Muscarnic Acetylcholine Receptor Regulates Phosphatidylcholine Phospholipase D in Canine Brain*

Zhuo Qian and Lester R. Drewes
From the Department of Biochemistry, School of Medicine, University of Minnesota, Duluth, Minnesota 55812

(Received for publication, July 7, 1989)

The hydrolytic activity of phosphatidylcholine phospholipase D in the synaptosomes from canine brain was examined using a radiochemical assay with 1,2-dipalmitoyl-sn-glycerol-3-phosphoryl[3H]choline as the exogenous substrate. The involvement of G protein(s) in regulation of this enzyme was demonstrated by a 2- to 3-fold stimulation of the basal activity (4.81 ± 0.44 nmol choline released/mg protein/h) with guanosine 5’- (3-O-thiol)triphosphate (GTPyS), guanyl-5’-yl(β,γmethylene)diphosphonate, aluminum fluoride, or chole- ra toxin. The stimulation of phospholipase D hydrolytic activity by GTPyS was inhibited by 2 mM guanosine 5’-(2-O-thiol)diphosphate. GTPyS at the maximum stimulatory concentration (10 μM) had an additive effect on the maximum chole- ra toxin stimulation of phospholipase D activity. However, the reverse was not true, thus indicating the possibility that more than one G protein may be involved. Furthermore, cholinergic agonists, including acetylcholine, carbachol, and muscarine, were able to increase the phospholipase D hydrolytic activity at low but not maximally stimulatory concentrations of guanine nu- cleotide. These cholinergic stimulations were antagonized by atropine, a muscarinic blocker. In addition, O-tetradecanoylphorbol 13-acetate, a protein kinase C activator, was able to stimulate the hydrolytic activity of phospholipase D more than 300% in the presence of 0.2 μM GTPyS. However, in the absence of GTPyS, stimulation was less than 60%. Our results not only indicate that the receptor-G protein-regulated phospholipase D may be directly responsible for the rapid accumulation of choline and phosphatidic acid in the central nervous system but also reveal that muscarinic acetylcholine receptor-G protein-regulated phospholipase D is a novel signal transduction process coupling the neuronal muscarinic receptor to cellular responses.

The muscarinic acetylcholine receptor is an important cholinergic receptor in a variety of cell types. Activation of these receptors can lead to various molecular responses, including inhibition of adenylate cyclase, increased levels of cGMP, modulation of potassium channels, and increased turnover rates in the phosphatidylinositol-phosphatidic acid cycle (1-4). Phosphatidic acid is rapidly produced during acetylcholine receptor-stimulated breakdown of phospholipids (5, 6), and according to the current hypothesis (7-9), 1,2-diacylglyceride formed by hydrolysis of phosphatidylcholine is a primary substrate for phospholipase D. The increase of phosphatidic acid is thus a secondary product of phosphatidylcholine breakdown by phospholipase D after stimulation by a variety of agonists including cholinergic agonists, such as acetylcholine, carbachol, muscarine, or oxotremorine (16-18). The muscarinic antagonist atropine was able to block the cholinergic agonist-induced choline increase (18). These observations indicate the possible involvement of cholinergic receptors in regulating phosphatidylcholine, phosphatidic acid, and choline metabolism by a phosphatidylcholine phospholipase D. Here we report for the first time the coupling relationship between the neuronal muscarinic receptor and phosphatidylcholine phospholipase D in canine brain.

EXPERIMENTAL PROCEDURES

Materials—1,2-Dipalmitoyl-sn-glycerol-3-phosphoryl[methyl-3H]choline (76 Ci/mmol), [methyl-3H]choline (75 Ci/mmol), and phospho[methyl-3H]choline (40 mCi/mmol) were purchased from Amersham Corp. HEPES, αTTP, αTP, cAMP, GTP-γS, GDP-βS, GMP-PCP, p-nitrophenolphosphate, Dowex-1 (1 × 8-200), sodium deoxycholate, sodium oleate, sodium linolate, CHAPS, oleylgluco- pyranoside, and Triton X-100 were from Sigma. Formic acid and ammonium formate were from Boehringer Mannheim. Inlet-activat- ing protein pertussis toxin and cholera toxin were purchased from ICN Biomedicals.

