M₁ Acetylcholine Receptor-Mediated Phosphatidylinositol Turnover in Adult and Senescent Rat Brain Slices

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ABSTRACT — Phosphatidylinositol (PI) turnover via muscarinic acetylcholine (mACh) receptor was investigated using the cerebral cortex from adult rats. Activities in the cerebral cortex, hippocampus and striatum from senescent rats were compared with those from adult rat. Carbachol (1 mM)-stimulated [³H]IP accumulation in the presence of 10 mM LiCl was inhibited by pirenzepine more potently than by AF-DX 116. Although the displacing activity of carbachol for [³H]pirenzepine binding was decreased by 50 μM GTPγS, pretreatment of slices with pertussis toxin (PTX, 0.01–1.0 μg/ml) did not affect the carbachol-induced [³H]IP accumulation. In the slices from all 3 tissues, cerebral cortex, hippocampus and striatum, both incorporation of [³H]inositol and carbachol-stimulated [³H]IP accumulation were reduced at 28 months compared to those at 2 months. Furthermore, the Bmax values of [³H]pirenzepine binding in membranes from these three regions were diminished at the senescent stage. Taken together, the results suggest that an M₁-subtype of muscarinic acetylcholine receptor could be involved in PI turnover via GTP-binding proteins insensitive to PTX. Age-related changes in M₁-receptor mediated PI turnover seem to be in part due to the decreased number of M₁-receptors with increasing age in the cerebral cortex, hippocampus and striatum; and some qualitative changes also seem to have occurred in the hippocampus of senescent rats in the mACh receptor-PI turnover system.

An initial observation by Macht (1) that centrally acting muscarinic acetylcholine (mACh) receptor antagonists, such as scopolamine and atropine, impaired learning has stimulated subsequent studies on the role of central acetylcholine (ACh) neurons in learning (2–5). The receptor heterogeneity concept regarding mACh receptors has been proposed and supported by experimental results using ligand binding (6–9). Recently, five distinct cDNAs of mACh receptors were identified by cloning (10–14). mACh antagonists may exert their effects through these distinct receptor populations which may couple to different signalling mechanisms (15): 1) inhibition of adenylate cyclase, 2) activation of guanylate cyclase and 3) activation of phospholipase C. However, little has been clarified regarding the roles of mACh receptor subtypes and the involvement of GTP-binding proteins (G proteins) in phospholipase C activation in the brain. The present study was designed to clarify which mACh receptor subtype couples with phosphatidylinositol (PI) turnover and

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whether G proteins mediate the signalling pathway in the cerebral cortex, hippocampus and striatum of rats. In addition, we investigated age-related changes in mACh receptor-mediated PI turnover.

MATERIALS AND METHODS

Animals
Wistar rats of both sexes were purchased from Nippon Rat, Ltd. Rats aged 2–3 months and 28–30 months were used as adult and senescent animals, respectively. The animals were housed two–three to a cage under a constant light-dark cycle (light on between 7:30 and 19:30) at 22 ± 2°C. Food and water were available ad lib.

Preparation of brain slices and treatment with pertussis toxin
Measurement of PI turnover activity using rat brain slices was essentially carried out as previously described (16–18). Adult or senescent rats were decapitated and the brains were rapidly removed. The brains were dissected into the cerebral cortex, hippocampus and striatum. Each section was chopped by a feather knife and pressed through a nylon mesh (300 × 300 μm). These chopped slices were incubated at 37°C for 30–45 min with gentle shaking in 20–30 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) having the following composition: 123 mM NaCl, 5.0 mM KCl, 1.4 mM KH2PO4, 0.8 mM CaCl2, 1.3 mM MgSO4, 26 mM NaHCO3, and 10 mM glucose. This solution was bubbled with O2/CO2 gas (95/5, v/v) before use and during incubation with tissues. Treatment with pertussis toxin (PTX) was carried out for 2 hr at 37°C.

Assay of cyclic AMP accumulation in cerebral cortical slices
After treatment with PTX for 2 hr as described above, the supernatant was removed, and the tissue slices (50 μl) were pipetted into tubes, each containing 150 μl of reaction buffer. The reaction buffer consisted of 0.5 mM RO 20-1724, a phosphodiesterase inhibitor, and 1 μM pirenzepine to suppress M1-receptor mediated cyclic AMP (cAMP) elevation (20) in the presence or absence of 1 μM forskolin/1 mM carbachol in Krebs-Ringer bicarbonate buffer. The reaction mixture was incubated for 15 min at 37°C, and then 0.2 ml of 0.2 N ice-cold HCl was added to stop the reaction and to elute cAMP. The mixture was boiled for 3 min and then centrifuged (15,000 × g for 5 min). The supernatant (100 μl) was
assayed using a Yamasa cAMP assay kit, a sensitive and specific radioimmunoassay method (21).

