Full Paper

Functional Activation of G-Proteins Coupled With Muscarinic Acetylcholine Receptors in Rat Brain Membranes

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Abstract. The functional activation of $G_{i/o}$ proteins coupled to muscarinic acetylcholine receptors (mAChRs) was investigated with the conventional guanosine-5'-O-(3-[³⁵S]thio)triphosphate ([³⁵S]GTP γ S) binding assay in rat brain membranes. The most efficacious stimulation elicited by acetylcholine or carbachol (CCh) was obtained in striatal membranes. The pharmacological properties of mAChR-mediated [³⁵S]GTP γ S binding determined with a series of muscarinic agonists and antagonists were almost identical among the three brain regions investigated, i.e., cerebral cortex, hippocampus, and striatum, except for the apparent partial agonist effects of (α R)- α -cyclopentyl- α -hydroxy-N-[1-(4-methyl-3-pentenyl)-4-piperidinyl]benzeneacetamide fumarate (J 104129) observed only in the hippocampus, but not in the other two regions. Among the muscarinic toxins investigated, only MT3 attenuated CCh-stimulated [³⁵S]GTP γ S binding. The highly selective allosteric potentiator at the M₄ mAChR subtype, 3-amino-N-[(4-chlorophenyl)methyl]-4,6-dimethylthieno[2,3-*b*]pyridine-2-carboxamide (VU 10010), shifted the concentration–response curve for CCh leftwards as well as upwards. On the other hand, neither thiochrome nor brucine *N*-oxide was effective. The increases induced by CCh and 5-HT were essentially additive, though not completely, indicating that the mAChRs and 5-HT_{1A} receptors were coupled independently to distinct pools of $G_{i/o}$ proteins. Collectively, all of the data suggest that functional activation of $G_{i/o}$ proteins coupled to mAChRs, especially the M₄ subtype, is detectable by means of CCh-stimulated [³⁵S]GTP γ S binding assay in rat discrete brain regions.

Keywords: muscarinic acetylcholine receptor, G-protein, [³⁵S]GTP γ S binding, muscarinic toxin, allosteric modulator

Introduction

Acetylcholine (ACh) plays vital roles as a neurotransmitter in modulating diverse central functions such as sleep, alertness, cognition, motor control, sensory processing, learning, and memory. There are two major families of receptors for ACh, the ionotropic nicotinic and metabotropic muscarinic receptors, and physiological functions of ACh are predominantly mediated through the latter in the central nervous system. The muscarinic ACh receptor (mAChR) family consists of five receptor subtypes termed M₁ – M₅, which are further separated into two groups based on G-protein coupling. The first

group (M₁, M₃, and M₅ mAChRs) activate phospholipase C and mobilize intracellular calcium through $G_{q/11}$, while the second group (M₂ and M₄ mAChRs) are coupled to $G_{i/o}$ and inhibit adenylate cyclase activity (1, 2).

An initial and common signaling step for G-protein-coupled receptors (GPCRs) including mAChRs is the agonist-induced binding of GTP to $G\alpha$ subunits, and this step, utilizing slowly hydrolysable guanosine-5'-O-(3-[³⁵S]thio)triphosphate ([³⁵S]GTP γ S), has been frequently used for investigations of receptor-ligand interactions (3). In general, this assay is experimentally more feasible for receptors coupled to the abundant $G_{i/o}$ proteins. As described above, the M₂ and M₄ receptors are known to be coupled to $G_{i/o}$ proteins, thus making the [³⁵S]GTP γ S binding assay a good candidate with which to study the pharmacological properties of these receptors. Due to overlapping expression profiles in the brain and lack of

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highly selective ligands for each mAChR subtypes, however, the pharmacological characteristics of the endogenously expressed M₂ and M₄ mAChRs have been poorly understood in native tissue to date.

Recently, Chapman et al. (4) reported that carbachol (CCh) stimulated specific [³⁵S]GTPγS binding in striatal membranes in M₂ mAChR-knockout mice as well as wild type, but not in M₄ mAChR-knockout mice, clearly indicating that M₄ mAChR was a predominant functional receptor subtype. They also utilized compound 28 (5), a selective M₄ mAChR antagonist, and concluded that CCh-stimulated [³⁵S]GTPγS binding in rat striatum was mediated through the M₄ mAChR subtype. As mentioned by themselves, however, it is far from satisfactory enough to use only one compound to characterize definitely a pharmacological response. It is also unknown whether this response can be extended to brain regions other than the striatum. In the present study, we determined mAChR-mediated [³⁵S]GTPγS binding not only in rat striatum, but also in the cerebral cortex and hippocampus, with the help of a number of mAChR-related ligands.

Materials and Methods

Materials

[³⁵S]GTPγS (NEG030H, 1,250 Ci/mmol) was purchased from PerkinElmer (Waltham, MA, USA). The following mAChR agonists and antagonists were obtained from Tocris Bioscience (Bristol, UK): xanomeline, oxotremorine, arecaidine but-2-ynyl ester tosylate, oxotremorine M, arecaidine propargyl ester tosylate, milameline, 5-methylfurmethiodide, 4-[[[(3-chlorophenyl)amino]carbonyl]oxy]-N,N,N-trimethyl-2-butyn-1-ammonium chloride (McN-A 343), pilocarpine, scopolamine, ipratropium, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP), biperiden, 11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one (AQ-RA 741), telenzepine, N-[2-[2-[(dipropylamino)methyl]-1-piperidinyl]ethyl]-5,6-dihydro-6-oxo-11H-pyrido[2,3-b][1,4]benzodiazepine-11-carboxamide (AF-DX 384), zamifenacin, tropicamide, 8-methyl-8-azabicyclo-3-endo[3.2.1]oct-3-yl-1,4-dihydro-2-oxo-3(2H)-quinazolinecarboxylic acid ester hydrochloride (DAU 5884), (S)-(+)-dimethindene, (αR)-α-cyclopentyl-α-hydroxy-N-[1-(4-methyl-3-pentenyl)-4-piperidinyl]benzeneacetamide fumarate (J 104129), nitrocaramiphen, pirenzepine, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one (AF-DX 116), 3,6a,11,14-tetrahydro-9-methoxy-2-methyl-(12H)-isoquino[1,2-b]pyrrolo[3,2-f][1,3]benzoxazine-1-carboxylic acid, ethyl ester (PD 102807), hexamethylene-bis-[dimethyl-(3-phthalimidopropyl)ammonium]dibromide (W-84), and