Preparation of Canine Cerebral Cortex Synaptosomes—The subcellular synaptosomal fraction from dog cerebral cortex was prepared essentially according to Natarajan et al. (19). Briefly, fresh dog cerebral cortex was minced and homogenized (10%, w/v) with a Potter-Elvehjem homogenizer in 0.32 M sucrose containing 1 mM EGTA. The homogenate was centrifuged initially at 1,000 × g for 10 min, and the postnuclear supernatant was centrifuged at 22,000 × g.

1 The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPyS, guanosine 5’-(3-O-thiol)triphos- phate; GMP-PCP, guanyl-5’-yl(β,γmethylene)diphosphonate; GDP-βS, guanosine 5’-(2-O-thiol)diphosphate; G protein, guanine nucleotide-binding protein; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; TPA, O-tetradecanoylphorbol 13-acetate.
for 30 min to obtain a crude membrane pellet, which contained myelin, synaptosomes, and mitochondria. The crude membrane fraction was resuspended in 0.32 M sucrose and centrifuged with a Beckman SW-27 rotor at 53,000 × g for 1 h on a discontinuous sucrose density gradient of 0.32, 0.85, and 1.2 M. The crude synaptosomal fraction was collected between the 0.85 and 1.2 M sucrose layer. The synaptosomal fraction containing the synaptosomes was diluted to a final sucrose concentration of 0.32 M and layered on top of a discontinuous sucrose gradient of 0.32, 0.85, and 1.2 M. After centrifugation at 75,000 × g for 30 min, the material at the interface of the 0.85 and 1.2 M sucrose layers was collected, diluted with 0.32 M sucrose, and centrifuged at 14,500 × g for 20 min to give the synaptosomal pellet. The pellet was then washed several times and resuspended in 0.32 M sucrose and stored at −20 °C until use. The protein content was determined according to Lowry et al. (20).

**Phospholipase D Assay**—To measure the phospholipase D hydrolytic activity, the exogenous substrate 1,2-dipalmitoyl-sn-glycerol-3-phosphoryl[3H]choline (specific activity: 1 × 10⁶ dpm/nmol) was used. The incubation medium in 0.25 ml contained 32 μM 1,2-dipalmitoyl-sn-glycerol-3-phosphoryl[3H]choline (8.0 × 10⁹ cpm), 40 mM HEPES buffer at pH 7.0, 0.1 mM MgCl₂, and 0.4% Triton X-100. The reaction was initiated by addition of cerebral cortex synaptosomes (200–300 μg of protein) as the enzyme source and incubated at 37 °C with shaking for 30 min. Reactions were stopped by adding methanol containing 1% acetic acid, and lipids were extracted. The radioactive choline and phosphorylcholine in the upper phase were separated with a Dowex-1 anion exchange column or separated by thin layer chromatography (silica gel-H) using methanol, 0.8% NaCl, concentrated ammonia (21). The radioactivity levels of the choline fractions were measured using liquid scintillation spectrometry. The amount of [3H]choline released, which was separated from phosphorylcholine, represents the hydrolytic activity of phospholipase D. To eliminate [3H]choline from hydrolysis of phosphoryl[3H]choline which was produced by phosphatidylcholine phospholipase C action, 5.0 mM cold phosphorylcholine or p-nitrophenylphosphate, a competitive inhibitor of phosphatases, was present in all the incubation media (22). The radioactivity in the choline fraction was always greater than 90% of the total radioactivity recovered in the aqueous phase after lipid extraction. On the other hand, less than 10% of the radioactivity was recovered in the phosphorylcholine fraction (data not shown). The enzyme activity was linear with incubation time (up to 30 min) and protein concentrations used in our experiments.

