Influence of Tissue Integrity on Pharmacological Phenotypes of Muscarinic Acetylcholine Receptors in the Rat Cerebral Cortex

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ABSTRACT

Distinct pharmacological phenotypes of muscarinic acetylcholine receptors (mAChRs) have been proposed. We compared the pharmacological profiles of mAChRs in intact segments and homogenates of rat cerebral cortex and other tissues by using radioligand binding assays with [3H]N-methylscopolamine ([3H]NMS). Recombinant M1 and M3 mAChRs were also examined. The density of mAChRs detected by [3H]NMS binding to rat cerebral cortex segments and homogenates was the same (approximately 1400 fmol/mg tissue protein), but the dissociation constant of [3H]NMS was significantly different (1400–1700 pM in segments and 260 pM in homogenates). A wide variation in [3H]NMS binding affinity was also observed among the segments of other tissues (ranging from 139 pM in urinary bladder muscle to 1130 pM in the hippocampus). The mAChRs of cerebral cortex were composed of M1, M2, M3, and M4 subtypes, which showed typical subtype pharmacology in the homogenates. However, in the cortex segments the M3 subtype showed a low selectivity for M3 antagonists (darifenacin, solifenacin) and was not distinguished by the M3 antagonists from the other subtypes. Recombinant M1 and M3 mAChRs showed high affinity for [3H]NMS and subtype-specific pharmacology for each tested ligand. The present binding study under conditions where tissue integrity was kept demonstrates a wide variation in [3H]NMS binding affinity among mAChRs of many rat tissues and the presence of an atypical M3 phenotype in the cerebral cortex, suggesting that the pharmacological properties of mAChRs are not necessarily constant, rather they may be significantly modified by tissue integrity and tissue type.

Introduction

It has been generally considered that antagonist affinities are constant for a given receptor subtype, regardless of the cell/tissue background in which the receptor is expressed. This concept has been referred to as the “antagonist assumption” (Kenakin et al., 1995; Nelson and Challiss, 2007). However, this traditional view has now been challenged by the observation of different pharmacological antagonist profiles for the same gene product in different cells/tissues (Kenakin, 2003; Baker and Hill, 2007; Nelson and Challiss, 2007; Muramatsu et al., 2008).

Muscarinic acetylcholine receptors (mAChRs) are widely distributed in mammals, including humans, and play a large number of important functions, such as in learning and memory in the central nervous system and in smooth muscle contraction or gland secretion in the periphery (Abrams et al., 2006; Wess et al., 2007). Five distinct mAChR genes (each encoding M1–M5 subtypes) have been cloned, and the pharmacological profiles of the recombinant mAChRs are generally consistent with those of the native receptors (Hulme et al., 1990; Dörje et al., 1991; Caulfield and Birdsall, 1998; Alexander et al., 2009). However, there have been several reports of “atypical” pharmacological profiles in which anomalous estimates of the functional affinity of receptors for antagonists in native tissues play a significant role in defining these atypical phenotypes of mAChRs (Nelson and Challiss, 2007; Dörje et al., 1991; Caulfield and Birdsall, 1998; Alexander et al., 2009).
liss, 2007). For example, in the guinea pig and rat uterus the functional affinities for mAChR antagonists are not in agreement with the affinities of $M_3$ mAChRs identified in radioligand binding studies, suggesting a possible involvement of an atypical operational mAChR in cholinergic contraction (Boxall et al., 1998; Munns and Pennefather, 1998). In addition, certain mAChR antagonists, such as darifenacin and solifenacin that are used in the management of overactive bladder, display in vivo selectivity for inhibition of mAChR-mediated urinary bladder contraction relative to salivary secretion, even though both responses are mediated by the same $M_3$ mAChR subtype (Abrams et al., 2006). Thus, it is likely that the same mAChR receptor gene product can yield a distinct pharmacological phenotype in different tissues or under different assay conditions.

Antagonist affinity has been estimated not only by using functional bioassays but also with radioligand binding assays. Tissue strips are used routinely to obtain bioassay-derived affinities, whereas tissue homogenates or membrane fractions have been used almost exclusively for most conventional radioligand binding assays (Bylund and Toews, 1993). Therefore, it has been speculated that receptor environments that differ between the preparations used for these two distinct assays (intact tissue versus membranes) may confer distinct receptor characteristics that can interact differently with the same antagonist (Christopoulos, 2000).

A binding assay that uses intact tissue segments has been shown to be a powerful method for analysis of the intrinsic properties of receptors present in native tissues (Muramatsu et al., 2005, 2009; Anisuzzaman et al., 2008a; Sathi et al., 2008; Su et al., 2008). When using the intact segment binding assay, in contrast to conventional membrane binding assays, it is not necessary to consider either the change of receptor characteristics that may occur upon tissue homogenization that eliminates the receptor’s natural environment or the selective loss of receptors upon isolating membranes by differential centrifugation (Muramatsu et al., 2005).

