Influence of tissue integrity on pharmacological phenotypes of muscarinic acetylcholine receptors in the rat cerebral cortex

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Running title page.

Running title: Distinct mAChR phenotypes in the rat cerebral cortex

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Abbreviations:
AF-DX 116; 11-([2-[(diethylamino) methyl]-1-piperidinyl]acetyl)-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one
CHO; Chinese hamster ovary
MT-7; muscarinic toxin 7
MT-3; muscarinic toxin 3
NMS; N-methylscopolamine
QNB; 3-quinuclidinylbenzilate

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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 $[^3]H$-N-methylscopolamine ($[^3]H$NMS). Recombinant M$_1$ and M$_3$ mAChRs were also examined. The density of mAChRs detected by $[^3]H$NMS binding to rat cerebral cortex segments and homogenates was the same (approximately 1400 fmol/mg tissue protein), but the dissociation constant of $[^3]H$NMS was significantly different (1400-1700 pM in segments and 260 pM in homogenates). A wide variation in $[^3]H$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 M$_1$, M$_2$, M$_3$ and M$_4$ subtypes, which showed typical subtype pharmacology in the homogenates. However, in the cortex segments the M$_3$ subtype showed a low selectivity for M$_3$ antagonists (darifenacin, solifenacin) and was not distinguished by the M$_3$ antagonists from the other subtypes. Recombinant M$_1$ and M$_3$ mAChRs showed high affinity for $[^3]H$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 $[^3]H$NMS binding affinity among mAChRs of many rat tissues and the presence of an atypical M$_3$.
phenotype in the cerebral cortex, suggesting that the pharmacological properties of mACHRs are not necessarily constant, rather 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, 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; Nelson and Challiss, 2007; Baker and Hill, 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; Dorje 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). For example, in the
guinea-pig and rat uterus the functional affinities for mAChR antagonists are not in
agreement with the affinities of M₂ 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 (OAB), display \textit{in vivo} selectivity for inhibition of
mAChR-mediated urinary bladder contraction relative to salivary secretion, even though
both responses are mediated by the same M₃ 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 using functional bioassays but also
using radioligand binding assays. Tissue strips are used routinely to obtain
bioassay-derived affinities, while 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 which differ
between the preparations used for these two distinct assays (intact tissue vs.
membranes) may confer distinct receptor characteristics that can interact differently
with the same antagonist (Christopoulos, 2000).

A recently developed 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; Anisuzzaman et al., 2008b; Su et al., 2008; Sathi et al., 2008; Muramatsu et al., 2009). 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 which 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 M₁, M₂, M₃ and M₄ mAChR subtypes have been clearly identified in rat cerebral cortex using conventional membrane binding assays (Ehlert and Tran., 1990; Waelbroeck et al., 1990; Ferrari-Dileo et al., 1994). We hypothesized that there would be differences in the mAChR phenotypes present in intact cortex tissue segments, as compared with the same receptors present in homogenates. To test this hypothesis, we used a hydrophilic radioligand [³H]NMS and re-evaluated the pharmacological profiles of mAChRs present in rat cerebral cortex 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 M₃ mAChR phenotype in rat cerebral cortex.
Methods:

Animals.

Male Wistar rats weighing 250-350 g (Charles River Japan, Inc., Yokohama, Japan) were used in the present study, which was conducted according to the Guidelines for Animal Experiments, University of Fukui (which is accredited by the Ministry of Education, Culture, Sports, Science and Technology, 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 (in mM): NaCl, 120.7; KCl, 5.9; MgCl₂, 1.2; CaCl₂, 2.0; NaH₂PO₄, 1.2; NaHCO₃, 25.5 and D-(-)-glucose, 11.5 (pH 7.4), which had been oxygenated with a mixture of 95% O₂ and 5% CO₂ and was 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 gastric body and the detrusor muscle of urinary bladder were used. 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 16-18 h in a final volume of 1 ml (Muramatsu et al., 2005; Anisuzzaman et al., 2008a). 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 figures). The incubation times for the association and dissociation experiments were recorded in the figures and their legends.