**Toxin Studies**—To determine the -ribosylation of cholera toxin-sensitive G protein, synaptosomes (200–400 μg of protein) were incubated for 20 min at 30 °C in a 250-μl mixture containing final concentrations of the following: 2.5 μM NAD, 1.5 μCi of [⁴⁰P]NAD, 1 mM ATP, 1 mM GTP, 10 mM thymidine, 1 mM dithiothreitol, 76 mM sodium phosphate, and 50 μg of activated cholera toxin. The reactions were stopped by addition of 250 μl of ice-cold 20% (w/v) trichloroacetic acid (23). Membranes were pelleted by centrifugation at 15,000 × g for 3 min, and then the membranes were washed with 50 mM KH₂PO₄/H₂PO₄, pH 2. After solubilization, proteins (50 μg of protein/lane) were separated by SDS-polyacrylamide gel electrophoresis. Dried gels were exposed to Kodak XAR-5 film at −70 °C. The radioactive bands were quantitated using a phosphorimager (24).

**RESULTS AND DISCUSSION**

To study the potential coupling between muscarinic receptors and phospholipase D, synaptosomes isolated from canine cerebral cortex were used. The hydrolytic activity of phosphatidylcholine phospholipase D in the synaptosomes was measured using a radiochemical assay with 1,2-dipalmitoyl-sn-glycerol-3-phosphoryl[3H]choline as the exogenous substrate. The basal level of the synaptosomal phospholipase D toward exogenous phosphatidylcholine was 4.81 ± 0.44 nmol of choline released/mg protein/h (n = 25). To examine the possible involvement of the G protein(s) in regulating phosphatidylcholine phospholipase D activity, GTP analogues were added to the incubation medium. A persistent concentration-dependent stimulation of phospholipase D hydrolytic activity was observed with GTPγS (Fig. 1A). However, the stimulation of phospholipase D hydrolytic activity by GTPγS was inhibited by 2 mM GDPβS. GDPβS itself was unable to stimulate phospholipase D activity. Another GTP analogue GDP-PCP was also capable of stimulating phosphatidylcholine phospholipase D by 2-fold but only at a concentration approaching 20 μM (data not shown). Zn²⁺ seems to have a direct inhibitory effect on the synaptosomal phospholipase D activity (Fig. 1A). Aluminum fluoride has been reported to activate certain known G proteins (24), and it is believed that aluminum fluoride may mimic the structure of the γ-phosphate group of GTP when it interacts with GDP. In the presence of fluoride plus aluminum (Fig. 1B), the synaptosomal phospholipase D hydrolytic activity was increased more than 2-fold.

To characterize further the G protein(s) involved, studies were conducted with cholera toxin and islet-activating protein pertussis toxin. These two toxins regulate the actions of G proteins on effectors through ADP-ribosyltransferase activity (24). Both toxins were thiol-activated (25). We observed that thiol-activated cholera toxin caused a 2–3-fold increase in phospholipase D hydrolytic activity in the presence of 0.1 μM GTPγS (Fig. 24), whereas islet-activating pertussis...
process and assay protein to stimulate hydrolytic activity of synaptosomal phospholipase D. Cholera toxin was activated for 20 min at 30°C with 0.1 PM GTPyS. The apparent molecular masses of these three polypeptides were estimated to be approximately 49, 43, and 24 kDa (Fig. 2B). Although these studies do not provide evidence that the observed radioactive bands are the G proteins which regulate phospholipase D activity, they do, however, demonstrate the presence of cholera toxin-sensitive G proteins in the synaptic plasma membranes.

In separate experiments, phospholipase D activity maximally stimulated by cholera toxin (20 µg/ml) was further enhanced with a maximal level of GTPyS (10 µM); however, the reverse was not true. Phospholipase D activity maximally stimulated by 10 µM GTPyS was not further increased with cholera toxin (data not shown). This suggests the possibility that more than one G protein may be involved. Further investigation is necessary to identify and characterize the G protein(s) coupled with phosphatidylinositol phospholipase D.