To date, the $M_1$, $M_2$, $M_9$, and $M_6$ mAChR subtypes have been clearly identified in rat cerebral cortex by using conventional membrane binding assays (Waelbroeck et al., 1990; Ehler and Tran, 1990; Ferrari-Dileo et al., 1994). We hypothesized that there would be differences in the mAChR phenotypes present in intact cortex tissue segments compared with the same receptors present in homogenates. To test this hypothesis, we used a hydrophilic radioligand $[3H]N$-methylscopolamine ($[3H]NMS$) and re-evaluated the pharmacological profiles of mAChRs present in rat cerebral cortex by using an intact tissue segment binding assay compared with a conventional measurement of binding in tissue homogenates. The results revealed a significant difference in binding profiles between intact segments and homogenates, as well as the presence of a unique $M_4$ mAChR phenotype in rat cerebral cortex.

**Materials and Methods**

**Animals.** Male Wistar rats weighing 250 to 350 g (Charles River Japan, Inc., Yokohama, Japan) were used in the present study, which was conducted according to the Guidelines for Animal Experiments of the University of Fukui (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan).

**Tissue Segment Binding Experiments Using $[3H]NMS$.** Rats were anesthetized with halothane and sacrificed by cervical dislocation. The brain, urinary bladder, stomach, and submaxillary gland were rapidly isolated and immersed in modified Krebs-Henseleit solution containing 120.7 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl$_2$, 2.0 mM CaCl$_2$, 1.2 mM Na$_2$HPO$_4$, 25.5 mM NaHCO$_3$, and 11.5 mM D(+)-glucose, pH 7.4, which had been oxygenated with a mixture of 95% O$_2$ and 5% CO$_2$ and kept at 0°C. The brain was cleaned from the pia mater and substantia alba. The gastric mucosa and epithelial layer of urinary bladder were removed, and the muscle layer of the urinary bladder, body and the detrusor muscle of urinary bladder were measured. The tissues were cut into small pieces (approximately 2 $\times$ 2 $\times$ 1 mm for cerebral cortex, hippocampus, and corpus striatum, approximately 1.5 $\times$ 1.5 $\times$ 1 mm for gastric muscle and bladder muscle, and approximately 4 $\times$ 3 $\times$ 3 mm for midbrain, pons, and submaxillary gland) and applied to tissue segment binding experiments. The binding incubation for tissue segments was routinely performed at 4°C for 16 to 18 h in a final volume of 1 ml (Muramatsu et al., 2005; Anisuzzaman et al., 2008b). To ensure equilibrium in binding, we adopted the fail-safe assumption, and experiments of longer incubation (32 or 48 h) were also performed (see Results and Supplemental Figs. 1–3). The incubation times for the association and dissociation experiments are shown in the figures and their legends. The incubation medium was similar to that of the modified Krebs-Henseleit solution, containing 135.7 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl$_2$, 2.0 mM CaCl$_2$, 1.2 mM Na$_2$HPO$_4$, 10.5 mM NaHCO$_3$, and 11.5 mM D(+)-glucose, pH 7.4 under equilibrated conditions with air. In saturation binding experiments, $[3H]NMS$ was used at concentrations ranging from 50 to 12,500 pM. In competition experiments, $[3H]NMS$ was simultaneously incubated with various concentrations of unlabeled antagonists. However, in some cases of competition by pirenzepine, darifenacin, and solifenacin, the cortex segments were also pretreated with the competitors for 24 h to ensure equilibrium and then incubated with $[3H]NMS$ for an additional 24 h (Berrie et al., 1985). After incubation, tissue segments were quickly moved into a plastic tube containing 1.5 ml of ice-cold incubation buffer (4°C) and carefully washed by vortex mixing for 1 min. This procedure resulted in the release of most of the unbound radioligand from the segments into the buffer medium and its absorption to the plastic tube. The tissue segments were then solubilized in 0.3 M NaOH solution to estimate the bound radioactivity and protein content. Nonspecific binding was determined in the presence of 1 uM atropine. The bound radioactivity was measured using a liquid scintillation counter (LSC-6100; Aloka, Tokyo, Japan). Protein concentration in each tissue segment was measured by the Coomassie Brilliant Blue G-250 binding method using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Experiments were done in duplicate at each concentration of $[3H]NMS$ for binding saturation experiments or at each concentration of competing ligand for competition binding experiments.

**Homogenate Binding Experiments Using $[3H]NMS$.** The isolated rat cerebral cortex, hippocampus, and detrusor muscle were minced with scissors and homogenized in 40 volumes (v/w) of Krebs incubation buffer by using a Polytron homogenizer (setting at 8, 5 x 20 s at 4°C) (Kinematica, Littau-Lucerne, Switzerland). The Krebs incubation buffer was the same as that used in the tissue segment binding experiments. To avoid yield loss, whole homogenates without fractionation were used. In binding experiments, the homogenates were incubated for 4 h at 4°C in 1 ml of Krebs incubation buffer. Binding equilibrium of $[3H]NMS$ was reached at approximately 2 h for the homogenate binding experiments. Increasing concentrations of $[3H]NMS$ (50–12,500 pM) were used for binding saturation experiments. In competition binding experiments, the homogenates were incubated under the same conditions (4 h at 4°C) with 500 pM $[3H]NMS$ in the absence or presence of unlabeled competing ligands. Reactions were terminated by rapid filtration, using a Brandel cell harvester (Brandel Inc., Gaithersburg, MD), onto Whatman GF/C filters (Whatman, Clifton, NJ) presoaked in...
0.3%polyethyleneimine for 15 min, and the filters were then washed three times with 5 ml of Krebs incubation buffer. The resulting filters were dried, and the trapped radioactivity was quantified by liquid scintillation counting. Nonspecific binding of [3H]NMS was defined as the binding in the presence of 1 μM atropine. Experiments were performed in duplicate at each concentration of radioligand for a binding saturation experiment or at each concentration of competing ligand for a competition binding experiment. The protein content of homogenates was measured as described above.