3-amino-N-[(4-chlorophenyl)methyl]-4,6-dimethylthieno[2,3-b]pyridine-2-carboxamide (VU 10010). N-Desmethylclozapine, arecoline, CCh, methacholine, ACh, trihexyphenidyl, thiochrome, brucine N-oxide, GDP, and GTPγS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Atropine was from Tokyo Chemical Industry Co. (Tokyo). Muscarinic toxins MT1, MT3, MT7, and MTα were from Peptide Institute, Inc. (Osaka), whereas MT2 was from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals used were of analytical grade.

Membrane preparation

The experimental protocols were reviewed and approved by the Animal Committee of Saitama Medical University, and the animal care and use procedures conformed to the Guiding Principles approved by The Japanese Pharmacological Society. Male Sprague-Dawley rats weighing 200–250 g were killed by decapitation and their brains were quickly removed. The cerebral cortex, hippocampus, or striatum, dissected from each rat, was homogenized in 5 ml of ice-cold TED buffer (5 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) containing 10% (w/v) sucrose by 20 strokes with a motor-driven Teflon/glass tissue grinder. All the following centrifuge procedures were carried out at 4°C. Subsequent to centrifugation of the homogenate at 1,000 × g for 10 min, the supernatant was decanted to another centrifuge tube. The pellet was vortexed in 5 ml of TED/sucrose buffer and centrifuged again at 1,000 × g for 10 min. The combined supernatant (10 ml) was centrifuged at 9,000 × g for 20 min and resuspended in 10 ml of TED buffer. After the same procedure was repeated, the homogenate was kept on ice for 30 min, followed by the final centrifugation at 35,000 × g for 10 min. The resulting pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) to produce the homogenate with a protein concentration ranging from 1.0 to 2.0 mg/ml. The homogenate was frozen quickly on fine-grained dry ice and stored at –80°C until use.

[³⁵S]GTPγS binding

The activation of specific [³⁵S]GTPγS binding to rat brain membranes mediated by mAChRs was determined as described previously (6). In brief, the brain membranes equivalent to 10–20 μg protein were incubated in duplicate at 30°C for 60 min in 500 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 0.2 nM [³⁵S]GTPγS, 20 μM GDP, 5 mM MgCl₂, 0.1 mM EDTA, 0.2 mM ethylene glycolbis(2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA), 0.2 mM dithiothreitol, 100 mM NaCl, and various concentrations of cholinergic ligands. After the incubation, the homogenate was filtered under vacuum through glass fiber filters (GF/B; Whatman International,

Kent, UK) using a Brandel cell harvester with 2×5 ml washing with ice-cold 50 mM Tris-HCl buffer (pH 7.4), and the radioactivity content retained on the filter was counted in 8 ml scintillation cocktail Emulsifier-Scintillator Plus (Packard Bioscience; Meriden, CT, USA) by a liquid scintillation counter. The non-specific binding was measured in the presence of 100 μ M unlabeled GTP γ S, which was subtracted from the total binding to define the specific [35 S]GTP γ S binding.

Data analysis

All results were presented as the mean $\pm$ S.E.M. of the indicated number of separate experiments, each performed in duplicate. The concentration-dependent increases in specific [35 S]GTP γ S binding by CCh and ACh were expressed as the percentage increase over the basal unstimulated value and analyzed using a nonlinear regression method with GraphPad Prism (GraphPad Software; La Jolla, CA, USA), to produce the concentration eliciting the half-maximal effect (EC_{50}) and the maximal percentage increase ($\%E_{max}$). In the case of other mAChR agonists, the increases in specific [35 S]GTP γ S binding over the basal unstimulated value were normalized with the increase elicited by 10 mM CCh regarded as 100%, and analyzed as well. The inhibition curve for mAChR antagonists against 100 μ M CCh was also analyzed by a nonlinear regression method, with the basal and the CCh-stimulated binding regarded as 0% and 100%, respectively, to generate the concentration that inhibited the binding to 50% (IC_{50}).

Results

Effects of mAChR agonists

As shown in Fig. 1, the specific [35 S]GTP γ S binding was increased by the endogenous neurotransmitter ACh as well as CCh in a concentration-dependent manner in the three brain regions, with EC_{50} values of 3–15 μ M. The $\%E_{max}$ values in striatal membranes (81.8 ± 3.2 and 80.6 ± 1.6 for ACh and CCh, respectively) were much higher than those in the other two brain regions, cerebral cortex (49.2 ± 2.7 and 45.9 ± 1.1 for ACh and CCh, respectively) and hippocampus (41.5 ± 1.5 and 47.2 ± 4.4 for ACh and CCh, respectively).

