Coexistence of Phosphatidylcholine-Specific Phospholipase C and Phospholipase D Activities in Rat Cerebral Cortex Synaptosomes

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ABSTRACT: DAG derived from phosphatidylcholine (PtdCho) acts as a lipid second messenger. It can be generated by the activation of phospholipase D (PLD) and the phosphatidic acid phosphohydrolase type 2 (PAP2) pathway or by a PtdCho-specific phospholipase C (PtdCho-PLC). Our purpose was to study PtdCho-PLC activity in rat cerebral cortex synaptosomes (CC Syn). DAG production was highly stimulated by detergents such as Triton X-100 and sodium deoxycholate. Ethanol and tricyclodecan-9-yl-xanthate potassium salt decreased DAG generation by 42 and 61%, respectively, at 20 min of incubation. These data demonstrate that both the PLD/PAP2 pathway and PtdCho-PLC contribute to DAG generation in CC Syn. PtdCho-PLC activity remained located mainly in the synaptosomal plasma membrane fraction. Kinetic studies showed K_m and V_{max} values of 350 μM and 3.7 nmol DAG × (mg protein × h)$^{-1}$, respectively. Western blot analysis with anti-PtdCho-PLC antibody showed a band of 66 KDa in CC Syn. Our results indicate the presence of a novel DAG-generating pathway in CC Syn in addition to the known PLD/PAP2 pathway.

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Phosphatidylcholine (PtdCho) is the most abundant class of glycerophospholipids in mammalian cell membranes, where it plays key roles in membrane structure, cell death, and cellular signaling (1). This phospholipid is also an important source of second lipid messengers such as lysophosphatidylcholine, phosphatidic acid (PtdOH), arachidonic acid, and DAG.

PtdCho hydrolysis can be elicited by growth factors, cytokines, neurotransmitters, hormones, and other extracellular signals (2–5). In response to several stimuli, DAG can be generated from PtdCho by subsequent activation of phospholipase D (PLD) and PtdOH phosphohydrolase type 2 (PAP2) or by a PtdCho-specific phospholipase C (PtdCho-PLC) (2,6). In contrast to DAG generated from phosphatidylinositol (4,5) bisphosphate ($PtdInsP_2$) by PtdIns-PLC activity, the wave of DAG elicited from PtdCho hydrolysis is generated more slowly and proceeds without elevation of intracellular Ca^{2+}. PtdCho-PLC activation might be related to a sustained activation of Ca^{2+}-independent protein kinase C (PKC) isoforms suggesting its involvement in slow and extended cell responses such as proliferation and differentiation (6,7).

PtdCho-PLC activity has been previously described in several rat tissues (8) as well as in rat platelets (9), and it has been isolated and characterized in bull seminal plasma (10). PtdCho-PLC activity is involved in many cellular events such as glutamate-induced nerve cell death (11) and in Fas-induced apoptosis (12,13). PtdCho-PLC also can be activated during cell mitogenic responses that are triggered by platelet-derived growth factor (14,15), thromboxane A_2 (16), and lipopolysaccharide (LPS) (17), leading to the activation of the mitogen-activated protein kinase pathway. Furthermore, PtdCho-PLC seems to be acting downstream Ras but upstream Raf-1 during mitogenic signal transduction (18,19).

Studies from our laboratory demonstrated the presence of the PLD/PAP2 pathway and its regulation by insulin in rat cerebral cortex (CC) synaptosomes (Syn) (20,21). Mammalian PLD isoforms (PLD1, PLD2) are present in neurons and in glial cells, and these enzymes are implicated in basic cellular functions such as vesicular trafficking as well as in brain development (22). However, the PtdCho-PLC pathway has not been thoroughly studied in the central nervous system (CNS), and no evidence has been reported to date about its presence in synaptic endings and its involvement in signal transduction events. In view of its wide distribution and its implication in such diverse cell responses, the aim of this work was to analyze PtdCho-PLC activity in rat CC Syn.

MATERIALS AND METHODS

Wistar-strain adult rats (4 mon old) were kept under constant environmental conditions and fed on a standard pellet diet *ad libitum* until decapitation.

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Abbreviations: Ab, antibody; CC, cerebral cortex; CM, crude mitochondrial pellet; CNS, central nervous system; D609, tricyclodecan-9-yl-xanthate potassium salt; DOC, sodium deoxycholate; DPPtdCho, dipalmitoylphosphatidylcholine; LPS, lipopolysaccharide; NK, natural killer; PAP2, phosphatidic acid phosphohydrolase type 2; PKC, protein kinase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdCho-PLC, phosphatidylcholine-specific phospholipase C; PtdEth, phosphatidylethanol; $PtdInsP_2$, phosphatidylinositol (4,5) bisphosphate; $PtdInsP_2$-PLC, phosphatidylinositol (4,5) bisphosphate phospholipase C; PtdOH, phosphatidic acid; PVDF, polyvinylidene difluoride; RHC80267, 1,6-bis(cyclohexyloximinocarbonylamino)hexane; SM, sphingomyelin; SMS, sphingomyelin synthase; Sol, soluble fraction; SPM, synaptosomal plasma membrane; Syn, synaptosomes, synaptosomal fraction; TH, total homogenate; TNF, tumor necrosis factor; T X-100, Triton X-100.