Recombinant M₁ and M₅ mAChRs in Chinese Hamster Ovary Cells. CHO cell lines stably expressing M₁ or M₅ mAChRs (CHO-M₁ or CHO-M₅, respectively) were provided by Dr. J. Wess (Molecular Signaling Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD) (Dörje et al., 1991). The cells were cultured in Dulbecco’s modified Eagle’s medium (Wako Pure Chemicals, Tokyo, Japan) supplemented with 10% fetal bovine serum in a humidified incubator under 5% CO₂ and 95% air. Cells were harvested by gentle scraping without using trypsin. The whole-cell binding assay was essentially the same as that of the homogenate binding experiment described above and previously (Nishimune et al., 2010). Incubation was carried out at 4°C for 4 h. Equilibrium of binding of [3H]NMS to the cells was reached at approximately 2 h.

Data Analysis. Binding data were analyzed using Prism (version 5.01; GraphPad Software Inc., San Diego, CA) as described previously (Muramatsu et al., 2005; Yoshiki et al., 2009). In brief, the data from saturation binding studies were fitted by a one-site saturation binding isotherm (binding-saturation equation in Prism), and the Kᵣ values and the binding capacity were then calculated. The abundance of the mAChRs is indicated as the maximum binding capacity per milligram of total tissue protein (B₉⁰max). For the competition studies, the data were analyzed using the binding-competitive equation of Prism. When different Kᵣ values were estimated for [3H]NMS from saturation experiments using tissue segments and homogenates, the corresponding Kᵣ value estimated for each preparation was used for analysis of the competition binding data to calculate Kᵣ values for antagonists and their proportions. A two-site model was adopted only when the residual sums of squares were significantly less (p < 0.05) for a two-site fit to the data than for a one-site by F test comparison. To validate the one-site and two-site models, Hill plot analyses were also performed. Kinetic experiments were also analyzed using the association kinetics equation (two or more ligand concentrations) of Prism. Data are shown as the mean ± S.E.M. of a number of experiments (n). Data were statistically analyzed using Student’s t test.

Reagents. The following drugs were used in the present study: [3H]NMS (specific activity 3.00 TBq/mmol) (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), atropine sulfate (Nacalai Tesque, Kyoto, Japan), darifenacin (Ono Pharmaceutical Co. Ltd., Osaka, Japan), solifenacin (a kind gift from Astellas Pharma Inc., Tokyo, Japan), pirenzepine (Sigma-Aldrich, St. Louis, MO), 11-(2-[diethylamino)methyl]-1-piperidinyl)acetyl)-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX 116) and methoctramine (Toctis Bioscience, Bristol, UK), and muscarinic toxin 7 (MT-7) and muscarinic toxin 3 (MT-3) (Peptide Institute Inc., Osaka, Japan).

Results

[3H]NMS Binding to Intact Segments and Homogenates of Rat Cerebral Cortex. The binding of [3H]NMS (50–12,500 pM) to the rat cerebral cortex preparations (16- to 18-h incubation at 4°C for tissue segments; 4-h incubation at 4°C for homogenates) was concentration-dependent (Fig. 1, A and B). Nonspecific binding of 2000 pM [3H]NMS in the presence of 1 μM atropine was less than 10 or 25% of the total binding in the segments and the homogenates, respectively. These results, together with the results obtained from binding competition experiments using other mAChR antagonists (see below), suggested that the specific binding of [3H]NMS to mAChRs could be estimated without nonspecific contamination or accumulation of the hydrophilic [3H]NMS receptor probe into the tissue segments. This point was confirmed further in the association and dissociation experiments using cortex segments (Fig. 1, D and E). The specific binding of

Fig. 1. Characterization of [3H]NMS binding to intact segments and homogenates of rat cerebral cortex at 4°C. A and B, representative saturation curves of [3H]NMS binding to intact segments (A) and homogenates (B) of rat cerebral cortex are shown. The ordinate represents the level of [3H]NMS binding per milligram of tissue protein. Specific binding (●) was determined by subtracting the amount of [3H]NMS bound in the presence of 1 μM atropine (nonspecific binding; ○) from the total binding (□). Each point represents the mean of duplicate determinations. C, the abundance of [3H]NMS binding sites estimated in the segments and homogenates of rat cerebral cortex (n = 7). D, association experiments using three different concentrations of [3H]NMS in rat cerebral cortex segments. The ordinate represents the specific binding of [3H]NMS binding per milligram of tissue protein. The abscissa represents the incubation time. E, dissociation experiment using rat cerebral cortex segments. After incubation with 2.0 nM [3H]NMS for 12 h, unlabeled NMS (1 μM) was added. The abscissa represents the time after the addition of NMS. The curves shown are representative of five experiments. Each point represents the mean of duplicate determinations and S.E.M. of representative experiments.
TABLE 1

Binding affinities for various muscarinic ligands estimated in intact tissue segments and homogenates of rat cerebral cortex