In the following experiments using mAChR agonists, the increase in specific [35 S]GTP γ S binding elicited by 10 mM CCh was always measured in parallel, and the increase in specific binding by the test compound was expressed as a normalized value, as exemplified in Fig. 2. Nonlinear regression analysis of the concentration–response curve for each compound yielded varied EC_{50} values and normalized $\%E_{max}$ values, as listed in Table 1. In addition to the compounds conventionally

Fig. 1. Effects of ACh and CCh on the specific [35 S]GTP γ S binding in rat brain membranes. The increases in specific [35 S]GTP γ S binding elicited by increasing concentrations of ACh (open circle, open triangle, open inverse triangle) and CCh (closed circle, closed triangle, closed inverse triangle) are expressed as percent over the basal binding in the cerebral cortex (open circle, closed circle), hippocampus (open triangle, closed triangle), and striatum (open inverse triangle, closed inverse triangle). The values represent the mean $\pm$ S.E.M. of 4–5 independent experiments, each performed in duplicate.

Fig. 2. Effects of several mAChR agonists on the specific [35 S]GTP γ S binding in rat striatal membranes. The increases in specific [35 S]GTP γ S binding elicited by increasing concentrations of *N*-desmethylozapine (open circle), oxotremorine M (closed circle), arecaine propargyl ester tosylate (open triangle), milameline (closed triangle), arecholine (open inverse triangle), McN-343 (closed inverse triangle), and pilocarpine (open diamond) are expressed as percent of the value induced by 10 mM CCh. The values represent the mean $\pm$ S.E.M. of 4 independent experiments, each performed in duplicate.

Table 1. Agonist properties of mAChR ligands in rat brain membranes

Compound	Cerebral cortex			Hippocampus			Striatum		
	pEC ₅₀	EC ₅₀ (μM)	%E _{max}	pEC ₅₀	EC ₅₀ (μM)	%E _{max}	pEC ₅₀	EC ₅₀ (μM)	%E _{max}
Xanomeline	6.27 ± 0.09	0.536	72.9 ± 13.3	5.55 ± 0.09	2.84	380.3 ± 43.1	6.54 ± 0.07	0.291	64.0 ± 5.4
N-Desmethylozapine	6.28 ± 0.07	0.526	84.4 ± 4.5	6.13 ± 0.10	0.738	149.7 ± 12.6	6.44 ± 0.04	0.361	46.1 ± 0.5
Oxotremorine	6.45 ± 0.07	0.352	90.8 ± 2.0	6.13 ± 0.12	0.740	102.4 ± 5.4	6.25 ± 0.07	0.566	77.4 ± 4.1
Arecaidine but-2-ynyl ester tosylate	6.42 ± 0.08	0.380	104.7 ± 7.0	6.12 ± 0.08	0.767	89.7 ± 2.8	6.19 ± 0.06	0.644	92.2 ± 1.3
Oxotremorine M	6.18 ± 0.04	0.656	128.4 ± 9.5	5.80 ± 0.06	1.58	152.4 ± 17.0	6.08 ± 0.02	0.826	118.0 ± 5.7
Arecaidine propargyl ester tosylate	5.97 ± 0.05	1.07	108.9 ± 2.6	6.09 ± 0.08	0.805	98.2 ± 6.5	5.97 ± 0.04	1.07	94.4 ± 4.5
Milameline	5.58 ± 0.17	2.63	58.5 ± 3.1	5.11 ± 0.17	7.83	64.8 ± 2.8	5.56 ± 0.06	2.79	56.1 ± 1.7
5-Methylflumethiodide	5.52 ± 0.08	2.99	87.5 ± 6.9	5.46 ± 0.03	3.51	84.0 ± 9.2	5.44 ± 0.06	3.63	92.1 ± 5.1
McN-A 343	— ^a	— ^a	— ^a	— ^a	— ^a	— ^a	5.37 ± 0.13	4.31	26.3 ± 3.4
Arecholine	5.45 ± 0.05	3.54	80.9 ± 5.7	5.00 ± 0.10	10.0	102.5 ± 8.0	5.21 ± 0.02	6.15	93.4 ± 1.4
Carbachol	5.30 ± 0.04	5.07	100	5.06 ± 0.07	8.63	100	5.08 ± 0.05	8.32	100
Methacholine	4.76 ± 0.13	17.3	121.7 ± 7.9	4.84 ± 0.02	14.6	120.3 ± 8.5	5.05 ± 0.08	9.02	112.5 ± 4.9
Acetylcholine	5.49 ± 0.10	3.21	100	5.43 ± 0.11	3.72	100	4.83 ± 0.12	14.9	100
Pilocarpine	— ^a	— ^a	— ^a	— ^a	— ^a	— ^a	— ^a	— ^a	— ^a

^aNot determinable because of low efficacy. Values are presented as the mean ± S.E.M. of 4–6 separate experiments.

classified as mAChR agonists, xanomeline, and N-desmethylozapine were included in the present study, following the previous report (4).

Effects of mAChR antagonists on CCh-stimulated [³⁵S]GTPγS binding

The inhibitory effects of a series of mAChR antagonists on CCh-stimulated [³⁵S]GTPγS binding were then analyzed in the three brain regions. The concentration-dependent inhibitory curves for several mAChR antagonists against the increase in specific [³⁵S]GTPγS binding elicited by 100 μM CCh were exemplified in Fig. 3. Almost all mAChR antagonists inhibited CCh-elicited increase to the basal unstimulated level, with a slope factor close to unity, and with the various IC₅₀ values shown in Table 2. An exception was the inhibitory curve of J 104129 in hippocampal membranes, which was extraordinarily shallow and apparently biphasic (Fig. 4A). To clarify the reason underlying its unique property in the hippocampus, the specific [³⁵S]GTPγS bindings were determined in the presence of increasing concentrations of J 104129 in the three brain regions. As shown in Fig. 4B, J 104129 per se was capable of stimulating the specific [³⁵S]GTPγS binding at micromolar concentrations only in the hippocampus, but not in the other two brain regions.