1-[^{14}C]Palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine ([^{14}C]DPPtdCho) (111 mCi/mmol), 1,2-dipalmitoyl-*sn*-glycero-3-phospho[methyl-^{3}H]choline ([^{3}H-choline] DPPtdCho) (43 Ci/mmol), and Omnifluor were obtained from New England Nuclear-DuPont (Boston, MA). Triton X-100 (T X-100), sodium deoxycholate (DOC), tricyclodecan-9-yl-xanthate potassium salt (D609), and 1,6-bis(cyclohexyloximinocarbonylamino)hexane (RHC80267) were obtained from Sigma-Aldrich (St. Louis, MO). Polyclonal anti-PtdCho-PLC antibody (Ab) raised against *Bacillus cereus* PtdCho-PLC was generously provided by Dr. Howard Goldfine, University of Pennsylvania (Philadelphia, PA). All other chemicals were of the highest purity available.

Preparation of synaptosomal fraction. Total homogenates were prepared from the CC of 4-mon-old rats. Rats were killed by decapitation and CC was immediately dissected (2–4 min after decapitation). All proceedings were in accordance with *Principles of Use of Animals and Guide for the Care and Use of Laboratory Animals* (National Institutes of Health regulation).

The synaptosomal fraction (Syn) was obtained as previously described by Cotman (23) with slight modifications (20). Briefly, CC homogenate was prepared in the following way: 20% (wt/vol) in a buffer containing 1 mM EDTA, 10 mM HEPES buffer (pH 7.4) in the presence of 1 mM DTT, 2 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin, and 0.1 mM PMSF.

The CC homogenate was centrifuged at 1,800 × *g* for 7.5 min using a JA-21 rotor in a Beckman J2-21 centrifuge, and the supernatant was carefully poured into another tube. The supernatant was subsequently centrifuged at 15,000 × *g* for 20 min to obtain the crude mitochondrial pellet (CM). The CM was washed and resuspended with 3 mL of the isolation medium and loaded onto a 8.5% Ficoll medium (6 mL) and 13% Ficoll medium (6 mL) discontinuous gradient. The Ficoll solutions were prepared in the isolation medium. The sample loaded onto the discontinuous gradient was centrifuged at 120,000 × *g* for 30 min using a SW 28.1 rotor in a Beckman L5-50 ultracentrifuge. The myelin fraction band was at the interface between the isolation medium and the 8.5% Ficoll medium, the Syn band at the interface between the 8.5% and the 13% Ficoll medium, and the free mitochondrial fraction was the pellet below the 13% Ficoll medium. Syn was centrifuged at 33,000 × *g* for 20 min using a JA-21 rotor in a Beckman J2-21 centrifuge and resuspended in the assay buffer.

Preparation of Syn plasma membrane (SPM). SPM isolation was performed according to a protocol previously described by Igbavboa *et al.* (24) with slight modifications. Briefly, the Syn fraction from one CC was resuspended in 4 mL of cold lysis buffer (5 mM Tris buffer, pH 8.5). The lysed suspension was vortex-mixed vigorously and incubated for 1 h at 4°C with repeated vortex mixing every 15–20 min. After 1 h the suspension was centrifuged at 33,000 × *g* for 30 min using a JA-21 rotor in a Beckman J2-21 centrifuge. The soluble fraction (Sol) from lysed Syn was collected; the pellet was resuspended in 4 mL of cold distilled water, layered onto 12 mL of 0.75 M sucrose containing 10 mM HEPES and 0.25 mM EDTA (pH 7.4), and centrifuged at 73,000 × *g* for 30 min using an SW 28.1 rotor in a Beckman L5-50 ultracentrifuge. The SPM suspension at the interface was removed and centrifuged at 33,000 × *g* for 30 min in a JA-21 rotor in a Beckman J2-21 centrifuge. The SPM pellet was resuspended in the assay buffer. To establish the purity of Syn subfractions, a 5′ nucleotidase assay was performed according to Widnell and Unkeless (25).

