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MODIFICATIONS OF PHOSPHOLIPID METABOLISM INDUCED BY CHLORPROMAZINE, DESMETHYLIMIPRAMINE AND PROPRANOLOL IN C6 GLIOMA CELLS

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Abstract—The effects of chlorpromazine (CPZ), desmethylimipramine (DMI) and propranolol (PRO) on phospholipid metabolism in C6 glioma cells were studied by following the incorporation of $^{32}P_i$, [U-^{14}C]glycerol, [2-^{3}H]glycerol and [1-^{14}C]oleate into lipids. The drugs produced a dose-dependent increase in the incorporation of $^{32}P_i$ and [U-^{14}C]glycerol, but not of [1-^{14}C] oleate, into total phospholipids, that reached a plateau at 200 μM CPZ and 500 μM DMI and PRO. The three drugs shifted the incorporation of precursors from neutral [phosphatidylcholine (PC) and phosphatidylethanolamine (PE)] to acidic phospholipids [phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylglycerol, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2)] in a dose-dependent, qualitatively similar manner. The incorporation of [2-^{3}H]glycerol into diacylglycerol was also depressed markedly by CPZ. Addition of 1 mM 1,2-dioleoylglycerol, 1-oleoyl-2-acetylglycerol or oleate only partially reversed the decrease in PC labeling caused by CPZ. 12-*O*-Tetradecanoylphorbol-13-acetate counteracted this effect of CPZ completely but greatly increased PC labeling even in the absence of the drug. Polyphosphoinositides rapidly incorporated $^{32}P_i$ at early times reaching a plateau in about 40 min. The labeling rate of PI was not parallel to that of PIP or PIP_2 and continued to increase even after the polyphosphoinositides had reached a plateau. CPZ increased PI labeling much more than that of PIP and PIP_2. These data suggest that cationic amphiphilic drugs may act by (1) inhibiting CTP:phosphocholine cytidylyltransferase, thus decreasing incorporation of precursors into PC and PE; (2) inhibiting PA phosphohydrolase with increased formation of phosphatidyl-CMP, the intermediate for the synthesis of acidic phospholipids; and (3) stimulating the inositol exchange reaction, forming a pool of PI that is not available for PIP and PIP_2 synthesis.

Many compounds commonly used in the treatment of a variety of diseases are cationic amphiphilic drugs (CADs).§ The common physicochemical and molecular features that they share, namely a bulky lipophilic ring system and a positively charged amino nitrogen separated from it by a short carbon chain, confer special properties on these compounds such as an ability to associate with negatively charged polar head groups of glycerophospholipids and to interact with the apolar moieties of these molecules [1–4]. This allows CADs to intercalate into phospholipid bilayers and thus change their properties, affecting fluidity, charge and degree of order of biological and artificial membranes [5–9]. Some of these drugs cause phospholipidosis as a side-effect when administered to patients, experimental animals or cells in culture for prolonged periods of time [10–13]. In addition to their capacity to alter membrane conformation, CADs affect phospholipid metabolism by redirecting the incorporation of radioactive precursors from the neutral lipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and triacylglycerol (TAG), to acidic ones, phosphatidic acid (PA), phosphatidyl-CMP (CMP-PA), phosphatidylinositol (PI), phosphatidylglycerol (PG) and diphosphatidylglycerol [for review see Refs. 14–16].

This report details studies on the effects of chlorpromazine (CPZ), desmethylimipramine (DMI) and propranolol (PRO) on the incorporation of radioactive precursors into C6 glioma cell lipids and on modification of these effects by diacylglycerol (DAG) and phorbol esters. Parts of these studies have been presented in preliminary form [17, 18].