Correlation of pEC₅₀ and pIC₅₀ values

The rank order of potencies of mAChR agonists (Table 1) and antagonists (Table 2) determined in the present study appeared very similar regardless of the brain regions. When the pEC₅₀ and pIC₅₀ values determined in the hippocampus were compared with those in the striatum (Fig. 5A), there was a highly significant

Fig. 3. Effects of several mAChR antagonists on the increase in specific [³⁵S]GTPγS binding elicited by 100 μM CCh in rat striatal membranes. The increases in specific [³⁵S]GTPγS binding elicited by 100 μM CCh in the presence of increasing concentrations of scopolamine (open circle), ipratropium (closed circle), 4-DAMP (open triangle), AF-DX 384 (closed triangle), (S)-(+)-dimethindene (open inverse triangle), AF-DX 116 (closed inverse triangle), and W-84 (open diamond) are expressed as percent of the value induced by 100 μM CCh alone. The values represent the mean ± S.E.M. of 4 independent experiments, each performed in duplicate.

correlation with a correlation coefficient of 0.95 and a slope of 0.95 ($P < 0.001$). Likewise, highly significant correlations between the cerebral cortex and striatum ($r = 0.94$, $P < 0.001$) (Fig. 5B) and between the cerebral cortex and hippocampus ($r = 0.93$, $P < 0.001$) (Fig. 5C) were obtained. In these comparisons, J 104129 was excluded because of its unique agonistic properties in

Table 2. Antagonistic properties of mAChR ligands in rat brain membranes

Compound	Cerebral cortex		Hippocampus		Striatum	
	pIC ₅₀	IC ₅₀ (nM)	pIC ₅₀	IC ₅₀ (nM)	pIC ₅₀	IC ₅₀ (nM)
Scopolamine	7.35 ± 0.13	44.4	7.71 ± 0.04	19.7	7.84 ± 0.02	14.4
Atropine	7.47 ± 0.03	34.0	7.65 ± 0.09	22.4	7.84 ± 0.05	14.5
Ipratropium	7.70 ± 0.07	20.0	7.80 ± 0.08	15.8	7.67 ± 0.05	21.4
4-DAMP	6.93 ± 0.06	118	7.17 ± 0.08	68.2	7.13 ± 0.04	74.5
Trihexyphenidyl	6.79 ± 0.08	162	6.85 ± 0.14	143	7.02 ± 0.02	95
Biperiden	6.76 ± 0.11	175	6.95 ± 0.06	111	6.99 ± 0.05	102
AQ-RA 741	6.68 ± 0.03	210	6.93 ± 0.04	118	6.75 ± 0.05	178
Telenzepine	6.65 ± 0.06	224	6.77 ± 0.05	169	6.73 ± 0.05	188
AF-DX 384	6.66 ± 0.20	218	6.73 ± 0.13	185	6.54 ± 0.05	292
Zamifenacin	6.13 ± 0.18	743	6.89 ± 0.08	130	6.53 ± 0.04	298
Tropicamide	6.28 ± 0.09	526	6.57 ± 0.04	270	6.52 ± 0.05	301
DAU 5884	6.10 ± 0.08	793	6.47 ± 0.05	341	6.51 ± 0.03	308
(S)-(+)-Dimethindene	5.86 ± 0.09	1390	6.16 ± 0.05	700	5.88 ± 0.03	1320
J 104129	5.05 ± 0.10	8930	— ^a	> 30000	5.76 ± 0.04	1730
Nitrocaramiphen	5.47 ± 0.04	3390	5.63 ± 0.11	2340	5.59 ± 0.06	2580
Pirenzepine	5.30 ± 0.09	5050	5.58 ± 0.06	2630	5.56 ± 0.06	2780
AF-DX 116	5.48 ± 0.11	3280	5.64 ± 0.08	2290	5.32 ± 0.07	4800
PD 102807	5.24 ± 0.09	5770	5.48 ± 0.14	3280	5.21 ± 0.02	6210
W-84	4.95 ± 0.10	11100	4.89 ± 0.13	13000	4.71 ± 0.08	19500

^aExtraordinarily shallow inhibitory curve with a slope factor of -0.32 ± 0.02 , as shown in Fig. 4A. Values are presented as the mean ± S.E.M. of 4 – 6 separate experiments.

Fig. 4. Effects of J 104129 on CCh-stimulated and basal [³⁵S]GTPγS binding in rat brain membranes. A) The increases in specific [³⁵S]GTPγS binding elicited by 100 μM CCh in the presence of increasing concentrations of J 104129 are expressed as percent of the value induced by 100 μM CCh alone in the cerebral cortex (open circle), hippocampus (closed circle), and striatum (open triangle). The values represent the mean ± S.E.M. of 4 independent experiments, each performed in duplicate. B) The increases in specific [³⁵S]GTPγS binding elicited by increasing concentrations of J 104129 are expressed as percent of the value induced by 10 mM CCh in the cerebral cortex (open circle), hippocampus (closed circle), and striatum (open triangle). The values represent the mean ± S.E.M. of 3 – 4 independent experiments, each performed in duplicate.

the hippocampus, as described above. Also, xanomeline and *N*-desmethylozapine were omitted, since the agonistic effects of these compounds in rat brain membranes were, at least in part, derived from the mecha-

nisms involving receptors other than mAChRs, as commented in detail in the Discussion.