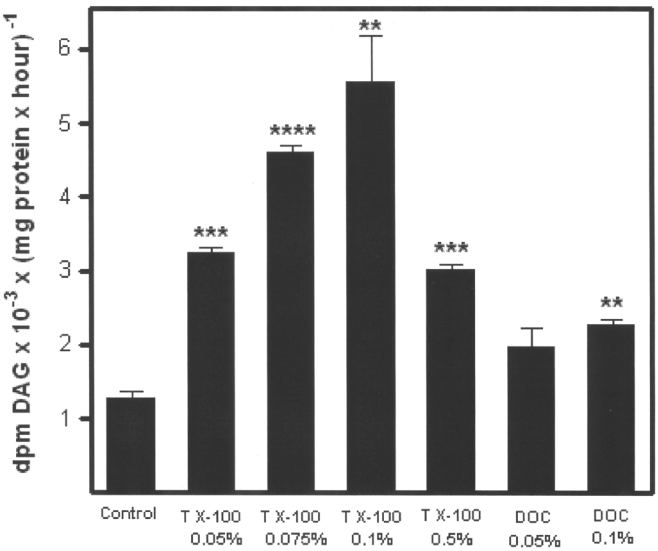

FIG. 1. Effect of detergents on synaptosomal DAG generation from phosphatidylcholine (PtdCho). Rat cerebral cortex (CC) synaptosomes (Syn) were incubated in a buffer containing 0.1 M Tris (pH 7.2) in the presence of 0.125 mM dipalmitoylphosphatidylcholine (DPPtdCho) (40,000 dpm of [^{14}C]DPPtdCho per assay) and different concentrations of Triton X-100 (T X-100; 0.05, 0.075, 0.1, and 0.5%) or sodium deoxycholate (DOC; 0.05 and 0.1%) as described in the Materials and Methods section. After 20 min, the enzyme reaction was stopped and lipids were extracted and isolated as described in the Materials and Methods section. Results are compared with the control condition without detergent, and the enzyme activity is expressed as dpm DAG × 10^{-3} × (mg protein × h)$^{-1}$. Each experiment was performed twice, using a pool of three animals on each occasion. Data are the mean ± SD of four samples (**$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$).

Determination of PtdCho-PLC activity. PtdCho hydrolysis by PtdCho-PLC was determined using lipid vesicles containing [^{14}C]DPPtdCho and cold DPPtdCho to yield 40,000 dpm and 0.125 mM per assay. These lipid vesicles (100 μL) were added to 100 μL of Syn, SPM, or Sol (150 μg of protein) in a final volume of 200 μL in a buffer containing 0.1M Tris (pH 7.2). The reaction was incubated at 37°C for 5 or 20 min and stopped by the addition of 5 mL of chloroform/methanol (2:1, vol/vol). Blanks were prepared identically, except that membranes were boiled for 5 min before being used. Lipids were extracted and separated as described below.

Determination of PLD activity. To assay PLD activity, transphosphatidylation reaction was measured in Syn and SPM. Lipid vesicles were prepared as described above and the assay was conducted in the presence of 2% ethanol. The reaction was incubated at 37°C for 5 or 20 min and stopped by the

addition of 5 mL of chloroform/methanol (2:1, vol/vol). Lipids were extracted and separated as described below.

Determination of sphingomyelin synthase (SMS) activity. SMS activity was assayed as described for PtdCho-PLC, but lipid vesicles were prepared with [^{3}H-choline]-DPPtdCho as labeled substrate. The reaction was incubated at 37°C for 5 or 20 min and stopped by the addition of 5 mL of chloroform/methanol (2:1, vol/vol). Lipids were extracted and separated as described below.

Extraction and isolation of lipids. Lipids were extracted according to Folch *et al.* (26). Briefly, the lipid extract was washed with 0.2 vol of 0.05% $CaCl_2$ and the lower phase was obtained after centrifugation at 900 × *g* for 5 min. Neutral lipids (MAG, DAG, and FFA) were then separated by 1-D TLC using silica gel G plates (Merck, Darmstadt, Germany) in a mobile phase consisting of hexane/diethyl ether/acetic acid (50:50:2.6, by vol). PtdCho was retained at the spotting site. Lipids were visualized by exposure of the plate to iodine vapors. DAG spots were scraped off for counting by liquid scintillation.

Phosphatidylethanol (PtdEth) was separated by 1-D TLC on silica gel H (Merck) and developed with chloroform/methanol/acetone/acetic acid/water (50:15:15:10:5, by vol) up to 70% of the plate. Then the plate was rechromatographed up to the top using hexane/diethyl ether/acetic acid (70:30:2.6, by vol) as described by Salvador and Giusto (27). Radioactivity of lipid spots was determined as previously described.

Sphingomyelin (SM) was separated by 1-D TLC on silica gel H (Merck) and developed with chloroform/methanol/27% ammonia (65:25:5, by vol). Radioactivity of lipid spots was determined as previously described.