Acetylcholine, carbachol, and muscarine were used to examine the effects of cholinergic agonists on the phospholipase D activity in canine cerebral cortex synaptosomes. These agonists all were able to increase the phospholipase D hydrolytic activity in the presence of 0.2 µM GTPyS, a concentration of guanine nucleotide which itself produces only mild stimulation (Fig. 3A). At maximally stimulatory concentrations of guanine nucleotide, cholinergic agonists were not able to stimulate phospholipase D hydrolytic activity further. Atropine was able to block acetylcholine stimulation (Fig. 3B). Thus, the above evidence demonstrates that a muscarinic acetylcholine receptor regulates phosphatidylinositol phospholipase D via the G protein(s) in cerebral cortex synaptosomes. It also strongly suggests that the muscarinic receptor-G protein-regulated phospholipase D directly contributes to the accumulation of choline and phosphatidic acid in acetylcholine-stimulated cells.

Phosphatidic acid is rapidly produced during receptor-stimulated breakdown of phospholipids (5, 6). It has been proposed that increased levels and/or turnover of phosphatidic acid is the result of phosphorylation of diacylglycerol by diacylglycerol kinase in the agonist-stimulated phosphatidylinositol-phosphatidic acid cycle. However, analyses (27-29) of the quantity and fatty acid composition of phosphatidic acid, diacylglycerol, and the inositol phospholipids in agonist-stimulated hepatocytes and kidney cells indicate that phosphatidylinositol, 4,5-bisphosphate is probably a minor source of the increased phosphatidic acid and diacylglycerol. The analyses suggest that the major source of phosphatidic acid and diacylglycerol is phosphatidylinositol. More recent studies (10-14) provided increasing evidence that breakdown of phosphatidylinositol via a phospholipase D mechanism is an alternative pathway for accumulation of phosphatidic acid after a variety of agonist stimulations. Using 1-palmitoyl-2-[9,10(9)-3H]palmitoyl-sn-glycerol-3-phosphorylcholine as the exoge-sylation of cholera toxin-sensitive G protein was performed and identified by SDS-polyacrylamide gel electrophoresis. The details are described under "Experimental Procedures." KD, kDa.
phospholipase D activity was determined in incubation medium containing
phosphatidic acid after acetylcholine stimulation. This supports
phosphatidylcholine phospholipase D was 4.81
accumulation of phosphatidic acid after acetylcholine stimu-
performed in triplicate.

The capability of atropine for blocking acetylcholine stimulation
activator, on the hydrolytic activity of phospholipase D in
the idea that phosphatidylcholine is an important source of
phosphatidic acid and that an alternative mechanism for the
activities of atropine. The incubation conditions and assay details are
described under "Experimental Procedures." The basal level of phosphatidyl-
choline phospholipase D was 4.81 ± 0.44 nmol of choline released/
mg of protein/h (n = 25), and data are expressed as percentages of
control. Values are the means ± S.D. of four separate experiments performed in triplicate. — , TPA/0.2 μM
GTPγS.

Various cellular responses have been attributed previously
to phosphatidylic acid (30, 31). The main function of phospha-
tidic acid seems to be related either to calcium influx through
the plasma membrane or intracellular calcium mobilization.
Exogenous phosphatidylic acid stimulates calcium uptake in
parotid cells, neuroblastoma cells, and liver cells (32, 33) and
also causes rapid accumulation of inositol 1,4,5-triphosphate, an intracellular signal for Ca²⁺ mobilization, in human A431
carcinoma cells (34). Recent reports show that phosphatidylic acid has growth factor-like action and is able to induce cell
proliferation (34) and that phosphatidylic acid also has an
inhibitory effect on adenylyl cyclase activity (35). In addi-
tion, it has been reported that phosphatidylic acid phosphatase
is abundant in synaptic plasma membranes (36), and phosphatidylic acid is dephosphorylated by this enzyme to form
diacylglycerol. Therefore, we postulate that formation of di-
acylglycerol through the receptor-regulated phosphatidylic-
line phospholipase D and phosphatidylic acid-phosphatase
pathway may also contribute to activation of protein kinase
C in the central nervous system. Phosphatidylic acid produced
by receptor-regulated phospholipase D may perform a role as
a second messenger in modulating various cellular physiological
responses.