<table>
<thead>
<tr>
<th>Antagonist (Subtype Selectivity)</th>
<th>Tissue Segments</th>
<th>Homogenates</th>
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<tr>
<td></td>
<td>$pK_{a}$ (R, % high)</td>
<td>$pK_{a}$</td>
</tr>
<tr>
<td>Atropine (NS)</td>
<td>8.34 ± 0.05</td>
<td>9.41 ± 0.03</td>
</tr>
<tr>
<td>NMS (NS)</td>
<td>8.66 ± 0.04</td>
<td>9.72 ± 0.03</td>
</tr>
<tr>
<td>Pirenzepine (M₁ &gt; M₄)</td>
<td>7.94 ± 0.37 (44 ± 5)</td>
<td>5.81 ± 0.15</td>
</tr>
<tr>
<td>MT-7 (M₁)</td>
<td>8.85 ± 0.44 (43 ± 6)</td>
<td>8.78 ± 0.17 (46 ± 2)</td>
</tr>
<tr>
<td>AF-DX 116 (M₄ &gt; M₁)</td>
<td>6.97 ± 0.10 (24 ± 3)</td>
<td>4.88 ± 0.03</td>
</tr>
<tr>
<td>Methoctramine (M₂ ≈ M₃)</td>
<td>7.90 ± 0.12 (34 ± 3)</td>
<td>5.31 ± 0.08</td>
</tr>
<tr>
<td>Darifenacin (M₃)</td>
<td>6.40 ± 0.20</td>
<td>6.60 ± 0.10</td>
</tr>
<tr>
<td>Solifenacin (M₃)</td>
<td>6.70 ± 0.10</td>
<td>6.70 ± 0.10</td>
</tr>
<tr>
<td>MT-3 (M₁)</td>
<td>8.84 ± 0.20 (15 ± 3)</td>
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</table>

NS, nonselective for subtypes; % high, percentage of high-affinity sites.

$\text{p}K_{a}$ high and $\text{p}K_{a}$ low, negative logarithm of the equilibrium constants ($\text{p}K_{a}$) at high and low-affinity sites for tested drugs.

$\text{p}K_{a}$ 7 and $\text{p}K_{a}$ 6 at concentrations up to 0.5 μM did not completely inhibit [³H]NMS binding.

[³H]NMS at three different concentrations slowly increased with an increase in incubation time. The addition of 1 μM unlabeled NMS after 12 h of incubation also slowly reduced the specific binding to the nonspecific binding level. The calculated association rate constant ($K_{on}$) was 1.01 ± 0.05 × 10⁶ M⁻¹·min⁻¹, and the dissociation rate constant ($K_{off}$) was 1.5 ± 0.11 × 10⁻⁵ min⁻¹, respectively, from the association experiments (n = 5). The $K_{off}$ value was calculated as 1.6 ± 0.14 × 10⁻³ min⁻¹ from the dissociation experiment (n = 5). The binding kinetics of [³H]NMS were more rapid for the homogenates than for the tissue segments; the $K_{on}$ and $K_{off}$ values in the homogenates were 6.0 ± 0.03 × 10⁷ M⁻¹·min⁻¹ and 21.0 ± 1.6 × 10⁻³ min⁻¹, respectively (n = 5).

Hill coefficients of saturation binding curves were 0.98 ± 0.09 (0.87–1.24) for tissue segments and 0.95 ± 0.07 (0.85–1.17) for homogenate preparations. The binding saturation isotherms revealed that the specific binding of [³H]NMS met the criteria for binding to a single set of sites. Of significance, the maximal binding capacities ($B_{max}$) for the tissue segments and the homogenate preparations did not differ appreciably: for segments, 1420 ± 82 fmol/mg tissue protein (n = 7), and for homogenates, 1380 ± 32 fmol/mg tissue protein (n = 7). However, the binding affinities ($K_{d}$) differed substantially: $K_{d} = 1420 ± 165$ pM ($K_{d} = 8.85 ± 0.05$) for tissue segments and $K_{d} = 260 ± 71$ pM ($K_{d} = 9.58 ± 0.12$) for homogenates. Therefore, in comparing the two preparations (segments versus homogenates), there was no difference in the abundance of [³H]NMS binding sites (Fig. 1C), but there was a significant difference in the dissociation constants of [³H]NMS between the segments and the homogenates ($p < 0.05$). The dissociation constants for [³H]NMS estimated from the saturation curves were in good agreement with the kinetically derived $K_{d}$ values in the segments (1490 ± 217 pM) and the homogenates (310 ± 81 pM). Such low affinity for [³H]NMS in the segments was estimated from the saturation experiments with longer incubation and at a higher temperature (1610 ± 79, 1760 ± 91, and 1680 ± 102 pM in 32-h incubation at 4°C, 48-h incubation at 4°C, and 4-h incubation at 37°C, respectively), whereas the $B_{max}$ values were not different among the incubation conditions (1510 ± 92, 1440 ± 186, and 1480 ± 112 fmol/mg tissue protein, respectively) (n = 4). The representative saturation curves under three incubation conditions are shown in Supplemental Fig. 1.