The incubation medium was similar to that of the modified Krebs-Henseleit solution, containing (in mM): NaCl, 135.7; KCl, 5.9; MgCl₂, 1.2; CaCl₂, 2.0; NaH₂PO₄, 1.2; NaHCO₃, 10.5 and D-(+)-glucose, 11.5 (pH = 7.4 under equilibrated conditions with air). In saturation binding experiments, [³H]NMS was used at concentrations ranging from 50–12,500 pM. In competition experiments, [³H]NMS was simultaneously incubated with various concentrations of unlabelled 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 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 were 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 μM 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 Inc., Hercules, CA, USA). 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 using a Polytron homogenizer (setting at 8, 5 × 20 s at 4 °C). The Krebs incubation buffer was the same as that used in the tissue segment binding experiments. In order 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 about 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 unlabelled competing ligands. Reactions were terminated by rapid filtration, using a Brandel cell harvester, onto Whatman GF/C filters 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 M<sub>1</sub> and M<sub>3</sub> mAChRs in Chinese hamster ovary (CHO) cells.**
CHO cell lines stably expressing M₁ or M₃ mAChRs (CHO-M₁ or CHO-M₃, 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, Maryland, USA) (Dorje et al., 1991). The cells were cultured in Dulbecco’s modified Eagle’s medium (Wako, 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 about 2 h.

Data analysis.

Binding data were analyzed using PRISM (Version 5.01, Graph Pad Software, La Jolla, CA, USA), as previously described (Muramatsu et al., 2005; Yoshiki et al., 2009). Briefly, the data from saturation binding studies were fitted by a one-site saturation binding isotherm (Binding-Saturation Equation in PRISM), and the Kd 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 (Bmax). For the competition studies, the data were analyzed using the Binding-Competitive Equation of PRISM. When different Kd values were estimated for [3H]NMS from saturation experiments using tissue segments and homogenates, the corresponding Kd value estimated for each preparation was used for analysis of the competition binding data to calculate Ki 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 model, 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: 1-[N-Methyl-3H]–scopolamine methyl chloride ([3H]NMS, specific activity 3.00 TBq/mmol) (Amersham Biosciences, 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 Co., St. Louis, MO, USA); AF-DX 116, methoctramine (Tocris Cookson Ltd., Bristol, UK); muscarinic toxin 7 (MT-7), muscarinic toxin 3 (MT-3) (Peptide Institute, 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 (16-18 h incubation at 4 °C for tissue segments; 4 h incubation at 4 °C for homogenates) was concentration-dependent (Figure 1A and 1B). Nonspecific binding of 2,000 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 (Figure 1D and 1E).

The specific binding of [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 $1.01 \pm 0.05 \times 10^6$ M$^{-1}$ min$^{-1}$ and the dissociation rate constant (K_{off}) was $1.5 \pm 0.11 \times 10^{-3}$ min$^{-1}$, respectively from the association experiments (n = 5). While the K_{off} value was calculated as $1.6 \pm 0.14 \times 10^{-3}$
min\(^{-1}\) from the dissociation experiment (n = 5). The binding kinetics of \(^{3}H\)NMS were
more rapid for the homogenates than for the tissue segments; the \(K_{on}\) and \(K_{off}\) values in
the homogenates were 6.8 \(\pm 0.03 \times 10^{7}\) M\(^{-1}\)min\(^{-1}\) and 21.0 \(\pm 1.6 \times 10^{-3}\) min\(^{-1}\) respectively
(n = 5).

Hill coefficients of saturation binding curves were 0.98 \(\pm 0.09\) (0.87 – 1.24) for
tissue segments and 0.95 \(\pm 0.07\) (0.85 – 1.17) for homogenate preparations. The binding
saturation isotherms revealed that the specific binding of \(^{3}H\)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 \(\pm 82\) fmol/mg tissue protein (n = 7), and for homogenates, 1380 \(\pm 32\)
fmol/mg tissue protein (n = 7). However, the binding affinities (K\(_{d}\)) differed
substantially: K\(_{d}\) = 1420 \(\pm 165\) pM (pK\(_{d}\) = 8.85 \(\pm 0.05\)) for tissue segments and K\(_{d}\) =
260 \(\pm 71\) pM (pK\(_{d}\) = 9.58 \(\pm 0.12\)) for homogenates. Therefore, in comparing the two
preparations (segments vs. homogenates), there was no difference in the abundance of
\(^{3}H\)NMS binding sites (Figure 1C), but there was a significant difference in the
dissociation constants of \(^{3}H\)NMS between the segments and the homogenates (p<0.05).
The dissociation constants for \(^{3}H\)NMS estimated from the saturation curves were in
good agreement with the kinetically derived K\(_{d}\) values in the segments (1490 \(\pm 217\)
pM) and in 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 pM, 1760 ± 91 pM and 1680 ± 102 pM in 32 h incubation at 4°C, in 48 h incubation at 4°C and in 4 h incubation at 37°C, respectively), while the Bmax values were not different among the incubation conditions (1510 ± 92 and 1440 ± 186, 1480 ± 112 fmol/mg tissue protein, respectively) (n = 4). The representative saturation curves under three incubation conditions are shown in supplemental figure 1.