Western blot assay of PtdCho-PLC. Syn samples were resuspended in Laemmli buffer and boiled for 3 min. Protein (60 µg) was loaded and resolved in a 10% SDS-PAGE following Laemmli (28) and transferred to a polyvinylidene difluoride (PVDF) membrane using a Mini Trans-Blot cell electroblotter (Bio-Rad Life Science Group, Hercules, CA) for 1 h. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (20 mM Tris, pH 7.5, 150 mM NaCl) containing 0.1% Tween-20 (2 h at room temperature) and then incubated with rabbit polyclonal anti-PtdCho-PLC Ab overnight at 4°C. Immunoreactions were detected with polyclonal horseradish peroxidase conjugated to total antirabbit Ab (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with enhanced chemiluminescence substrates (New England Nuclear-Dupont).

Other methods. Protein concentration was determined according to Bradford (29).

Statistical analysis. Statistical analysis was performed using Student's *t*-test.

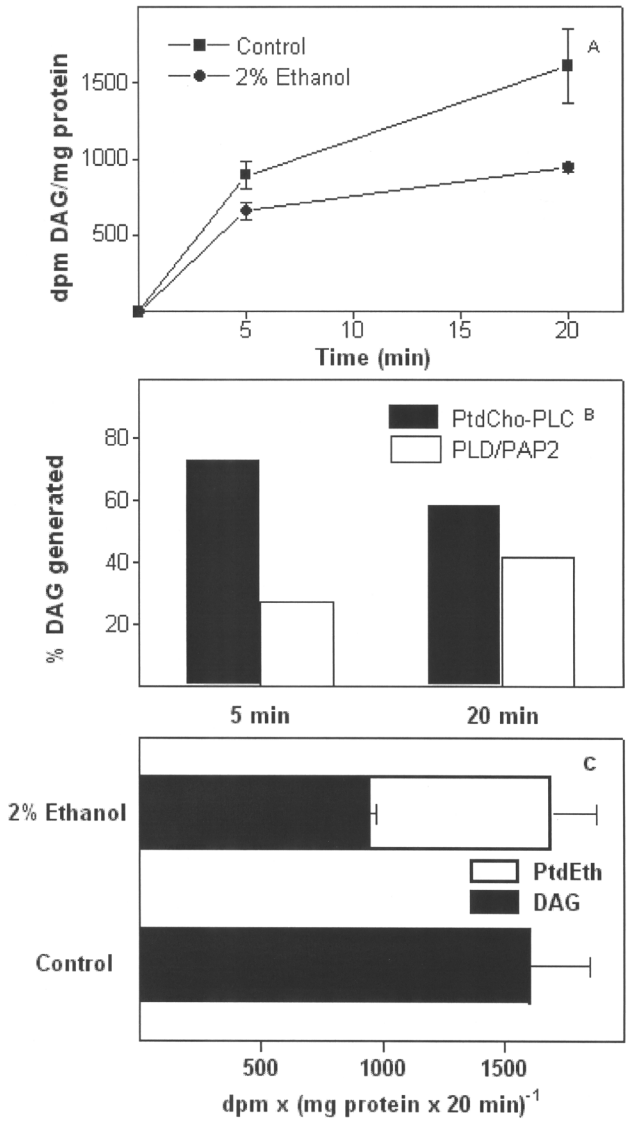

FIG. 2. (A) Contribution of PtdCho-PLC (PtdCho-specific phospholipase C) and phospholipase D/phosphatidic acid phosphohydrolase type 2 (PLD/PAP2) pathways to synaptosomal DAG generation. The enzyme assay was carried out as described in Figure 1 in a buffer containing 0.1% T X-100 with 2% ethanol and without ethanol (control condition). DAG generation was evaluated as a time function (5 and 20 min), and results are expressed as dpm DAG/mg protein. (B) Contribution of PtdCho-PLC and PLD/PAP2 pathways to synaptosomal DAG formation as a time function. Results are expressed as the percentage of the total DAG generated. (C) Synaptosomal phosphatidylethanol (PtdEth) formation. PtdEth production was measured as described in the Materials and Methods section. DAG and PtdEth levels obtained in Syn incubated with 2% ethanol are compared with DAG levels obtained under control condition at 20 min incubation. Results are expressed as dpm × (mg protein × 20 min)$^{-1}$. Each experiment was performed twice, using a pool of three animals on each occasion. Data are the mean ± SD of four samples. For other abbreviations see Figure 1.