**Receptor binding assay using [3H]pirenzipine**

**Preparation of crude synaptic membranes:** P2 membranes were prepared according to the method of Nomura et al. (22). Briefly, brain tissues of male rats were homogenized in 10 volumes of ice-cold 25 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose using a Potter glass/teflon homogenizer. The homogenates were centrifuged at 1,000 × g for 10 min, and the supernatant was then centrifuged at 50,000 × g for 10 min. The pellet was re-suspended in 25 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl. This suspension was stored at −80°C until assay.

**Effect of GTPγS on carbachol binding:** A 100-μg portion of the cerebral cortical P2 fractions (200 μl) was suspended in 25 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl and 5 mM MgCl₂ and then incubated at 25°C for 1.5 hr together with [3H]pirenzipine and increasing concentrations of carbachol (1 μM - 10 mM), in the presence or absence of 50 μM GTPγS. The non-specific binding of [3H]pirenzipine was defined as the radioactivity bound in the pellets in the presence of 1.0 μM atropine. Reactions were terminated by rapid filtration through Whatman GF/B glass filters, which were washed three times with 1.5 ml of ice-cold reaction buffer. Radioactivities on the filters were measured as described above. Data were analyzed by a previously described two-site computer model from our laboratory (23).

**[3H]pirenzipine binding to P2 membranes:** Crude membranes of approximately 100 μg protein (cerebral cortex) or 50 μg protein (hippocampus and striatum) were incubated in 25 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl (final volume of 200 μl) in the presence of 10 nM [3H]pirenzipine and increasing concentrations of pirenzipine (5 - 500 nM) at 25°C for 1.5 hr. The bound and free ligand were separated by rapid filtration through Whatman GF/B filters. Radioactivities on the filters were measured. The specific binding of pirenzipine was defined as the binding inhibited by the addition of 1 μM atropine.

**Protein assay**

The protein concentration of each sample of brain slices and P2 membranes was determined by the method of Lowry et al. (24) with bovine serum albumin as the standard.

**Drugs**

Myo-[2-3H(N)]inositol (12.8 Ci/mmol) and [N-methyl-3H]pirenzipine (82.0 Ci/mmol) were obtained from New England Nuclear. Drugs and an assay kit were kindly donated to us as follows: 11-[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one, AF-DX 116, from Dr. Thomae GmbH (Biberach, F.R.G.); PTX was from Kaken Pharmaceutical Co., Ltd. (Shiga, Japan); RO 20-1724 was from Nippon Roche Research Center (Kanagawa, Japan); and a Yamasa cAMP assay kit from Yamasa Shoyu Co. (Choshi, Japan). Other drugs were obtained as follows: carbamylcholine chloride, pirenzipine dihydrochloride and bovine serum albumin from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A); other drugs were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

**Statistical significance**

Student’s t-test was used for the evaluation of statistical significance.

**RESULTS**

**Effects of high potassium and physostigmine on [3H]inositol 1-phosphate accumulation in cerebral cortical slices**

Accumulations of [3H]inositol 1-phosphate ([3H]IP) formed from phosphatidylinositol labelled by [3H]inositol were measured in cerebral cortical slices in Krebs-Ringer solution containing 10 mM LiCl. The addition of 50 mM KCl significantly (P < 0.01) enhanced [3H]IP accumulation compared with the accu-
mulation in normal buffer containing 5 mM KCl (Fig. 1). Atropine (10 μM) significantly (P < 0.01) lowered the accumulation enhanced by high KCl. Although physostigmine (10 μM) alone did not affect [3H]IP accumulation, physostigmine enhanced the accumulation by high KCl. This enhancement by physostigmine was diminished by the addition of 10 μM atropine.

Fig. 1. The effects of physostigmine and atropine on high K+-evoked [3H]inositol 1-phosphate accumulation. After incubation with [3H]inositol, cerebral cortical slices were incubated in modified Krebs-Ringer buffer containing 50 mM KCI, 10 μM atropine and 10 μM physostigmine for 1 hr at 37°C. Accumulation of [3H]IP is expressed as a percentage of the accumulation in slices in normal buffer. Significance: a)P < 0.001 vs. control and b)P < 0.01 vs. high KCl.

Effects of pirenzepine and AF-DX116 on carbachol-induced [3H]inositol 1-phosphate accumulation

Inhibitory effects of pirenzepine (8) and AF-DX 116 (9) on carbachol-induced [3H]IP formation were examined in cerebral cortical slices. Pirenzepine (0.1 mM) completely blocked the submaximal response induced by 1 mM carbachol, and the IC50 value was estimated as 1.15 μM (Fig. 2). AF-DX 116 also inhibited

Fig. 2. Concentration-dependent inhibition by pirenzepine (○) and AF-DX 116 (•) on 1 mM carbachol-induced [3H]IP accumulation in cerebral cortical slices. Antagonists in Krebs buffer containing 10 mM LiCl were added into the reaction mixture 5 min before addition of 1 mM carbachol.
the response with an IC₅₀ value of 9.66 µM. The Hill coefficients of pirenzepine and AF-DX 116 were 0.999 and 0.702, respectively.