Since 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 non-radiolabeled NMS in the tissue segments were more than 10 times lower than those in the homogenates (Table 1). The estimated pKi values for NMS in the segments and homogenates were respectively in good agreement with the pKd 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 Figures 2A, 2B and 2C (pirenzepine for M₁ subtype, AF-DX 116 for M₂ subtype and MT-3 for M₄ subtype) (Wess et al., 2007; Alexander et al., 2009). Competition curves for pirenzepine and AF-DX 116 in the tissue segments and in the homogenates were shallow, and better fitted a two-site model in computer analysis (Figure 2A and 2B). 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 (Figure 2C). MT-7 (M₁ 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 M₂ and M₄ subtypes) showed biphasic competition, and had a pKi 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 M₁ receptor subtype (approximately 45% of total binding), the [³H]NMS binding sites in rat cerebral cortex also included significant amounts of M₂ and M₄ subtypes (25% and 15%, respectively). The estimated proportions of the three
subtypes (M₁, M₂, M₄) and the pKi values for their subtype-selective antagonists tested were not significantly different between tissue segments and homogenates (Figure 2F, Table 1), and were well consistent with previous reports (Waelbroeck et al., 1990).

In contrast, the binding competition curves for darifenacin and solifenacin (M₃ selective antagonists) appeared to differ between tissue segments and homogenates. As shown for the tissue segments in Figure 2D and 2E, both antagonists at relatively high concentrations monotonically competed with [³H]NMS for binding (Hill slope: -0.92 ± 0.07 for darifenacin and -0.89 ± 0.04 for solifenacin, n = 7 in each). In contrast, in the homogenates (open symbols, Figures 2D and 2E), these M₃ antagonists competed with [³H]NMS for binding to the homogenates in a biphasic manner over a wide range of concentrations (Hill slope: -0.64 ± 0.05 for darifenacin and -0.67 ± 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 M₃ antagonists. These results suggested that the M₃ mAChR subtype of rat cerebral cortex shows an atypical binding profile in the segments but that its pharmacological property changes to a typical M₃ profile after homogenization. The proportions of mAChR subtypes identified in the segments and homogenates of rat
cerebral cortex are shown in Figure 2F and Table 1.

The above results in the segments were obtained in 16-18 h incubation at 4 °C. Since binding kinetics was very slow at low temperature, no detection of M₃ subtype in the segments may reflect extremely slow association of the M₃ 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 figure 2). Second, the cortex segments were pretreated with competitors for 24 h and then incubated with [³H]NMS for additional 24 h in the presence of competitor (supplemental figure 3). In both cases, three tested competitors showed the same competition curves as those in 16-18 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 M₃ mAChR in this tissue.

[³H]NMS binding to intact segments and homogenates of other tissues.

Since unique binding parameters for [³H]NMS, darifenacin and solifenacin were detected in the intact segments of rat cerebral cortex, we then examined if such unique profiles could be detected in the segments of other tissues (hippocampus, corpus striatum, and pituitary gland).
striatum, midbrain, pons, urinary bladder detrusor muscle, gastric muscle and submaxillary gland). The dissociation constants and maximal binding capacities of $[^3\text{H}]\text{NMS}$ in segments of various tissues are shown in Table 2. Large variations, not only in the density of mACHRs, but also in $[^3\text{H}]\text{NMS}$ binding affinity, were observed between the tissues. Representative competition binding curves for darifenacin and solifenacin are shown in Figure 3, which 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 (Figure 3). M$_3$ mACHR subtype showing high affinity for darifenacin and solifenacin was identified in the segments of all tested tissues except for cerebral cortex, and the high and low-affinity estimates were relatively constant for these tissues (Table 3).