RESULTS

Effect of detergents on DAG generation in CC Syn. The effect of detergents such as DOC and T X-100 on synaptosomal DAG generation was studied. Syn were incubated in the presence of 0.125 mM DPPtdCho (40,000 dpm of [^{14}C]DPPtdCho per assay) and in different concentrations of T X-100 (0.05, 0.075, 0.1, and 0.5%) or DOC (0.05 and 0.1%) as described in the Materials and Methods section. The effect of these tensioactive

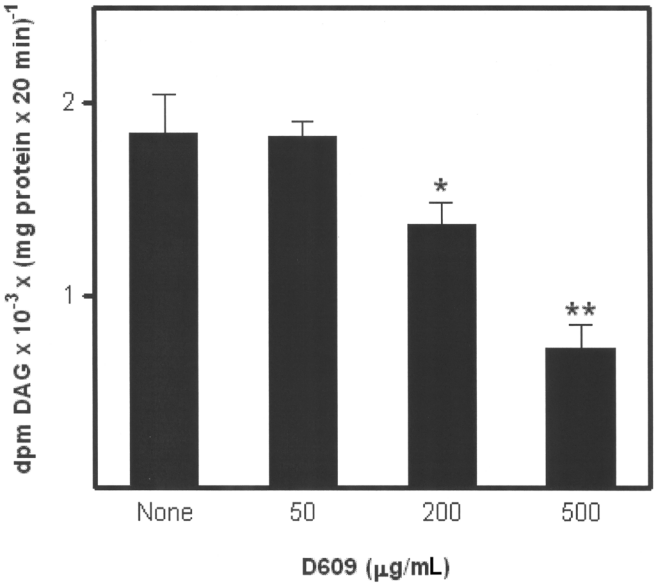

FIG. 3. Effect of tricyclodecan-9-yl-xanthate potassium salt (D609) on synaptosomal DAG formation. Syn were preincubated at 4°C for 20 min in the presence of different concentrations of D609 (50, 200, and 500 μg/mL). The enzyme reaction was carried out as described in Figure 2. Results are compared with the control condition, in which samples were preincubated without the inhibitor, and are expressed as dpm DAG × (mg protein × 20 min)$^{-1}$. Each experiment was performed twice, using a pool of three animals on each occasion. Data are the mean ± SD of four samples (*$P < 0.025$; **$P < 0.01$). For abbreviation see Figure 1.

agents on synaptosomal DAG generation is shown in Figure 1. Both detergents stimulated DAG generation at all concentrations assayed when the activity was compared with the control condition (without any detergent). The major DAG generation was observed with 0.1% T X-100: DAG formation increased by 330% with respect to the control condition. At the same concentration, DOC increased DAG generation by only 77% with respect to the control condition. Based on these results, all subsequent experiments were conducted in the presence of 0.1% T X-100.

Contribution of PtdCho-PLC and PLD/PAP2 pathways to synaptosomal DAG generation. To study the contribution of PtdCho-PLC and PLD/PAP2 pathways to synaptosomal DAG generation, ethanol was used as a marker of PLD activity. The enzyme assay was conducted in the presence of 2% ethanol, and DAG generation was evaluated as a time function (5 and 20 min) and compared with the control condition (without ethanol) (Fig. 2A).

Figure 2B shows the contribution of PtdCho-PLC and PLD/PAP2 pathways to DAG formation in CC Syn. At 5 min incubation, 73% of the total DAG generated is originated by PtdCho-PLC activity while the remaining 27% is generated by the PLD/PAP2 pathway. At 20 min incubation, 58% of DAG is generated by PtdCho-PLC activity and 42% is from the PLD/PAP2 pathway, respectively. In the presence of ethanol, DAG and PtdEth levels correlated to total DAG levels generated under control condition (Fig. 2C), indicating that PtdOH generated by PLD was converted to PtdEth.