MATERIALS AND METHODS

Materials. Chlorpromazine · HCl was obtained

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§ Abbreviations: CAD, cationic amphiphilic drug; CMP-PA, phosphatidyl-CMP; CPZ, chlorpromazine; DAG, diacylglycerol; diolein, 1,2-dioleoylglycerol; DMI, desmethylimipramine; EPG, ethanolamine phosphoglycerides; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Me_2SO, dimethyl sulfoxide; OAG, 1-oleoyl-2-acetylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; POPOP, 1,4-bis[5-phenyl-2-oxazolyl]-benzene; PPO, 2,5-diphenyloxazole; PRO, propranolol; TAG, triacylglycerol; and TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

from Smith Kline & French Laboratories, Philadelphia, PA; desmethylimipramine·HCl was purchased from Merrell Dow Pharmaceuticals, Inc., Cincinnati, OH; *d,l*-propranolol·HCl, sodium oleate, 2,5-diphenyloxazole (PPO), 1,4-bis[5-phenyl-2-oxazolyl]benzene (POPOP), Triton X-100, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) were purchased from the Sigma Chemical Co., St. Louis, MO; 1,2-dioleoylglycerol (diolein) and 1-oleoyl-2-acetylglycerol (OAG) were bought from Serdary Research Laboratories, London, Ontario. TPA, diolein and OAG were suspended in dimethyl sulfoxide (Me_2SO) and added to the culture media to give a final Me_2SO concentration of 1% (v/v). All other chemicals and solvents were analytical grade and were obtained from the Fisher Scientific Co., Pittsburgh, PA. Chloroform was redistilled.

[U-^{14}C]Glycerol (sp. act. 30 mCi/mmole) and [1-^{14}C]oleate (sp. act. 50 mCi/mmole) were obtained from ICN Pharmaceuticals Inc., Irvine, CA; [2-^{3}H]glycerol (sp. act. 200 mCi/mmole) and [^{32}P]orthophosphoric acid (carrier-free) were supplied by New England Nuclear, Boston, MA.

Cell culture conditions and lipid labeling. Rat C6 glioma cells [19] were grown in six-well cluster dishes (CoStar, Cambridge, MA) under conditions previously described [20] with 15 μg/ml gentamycin. Serum was withdrawn 24 hr before use. Culture media were removed and cells were rinsed twice with 2 ml of the following buffer at 37°: 122 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM $MgSO_4$, 10 mM $NaHCO_3$, 5 mM glucose, 25 mM HEPES, pH 7.4. Seven hundred microliters of the same buffer containing drugs and lipids as indicated, and either about 5 μCi of $^{32}P_i$, 2 μCi of [U-^{14}C]glycerol or [1-^{14}C]oleate, or 10 μCi of [2-^{3}H]glycerol per well were added, and the cultures were incubated at 37° for the times indicated.

At the end of the incubation period the media were rapidly removed, and the cells were rinsed once with 2 ml of ice-cold buffer, followed by 2 ml of ice-cold methanol in which the cells were harvested after scraping with a rubber policeman.

Lipid extraction and analysis. Lipids were extracted and the extracts washed according to Folch *et al.* [21]. The extracted cell residues were recovered from the upper phases by filtration through small wads of cotton inserted into Pasteur pipettes, re-extracted twice for 20 min at 37° with 4- and 2-ml aliquots of chloroform–methanol (2:1) containing 0.25% 12 M HCl and washed as described previously [22]. The separate extractions were done to avoid degradation by acid of alkenyl-acyl-glycerophosphoethanolamine which constitutes about 40% of the ethanolamine phosphoglycerides (EPG) of this cell line. The neutral and acidic lipid extracts from each sample were pooled and phospholipids were separated on silica gel HL plates (Analtech, Inc., Newark, DE) with chloroform–methanol–concentrated ammonia–water (45:45:11:5.5) [23]. Since PG and EPG comigrate in this system, the appropriate radioactive areas were scraped, the lipids eluted from the gel according to Arvidsson [24], and PG and EPG separated on silica gel G plates with chloroform–methanol–water (14:6:1) [25]. To separate alkenyl-acyl-glycerophosphoethanolamine from the diacyl-compound, the TLC plates were exposed to HCl vapors for 5 min after spotting. Radioactivity in lysoPE spots was taken as a measure of alkenyl-acyl-glycerophosphoethanolamine labeling [26].