$[^3\text{H}]\text{NMS}$ binding to CHO-M$_1$ or CHO-M$_3$ mACHRs

Since a predominant mACHR in central nervous system is the M$_1$ subtype (present study and Abrams et al., 2006) and since the M$_3$ mACHRs in rat cortex showed an

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atypical phenotype, the pharmacological properties of recombinant M₁ and M₃ mAChRs expressed in CHO cells were also examined under the same experimental conditions as for the tissue segments and homogenates. Whole cell binding experiments at 4 °C revealed high affinity Kd values for [³H]NMS for both M₁ and M₃ mAChR subtypes (270 ± 25 pM and 290 ± 15 pM, respectively, n = 4). The recombinant M₁ subtype showed high affinities for atropine, NMS, pirenzepine and MT-7, while the recombinant M₃ subtype showed high affinities for atropine, NMS, darifenacin and solifenacin (Table 4).
Discussion:

The main finding of our study was that measurements of $[^3]$H]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 $[^3]$H]NMS was essentially the same in both homogenate and tissue segment preparations. Under comparable binding conditions, variable affinities for M$_3$ binding were also observed in the cortex.

In order to evaluate the native profile of mAChRs, the tissue segment binding assay was first validated using rat cerebral cortex segments. Nanomolar concentrations of $[^3]$H]NMS slowly but specifically bound to cortex segments. This binding was saturable and reversible, and was selectively inhibited by several mAChR ligands including atropine. Since the binding kinetic analysis revealed a rate constant for dissociation of $[^3]$H]NMS from intact cortex segments of $1.6 \pm 0.14 \times 10^{-3}$ min$^{-1}$, which translates into a half time of 7.2 h, the segments were incubated for long periods (16-18 h, 32 h and 48 h) and binding equilibrium of $[^3]$H]NMS was confirmed.

Two significant differences were observed between the tissue segment binding and the homogenate binding. The first difference was that $[^3]$H]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 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 $[^3]$H]NMS binding was extremely slow in the segments, as compared with the homogenates. $[^3]$H]NMS has been classically recognized as a subtype-nonselective 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; Ehlert and Tran, 1990; Muramatsu et al., 2005; Alexander et al., 2009). These high affinities for $[^3]$H]NMS were all 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 also 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 modify 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 [3H]prazosin at \( \alpha_1 \)-adrenoceptor, Morishima et al., 2008; 204 pM for [3H]epibatidine at nicotinic acetylcholine receptor, Muramatsu et al., 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 M1, M2, M3 and M4 subtypes roughly in a 45 : 25 : 15 : 15 ratio, although there are some limitation in the estimation by subtype-selectivity of available antagonists (Table 1). However, high-affinity binding for M3-selective antagonists (darifenacin and solifenacin) was not observed in the segments and the M3 subtype could not be distinguished from the other mAChR
subtypes by the M₃ antagonists. The expression of M₃ mAChRs in the rat cerebral
cortex has been previously demonstrated at both protein and mRNA levels
(conventional membrane binding: Waelbroeck et al., 1990; western blotting: Levey et
al., 1991; mRNA: Krejcí and Tucek, 2002). Thus, since the total density of mAChRs
and the proportions of M₁, M₂ and M₄ subtypes were not different between the
segments and the homogenates, the disappearance of high-affinity binding for M₃
antagonists suggests the presence of an atypical phenotype of M₃ mAChRs under the
segmental conditions. Among the tested tissues, such an atypical M₃-phenotype was
unique to the cerebral cortex segments (Table 3, Figures 2 and 3).

It is interesting to note that both of the significant differences (a variety of
[^3]H[NMS affinities and an atypical M₃ 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 M₁ and M₃ 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
(Dorje et al., 1991; Caulfield and Birdsall, 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 H₂-receptor-mediated stimulation of cAMP formation in the homogenates of guinea-pig hippocampus but failed to affect the cAMP formation in the slice preparations (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, M₁-mAChRs could be detected in the tissue segments but were undetectable in the homogenates (Anisuzzaman et al., 2008b).