The pEC₅₀ and pIC₅₀ values for a series of mAChR ligands in the cerebral cortex determined in the present

Fig. 5. Correlation of pEC₅₀ or pIC₅₀ values of a series of mACHR ligands. A) The pEC₅₀ (open circle) and pIC₅₀ (closed circle) values determined in the hippocampus are correlated with those determined in the striatum as to a series of mACHR agonists and antagonists except for J 104129, xanomeline, and *N*-desmethylozapine. B) The pEC₅₀ (open circle) and pIC₅₀ (closed circle) values determined in the cerebral cortex are correlated with those determined in the striatum as to a series of mACHR agonists and antagonists except for J 104129, xanomeline, and *N*-desmethylozapine. C) The pEC₅₀ (open circle) and pIC₅₀ (closed circle) values determined in the cerebral cortex are correlated with those determined in the hippocampus as to a series of mACHR agonists and antagonists except for J 104129, xanomeline, and *N*-desmethylozapine. D) The pEC₅₀ (open circle) and pIC₅₀ (closed circle) values determined in the cerebral cortex in the present study are correlated with those determined in the cerebral cortex by using antibody-capture SPA/[³⁵S]GTPγS binding with anti-Gα_q antibody, the method for functional activation of Gα_q coupled with M₁ mACHR (7).

study were also correlated with those determined by means of antibody-capture scintillation proximity assay (SPA)/[³⁵S]GTPγS binding using anti-Gα_q antibody in the same brain region (Fig. 5D). As reported recently (7), M₁ mACHR-mediated activation of Gα_q is detectable by this method. Although still significant ($P < 0.001$), the correlation coefficient (0.80) was much lower than those described above. Furthermore, the slope of the regression line was 0.39, far from unity.

Effects of muscarinic toxins on CCh-stimulated [³⁵S]GTPγS binding

Muscarinic toxins serve as useful probes in mACHR research (8). As shown in Fig. 6, the increase in specific [³⁵S]GTPγS binding elicited by 100 μM CCh was barely affected by the addition of MT1, MT2, MT7, and MTα up to 1 μM in rat cerebral cortical membranes. On the other hand, MT3 inhibited CCh-stimulated [³⁵S]GTPγS binding at the highest concentration, 1 μM, to 61.3% ± 3.7 % of the value induced by 100 μM CCh alone. The inset of Fig. 6 represents the concentration–response curves for CCh in the absence and presence of 1 μM MT3.

Fig. 6. Effects of muscarinic toxins on CCh-stimulated [35 S]GTP γ S binding in rat cerebral cortical membranes. The increases in specific [35 S]GTP γ S binding elicited by 100 μ M CCh in the presence of increasing concentrations of MT1 (open circle), MT2 (closed circle), MT3 (open triangle), MT7 (closed triangle), and MT α (open inverse triangle) are expressed as percent of the value induced by 100 μ M CCh alone. The values represent the mean $\pm$ S.E.M. of 3 independent experiments, each performed in duplicate. (Inset) Effects of MT3 on CCh-stimulated [35 S]GTP γ S binding in rat cerebral cortical membranes. The increases in specific [35 S]GTP γ S binding elicited by increasing concentrations of CCh are expressed as percent over the basal binding in the cerebral cortex, in the absence (open circle) and presence of 1 μ M (closed circle) MT3. The values represent the mean $\pm$ S.E.M. of 3 independent experiments, each performed in duplicate.

The %E_{max} value was unaltered ($36.6\% \pm 1.3\%$ and $35.3\% \pm 1.2\%$ in the absence and presence of 1 μ M MT3, respectively; $n = 3$, $P > 0.05$, paired Student's t -test), while the EC₅₀ value of CCh was significantly ($P < 0.01$) increased from 5.05 μ M (pEC₅₀ = 5.30 ± 0.03) to 48.8 μ M (pEC₅₀ = 4.31 ± 0.08) and the Hill slope was significantly ($P < 0.01$) reduced from 1.07 ± 0.08 to 0.54 ± 0.03 by the addition of 1 μ M MT3.

Effects of M_4 mAChR allosteric modulators on CCh-stimulated [35 S]GTP γ S binding

The concentration-response curves for CCh were then determined in the absence and presence of the compounds that had been reported to act as allosteric modulators of M_4 mAChRs, i.e., VU 10010 (9), thiochrome (10), and brucine N -oxide (11). As shown in Fig. 7A, the addition of increasing concentrations of VU 10010 shifted the curve leftwards as well as upwards in rat striatal membranes. The EC₅₀ value was shifted from 9.40 μ M in the absence of VU 10010 (pEC₅₀ = 5.03 ± 0.02)

to 3.63 μ M (pEC₅₀ = 5.44 ± 0.06) and 2.11 μ M (pEC₅₀ = 5.68 ± 0.02) in the presence of 1 and 10 μ M VU 10010, respectively ($P < 0.001$, one-way repeated measures ANOVA). The %E_{max} value in the absence of VU 10010 (102.6 ± 2.2) was increased significantly ($P < 0.01$) to 116.4 ± 6.1 and 130.2 ± 6.0 in the presence of 1 and 10 μ M VU 10010, respectively. The similar results were obtained also in rat cerebral cortical membranes (data not shown). On the other hand, the addition of neither thiochrome (10 and 100 μ M) (Fig. 7B) nor brucine N -oxide (10 and 100 μ M) shifted the CCh-stimulated [35 S]GTP γ S binding significantly.