Neutral lipids were separated on silica gel G plates with ethyl ether–benzene–ethanol–acetic acid (40:50:2:0.2) followed in the same direction by ethyl ether–hexane (6:94) [27].

Lipids were visualized by radioautography using X-Omat RP film (Eastman Kodak Co., Rochester, NY). For tritiated lipids, fluorography was done by spraying the plates with 0.4% PPO in 2-methyl napthalene containing 10% toluene and exposing the radioautograms at −75° for 1–3 weeks [28].

Radioactive areas were scraped from the plates and transferred into vials. Ten milliliters of toluene scintillation fluid [0.4% PPO and 0.005% POPOP (w/v) in toluene], containing 9% (v/v) Triton X-100 for tritiated lipids, was added and radioactivity was measured in a Packard Tri-Carb scintillation spectrometer.

RESULTS

CPZ, PRO and DMI increased the incorporation of radiolabeled precursors into total phospholipids of C6 glioma cells. The drugs increased the incorporation of $^{32}P_i$ and [U-^{14}C]glycerol into total phospholipids by 50–70%, but when [1-^{14}C]oleate was used, the increase was only 10% in 1 hr (Table 1).

Dose–response curves obtained by treating the cells with increasing concentrations of the drugs and measuring the incorporation of $^{32}P_i$ into total phospholipids showed that the process is saturable,

Table 1. Incorporation of precursors into phospholipids

	$^{32}P_i$	[U-^{14}C]Glycerol (dpm × 10^{-3}/hr/μCi added per well)	[1-^{14}C]Oleate
Control	21.1 ± 1.4	1.5 ± 0.1	35.0 ± 2.0
CPZ	34.6 ± 3.0	2.2 ± 0.1	39.0 ± 1.2
DMI	34.6 ± 3.5	2.4 ± 0.1	
PRO	31.7 ± 3.8	2.5 ± 0.1	

Cells were labeled for 60 min in the presence of a 100 μM concentration of each drug. Values are means ± SD of the sums of the radioactivity incorporated into individual phospholipid classes for three cell cultures.

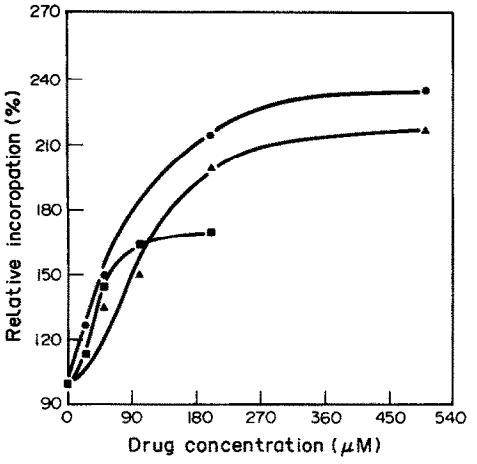

Fig. 1. Dose–response curves of cationic amphiphilic drugs for $^{32}P_i$ incorporation into total phospholipids in C6 glioma cells. Cells were incubated with $^{32}P_i$ for 60 min as indicated in Materials and Methods. Values are means of the radioactivity in total phospholipids (sum of the individual phospholipid classes) expressed as percentage of control, in three different cell cultures. Key: CPZ (■); DMI (●); and PRO (▲).