In the α₁-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 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 previously predicted. And, such tissue-specific environment would not be easily reconstructed by simple expression of each receptor cDNA in cell culture system.

Recently, the pharmacological and biochemical properties of G protein-coupled receptors (GPCRs) 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 GPCRs possess not only orthosteric binding sites but also additional binding sites such as allosteric sites, form multiple conformations, and activate different signaling pathways. The atypical M₃ phenotype detected in the cortex segments may be related to a distinct isoform or a distinct conformation of M₃ mAChR. It is possible that the distinct phenotype/conformation may be produced by interaction with many associated proteins such as cytoskeleton and CRELD 1α. However, further studies are...
recommended to clarify the mechanisms underlying the atypical M₃ mAChR phenotype and the variation in [³H]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-dependency of pharmacological phenotype’ would provide very valuable information for the study of in vivo pharmacology and for other in vivo receptor analyses such as single photon emission tomography or positron emission tomography.
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Authorship Contributions:

Participated in research design: Anisuzzaman, Nishimune, Uwada, and Muramatsu.

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.
References:


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Footnotes:

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A.S.M.A is supported by the Headquarters for the Advancement of High Priority Research grant, University of Fukui.
Legends for figures:

Figure 1: Characterization of $[^3]H$NMS binding to intact segments and homogenates of rat cerebral cortex at 4°C. Representative saturation curves of $[^3]H$NMS binding to intact segments (A) and homogenates (B) of rat cerebral cortex are shown. The ordinate represents the level of $[^3]H$NMS binding per mg tissue protein. Specific binding (closed circles) was determined by subtracting the amount of $[^3]H$NMS bound in the presence of 1 μM atropine (nonspecific binding, open circles) from the total binding (open squares). Each point represents the mean of duplicate determinations. C: The abundance of $[^3]H$NMS binding sites estimated in the segments and homogenates of rat cerebral cortex (n = 7). D: Association experiments using three different concentrations of $[^3]H$NMS in rat cerebral cortex segments. The ordinate represents the specific binding of $[^3]H$NMS binding per mg tissue protein. The abscissa represents the incubation time. E: Dissociation experiment using rat cerebral cortex segments. After incubation with 2.0 nM $[^3]H$NMS for 12 h, unlabelled 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 SEM of representative experiment.
Figure 2: Pharmacological profile of \[^3\text{H}\]NMS binding sites in rat cerebral cortex. A-E: Competition curves for pirenzepine, AF-DX 116, MT-3, darifenacin and solifenacin in segments (closed circles) and homogenates (open circles) of rat cerebral cortex. The concentrations of \[^3\text{H}\]NMS used were 2000 and 500 pM for the intact segments and homogenates, respectively. F: Percentage of mAChR subtype in the segments and homogenates of rat cerebral cortex, estimated from competition binding experiments with various antagonists (Table 1).

Figure 3: Competition curves of darifenacin and solifenacin at \[^3\text{H}\]NMS binding sites in the segments and homogenates of hippocampus and urinary bladder detrusor muscle. Representative competition binding curves for darifenacin (A, C) and solifenacin (B, D) using intact segments and homogenates are shown. The concentrations of \[^3\text{H}\]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.
TABLE 1: Binding affinities for various muscarinic ligands estimated in intact tissue segments and homogenates of rat cerebral cortex.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Tissue segments</th>
<th>Homogenates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKᵢ&lt;sub&gt;high&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (%) high &lt;sup&gt;c&lt;/sup&gt;</td>
<td>pKᵢ&lt;sub&gt;low&lt;/sub&gt; (%) high &lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Atropine (NS&lt;sup&gt;e&lt;/sup&gt;)</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</td>
<td>5.81 ± 0.15</td>
</tr>
<tr>
<td>MT-7 (M₁)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.85 ± 0.44</td>
<td>4.93 ± 0.03</td>
</tr>
<tr>
<td>AF-DX 116 (M₂&gt;M₄)</td>
<td>6.97 ± 0.10</td>
<td>4.93 ± 0.03</td>
</tr>
<tr>
<td>Methoctramine (M₂≥ M₄)</td>
<td>7.90 ± 0.12</td>
<td>5.31 ± 0.08</td>
</tr>
<tr>
<td>Darifenacin (M₃)</td>
<td>6.40 ± 0.20</td>
<td>5.31 ± 0.08</td>
</tr>
<tr>
<td>Solifenacin (M₃)</td>
<td>6.70 ± 0.10</td>
<td>6.60 ± 0.08</td>
</tr>
<tr>
<td>MT-3 (M₄)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.84 ± 0.20</td>
<td>9.20 ± 0.20</td>
</tr>
</tbody>
</table>