Interaction between mAChR- and 5-HT_{1A} receptor-mediated G-protein activation

We previously showed that 5-HT-stimulated [35 S]GTP γ S binding in rat cerebral cortical membranes was mediated through 5-HT_{1A} receptors (12). In the present study, the increase in specific [35 S]GTP γ S binding elicited by CCh was determined in the absence and presence of 5-HT at a maximally effective concentration, 100 μ M (Fig. 8). In the absence of 5-HT, CCh stimulated the specific [35 S]GTP γ S binding with a mean EC₅₀ of 6.19 μ M (pEC₅₀ = 5.21 ± 0.05) to the %E_{max} value of $37.9\% \pm 2.0\%$ ($n = 4$). In the presence of 100 μ M 5-HT, the specific binding was increased by $38.9\% \pm 2.5\%$ and the addition of increasing concentrations of CCh further augmented the binding to $67.3\% \pm 1.9\%$ with a mean EC₅₀ of 9.64 μ M (pEC₅₀ = 5.02 ± 0.09). When the percent increase elicited by 100 μ M 5-HT and the %E_{max} value determined with CCh alone was simply added in each experiment, the increase by $76.9\% \pm 1.8\%$ was obtained as the theoretical value on the assumption that both mAChRs and 5-HT_{1A} receptors stimulated completely distinct pools of G-proteins without any interaction. In fact, there was statistically significant difference among the %E_{max} for CCh alone, the %E_{max} for CCh in the presence of 100 μ M 5-HT, and the theoretical additive value ($P < 0.001$, one-way repeated measures ANOVA). Although not completely ($P < 0.05$, Tukey's multiple comparison test, between the measured %E_{max} for CCh in the presence of 100 μ M 5-HT and the theoretical additive value), there appeared additive effects between mAChR-mediated and 5-HT_{1A} receptor-mediated G-protein activation ($P < 0.001$, Tukey's multiple comparison test, between the measured %E_{max} for CCh alone and the measured %E_{max} for CCh in the presence of 100 μ M 5-HT).

Discussion

In the current study, we tried to characterize pharmacologically the functional activation of G-proteins coupled

Fig. 7. Effects of VU 10010 and thiochrome on CCh-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in rat striatal membranes. A) The increases in specific $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding elicited by increasing concentrations of CCh are expressed as percent over the basal binding in the striatum, in the absence (open circle) and presence of $1 \mu\text{M}$ (closed circle) and $10 \mu\text{M}$ (open triangle) VU 10010. The values represent the mean $\pm$ S.E.M. of 4 independent experiments, each performed in duplicate. B) The increases in specific $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding elicited by increasing concentrations of CCh are expressed as percent over the basal binding in the striatum, in the absence (open circle) and presence of $10 \mu\text{M}$ (closed circle) and $100 \mu\text{M}$ (open triangle) thiochrome. The values represent the mean $\pm$ S.E.M. of 4 independent experiments, each performed in duplicate.

Fig. 8. Effects of CCh on the specific $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in rat cerebral cortical membranes, alone or in combination with 5-HT at a maximally effective concentration. The increases in specific $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding elicited by increasing concentrations of CCh, either in the absence (open circle) or presence (closed circle) of $100 \mu\text{M}$ 5-HT, are expressed as percent over the basal binding in the cerebral cortex. The values represent the mean $\pm$ S.E.M. of 4 independent experiments, each performed in duplicate. The values (mean $\pm$ S.E.M.) calculated by simple addition of the $\%E_{\text{max}}$ elicited by CCh to the 5-HT-induced increase through $5\text{-HT}_{1\text{A}}$ receptors (12) are indicated as hatching.

to mAChRs detected by means of the conventional $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assay in three discrete brain regions of rats. The $\%E_{\text{max}}$ values determined by using the endogenous ligand ACh as well as the full agonist CCh were the highest in the striatum. Nevertheless, the responses in the cerebral cortex and hippocampus were still enough high to perform further detailed pharmacological investigations.

The distribution of mAChR subtypes in the brain has been investigated using several different approaches, i.e., quantitative receptor binding autoradiography, in situ hybridization, and immunohistochemistry. By using immunoprecipitation with subtype selective antibodies, Levy et al. (13) showed that M_1 , M_2 , and M_4 mAChRs are the predominant subtypes expressed in the brain, with M_3 and M_5 mAChRs also being expressed throughout the brain in low abundance. Among five mAChR subtypes, M_2 mAChR immunoreactivity is abundantly expressed in basal forebrain, scattered striatal neurons, mesopontine tegmentum, and cranial motor nuclei, while M_4 mAChR immunoreactivity is enriched in neostriatum, olfactory tubercle, and islands of Calleja (13). Quantitative immunoprecipitation study demonstrates that of the total mAChRs, M_1 , M_2 , and M_4 mAChR subtypes represent approximately 31%, 29%, and 29%, respectively, in the striatum and 40%, 37%, and 15%, respectively, in the cortex (13). Vilaró et al. (14) demonstrated that M_4

mRNA was the most abundant, followed by M_1 mRNA, with M_2 and M_3 mRNAs much less abundantly expressed, in rat neostriatum. The highest % $E_{\max}$ values for CCh and ACh in the striatum among the three discrete brain regions examined in the present study appear to correspond well to the expression pattern of M_4 mAChR subtype (13).

