors in the rat prostate belong mainly to the M3 subtype. Furthermore, $B_{\text{max}}$ values for muscarinic cholinoceptors in the aged rat prostate (approximately 1-year-old) were smaller than those in the young rat prostate (6- to 8-week-old) ($87 \pm 13$ vs. $183 \pm 32 \text{ fmol/mg protein}$). However, $K_D$ values for muscarinic cholinoceptors, and $B_{\text{max}}$ and $K_D$ values for $\alpha$-adrenoceptors showed no change. These results suggest that the number of prostatic muscarinic cholinoceptors decreases with aging.

Keywords: Prostate (rat), Muscarinic cholinoceptor subtype (M3), Aging
brain, salivary gland, heart and prostate were removed. Tissues were finely minced with scissors and homogenized in 10 vol. of ice-cold homogenization buffer (0.25 M sucrose containing 50 mM tris-HCl pH 7.5) with a polytron (Kinematica, Lucerne, Switzerland). The homogenate was centrifuged at 600 × g for 15 min at 4°C. The supernatant was filtered through a single layer of nylon mesh and recentrifuged at 40,000 × g for 20 min at 4°C. The resulting pellets were washed twice with ice-cold incubation buffer (50 mM tris-HCl pH 7.5 containing 10 mM MgCl₂). The final pellet was resuspended with 3–10 vol. of ice-cold incubation buffer and stored at −80°C until use.

Binding assay
Muscarinic cholinoceptor density was determined in saturation experiments by incubating aliquots of membrane preparation (200 μg protein) with increasing concentrations of [³H]-methyl-quinuclidinyl benzilate (QNB) (0.03–1.4 nM) in a final volume of 0.5 ml for 60 min at 25°C. In the competition experiments, a single concentration (0.1 nM) and 5 to 7 concentrations of antagonists were used. Incubation was terminated by rapid filtration through Whatman GF/C filters using a Brandel cell harvester. The filter was then rinsed 3 times with 3-ml aliquots of ice-cold incubation buffer. Radioactivity retained on the filter was counted by a liquid scintillation counter (2000CA, Packard, Meriden, CT, U.S.A.). Nonspecific binding was determined in the presence of 10 μM atropine; it was found to be less than 10% of the total binding at 0.1 nM [³H]-methyl-QNB under all conditions.

β-Adrenoceptor binding density was determined in saturation experiments by incubating aliquots (200 μg protein) of membrane prepared from rat prostate with increasing concentrations of [³H]-dihydroalprenolol (DHA) (0.03–1.4 nM) for 10 min at 30°C. Nonspecific binding was determined in the presence of 10 μM isoproteanol. Other details were the same as those for the muscarinic binding study. The protein content of each membrane suspension was measured by the Bradford method (19), with bovine serum albumin as the standard.

Aging study
[³H]-Methyl-QNB binding was examined in prostates obtained from male Sprague-Dawley rats aged 6 to 8 weeks (young rats) and 52 to 60 weeks (old rats) (520–690 g). The tissue was prepared and assayed as above. Six separate assays were performed in duplicate for each age group.

Statistical analyses
The data were analyzed as previously reported (20). Results were expressed as the mean ± S.E.M. or the mean with 95% confidence limits. Statistical significance was assessed by the non-paired Student’s t-test (P < 0.01).

Drugs
The following drugs were used: [³H]-methyl-QNB (3200.5 GBq/mmole) and [³H]-DHA (2338.4 GBq/mmole) were purchased from New England Nuclear (Boston, MA, U.S.A.). Atropine sulfate, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), pirenzepine dihydrochloride, hexahydro-sila-difenidol hydrochloride p-fluoroanalog (p-F-HHSiD) and methoctramine tetrahydrochloride were purchased from Research Biochemicals, Inc. (Natic, MA, U.S.A.). [11-((2-Dimethylamino)methyl)-1-piperidinyl]acetyl)-5,11-dihydro-6H-pyrido[2,3-b](1,4)benzodiazepine-6-one (AF-DX 116) was synthesized by Yamanouchi Pharmaceutical Co., Ltd. Other chemicals used were of analytical grade.

RESULTS
Radioligand binding studies were carried out on rat prostate membrane. Figure 1a illustrates the specific and nonspecific binding of [³H]-methyl-QNB at the concentration range of 0.03 to 1.4 nM in rat prostate membrane. Nonspecific binding of [³H]-methyl-QNB to the membrane increased linearly with increasing ligand concentration. However, specific binding was saturable with relatively low nonspecific binding. Scatchard analysis revealed that its binding profile was monocomponent with high affinity (K_D = 0.24 ± 0.04 nM) and low capacity (B_max = 219 ± 65 fmol/mg protein) (Fig. 1b). Compared with other novel muscarinic cholinoceptor subtypes (M₁, brain; M₂, heart; M₃, salivary gland), the K_D values for the rat prostate were closer to those for the salivary gland than to those for the brain and heart (Table 1). Six muscarinic cholinoceptor antagonists inhibited the [³H]-methyl-QNB binding of rat prostate membrane and tissues. The ranking order of inhibitory activity was: atropine > 4-DAMP > p-F-HHSiD > pirenzepine > methoctra-