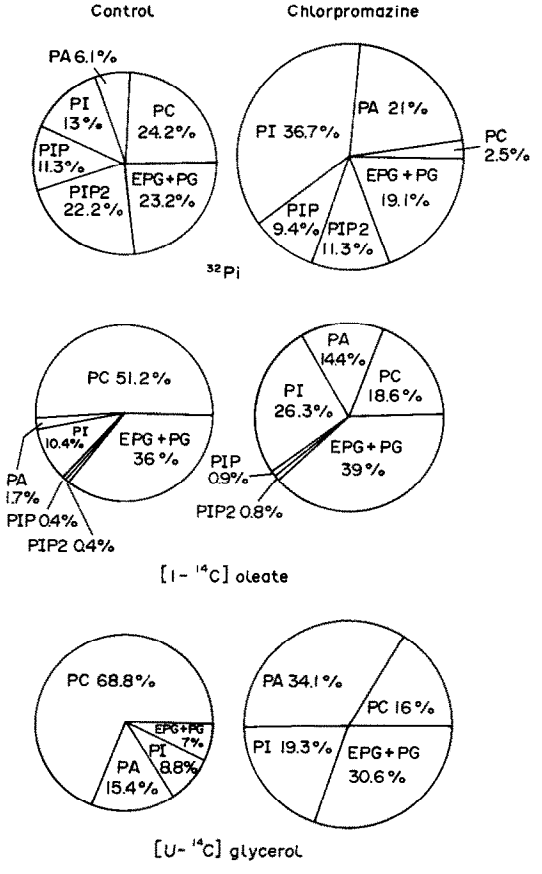

Fig. 2. Labeling patterns of phospholipids in C6 cells. Cells were labeled with radioactive precursors with or without 100 μM CPZ for 60 min. Values are radioactivity incorporated into each phospholipid class expressed as percent of the incorporation into total phospholipids, in three different cell cultures. The areas of the circles are proportional to the total radioactivity incorporated into phospholipids.

with a plateau at about 300 μM for DMI and PRO and at about 100 μM for CPZ (Fig. 1).

Different labeling patterns in individual phospholipid classes were observed with [U-^{14}C]glycerol, $^{32}P_i$ and [1-^{14}C]oleate in the absence of drug (Fig. 2), but the changes induced by 100 μM CPZ in 1 hr were qualitatively similar for each precursor and consisted of a pronounced decrease in PC and a dramatic increase in PA and PI labeling (Fig. 2). Dose–response curves of the incorporation of $^{32}P_i$ into individual phospholipid classes showed that the changes caused by the three drugs were dose-dependent and that the rank order of potency was the same for inhibition of PC labeling and stimulation of PA and PI labeling. Fifty percent inhibition of PC labeling was achieved with 50 μM CPZ, 100 μM PRO or 250 μM DMI (Fig. 3). In one experiment where EPG and PG were separated, the changes induced by increasing DMI concentration from 25 to 500 μM were similar to those observed in PC and PI respectively (data not shown). Changes in PE and the alkenyl-acyl-glycerophosphoethanolamine species were parallel, with the latter consistently incorporating about 40% of the label in EPG.

The radioactivity of [2-^{3}H]glycerol and $^{32}P_i$ appeared in PA at early times and plateaued in 20–30 min, whereas the incorporation of both precursors into PC started after a lag of about 5 min. When CPZ was added at the beginning of the incubations, it was able to prevent PC labeling and greatly stimulate that of PA (Fig. 4). When the radioactivity was expressed as a percentage of the incorporation into total phospholipids, PA reached a maximum in about 10 min and then decreased, whereas incorporation into PC became progressively more pronounced. CPZ maintained PA labeling at its maximum level and prevented PC labeling virtually completely (Fig. 5A).

Polyphosphoinositides rapidly incorporated $^{32}P_i$ and reached a plateau at 60 min, whereas PI label

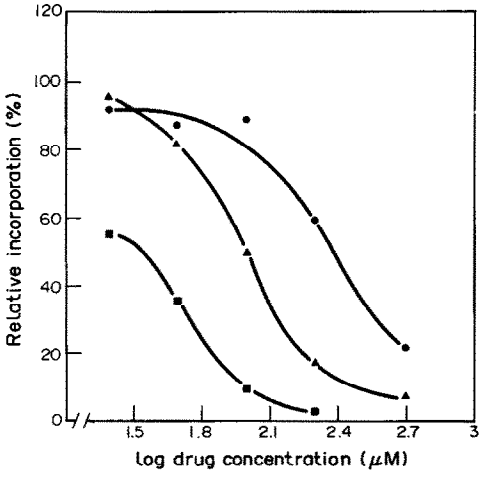

Fig. 3. Dose–response curves of cationic amphiphilic drugs for $^{32}P_i$ incorporation into PC. Cells were labeled with $^{32}P_i$ as indicated in Materials and Methods. Values are means of the radioactivity incorporated as percent of the radioactivity in the controls, in three different cell cultures. Key: CPZ (■); DMI (●); and PRO (▲).