The pharmacological profiles investigated with a series of mAChR agonists and antagonists in the three discrete brain regions in the current study are essentially identical to one another, indicating that the same mAChR subtype is involved. As already mentioned in the Introduction, Chapman et al. (4) identified the mAChR involved in CCh-stimulated [35 S]GTP γ S binding in the striatum as the M_4 subtype, based on the experiments using knockout mouse tissue as well as a selective M_4 mAChR antagonist, compound 28 (5). Although we were unable to include this commercially unavailable compound in the present study, the pharmacological characteristics were not inconsistent with the involvement of M_4 mAChR. However there are a few discrepancies between the results reported by Chapman et al. (4) and ours. The most prominent one is the potency of ACh, which is extraordinarily low ($pEC_{50} = 3.0 \pm 0.09$) in the report of Chapman et al. (4), in comparison with that in our study ($pEC_{50} = 4.83 \pm 0.12$). They mentioned the possibility of the presence of endogenous acetylcholinesterase in their sample tissue, and this is likely because they used whole membrane preparation after washing only two times.

In accordance with the report of Chapman et al. (4), we also tested xanomeline and *N*-desmethylozapine as agonists in this assay system. Both compounds stimulated the specific [35 S]GTP γ S binding with submicromolar to micromolar order EC_{50} values in all three brain regions. Surprisingly, the % $E_{\max}$ values for both compounds overshoot the % $E_{\max}$ of the full mAChR agonist CCh in the hippocampus. These results raised doubts about the origin of the stimulatory effects of both compounds. It has been reported that xanomeline behaves as an agonist at 5-HT $_{1A}$ receptors (15) and that *N*-desmethylozapine acts as an agonist at 5-HT $_{1A}$ receptors as well as δ -opioid receptors (16). The stimulatory effects of xanomeline and *N*-desmethylozapine detected in the present study may include the effects mediated by these $G_{i/o}$ -coupled receptors other than mAChRs. This should be ascertained by the experiments using a series of antagonists against xanomeline- and *N*-desmethylozapine-stimulated [35 S]GTP γ S binding, which are now in progress in our laboratory. At present, it appears problematic to include these two compounds as pure mAChR agonists, and this is the reason we excluded these reagents from the pEC_{50}/pIC_{50} correlation analyses.

Another compound that was not included in the pEC_{50}/pIC_{50} correlation analyses was J 104129, the compound reportedly referred to as a selective M_3 mAChR antagonist (17). Although we are unaware of the reports with regard to its pharmacological properties besides it being an mAChRs antagonist, the present data clearly indicate that J 104129 stimulates specific [35 S]GTP γ S binding in the hippocampus, probably mediated through the receptors that are coupled to $G_{i/o}$ and expressed in abundance especially in this brain area.

The pEC_{50} and pIC_{50} values determined in the present study were still significantly correlated with those determined by antibody-capture SPA/[35 S]GTP γ S binding using an anti- $G\alpha_q$ antibody, which were mediated through M_1 mAChRs (7). This means that most mAChR ligands do not possess high subtype selectivity to discriminate between $G\alpha_q$ -coupled M_1 mAChRs and $G\alpha_{i/o}$ -coupled mAChR subtype involved in the present study. However, several antagonists showed substantial preference for M_1 mAChRs over the mAChRs of interest in the present study, with > 100-fold selectivity for pirenzepine and > 40-fold selectivity for DAU 5884, scopolamine, and telenzepine. These results appear quite reasonable regarding pirenzepine, DAU 5884, and telenzepine, because it has been reported that these three antagonists show the highest affinities at M_1 mAChR subtype over the other mAChRs (18–20). In contrast to these compounds, scopolamine is usually referred to as a non-selective mAChR antagonist, with roughly equivalent affinities to all five mAChR subtypes (19). However, this result was derived from the experiment using cloned human mAChR subtypes expressed in Chinese hamster ovary (CHO) cells, and it was reported that scopolamine had substantial selectivity to M_1 mAChRs in rat brain (21). There may be some differences in pharmacological properties of mAChRs between human and rat, as suggested by Van den Beukel et al. (22).

Currently, there is a lack of highly selective orthosteric antagonists at the M_4 mAChR subtype, although some compounds such as tropicamide (23, 24) and PD 102807 (25, 26) are often referred to as selective M_4 antagonists. However, the selectivity of tropicamide for M_4 subtype has not been confirmed in the subsequent reports (27–29). In the present study, PD 102807 behaved as a rather weak antagonist with micromolar order IC_{50} values, and these results apparently exclude the involvement of M_4 mAChRs, considering the reported pK_B value of 7.40 at cloned human M_4 mAChRs in CHO cells (26). In the report of Chapman et al. (4), however, the similar discrepancies were found as to the antagonistic effects of compound 28, a benzoxazine derivative synthesized subsequent to PD 102807 (5). The K_B values of compound 28 determined by [35 S]GTP γ S binding assay in

mouse and rat striatal membranes ($pK_B = 7.11$ and 7.12 , respectively) were substantially less potent than the reported K_i value determined by receptor binding assay for cloned human M_4 mAChRs in CHO cells ($pK_i = 9.0$) (5) and also than that determined by themselves ($pK_i = 7.8$). Further studies are needed as to the antagonistic properties of these benzoxazine derivatives at native mAChRs.