**Inhibitory effects of carbachol on [³H]pirenzepine binding in the presence or absence of GTPγS**

Using cerebral cortical membranes, the inhibitory effects of carbachol on [³H]pirenzepine binding were examined in the presence or absence of 50 µM GTPγS. Total binding of [³H]pirenzepine in the presence of GTPγS was almost equal to that in the absence of GTPγS, but the carbachol displacement curve was shifted to the right (Fig. 3). The proportion of high affinity sites for carbachol was calculated by the two-site computer model (23) as 43.3% in the absence of GTPγS. It was decreased to 26.8% in the presence of GTPγS.

**Effects of pertussis toxin on carbachol-stimulated accumulation of inositol 1-phosphate and inhibited formation of cAMP**

Forskolin (1 µM)-stimulated cAMP formation was inhibited by 62.8% by 1 mM carbachol (Fig. 4A). The inhibitory effect was suppressed by pretreatment with PTX for 2 hr in a concentration-dependent manner (Fig. 4A). In contrast, carbachol (3 mM)-induced accumulation of [³H]IP was not significantly affected by pretreatment with PTX for 2 hr in cerebral cortical slices. However, the accumulation was slightly enhanced by PTX (10–100 ng/ml) in a concentration-dependent manner (Fig. 4B).

**Age-related changes**

**Incorporation of [³H]inositol into phospholipids:** Incorporation of [³H]inositol into slices of cerebral cortex, hippocampus and striatum was examined in adult and senescent animals. In senescent rats, incorporation in each of the three regions was significantly decreased compared to those in adults: 8.7% (cerebral cortex), 18.1% (striatum) and 21.6% (hippocampus) (Fig. 5).

**Carbachol-stimulated [³H]inositol 1-phosphate accumulation:** Concentration-response curves of carbachol-evoked [³H]IP accumulation in the three brain regions of senescent rats were compared with those of adult rats. The curves obtained from the cerebral cortex and striatum data of senescent animals were

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**Fig. 3.** The displacement of [³H]pirenzepine binding by carbachol in cortical membrane fractions in the absence (○) and presence (●) of 50 µM GTPγS. Proportion of high affinity sites for carbachol changed from 43.3% (control) to 26.8% (GTPγS). Data were analyzed with the two-site computer model previously reported by Kitamura and Nomura (23).
parallel with those obtained in adults, but were significantly shifted downward (Fig. 6, A and C). In the hippocampus of senescent rats, the basal level of \(^{3}H\)IP formation was slightly but not significantly decreased (Fig. 6B). Carbachol-induced \(^{3}H\)IP accumulation was significantly (\(P < 0.01\) or \(P < 0.001\)) lowered in comparison to that in adults (Fig. 6B).
Carbachol-induced $[^{3}H]$IP accumulation in cerebral cortical (A), hippocampal (B) and striatal (C) slices of adult (○) and senescent rat (●) and in cerebral cortical slices of 22 month-old rats (▲) in panel A. These data were obtained from 3–5 adult and 3 senescent rats. Data are shown as a percentage calculated as follows: $(\frac{[^{3}H]IP}{\text{incorporation of}[^{3}H]\text{-inositol}}) \times 100$. Significance: *P < 0.05, **P < 0.01 and ***P < 0.001 vs. adult.

We estimated the activity of PI turnover in cortical slices obtained from 22 month-old rats, but no significant difference was found between young rats and ones of this age (Fig. 6A).

$[^{3}H]$pirenzepine binding to crude membranes: To elucidate age-related changes in ligand affinity and the number of binding sites of M1-receptors, Scatchard analysis of $[^{3}H]$pirenzepine binding was carried out using crude synaptic membranes. The $K_d$ was not different between adult and senescent rats in each region. However, the $B_{max}$ values at the senescent stage were decreased in all regions compared to adults: 35.5% in the cerebral cortex, 34.9% in the striatum and 45.5% in the hippocampus (Table 1).