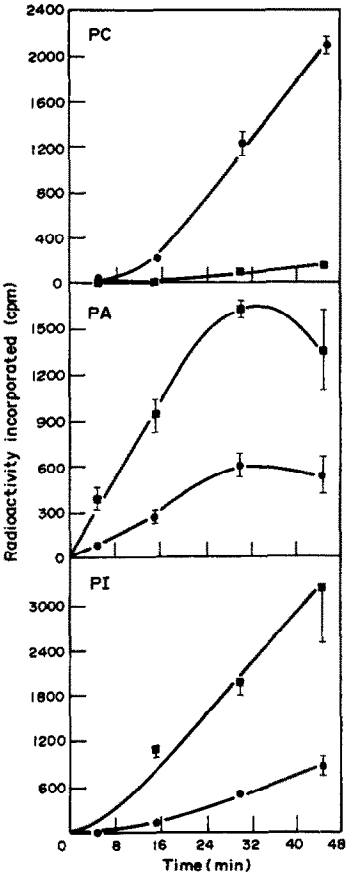

Fig. 4. Time-course of the incorporation of [2-^{3}H]glycerol into individual phospholipid classes. Cells were labeled in the absence (●) or presence (■) of 100 μM CPZ for the times indicated. Values are means ± SD of the radioactivity incorporated into three different cell cultures.

was still increasing at that time point. Phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-biphosphate (PIP_2) contained about 18 and 48% of the phospholipid radioactivity at early times and then decreased progressively, whereas PI increased during the same time period (Fig. 5B). CPZ greatly elevated [2-^{3}H]glycerol incorporation into PI (Fig. 4), but the effect on $^{32}P_i$ incorporation into PIP and PIP_2 was much less pronounced (Fig. 5B). When the labeling rates of these three lipids were plotted (Fig. 6), PIP and PIP_2 gave parallel curves with negative slopes whereas that for PI was consistently positive. CPZ greatly enhanced this difference by dramatically increasing the PI labeling rate without any influence on that of the polyphosphoinositides.

It seemed particularly important to monitor the labeling behavior of DAG in the presence of the drugs, because this compound is the intermediate for the synthesis of PC and PE and is formed primarily by the action of PA phosphohydrolase, an enzyme that is greatly inhibited by CADs in C6 cells [29] as it is in other systems [14, 15]. In the presence of 100 μM CPZ, the incorporation of [2-^{3}H]glycerol into DAG was decreased markedly at each time point (Fig. 7).

If a lack of DAG is responsible for the reduced labeling of PC and PE, supplementation of the medium might be able to overcome this deficit. When diolein or OAG (1 mM) was added to the culture media, the incorporation of $^{32}P_i$ into PC, which was reduced by CPZ to about 20% of the control values, was increased to 45%. However, both diolein and OAG increased the incorporation of $^{32}P_i$ into PC by about 25% even in the absence of drug.

Oleate has been shown to restore the activity of CTP:phosphocholine cytidylyltransferase, the regulatory step for PC biosynthesis, when it is inhibited