Compared to the conventional orthosteric mAChR antagonists described above, some toxins isolated from the mamba snake venoms show a truly high selectivity to one of the five mAChR subtypes (8). For the M_4 mAChR subtype, MT3 shows high affinity with a pK_i value of 8.70 , as compared to much lower affinities to the other mAChRs ($pK_i = 7.11$ and < 6.0 for M_1 mAChR and the others, respectively) (30). On the other hand, MT7 is a highly potent and selective antagonist for the M_1 mAChR subtype (31, 32). In the present study, the increase in specific [35 S]GTP γ S binding elicited by $100 \mu\text{M}$ CCh was inhibited by MT3 at the highest concentration investigated ($1 \mu\text{M}$), but not by the other toxins. As shown in the inset of Fig. 6, the inhibitory manner of MT3 in the present study was inconsistent with the typical competitive antagonism shown in the mAChR-mediated adenylate cyclase inhibition assay in rat striatal (33) and olfactory tubercle (34) membranes, and in [35 S]GTP γ S binding assay in CHO cells expressing M_4 mAChRs (35). Although a reasonable explanation for this discrepancy is unclear, the inhibitory effects of MT3 indicate that the M_4 mAChR subtype is certainly involved, if not exclusively, in the response investigated in the present study.

Recently, several allosteric modulators have been developed with high selectivity for one of the mAChR subtypes, especially for M_1 and M_4 mAChRs (36). Among them, VU 10010 is particularly useful in investigations of M_4 mAChRs because this compound has been shown to act as a highly selective allosteric potentiator at the M_4 subtype with no activity at any other mAChR subtypes (9). In the present study, VU 10010 potentiated the effects of CCh, by leftward shifting of the concentration–response curve as well as by enhancing the maximal response. These bimodal effects of VU 10010 are consistent with the previous functional study using CHO cells expressing M_4 mAChRs (9). Interestingly, the basal [35 S]GTP γ S binding was slightly increased by the addition of VU 10010 alone, indicating that this compound might behave, by itself, as an agonist at M_4 mAChRs. Although we were unable to detect significant effects of thiochrome and brucine *N*-oxide, which were reported as positive allosteric potentiators at M_4 mAChRs (10, 11), the results with regard to VU 10010 clearly indicated the involvement of the M_4 mAChR subtype in

the present study.

Overall, it can be concluded that CCh-stimulated [35 S]GTP γ S binding detected by conventional filtration techniques is mediated mainly via the M_4 mAChR subtype in rat cerebral cortical, hippocampal, and striatal membranes. These M_4 mAChRs could be identical to the receptors that have been reported to control cyclic AMP formation in an inhibitory manner in rat striatum (33, 34, 37–39) and in hippocampus (40). Nevertheless, minor contribution of M_2 mAChRs to the responses cannot be completely ruled out because a functional autoradiographic study using [35 S]GTP γ S binding in gerbil brain indicates the involvement of both M_2 and M_4 mAChRs (41). Development of the ligands that can be utilized to discriminate between M_2 and M_4 mAChRs is demanded.

The interaction between CCh- and 5-HT-stimulated [35 S]GTP γ S bindings indicates that both responses are additive, even though not entirely. Since 5-HT-stimulated [35 S]GTP γ S binding in rat cerebral cortical membranes is mediated through 5-HT $_{1A}$ receptors (12), these results imply that the mAChRs and 5-HT $_{1A}$ receptors are coupled to distinct pools of $G_{i/o}$ proteins. These results are consistent with our previous data on the interaction between several GPCRs (i.e., 5-HT $_{1A}$, GABA $_B$, dopamine D $_2$, adenosine A $_1$, and pirenzepine-insensitive mAChRs) and their coupled G-proteins assessed by agonist-induced high-affinity GTPase activity in rat brain membranes (42–44). Humbert-Claude et al. (45) also reported the lack of interactions between histamine H $_3$ receptor- and dopamine D $_2$ receptor-mediated [35 S]GTP γ S binding in rat striatum. In the case of the receptors coupled with $G_{i/o}$, there may be compartmentalization that limits the free access of the receptors to the full complement of G-proteins (46). Alternatively, the additive effects may be explained by the stoichiometric ratio between the receptors and G-proteins under the collision coupling model (47). According to the theory by Brinkerhoff et al. (47), the % competitive is calculated to be 74.9% for both receptors, indicating one-fourth of 5-HT $_{1A}$ receptors and mAChRs share the common $G_{i/o}$ proteins. Whatever the underlying molecular mechanisms, these results are in marked contrast to the complete competition of G_q proteins by M_1 mAChRs and 5-HT $_{2A}$ receptors in rat cerebral cortical membranes revealed by antibody-capture SPA/[35 S]GTP γ S binding using anti- G_{α_q} antibody (48) and immunoprecipitation (49). Further investigations are necessary to reveal the implications of these phenomena in brain functions controlled by the GPCR-mediated signal transductions.

In conclusion, the present study describes the pharmacological profile of mAChR-mediated [35 S]GTP γ S binding determined by the conventional filtration assay

in rat cerebral cortical, hippocampal, and striatal membranes in detail. All of the data using mAChR ligands, muscarinic toxins, and allosteric modulators support the notion that M₄ mAChR coupled to G_{i/o} is the principal mediator of the response, although minor contribution of M₂ mAChR is not completely ruled out. In addition, several issues have been raised that need to be addressed, e.g., possible involvement of other GPCRs than mAChRs in the effects of xanomeline and *N*-desmethylozapine, the properties of tropicamide and PD 102807 as selective M₄ mAChR antagonists, the partial agonistic effect of J 104129 in the hippocampus, the unusual inhibitory manner of MT3, and the apparent lack of allosteric effects of thiocholine and brucine *N*-oxide. Muscarinic agonist-stimulated [³⁵S]GTPγS binding in the membranes prepared from postmortem human brains have been utilized to unravel the pathological grounds for Alzheimer's disease (50, 51), schizophrenia (52), and mood disorders (53). We intend to adapt the method described in the present study to postmortem human brains to investigate possible alterations in mAChR/G_{i/o} interaction in mental disorders in the future.

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Conflicts of Interest

The authors declare that they have no conflicts of interest to disclose.

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