Because the dissociation constant and binding kinetics for [³H]NMS were significantly different between the segments...
and the homogenates of rat cortex, the pharmacological profile of $[^3H]$NMS binding sites in both preparations was examined in competition binding studies using several drugs. Atropine and NMS monophasically competed with $[^3H]$NMS for binding, indicating a single binding affinity. Thus, both antagonists recognized the $[^3H]$NMS binding sites as a single set. However, the affinity estimates for atropine or nonradio-labeled NMS in the tissue segments were more than 10 times lower than those in the homogenates (Table 1). The estimated $pK_a$ values for NMS in the segments and homogenates were in good agreement with the $pK_a$ values for $[^3H]$NMS mentioned above.

Representative competition curves for the three mAChR antagonists that have been widely used for the determination of different muscarinic receptor subtypes are shown in Fig. 2, A to C (pirenzepine for $M_1$ subtype, AF-DX 116 for $M_2$ subtype, and MT-3 for $M_4$ subtype) (Wess et al., 2007; Alexander et al., 2009). Competition curves for pirenzepine and AF-DX 116 in the tissue segments and the homogenates were shallower and better fitted a two-site model in computer analysis (Fig. 2, A and B). The percentages of high-affinity sites for pirenzepine and AF-DX 116 were approximately 45 and 25%, respectively (Table 1). However, competition by MT-3 was monophasic and incomplete; approximately 15% of the binding sites were calculated to be MT-3-sensitive sites in both segments and homogenates (Fig. 2C). MT-7 ($M_1$-specific antagonist, 0.1–300 nM) also competed for approximately 45% of the $[^3H]$NMS binding sites in both preparations. Methoctramine (which is relatively selective for $M_3$ and $M_4$ subtypes) showed biphasic competition and had a $K_i$ of approximately 8.0 for the high-affinity sites, which represented approximately 35% of the total binding sites, regardless of whether measured in the segments or the homogenates. Thus, in addition to the predominant $M_1$ receptor subtype (approximately 45% of total binding), the $[^3H]$NMS binding sites in rat cerebral cortex included significant amounts of $M_2$ and $M_4$ subtypes (25 and 15%, respectively). The estimated proportions of the three subtypes ($M_1$, $M_2$, $M_4$) and the $pK_a$ values for their subtype-selective antagonists tested were not significantly different between tissue segments and homogenates (Fig. 2F and Table 1) and were well consistent with previous reports (Waelbroeck et al., 1990).

In contrast, the binding competition curves for darifenacin and solifenacin ($M_3$-selective antagonists) seemed to differ between tissue segments and homogenates. As shown for the tissue segments in Fig. 2, D and E, both antagonists at relatively high concentrations monotonically competed with $[^3H]$NMS binding (Hill slope: $-0.92 \pm 0.07$ for darifenacin and $-0.89 \pm 0.04$ for solifenacin; $n = 7$ in each). In contrast, in the homogenates (Fig. 2, D and E, open symbols), these $M_3$ antagonists competed with $[^3H]$NMS for binding to the homogenates in a biphasic manner over a wide range of concentrations (Hill slope: $-0.64 \pm 0.05$ for darifenacin and $-0.67 \pm 0.04$ for solifenacin; $n = 7$ in each). Therefore, the $[^3H]$NMS binding sites were detected totally as low-affinity sites for darifenacin and solifenacin in the segments, whereas in the homogenates approximately 15% of the total binding showed an apparently higher affinity for the $M_3$ antagonists. These results suggested that the $M_3$ mAChR subtype of rat cerebral cortex shows an atypical binding profile in the segments but its pharmacological property changes to a typical $M_3$ profile after homogenization. The proportions of mAChR subtypes identified in the segments and homogenates of rat cerebral cortex are shown in Fig. 2F and Table 1.

The above results in the segments were obtained in 16- to 18-h incubation at 4 °C. Because binding kinetics was very slow at low temperature, no detection of $M_3$ subtype in the segments may reflect extremely slow association of the $M_3$ antagonists. This possibility was examined in two types of experiments. At first, competitors (darifenacin, solifenacin, and pirenzepine) were simultaneously incubated with $[^3H]$NMS for longer periods (32 and 48 h; Supplemental Fig. 2). Second, the cortex segments were pretreated with competitors for 24 h and then incubated with $[^3H]$NMS for an additional 24 h in the presence of competitor (Supplemental Fig. 3). In both cases, three tested competitors showed the same competition curves as those in 16- to 18-h incubation, and the similar affinities were estimated. Therefore, it was likely that lack of high-affinity sites for darifenacin and solifenacin in cerebral cortex segments reflects the inherent property of $M_3$ mAChR in this tissue.

$[^3H]$NMS Binding to Intact Segments and Homogenates of Other Tissues. Because unique binding parameters for $[^3H]$NMS, darifenacin, and solifenacin were detected in the intact segments of rat cerebral cortex, we then examined whether such unique profiles could be detected in the segments of other tissues (hippocampus, corpus striatum, midbrain, pons, urinary bladder detrusor muscle, gastric muscle, and submaxillary gland). The dissociation constants and maximal binding capacities of $[^3H]$NMS in segments of various tissues are shown in Table 2. Large variations, not only in the density of mAChRs, but also in $[^3H]$NMS binding affinity, were observed between the tissues. Representative competition binding curves for darifenacin and solifenacin shown in Fig. 3 were obtained using segments of hippocampus and urinary bladder detrusor muscle. In contrast with cerebral cortex, the slope factors in the pseudo-Hill plot analyses for darifenacin and solifenacin were different from unity: $-0.58 \pm 0.05$ and $-0.56 \pm 0.06$ in the hippocampus and $-0.43 \pm 0.09$ and $-0.48 \pm 0.06$ in urinary bladder detrusor muscle, respectively. In homogenates, similar competition curves were obtained for both tissues (Fig. 3). $M_3$ mAChR subtype showing high affinity for darifenacin and solifenacin was identified in the segments of all tested tissues except cerebral cortex, and the high- and low-affinity estimates were relatively constant for these tissues (Table 3).