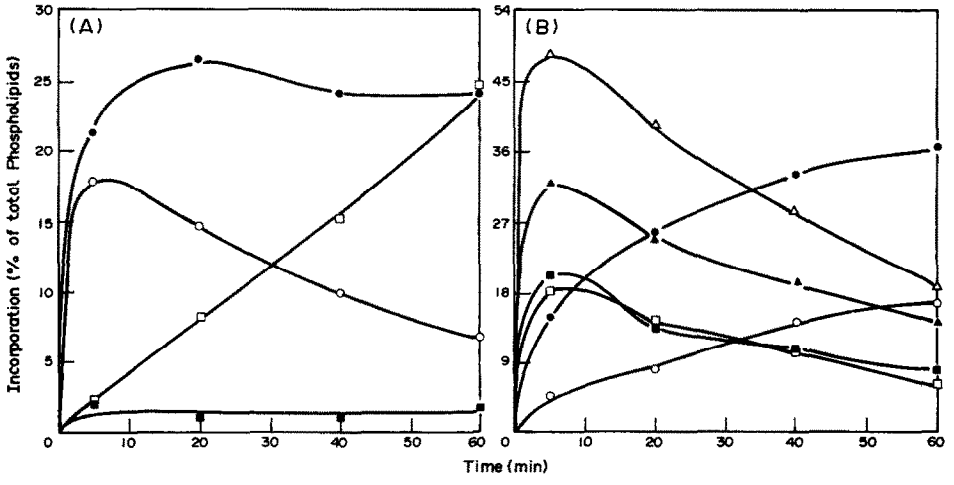

Fig. 5. Effects of CPZ on the time-course of $^{32}P_i$ incorporation into phospholipids. Values are percentages of the radioactivity incorporated in total phospholipids. Control (open symbols); 100 μM CPZ (solid symbols). In panel A, PC = □,■; and PA = ○,●; in panel B, PI = ○,●; PIP = □,■; and PIP_2 = △,▲.

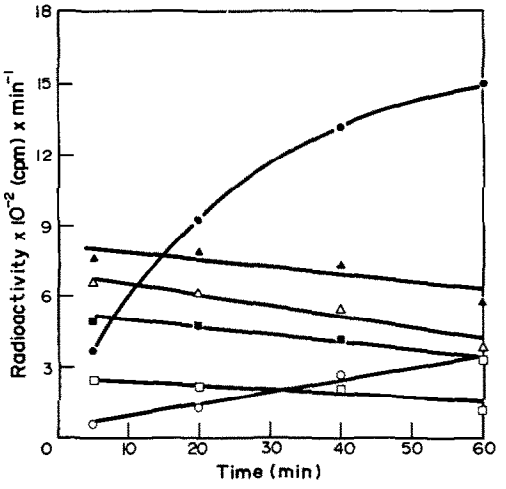

Fig. 6. Rate of incorporation of $^{32}P_i$ into inositol-containing lipids. Data of Fig. 5B are replotted to demonstrate the differences in the rate of formation of PI and the polyphosphoinositides. Control (open symbols); 100 μM CPZ (solid symbols). PI = ○,●; PIP = □,■; and PIP_2 = △,▲.

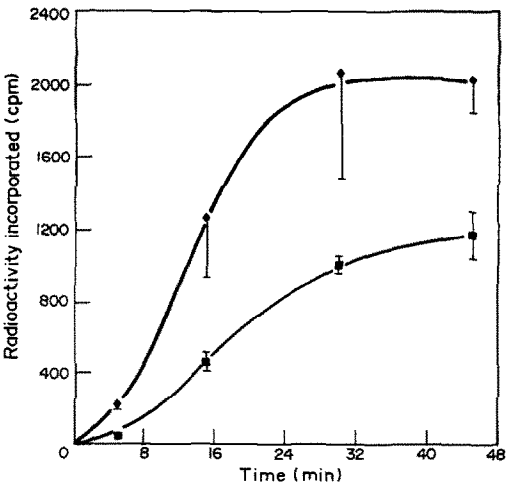

Fig. 7. Effects of CPZ on the time–course of incorporation of [2-^{3}H]glycerol into DAG. Cells were treated as in Fig. 4. Values are means ± SD of the radioactivity incorporated into DAG in three different cell cultures. Key: control (◆); and 100 μM CPZ (■).

by CPZ in HeLa cells [30]. This may be due to the capacity of oleate to promote the association of this enzyme with microsomal membranes. When we treated C6 cells with 1 mM oleate, the labeling of PC, which was decreased by 85% by 100 μM CPZ in this experiment, was raised almost to the control level.