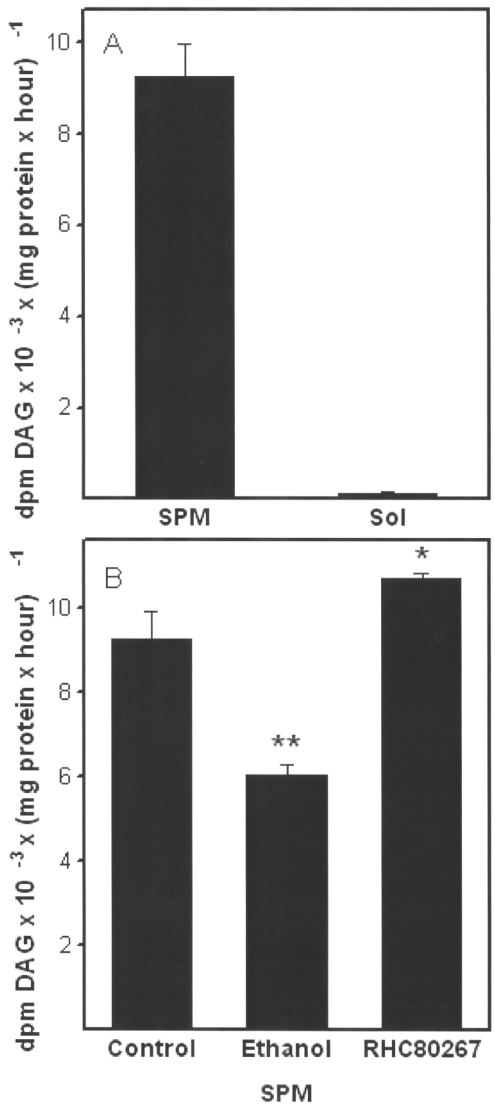

FIG. 4. (A) Localization of PtdCho-PLC activity. DAG formation was measured in the synaptosomal plasma membrane (SPM) and soluble fractions (Sol) obtained from Syn. SPM and Sol were isolated as described in the Materials and Methods section, and the enzyme reaction was carried out as described in Figure 2. (B) Effect of ethanol and 1,6-bis(cyclohexyloximinocarbonylamino)hexane (RHC80267) on DAG generation in SPM. DAG formation was measured under control conditions and in the presence of either 2% ethanol or 30 μM RHC80267 using SPM as the enzyme source. Results are expressed as dpm DAG × 10^{-3} × (mg protein x h)$^{-1}$. Each experiment was performed twice, using a pool of three animals on each occasion. Data are the mean ± SD of four samples (*$P < 0.025$; **$P < 0.01$). For other abbreviations see Figure 1.

Effect of D609 on DAG formation. To evaluate the effect of the PtdCho-PLC inhibitor D609, Syn were preincubated at 4°C for 20 min in the presence of increasing concentrations of D609 (50, 200, and 500 μg/mL) before starting the enzyme reaction. Results in Figure 3 show that D609 inhibited DAG formation in a dose-dependent manner: 50 μg/mL had no effect on DAG generation whereas 200 μg/mL decreased DAG formation by

TABLE 1
Protein and 5′ Nucleotidase Distribution in Total Homogenate, Synaptosomes, Synaptosomal Plasma Membrane, and Soluble Fractions Prepared from Rat Cerebral Cortex[a]

Subcellular fraction	mg protein/g CC	5´ Nucleotidase activity	Nucleotidase activity/ TH nucleotidase activity
TH	145.8	352 ± 56	1
Syn	12.02	684 ± 96	1.94
SPM	2.56	1500 ± 2.4	4.26
Sol	8.7	40 ± 16	0.11

[a]Subcellular fractions were prepared as described in the Materials and Methods section. The 5′ nucleotidase activity is expressed as nmol Pi × (mg protein × h)$^{-1}$. Data are the mean ± SD of four samples. TH, total homogenate; Syn, synaptosomes; SPM, synaptosomal plasma membrane; Sol, soluble fraction; Pi, inorganic phosphorus.

26% and 500 μg/mL decreased DAG formation by 61% with respect to the control condition (without the inhibitor).

Localization of PtdCho-PLC activity. For determining the localization of PtdCho-PLC activity, PtdCho hydrolysis was measured in the plasma membrane fraction and in the Sol, obtained after the lysis of Syn. SPM and Sol were isolated as described in the Materials and Methods section, and the enzyme reaction was carried out as previously described.

Figure 4A shows that PtdCho-PLC activity was present in the SPM fraction whereas enzyme activity was negligible in Sol. These results are evidence that PtdCho-PLC is located in the SPM fraction. To evaluate the contribution of the PLD/PAP2 pathway to DAG formation in SPM, enzyme assays were carried out in the presence of 2% ethanol (Fig. 4B). At 20 min incubation, ethanol inhibited DAG generation by 35%. These results demonstrated that 65% of DAG was generated by PtdCho-PLC activity and 35% was from PLD/PAP2 pathway in the plasma membrane fraction.

As DAG lipase, which is a very active enzyme, is also located in the plasma membrane, DAG formation was evaluated in the presence of the DAG lipase inhibitor, RHC80267. DAG formation in the presence of 30 μM RHC80267 was increased by 16% in SPM with respect to the control condition (Fig. 4B). The activity of 5′ nucleotidase was measured in Syn, SPM, Sol, and total homogenate (TH), for evaluating purity of fractions (Table 1). The specific activity of 5′ nucleotidase in SPM was 4.26-fold higher than the specific activity present in TH.

Determination of PtdCho-PLC K_m and V_{max}. To determine PtdCho-PLC K_m and V_{max} values, enzyme reactions were carried out in the presence of increasing concentrations of exogenous DPPtdCho. DAG generation was measured using 50, 75, 125, 200, 300, 500, 700, and 900 μM of DPPtdCho (150,000 dpm per assay) in the presence of 2% ethanol. DAG generation increased linearly up to 200 μM DPPtdCho; at higher concentrations PtdCho-PLC showed a saturation behavior (Fig. 5A). K_m and V_{max} values calculated from a Lineweaver-Burk plot were 350 μM and 3.7 nmol DAG × (mg protein × h)$^{-1}$, respectively (Fig. 5B).