Choline, another degradation product of phosphatidylic acid
hydrolisis by phospholipase D, has been suggested to be an
important source of free choline by acetylcholine synthesis in
brain (15, 37). The significance of this regulated process
for supplying free choline for acetylcholine synthesis is espe-
cially evident when hydrolysis of acetylcholine to choline is
blocked by acetylcholinesterase inhibitors. Therefore, we prop-
ose that the dynamic interrelationship between choline in

Fig. 3. Cholinergic agonist and antagonist effects on the
hydrolytic activity of phospholipase D in synaptosomes from
canine cerebral cortex. A, various concentrations of acetylcholine,
carbachol, or muscarine were added to the incubation medium (0.25
ml) containing 32 μM 1,2-dipalmitoyl-sn-glycerol-3-phosphoryl[³H]
choline (8.0 × 10⁴ dpm), 40 mM HEPES buffer at pH 7.0, 0.1 mM
MgCl₂, 0.4% Triton X-100, and 0.2 μM GTPγS. B, the dose-response
capability of atropine for blocking acetylcholine stimulation of
phospholipase D activity was determined in incubation medium containing
32 μM 1,2-dipalmitoyl-sn-glycerol-3-phosphoryl[³H]choline (8.0 × 10⁴
dpm), 40 mM HEPES buffer at pH 7.0, 0.1 mM MgCl₂, 0.4% Triton
X-100, 0.2 μM GTPγS, 0.6 mM acetylcholine, and various concentra-
tions of atropine. The incubation conditions and assay details are
described under "Experimental Procedures." The basal level of phos-
phatidylic acid phospholipase D was 4.81 ± 0.44 nmol of choline released/mg of protein/h (n = 25), and data are expressed as specific
activity. Values are the means ± S.D. of six separate experiments performed in triplicate.

musnous substrate, we observed the accumulation of [³H]phos-
phatidylic acid after acetylcholine stimulation. This supports
the idea that phosphatidylic acid is an important source of phosphatidylic acid and that an alternative mechanism for the
accumulation of phosphatidic acid after acetylcholine stimu-
lization is present in the central nervous system.

We also tested the effects of TPA, a protein kinase C
activator, on the hydrolytic activity of phospholipase D in
isolated synaptosomes. When synaptosomes were incubated
with various concentrations of TPA in the presence of 0.2 μM
GTPγS, significant stimulations up to 350% were observed
(Fig. 4). Less than 50% stimulation was found in the absence of
GTPγS. ATP (0.1 mM) had no effect on the hydrolytic activity of phospholipase D (data not shown). Consequently,
this evidence suggests that activated protein kinase C may
directly or indirectly modulate the G protein(s) to enhance
the phospholipase D hydrolytic activity.

Z. Qian and L. R. Drewes, unpublished observations.
Acetylcholine and choline in membrane phosphatidylcholine is delicately controlled. This regulated synaptosomal phosphatidylcholine phospholipase D may play an important role in providing choline for acetylcholine synthesis in brain. Evidence provided here demonstrates that a muscarinic acetylcholine receptor is coupled to phosphatidylcholine phospholipase D via the G protein(s), and this mechanism is a novel signal transduction process coupling the neuronal muscarinic receptor to cellular responses.

Acknowledgment—We wish to acknowledge the expert editorial assistance of Carolyn Clark in the preparation of this manuscript.

REFERENCES