Phorbol esters have also been shown to promote the association of bimodally distributed enzymes, such as protein kinase C [31], PA phosphohydrolase [32] and CTP:phosphocholine cytidylyltransferase [33], with membranes and in some cases to cause stimulation when the membrane-bound is the active form of the enzyme. Treatment of the cells with TPA (10 μM) reversed the effect of the drug on PC labeling. However, TPA alone had a dramatic stimulatory effect on the incorporation of $^{32}P_i$ into PC (Table 2).

DISCUSSION

The CADs, CPZ, DMI and PRO, dramatically increased the incorporation of $^{32}P_i$ and [U-^{14}C]glycerol, but not of [1-^{14}C]oleate, into total phospholipids of C6 glioma cells. They also shifted the incorporation of these precursors from neutral to acidic phospholipids by enhancing the labeling of PA, PI and PG and by decreasing the labeling of PC and PE analogous to results obtained with other tissues.

If the overall effects of these drugs were only due to a stimulation of precursor uptake, the percent changes in radioactivity would be parallel in all phospholipid classes. On the other hand, if the increase in total labeling only reflects the increase in acidic phospholipid radioactivity, this would also be observed with [1-^{14}C]oleate as precursor. However, the three drugs elicited labeling patterns with this precursor similar to those with $^{32}P_i$ and [1-^{14}C]glycerol, even though the total incorporation was not enhanced. The two phenomena must therefore be due to two different effects of the drugs.

CPZ, DMI and PRO have been shown to inhibit rat brain Na^+/K^+-ATPase [34–36], mitochondrial Mg^{2+}-ATPase [37] and sarcoplasmic Ca^{2+}-ATPase [36, 38, 39]. Although we have not yet assayed ATPases in our system, we propose that a part of the total labeling increase of phospholipids by $^{32}P_i$ in the presence of the drugs may be due to increased [^{32}P]ATP, possibly caused by the inhibition of ATPase.

When [^{14}C]glycerol was the precursor, incor-

Table 2. Effects of TPA on $^{32}P_i$ incorporation into PC

	Radioactivity incorporated (cpm × 10^{-3}/hr/μCi added)	(% of control)
Control	3.00 ± 0.46	100
TPA	15.60 ± 2.28	512
CPZ	0.93 ± 0.13	31
TPA + CPZ	3.80 ± 0.64	125

Cells were incubated for 60 min with 100 μM CPZ and 10 μM TPA as indicated in Materials and Methods. Values are means ± SD of the radioactivity incorporated into PC in three cell cultures.

poration into total phospholipids was also greatly enhanced by the three drugs. Glycerol uptake is mediated by passive or by facilitated diffusion. PRO and local anesthetics inhibit the facilitated transport, but enhance the passive diffusion owing to their capacity for perturbing cell membranes [40]. It has been suggested that the facilitated transport is due to a membrane protein and that the passive diffusion is regulated by the physicochemical state of the membranes [40]. Although no data are available about the transport mechanisms of glycerol in C6 glioma cells, the enhanced overall labeling of phospholipids from this precursor may be due to uptake by passive diffusion that is increased by drug–membrane interaction.

An explanation for the shift in the incorporation of precursors toward acidic phospholipids must be sought in changes in the activity of one or more of the enzymes responsible for the flow of precursors under normal physiological conditions. One of the key enzymes in this pathway, PA phosphohydrolase, has been implicated in the observed redirection by virtue of the ability of several CADs to reduce its activity in cell-free preparations from liver [41], brain [42] and C6 glioma cells in culture [29]. If this were to occur in the intact system, it might be responsible for a decrease in DAG required for PC and PE synthesis, and this mechanism could explain the increased labeling observed in PA. PA would then be available to react with CTP, catalyzed by CTP:PA cytidylyltransferase, to give CMP-PA, the common intermediate for the synthesis of PI and PG. This may explain our results of a marked decrease in DAG labeling and increased PA labeling, which are translated into decreased labeling of PC and EPG and increased labeling of PI and PG respectively.