Detection of synaptosomal PtdCho-PLC by Western blot assay. Synaptosomal proteins were resolved in a 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was then incubated with a polyclonal Ab raised against *B. cereus* PtdCho-PLC (Fig. 6). *Clostridium perfringens* PtdCho-PLC (line A, Fig. 6) was used as a positive control for the Ab since the N-terminal domain of this protein shows structural similarity to *B. cereus* PtdCho-PLC (30). CC Syn from adult rats (line B) showed a band corresponding to 66 kDa.

DISCUSSION

DAG was discovered as a lipid second messenger through its involvement in PKC activation (31,32). At present there is increasing interest in the question as to whether DAG derived from PtdCho may function as a lipid second messenger and regulate PKC analogous to DAG derived from $PtdInsP_2$. Among the pathways that generate lipid second messengers from PtdCho, PtdCho-PLC is one of the least characterized. It has been reported that PtdCho-PLC is involved in the activation of the nuclear transcription factor κB in response to the tumor necrosis factor α (TNF-α) (33,34) as well as in the activation of the signal transducer and activator of transcription 6 (STAT6) induced by interleukin-4 (35). In immune responses and inflammatory processes, PtdCho-PLC may play important roles because it co-localizes with perforin-carrying granules and it is involved in natural killer (NK)-mediated lytic activity (36). In addition, PtdCho-PLC inhibition blocks interleukin-6 and TNF release in LPS-stimulated human alveolar macrophages (17). However, little is known about PtdCho-PLC implications in signal transduction pathways in CNS.

In this work we studied the existence of the PtdCho-PLC pathway in rat CC Syn. Tensioactive agents such as T X-100 and DOC stimulated synaptosomal DAG formation. Maximal DAG generation was observed at 0.1% T X-100, and DAG formation increased by 330% with respect to the control condition (Fig. 1). Our results agree with data regarding PtdCho-PLC activity in rat platelets (9). However, rat platelet PtdCho-PLC activity was highest at 0.01% DOC.

To corroborate the source of synaptosomal DAG generation, ethanol was used as a marker of PLD activity. It is known that PLD in the presence of primary alcohols, such as ethanol or 1-butanol, not only catalyzes PtdCho hydrolysis yielding PtdOH but also catalyzes a competitive transphosphatidylation reaction leading to the formation of either PtdEth or phosphatidylbutanol with the accompanying suppression of DAG formation from PtdOH (37,38). In the presence of 2% ethanol, DAG formation decreased by 27 and 42% at 5

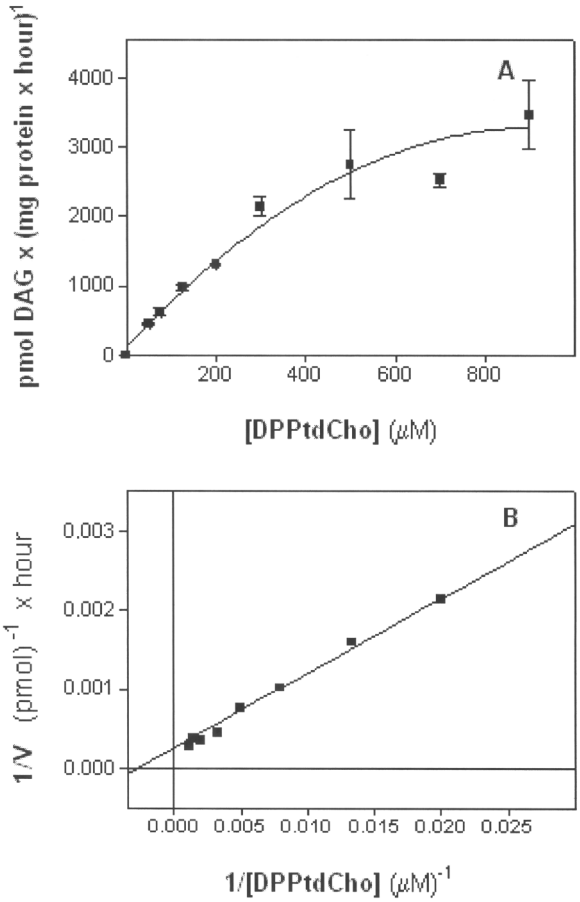

FIG. 5. (A) Determination of PtdCho-PLC K_m and V_{max}. Enzyme reactions were carried out in the presence of increasing concentrations of exogenous dipalmitoylphosphatidylcholine (DPPtdCho; 50, 75, 125, 200, 300, 500, 700, and 900 μM), 150,000 dpm of [^{14}C]DPPtdCho per assay in the presence of 2% ethanol, and 0.1% T X-100. Results are expressed as pmol DAG × (mg protein × h)$^{-1}$. (B) Lineweaver-Burk plot from the data in Figure 5A. Each experiment was performed twice, using a pool of three animals on each occasion. Data are the mean ± SD of four samples. For other abbreviations see Figure 1.