TABLE 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$B_{max}$ (fmol/mg of protein)</th>
<th>$K_i$ (pM)</th>
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<tbody>
<tr>
<td>Cerebral cortex</td>
<td>1420 ± 82</td>
<td>1420 ± 165</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1349 ± 67</td>
<td>1130 ± 53</td>
</tr>
<tr>
<td>Corpus striatum</td>
<td>1337 ± 49</td>
<td>1045 ± 29</td>
</tr>
<tr>
<td>Midbrain</td>
<td>240 ± 31</td>
<td>713 ± 24</td>
</tr>
<tr>
<td>Pons</td>
<td>412 ± 17</td>
<td>936 ± 46</td>
</tr>
<tr>
<td>Urinary bladder muscle</td>
<td>621 ± 36</td>
<td>139 ± 27</td>
</tr>
<tr>
<td>Gastric muscle</td>
<td>1417 ± 58</td>
<td>560 ± 72</td>
</tr>
<tr>
<td>Submaxillary gland</td>
<td>326 ± 21</td>
<td>262 ± 17</td>
</tr>
</tbody>
</table>

The dissociation constants and maximal binding capacities of $[^3H]$NMS in intact segments of various tissues.

Data represent means ± S.E.M. of four to seven experiments. Saturation binding experiments with intact tissue segments were carried out at 4°C.
Distinct mACHR Phenotypes in the Rat Cerebral Cortex

Fig. 3. Competition curves of darifenacin and solifenacin at [3H]NMS binding sites in the segments and homogenates of hippocampus and urinary bladder detrusor muscle. Representative competition binding curves for darifenacin (A and C) and solifenacin (B and D) using intact segments and homogenates are shown. The concentrations of [3H]NMS used were 2000 and 500 pM in the hippocampus intact segments and homogenates, respectively, and 500 pM in both the detrusor muscle segments and homogenates.

Discussion

The main finding of our study was that measurements of [3H]NMS binding revealed an unexpectedly low-affinity phenotype for the mACHRs in intact cortex tissue segments compared with homogenates. However, the density of mACHRs detected by [3H]NMS was essentially the same in both homogenate and tissue segment preparations. Under comparable binding conditions, variable affinities for M3 binding were also observed in the cortex.

To evaluate the native profile of mACHRs, the tissue segment binding assay was first validated using rat cerebral cortex segments. Nanomolar concentrations of [3H]NMS slowly but specifically bound to cortex segments. This binding was saturable and reversible and was selectively inhibited by several mACHR ligands including atropine. Because the binding kinetic analysis revealed a rate constant for dissociation of [3H]NMS from intact cortex segments of 1.6 ± 0.14 × 10⁻³ min⁻¹, which translates into a half-time of 7.2 h, the segments were incubated for long periods (16–18, 32, and 48 h) and binding equilibrium of [3H]NMS was confirmed.

Two significant differences were observed between the tissue segment binding and the homogenate binding. The first difference was that [3H]NMS displayed distinct dissociation constants when assayed using segments and homogenates of rat cerebral cortex (1400–1700 pM in segments and 260 pM in homogenates). Such a difference in dissociation constant was estimated from not only binding saturation experiments but also from kinetic measurements of the on and off rates. Furthermore, the time course of [3H]NMS binding was extremely slow in the segments compared with the homogenates. [3H]NMS has been classically recognized as a subtype-nonspecific but specific radioligand of mACHRs showing a relatively constant and high (subnanomolar) affinity in various tissues (Alexander et al., 2009), for example, 270 pM in rat cerebral cortex, 290 pM in rat hippocampus, 230 pM in rat corpus striatum, and 547 pM in rat gastric muscle (Delmendo et al., 1989; Ehler and Tran, 1990; Muramatsu et al., 2006) and Abrams et al., 1989; Ehlert and Tran, 1990; Muramatsu et al., 1989; Ehlert and Tran, 1990; Muramatsu et al., 1989).