However, other steps may also be modified by CADs. The regulatory enzyme for the synthesis of PC, CTP:phosphocholine cytidylyltransferase, which is present in the cell in both soluble and membrane-bound form is activated by translocation from the cytosolic to a membrane compartment [43–45]. This reaction has been shown to be inhibited in HeLa cells by CPZ [30], but oleate, which can promote the translocation, is able to counteract the action of the drug [30]. Phorbol esters and DAG are also able to stimulate this enzyme by the same mechanism [32, 46]. Further, treatment of Chinese hamster ovary cells in culture with phospholipase C has been shown recently to stimulate PC synthesis by indirectly activating this enzyme [45, 47]. These considerations support the suggestion that CTP:phosphocholine cytidylyltransferase could be inhibited by CADs in C6 glioma cells as well.

The fact that DAG was able to counteract inhibition of PC labeling by CPZ only partly in our experiments leads to the conclusion that a decrease in DAG availability is not the factor responsible for the drug effect on PC. DAG seems to become limiting only under conditions where the activity of CTP:phosphocholine cytidylyltransferase, decreased by CPZ, is restored to normal by translocating agents such as oleate [48] or TPA. Although translocation may also play a role in the function of PA phosphohydrolase [32], the critical feature for this enzyme may be the formation of a drug–phospholipid–protein complex which might prevent reversal by phorbol esters or oleate of the inhibition by CADs. This would be in contrast to the situation with the cytidylyltransferase which does not act on a lipid substrate, and can therefore not be subject to the same type of inhibition. The great increase in PC labeling induced by TPA in the absence of drugs has been reported also in other systems [49] and remains unexplained.

The effects of CPZ on the metabolism of inositol-containing lipids reported here consist of a dramatic uncoupling of the labeling rate of PI from that of the polyphosphoinositides, and of a high early labeling of PIP and PIP_2 that is not substantially modified by the drug. The very rapid turnover of the 4- and 5-monoesterified phosphates of PIP and PIP_2 [50] can explain the early appearance of $^{32}P_i$ in these phospholipids but, eventually, the diesterified phosphate should also become more heavily labeled because the radioactivity of this group in precursor PI is greatly increased. This would be expected to lead to a further proportionate increase in the radioactivity incorporated into PIP and PIP_2.

Our data suggest that only a fraction of PI is available for the synthesis of polyphosphoinositides even under control conditions. This may be due to the existence of two separate PI pools in the cells, of which only one is available for the synthesis of polyphosphoinositides. PI is synthesized in animal cells not only by CMP-PA:inositol phosphatidyltransferase but also by a Mn^{2+}-dependent inositol exchange reaction [51]. Mn^{2+} stimulates the incorporation of [2-^{3}H]inositol into PI, but not into PIP or PIP_2, in rat brain cortex slices [52]. This has led to the conclusion that a pool of PI, which cannot be converted to PIP or PIP_2 is formed in brain by this pathway [52, 53]. Thus, CADs might stimulate the exchange reaction, leading to an accumulation of a distinct radioactive pool of PI. This is in agreement with the reported stimulation of the Mn^{2+}-dependent inositol exchange reaction by CPZ in rat liver microsomes [54].

Another explanation of these findings arises from the differences in subcellular localization of the enzymes for the synthesis of PI, PIP and PIP_2. This requires that PI be transferred from one locus to another within the cell for further processing, a function carried out by a protein with high specificity [55]. This transport is inhibited by CPZ, PRO and other CADs [56] which could reduce the availability of PI for the synthesis of polyphosphoinositides, producing the observed results.

In summary, CPZ, PRO and DMI could affect phospholipid metabolism in C6 glioma cells by possibly decreasing ATP hydrolysis, by inhibiting PA phosphohydrolase and CTP:phosphocholine cytidylyltransferase, and perhaps also by stimulating inositol exchange. These diverse actions indicate the capacity of CADs to interfere with fundamental physiological cell functions such as membrane homeostasis and signal transmission mechanisms. Studies with cell-free preparations are in progress in this laboratory to provide more direct evidence on the influence of CADs on specific enzymatic reactions.

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