DISCUSSION

The results presented here show that high concentrations of KCl lead to $[^{3}H]$IP accumulation in cerebral cortical slices. This enhancement was lowered by atropine and further increased by physostigmine, suggesting that endogenous ACh released by depolarizing stimulation increases $[^{3}H]$IP accumulation. Since atropine did not completely inhibit the accumulation enhanced by high KCl, it is possible that other neurotransmitters may also be liberated, resulting in enhanced $[^{3}H]$IP accumulation. Under this experimental condition, Ca$^{2+}$ may be elevated via two mechanisms as follows: 1) entry of Ca$^{2+}$ through voltage-dependent Ca channels activated by high K+-induced depolarization and 2) intracellular mobilization of Ca$^{2+}$ by inositol 1,4,5-trisphosphate (IP$_3$) formed by receptor stimulation. It is reported that here are several isomers of phospholipase C (PLC) and some types of PLC involved in hydrolysis of PI into IP in the presence of high concentration of Ca$^{2+}$ (25–28). Intracellular Ca$^{2+}$ increased by high K$^+$ may also enhance $[^{3}H]$IP accumulation. In the present data, atropine could not inhibit $[^{3}H]$IP accumulation by high K$^+$, suggesting that other neurotransmitters and increased intracellular Ca$^{2+}$ also stimulate PI turnover. However, ACh-evoked $[^{3}H]$IP accumulation is perhaps dominant in neurotransmitter receptor-mediated $[^{3}H]$IP accumulation in the cerebral cortex, since atropine markedly
inhibited [\(^{3}\text{H}\)]IP accumulation by high K\(^{+}\).

Identification of five distinct cDNAs of mACh receptors and the expression of cloned mACh receptors have been reported (10–14, 29–31). The mACh receptor has been pharmacologically classified into three subclasses, M\(_1\), M\(_2\), and M\(_3\), according to the affinity for pirenzepine and AF-DX 116. Since the Hill coefficient of pirenzepine was unity, it is possible that pirenzepine-sensitive receptors couple to PI turnover. In contrast, the Hill coefficient of AF-DX 116 was less than unity, suggesting that 1) AF-DX 116-sensitive receptors are coupled to PI turnover with negative cooperativity, or 2) that at least two different types of receptors which have close affinities for pirenzepine and different affinities for AF-DX 116 are linked to PI turnover.

Purified mACh receptors have been shown to couple with G\(_{i}/G\(_{o}\) (32). Among G proteins capable of coupling receptors to PLC, which are termed G\(_{p}\) (33), some are PTX-sensitive (34–36) and others are insensitive (37–39). GTP\(_{\gamma}\)S-induced reduction in carbachol affinity for [\(^{3}\text{H}\)]pirenepine binding suggests that M\(_1\)-receptors interact with G proteins. Although carbachol-stimulated PI turnover was not significantly affected by PTX treatment, PTX slightly enhanced the reactivity to carbachol.

In contrast, the inhibitory effect of carbachol on forskolin-induced cAMP accumulation was suppressed by PTX, suggesting that PTX caused ADP-ribosylation of G\(_{i}/G\(_{o}\) under the present conditions. Ashkenazi et al. (20, 40) proposed that specific regulation of PLC activity may be achieved by selective coupling of different receptors by multiple G\(_{p}\), which activate PLC to different levels. The PTX-insensitive G\(_{p}\) are substantially greater activators of PLC than PTX-sensitive G\(_{p}\). The m1 and m3 receptors appear to couple to PTX-insensitive G\(_{p}\) dominantly; on the other hand, the m2 and m4 receptors appear to couple to the PTX-sensitive G\(_{p}\) and G\(_{i}/G\(_{o}\). If the sensitivity to PTX of PTX-sensitive G\(_{p}\) is lower than that of G\(_{i}/G\(_{o}\), G\(_{i}/G\(_{o}\) are preferentially ADP-ribosylated compared to G\(_{p}\). Uncoupling of G\(_{i}/G\(_{o}\) with m2 and m4 receptors by ADP-ribosylation may facilitate the signalling pathway from the receptors to PLC via G\(_{p}\). This facilitation results in slight enhancement of the reactivity to carbachol.

Two different pathways of uptake, diffusion and active transport of inositol, have been reported (41–43). Decrease in the fluidity of lipid bilayer membranes and surplus formation of phospholipid peroxides as a product of reactions of radicals (44) may disturb the inositol uptake pathway at the senescent stage.

The concentration-response curves of carbachol-induced [\(^{3}\text{H}\)]IP accumulation in senescent cerebral cortical and striatal slices were shifted downward but paralleled adult curves. Such findings suggest that these age-related
changes are quantitative and can be explained in part by a decrease in M₁-receptors and/or neuronal population in the regions. In contrast, both basal activity and reactivity of IP accumulation to carbachol-stimulation declined in the hippocampus. The changes in PI response to M₁-receptor stimulation in the hippocampus could be due to: 1) a decrease in the M₁-receptor population, 2) a decrease in fluidity of plasma membranes (44) followed by a decline of coupling between receptors, G proteins and PLC, and 3) structural changes in G proteins and/or PLC.

Reduction of [3H]inositol incorporation into senescent slices suggests a reduction in the formation of substrates for PLCs. The decline in biosynthetic activities of phosphatidylinositol phosphates and their hydrolysis and decrease in the number of M₁-receptors might occur in the nervous system of rats during aging.

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