TABLE 3

Binding affinities for M3-selective mACH receptor antagonists (darifenacin and solifenacin) estimated in intact segments of various tissues of rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>[3H]NMS pKᵢ (% high)</th>
<th>[3H]NMS pKᵢ (% high)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>8.3 ± 0.2 (15 ± 3)</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>8.5 ± 0.1 (19 ± 2)</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>Corpus striatum</td>
<td>8.6 ± 0.2 (32 ± 5)</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td>Midbrain</td>
<td>8.3 ± 0.3 (35 ± 5)</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>Pons</td>
<td>8.2 ± 0.2 (22 ± 3)</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>Urinary bladder muscle</td>
<td>8.3 ± 0.2 (25 ± 3)</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>Gastric muscle</td>
<td>8.6 ± 0.1 (27 ± 3)</td>
<td>6.2 ± 0.3</td>
</tr>
<tr>
<td>Submaxillary gland</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 4

Binding affinities of various muscarinic antagonists for recombinant M1 and M3 mACHRs

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>M1 mACHRs</th>
<th>M3 mACHRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>9.20 ± 0.20</td>
<td>9.30 ± 0.20</td>
</tr>
<tr>
<td>NMS</td>
<td>9.47 ± 0.18</td>
<td>9.70 ± 0.30</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>8.25 ± 0.28</td>
<td>6.80 ± 0.20</td>
</tr>
<tr>
<td>MT-7</td>
<td>9.10 ± 0.39</td>
<td>N.D.</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>6.15 ± 0.07</td>
<td>6.00 ± 0.20</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>7.10 ± 0.20</td>
<td>6.43 ± 0.05</td>
</tr>
<tr>
<td>Darifenacin</td>
<td>6.90 ± 0.20</td>
<td>8.40 ± 0.20</td>
</tr>
<tr>
<td>Solifenacin</td>
<td>7.10 ± 0.30</td>
<td>8.10 ± 0.20</td>
</tr>
</tbody>
</table>

N.D., not determined; pKᵢ, negative logarithm of the equilibrium constant for tested drugs.
2005; Alexander et al., 2009). These high affinities for \[^{3}H\]NMS all were estimated in the conventional membrane binding studies. A similar high affinity was obtained from the present homogenate binding, but a wide variation of \[^{3}H\]NMS dissociation constants was observed in the segments of rat cerebral cortex and other tissues (Table 2). Thus, the difference in ligand affinities between segments and homogenates or among tissues suggests that both of binding parameters (affinity and kinetics) are significantly affected by receptor environments, especially tissue integrity.

In addition to the saturation binding experiments, competition experiments revealed an approximately 10 times lower affinity for NMS and atropine in the cortex segments compared with the homogenates. However, such a large deviation was not detected in the affinities of segments and homogenates for other competitive ligands (pirenzepine, AF-DX 116, MT-7, methoctramine, and MT-3). Therefore, it is likely that receptor environment does not uniformly bind the binding of all ligands. Indeed, this conclusion is supported by relatively high binding affinities for other tritium radioligands in the rat cortex segments (281 pM for \[^{3}H\]prazosin at \(\alpha_{1}\)-adrenoceptor, Morishima et al., 2008; 204 pM for \[^{3}H\]epibatidine at nicotinic acetylcholine receptor, M.-H. Wang, H. Yoshiki, A. S. M. Anisuzzaman, and I. Muramatsu, unpublished observations).

Another difference between segments and homogenates was observed during mACHR subtype analysis of rat cerebral cortex. As reported previously (Ehlert and Tran, 1990; Waelbroeck et al., 1990; Ferrari-Dileo et al., 1994), the mACHRs in the homogenates of rat cerebral cortex were composed of M\(_1\), M\(_2\), M\(_3\), and M\(_4\) subtypes in an approximately 45:25:15:15 ratio, although there are some limitations in the estimation by subtype selectivity of available antagonists (Table 1). However, high-affinity binding for M\(_2\)-selective antagonists (darifenacin and solifenacin) was not observed in the segments, and the M\(_3\) subtype could not be distinguished from the other mACHR subtypes by the M\(_3\) antagonists. The expression of M\(_3\) mACHRs in the rat cerebral cortex has been demonstrated previously at both protein and mRNA levels (conventional membrane binding, Waelbroeck et al., 1990; Western blotting, Levey et al., 1991; mRNA: Krejci and Tucek, 2002). Thus, because the total density of mACHRs and the proportions of M\(_1\), M\(_2\), M\(_3\), and M\(_4\) subtypes were not different between the segments and the homogenates the disappearance of high-affinity binding for M\(_3\) antagonists suggests the presence of an atypical phenotype of M\(_3\) mACHRs under the segmental conditions. Among the tested tissues, such an atypical M\(_3\) phenotype was unique to the cerebral cortex segments (Table 3 and Figs. 2 and 3).

It is interesting to note that both of the significant differences (a variety of \[^{3}H\]NMS affinities and an atypical M\(_3\) phenotype) were observed only in the segments and failed to be detected in the homogenates. Furthermore, such unique profiles were also not observed when recombinant M\(_1\) and M\(_3\) mACHRs were assayed under whole-cell conditions (Table 4). No divergent pharmacology has been reported in conventional membrane binding assays using native and recombinant mACHRs (Dörje et al., 1991; Caulfield and Birdsell, 1998; Alexander et al., 2009). Thus, it was considered that the unique pharmacological profiles are extremely dependent on their endogenous expression in native tissues and/or on their natural receptor environment.