Table 1. Muscarinic receptor density (B_max) and equilibrium dissociation constant (K_D) derived from [³H]-methyl-QNB saturation experiments in rat brain, heart, salivary gland and prostate membranes

<table>
<thead>
<tr>
<th>Tissue</th>
<th>K_D (nM)</th>
<th>B_max (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.18 ± 0.02</td>
<td>212 ± 45</td>
</tr>
<tr>
<td>Heart</td>
<td>0.17 ± 0.02</td>
<td>73 ± 3</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>0.32 ± 0.02</td>
<td>190 ± 27</td>
</tr>
<tr>
<td>Prostate</td>
<td>0.24 ± 0.04</td>
<td>219 ± 65</td>
</tr>
</tbody>
</table>

Figures represent the mean ± S.E.M. of five separate experiments.
Fig. 1. Specific [$^3$H]-methyl-QNB binding to rat prostate membrane as a function of increasing concentrations of [$^3$H]-methyl-QNB. a: Specific [$^3$H]-methyl-QNB binding (/) was determined experimentally as the difference between the total and non-specific binding (O) in parallel assays in the absence and presence of 10 nM atropine. Each point represents the average of four determinations. b: Scatchard plot derived from the same data. Ordinate, bound to free (B/F) [$^3$H]-methyl-QNB (fmol/mg protein/nM). Abscissa, bound [$^3$H]-methyl-QNB (fmol/mg protein).

Fig. 2. The displacement of [$^3$H]-methyl-QNB binding by atropine (■), 4-DAMP (○), p-F-HHSiD (□), pirenzepine (▲), methoctramine (●), and AF-DX 116 (□) in rat prostate membrane fraction.

Fig. 3. Correlation of the pKᵢ values of six muscarinic cholinoreceptor antagonists for [$^3$H]-methyl-QNB binding to the receptor in the rat salivary gland and prostate. The abscissa indicates the pKᵢ value for [$^3$H]-methyl-QNB binding to the salivary gland membrane and the ordinate indicates pKᵢ value for binding to the prostate membrane.

[Graphs and figures as described in the text]
The major aim of the present study was to characterize the binding of $[^3H]$-methyl-QNB to muscarinic cholinoreceptors present in the rat prostate. As in other rat tissues, $[^3H]$-methyl-QNB binding to prostate membranes appears to involve a single population of binding sites. The $K_D$ value and maximal density of binding sites were $0.24 \pm 0.04$ nM and $219 \pm 65$ fmol/mg protein, respectively. $[^3H]$-Methyl-QNB affinity for these binding sites was similar to values found in the rat salivary gland ($0.32 \pm 0.02$ nM). Atropine, pirenzepine (21), AF-DX 116 (22) and methoctramine (23), and p-F-HHSiD (24) and 4-DAMP (25), respectively, were used as non-selective, $M_1$-selective, $M_2$-selective and $M_3$-selective muscarinic antagonists in competition experiments against $[^3H]$-methyl-QNB binding. All these muscarinic antagonists inhibit the binding of $[^3H]$-methyl-QNB to the binding sites in rat tissue membranes. Three different pharmacologically identifiable receptors have been proposed: $M_1$ (neuronal tissue), $M_2$ (heart tissue) and $M_3$ (exocrine glands), with the classification based on the binding characteristics of three selective ligands, pirenzepine ($M_1$), AF-DX 116 ($M_2$) and p-F-HHSiD ($M_3$). We thus used brain ($M_1$-rich membrane), heart ($M_2$-rich membrane) and salivary gland ($M_3$-rich membrane) (25) in our study. Recently, five muscarinic cholinoreceptors have been cloned ($m_1$–$m_5$), but the expression of $m_4$ and $m_5$ muscarinic cholinoreceptors remained unclear. Hill coefficients of 5 antagonists (ex-

Table 2. Inhibition of $[^3H]$-methyl-QNB binding sites by muscarinic cholinoreceptor antagonists in rat tissues

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Brain</th>
<th>Heart</th>
<th>Salivary gland</th>
<th>Prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pK_i$</td>
<td>Hill coefficient</td>
<td>$n$</td>
<td>$pK_i$</td>
</tr>
<tr>
<td>Atropine</td>
<td>9.46 (9.43–9.49)</td>
<td>0.99 (0.88–1.09)</td>
<td>22</td>
<td>9.31 (9.30–9.32)</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>8.96 (8.94–8.99)</td>
<td>1.01 (0.91–1.11)</td>
<td>21</td>
<td>8.28 (8.26–8.30)</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>7.72 (7.68–7.75)</td>
<td>0.76 (0.67–0.84)</td>
<td>26</td>
<td>6.67 (6.66–6.69)</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>6.84 (6.83–6.86)</td>
<td>0.85 (0.74–0.97)</td>
<td>33</td>
<td>8.09 (8.02–8.15)</td>
</tr>
<tr>
<td>p-F-HHSiD</td>
<td>7.47 (7.45–7.49)</td>
<td>0.88 (0.80–0.97)</td>
<td>25</td>
<td>6.56 (6.52–6.60)</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>5.83 (5.81–5.85)</td>
<td>0.74 (0.68–0.80)</td>
<td>34</td>
<td>6.57 (6.54–6.59)</td>
</tr>
</tbody>
</table>

Data are the mean and 95% confidence limits. $n$ = number of data points.