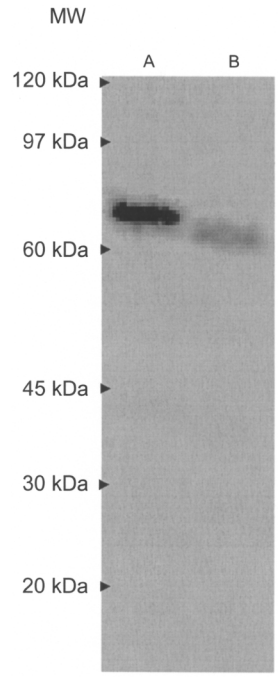

FIG. 6. Detection of synaptosomal PtdCho-PLC by Western blot assay. Synaptosomal proteins (60 μg) were resolved in a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was then incubated with rabbit polyclonal anti-PtdCho-PLC antibody (Ab), and immunoreactions were detected with polyclonal horseradish peroxidase conjugated to total antirabbit Ab. *Clostridium perfringens* PtdCho-PLC was used as a positive control for the anti-PtdCho-PLC Ab (A). Synaptosomal proteins from adult rats (4-mon-old) were loaded on the second line (B). For other abbreviations see Figure 1.

and 20 min, respectively (Fig. 2A). Consequently, the PtdCho-PLC contribution decreased from 73 to 58% as a time function. This decrease in DAG generation induced by ethanol demonstrates that PLD/PAP2 and PtdCho-PLC pathways differentially contribute to DAG production as a time function in CC synaptic endings (Fig. 2B). Our experiments suggest that PtdCho-PLC could be activated upstream from the PLD/PAP2 as it has been reported in other experimental systems (12,13,39).

For additional confirmation of the origin of DAG in CC Syn, enzyme assays were conducted in the presence of D609. This antiviral and antitumoral potassium xanthate is widely accepted as a selective inhibitor of PtdCho-PLC (15,40–42), and it has been proposed that it behaves as a competitive inhibitor of the enzyme (41,43). D609 inhibited synaptosomal DAG generation in a dose-dependent manner (Fig. 3). At 20 min of incubation, 500 μg/mL D609 decreased DAG formation by 61%, which was correlated with the PtdCho-PLC contribution observed in the presence of 2% ethanol.

Previous studies suggest that some of the biological events that had been attributed to PtdCho-PLC could be due to SMS (44,45). SMS is an enzyme involved in SM and ceramide metabolism that transfers a phosphocholine group from PtdCho to ceramide to generate SM and DAG (46,47). However, the kinetics of DAG production by SMS is significantly different from that of DAG production by PtdCho-PLC or PLD/PAP2 pathways because SMS-mediated DAG production is a slow process. Moreover, when [^{3}H-choline]DPPtdCho was used as a labeled substrate, no SM formation from PtdCho was detected under our experimental conditions (data not shown).

Experiments performed in the SPM and Sol fractions from Syn provide evidence that PtdCho-PLC is located in the plasma membrane (Fig. 4A). PtdCho-PLC localization in the membrane fraction is in accordance with previously published data observed in NK cells (36) and in NIH-3T3 fibroblasts (15). The inhibition of DAG generation by ethanol demonstrates that both PLD/PAP2 and PtdCho-PLC pathways contribute to DAG generation in SPM.

Although the molecular structure of eukaryotic PtdCho-PLC is still unknown, Clark *et al.* (48) demonstrated that antibodies prepared to *B. cereus* PtdCho-PLC cross-react with a

PtdCho-PLC from mammalian cells. Detection of synaptosomal PtdCho-PLC by Western blot assay with a polyclonal Ab raised against *B. cereus* PtdCho-PLC showed a band corresponding to 66 kDa (Fig. 6). Using also a polyclonal Ab directed to *B. cereus* PtdCho-PLC, a 66 kDa band was detected in NIH-3T3 fibroblasts (15) and in human NK cells (36). Our results also correlate to those obtained by Sheikhnejad and Srivastava (10), who determined the M.W. of an isolated PtdCho-PLC from bull seminal plasma by SDS-gel electrophoresis and reported two bands of 69 and 55 kDa.

Our experiments demonstrate the existence of a novel synaptosomal DAG generation pathway, thus confirming the first evidence for the presence of PtdCho-PLC in rat CC Syn. The existence of this novel lipid second messenger pathway in CNS is the starting point for the study of its involvement in neuronal signal transduction.

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