Evidence on the receptors showing distinct pharmacological profiles under different assay conditions has been gradually accumulating. An early example is that several antidepressants such as chlorimipramine potently inhibited H\(_2\) receptor-mediated stimulation of cAMP formation in the homogenates of guinea pig hippocampus but failed to affect the cAMP formation in the slice preparations (Dam Trung Tuong et al., 1980). The pharmacological profile of mACHRs mediating carbachol-induced contraction in the guinea pig and rat uterus was not in agreement with the profile obtained from radioligand binding studies with the homogenates (Boxall et al., 1998; Munns and Pennefather, 1998). In the rat stomach mucosa, M\(_3\) mACHRs could be detected in the tissue segments but were undetectable in the homogenates (Anisuzzaman et al., 2008a). In the \(\alpha_{1}\)-adrenoceptors of rat cortex and rabbit prostate, the pharmacological profile showing low affinity for prazosin changed to high affinity after homogenization (Morishima et al., 2008; Su et al., 2008). These lines of evidence strongly suggest that tissue integrity is significantly attributed to the native pharmacological profile of receptors.

The question remains as to the mechanisms that underlie the unique pharmacological properties of mACHRs in intact segments. The simplest assumption is that the receptor conformation and therefore its interaction with common antagonists are different between the intact tissue and the disrupted membrane. The intact tissue can provide for distinct submembrane effector interactions in the presence of a constrained membrane architecture. This condition may modify the receptor-ligand association or inhibit the accessibility of ligand to receptor. In this scenario, tissue-specific environment is easily destroyed by homogenization, resulting in relatively universal properties for each mACHR subtype, which have been predicted previously. And, such a tissue-specific environment would not be easily reconstructed by simple expression of each receptor cDNA in a cell culture system.

The pharmacological and biochemical properties of G protein-coupled receptors, including mACHRs, were demonstrated to be more complex than originally supposed (Christopoulos and Kenakin, 2002; van Koppen and Kaiser, 2003; Bockaert et al., 2004; Baker and Hill, 2007; Ramachandran et al., 2009; Nishimune et al., 2010). Most G protein-coupled receptors possess not only orthosteric binding sites but additional binding sites such as allosteric sites, form multiple conformations, and activate different signaling pathways. The atypical M\(_3\) phenotype detected in the cortex segments may be related to a distinct isoform or a distinct conformation of M\(_3\) mACHR. It is possible that the distinct phenotype/conformation may be produced by interaction with many associated proteins such as cytoskeleton and CRELD 1a (cysteine-rich with epidermal growth factor-like domain protein 1a). However, further studies are recommended to clarify the mechanisms underlying the atypical M\(_3\) mACHR phenotype and the variation in \[^{3}H\]NMS affinity.

In conclusion, the present study on mACHRs suggests that antagonist affinity is not necessarily constant, but is rather changeable depending on the cell/tissue environment and tissue integrity. Tissue dependence of pharmacological phenotype would provide very valuable information for the study of in vivo pharmacology and other in vivo receptor analyses.
such as single-photon emission tomography or positron emission tomography.

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Authorship Contributions

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Conducted experiments: Anisuzzaman, Yoshiki, and Muramatsu.

Contributed new reagents or analytic tools: Uwada.

Performed data analysis: Anisuzzaman, Nishimune, and Muramatsu.

Wrote or contributed to the writing of the manuscript: Anisuzzaman and Muramatsu.

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Journal title: Journal of Pharmacology and Experimental Therapeutics

Supplemental figure 1. Representative saturation curves of $[^3\text{H}]$NMS binding to rat cerebral cortex segments in the incubation for 32 h at 4°C (A), 48 h at 4°C (B) and 4 h at 37°C (C). The specific binding (open circle) was determined by subtracting the amount of $[^3\text{H}]$NMS binding in the presence of 1μM of atropine (closed triangle) from the total binding (closed circle).
Supplemental figure 2. Competition curves of pirenzepine (A), darifenacin (B) and solifenacin (C) at [3H]NMS binding sites in rat cerebral cortex segments (n = 4). Competition experiments were carried out at 4°C for 32 h (■) and 48 h (○). The concentration of [3H]NMS was 2.0 nM. The Hill-slopes in the incubation for 32 h and 48 h were -0.43 ± 0.06 and -0.48 ± 0.04 for pirenzepine, -0.93 ± 0.08 and -1.09 ± 0.11 for darifenacin; and -0.91 ± 0.05 and -0.95 ± 0.07 for solifenacin, respectively. The affinity estimates (pKi) and proportions (%) of high affinity sites for pirenzepine were 8.06 ± 0.13 (45 ± 5 %) and 8.10 ± 0.15 (42 ± 7 %) for 32 and 48 h incubation, respectively. The pKi value in 32 and 48 h incubation for darifenacin were 6.28 ± 0.17 and 6.30 ± 0.10; and those for solifenacin were 6.40 ± 0.20 and 6.27 ± 0.13, respectively.
A. Pirenzepine

Supplemental figure 3. Competition curves of pirenzepine (A), darifenacin (B) and solifenacin (C) at [³H]NMS binding sites in rat cerebral cortex segments. The segments were pretreated with the competitor for 24 h and then incubated for subsequent 24 h with [³H]NMS at 4°C (n = 4). The concentration of [³H]NMS was 2.0 nM. The Hill-slopes were -0.39 ± 0.12, -1.08 ± 0.05 and -0.89 ± 0.10 for pirenzepine, darifenacin and solifenacin, respectively. The affinity estimates (pKi) and proportions of high affinity sites for pirenzepine were 8.10 ± 0.20 (51 ± 6 %), and the pKi values for darifenacin and solifenacin were 6.18 ± 0.14 and 5.95 ± 0.20, respectively.