Table 3. Muscarinic cholinoreceptor and $\beta$-adrenoceptor densities ($B_{max}$) and equilibrium dissociation constants ($K_D$) derived from $[^3H]$-methyl-QNB and $[^3H]$-dihydroalprenolol saturation experiments in young and old rat prostate membranes, respectively

<table>
<thead>
<tr>
<th></th>
<th>Young rats</th>
<th>Old rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_D$ (nM)</td>
<td>$B_{max}$ (fmol/mg protein)</td>
</tr>
<tr>
<td>Muscarinic cholinoreceptor</td>
<td>0.35±0.07</td>
<td>183±32</td>
</tr>
<tr>
<td>$\beta$-Adrenoceptor</td>
<td>0.28±0.04</td>
<td>340±49</td>
</tr>
</tbody>
</table>

Each figure represents the mean ± S.E.M. of 6 separate experiments. **significantly less than young rat prostate membrane (Student’s $t$-test) ($P<0.01$).
cept atropine) in heart membrane were smaller than 1. This reason might be explained by the existence of another subtype of muscarinic cholinceptor. The ranking order of these ligands for the M1 receptor subtype was atropine > 4-DAMP > p-F-HHSiD > pirenzepine > AF-DX 116. Ranking for the M2 receptor was atropine > 4-DAMP > AF-DX 116 > p-F-HHSiD > pirenzepine, while that for the M3 receptor was atropine > 4-DAMP > p-F-HHSiD > pirenzepine > AF-DX 116 (26). The displacement ranking order of these antagonists against [3H]-methyl-QNB in rat prostate membrane (atropine > 4-DAMP > p-F-HHSiD > pirenzepine > methoctramine > AF-DX 116) was similar to that described above for the M1-muscarinic cholinceptor subtype and for the salivary gland in this study. This order agrees with that (27) for the inhibition of the maximum acetylcholine-induced contraction of guinea pig ileum smooth muscle strips (pA2 values). Furthermore, both M3-selective antagonists, 4-DAMP and p-F-HHSiD, gave the expected affinity profile: prostate ≥ salivary gland ≥ brain ≥ heart. We could not, however, clearly discriminate between M1 (brain) and M3 (salivary gland) cholinceptor because of the weak selectivity (M3/M1) of these compounds (13, 25), and the heterogeneity of muscarinic receptors in these tissues. Another study used m1 and m3 receptors expressed by CHO-K1 cells (28) to show that pirenzepine (M1-selective antagonist) was 10-fold more selective for M1 receptors than M3 receptors. We saw a similar tendency in M1/M3 selectivity among these compounds (brain: pK1 = 7.72, salivary gland: 7.28, prostate: 7.43) (Table 2). Furthermore, the M2-selective (M2 > M1 > M3) antagonist methoctramine showed the same rank order in this study: heart (pK1 = 8.09) > brain (6.84) > salivary gland (6.22) = prostate (6.42). Muscarinic cholinceptors in the rat prostate may therefore be mainly M3-muscarinic cholinceptors.

The number of autonomic receptors in prostate homogenate from aged rats was measured to study the effect of aging on autonomic receptors in this gland. δ-Adrenoceptors and muscarinic cholinceptors were counted using [3H]-DHA and [3H]-methyl-QNB, respectively. Results showed an apparent reduction in the number of muscarinic cholinceptors with advancing age. Surprisingly, there have been only a few reports that age affects muscarinic cholinergic receptors in the rat prostate. Three reports (29–31) have examined muscarinic receptors in the aging rat urinary bladder. Two showed an increase (29, 30) in the density of receptors, while the other showed no change (31). However, these discrepancies might be explained by possible differences in the nature of the tissue samples and/or rat strain. No consensus view on this question was reached. However, in other tissues (rat hippocampus (32), cow trachea (33), some reports indicated that there was a reduction of muscarinic cholinceptors with advancing age.

Acetylcholine causes a significant contractile response in human prostatic capsule; this response is inhibited by atropine (34). Autoradiographic studies have localized the muscarinic cholinceptors to the glandular epithelium (35). The possible functional relevance of these muscarinic receptors is evidenced by studies in which pilocarpine was shown to significantly enhance the basal output of prostatic secretions in the dog (10). This pilocarpine-induced increase in prostatic secretion has not been observed in castrated dogs (11). The present study showed a similar down-regulation of muscarinic receptors in aged rat prostate.

In summary, our data showed a single muscarinic cholinceptor subpopulation in the rat prostate which is similar to the M3 subtype. Furthermore, aged rats showed an apparent reduction in the number of muscarinic receptors.

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