Competition binding experiments using rat cerebral cortex were carried out at 4°C. The concentrations of [³H]NMS used were 1000 or 2000 pM for intact tissue segments and 500 pM for homogenates. Data represent means ± S.E.M. of 4 - 7 experiments.

<sup>a</sup> Subtype selectivity was referred from Dorje et al. (1991); Caulfield and Birdsell (1998) and Alexander et al. (2009).

<sup>b</sup> pKᵢ<sub>high</sub> and pKᵢ<sub>low</sub>: negative logarithm of the equilibrium constants (pKi) at high and low-affinity sites for tested drugs.

<sup>c</sup> % high: percentage of high-affinity sites.
MT-7 and MT-3 at concentrations up to 0.3 μM did not completely inhibit \[^3H\]NMS binding.

NS: non-selective for subtypes.
TABLE 2: The dissociation constants and maximal binding capacities of [³H]NMS in intact segments of various tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Bmax  (fmol/mg of protein)</th>
<th>Kd  (pM)</th>
</tr>
</thead>
<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>

Data represent means ± S.E.M. of 4 – 7 experiments.

Saturation binding experiments with intact tissue segments were carried out at 4°C.
TABLE 3: Binding affinities for M₃-selective mAChR antagonists (darifenacin and solifenacin) estimated in intact segments of various tissues of rats.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Darifenacin</th>
<th>Solifenacin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pK&lt;sub&gt;ihigh&lt;/sub&gt;</td>
<td>pK&lt;sub&gt;ilow&lt;/sub&gt;</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>6.4 ± 0.2</td>
<td>6.7 ± 0.1</td>
</tr>
<tr>
<td>hippocampus</td>
<td>8.3 ± 0.2</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>corpus striatum</td>
<td>8.5 ± 0.1</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>Midbrain</td>
<td>8.6 ± 0.2</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td>Pons</td>
<td>8.3 ± 0.3</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>Urinary bladder muscle</td>
<td>8.3 ± 0.2</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>Gastric muscle</td>
<td>8.3 ± 0.2</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>Submaxillary gland</td>
<td>8.6 ± 0.1</td>
<td>6.2 ± 0.3</td>
</tr>
</tbody>
</table>

Intact tissue segment binding experiments were carried out at 4°C using 1000 or 2000 pM [³H]NMS for cerebral cortex, hippocampus and corpus striatum and 500 pM for the other tissues.

The other experimental conditions are the same as those in Table 1.

Data represent means ± S.E.M. of 4 – 7 experiments.
TABLE 4: Binding affinities of various muscarinic antagonists for recombinant M₁ and M₃ mAChRs.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>M₁ mAChRs pKᵢᵃ</th>
<th>M₃ mAChRs pKᵢᵃ</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>NDᵇ</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>

Competition binding experiments using CHO-M₁ and CHO-M₃ cells were carried out at 4°C under whole cell conditions. The concentration of [³H]NMS used was 500 pM.

All of the competition curves were fitted to a one site model. Data represent means ± S.E.M. of 3 – 4 experiments.

ᵃpKᵢ: negative logarithm of the equilibrium constant for tested drugs.

ᵇND: Not determined.
Figure 1: Muscarinic acetylcholine receptor in the rat cerebral cortex

A. Segments

B. Homogenates

C. Abundance
Figure 1  D, E

D. Association in the segments

E. Dissociation in the segments
Figure 2: Muscarinic receptor subtypes in the rat cerebral cortex

A. Pirenzepine

B. AF-DX 116

C. MT-3

D. Darifenacin

E. Solifenacin

F. Subtypes
Figure 3: Darifenacin and solifenacin competition binding on hippocampus and detrusor muscle segments and homogenates

A. Hippocampus

B. Hippocampus

C. Detrusor muscle

D. Detrusor muscle