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MECHANISM OF DEPRESSION OF BRAIN PHOSPHOLIPID LEVELS BY AN EPILEPTOGENIC DRUG

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Abstract—Treatment of the rat with U18666A [3β-(2-diethylaminoethoxy) androst-5-en-17-one HCl] resulted in development of a chronic seizure state and 20–40% reductions in the concentration of all major phospholipid in whole brain. The mechanism of the phospholipid changes was explored in the present study. Incorporation of intracerebrally injected [1,3-^{3}H]glycerol and [^{32}P]orthophosphate into glycerolipids was decreased by 30–40% in treated rats. U18666A added *in vitro* to brain slices totally blocked glycerolipid synthesis at a high drug level (10^{-3} M) but stimulated incorporation into diacylglycerol, phosphatidic acid and phosphatidylinositol at a lower level (10^{-4} M). When added *in vitro* to cell fractions from liver or brain, U18666A readily inhibited phosphatidate phosphohydrolase and the acyltransferase enzymes which convert glycerolphosphate to phosphatidic acid and which convert diacylglycerol to triacylglycerol. Fifty percent inhibition of all three enzymes occurred at drug concentrations of between 0.4 and 1.0 mM. Phosphatidate cytidylyltransferase, an enzyme important to formation of phosphatidylinositol, was comparatively resistant to inhibition. Taken together, the results indicate that the marked reduction in the concentration of brain phospholipids caused by treatment of the young rat with U18666A is likely due to decreased phospholipid synthesis secondary to inhibition of several key enzymes in glycerolipid synthesis and, particularly, to inhibition of glycerolphosphate acyltransferase and phosphatidate phosphohydrolase.

Treatment of the rat with U18666A [3β-(2-diethylaminoethoxy) androst-5-en-17-one HCl] resulted in production of a chronic seizure state which was similar to absence or petit mal epilepsy in man [1, 2]. The epileptiform condition was accompanied by 20–40% reductions in the concentration of the major phospholipids in brain microsomal, synaptosomal and crude myelin fractions [3]. Maintenance of the perturbations of phospholipids could be a precondition for development and expression of the disorder. To our knowledge, drug-induced reductions of brain phospholipids of this magnitude have not been reported previously.

U18666A is an example of a cationic amphiphilic drug, one containing a hydrophobic region and a substituted amine that can carry a positive charge. Similar compounds have been shown to be potent inhibitors of phospholipid biosynthesis [4–6]. U18666A is also reported to inhibit cholesterol biosynthesis at late metabolic steps [7, 8]. Fatty acid synthesis was not inhibited [9]. In an effort to determine the mechanism by which exposure to U18666A markedly lowered the concentration of brain phospholipids, we examined the ability of U18666A to influence formation of cerebral phospholipids both *in vivo* and *in vitro* and to affect the activity of enzymes important in glycerolipid synthesis.

METHODS

Animals and treatment. Beginning at one day after birth, Sprague–Dawley rats (pregnant females purchased from Hilltop Lab Animals, Inc., Scottdale, PA) were injected (s.c.) every fourth day with 10 mg/kg of U18666A suspended in olive oil. Treated and untreated control rats were decapitated at about 3 and 6 weeks of age. Total lipids of whole brain were extracted by homogenization in 20 vol. of chloroform–methanol (2:1, v/v) as described before [10]. Individual phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silica gel H [11]; the separated phospholipids were recovered from the plates and quantitated by colorimetric assay [12].

Incorporation in vitro *of* [1,3-^{3}*H*]*glycerol into brain phospholipids.* Whole brains from untreated Sprague–Dawley rats (male and female, approximately 150 g) were chilled in ice-cold isotonic saline, blotted dry, and sliced horizontally into 0.5 mm sections (Stadie–Riggs tissue slicer, Arthur H. Thomas Co., Philadelphia, PA). Brain slices (200–250 mg) were transferred to 18 × 150 mm test tubes and preincubated at 37° for 10 min in 2.3 ml of Krebs phosphate buffer (pH 7.4) plus 0.1 ml of 50% propylene glycol (PG)–water (v/v) containing U18666A at 0, 2.5×10^{-3} M, 1.25×10^{-2} M or 2.5×10^{-2} M. Incubations at each concentration were conducted in triplicate. Twenty microcuries of [1,3-^{3}H]glycerol (80 μCi/μmole, Amersham Corp., Arlington Heights, IL) was added in 0.1 ml of isotonic saline; the tubes were gassed with oxygen and sealed, and the slices were incubated for 30 additional min at 37° with shaking (120 cycles/min). The final concentration of U18666A in the incubation mixture was either 0, 1×10^{-4} M, 5×10^{-4} M or 1×10^{-3} M. The reactions were stopped by addition of 15 ml of ice-

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cold isotonic saline, and the tubes were centrifuged immediately for 5 min at about 1000 *g*. The saline wash was aspirated, and the slices were washed a second time with saline. Total lipids were extracted from the slices into chloroform by the method of Hajra *et al.* [13], and the chloroform layer was washed twice with upper phase mixture containing glycerol and glycerol-3-phosphate [4]. The recovered lipids were fractionated by two separate TLC systems. Diacylglycerols and triacylglycerols were separated on silica gel G plates using a solvent of hexane–glacial acetic acid–diethylether (73:2:25, by vol.). Individual phospholipids were separated by a two-dimensional TLC system using silica gel H [11]. The silica gel zones containing the various phospholipids or neutral lipids were transferred to scintillation counting vials. The gels were inactivated by addition of 0.5 ml water followed by 1.0 ml methanol. The ^{3}H-content of each lipid fraction was measured following addition of 15 ml of scintillation mixture containing 11% BioSolve (Beckman Corp., Fullerton, CA). The results were expressed as dpm incorporated/g brain (wet weight). The incorporation of ^{3}H from [1,3-^{3}H]glycerol into triacylglycerols (TG) and phosphatidylcholine (PC) was essentially linear over a 30-min incubation. Radiolabeled [1,3-^{3}H]glycerol rather than [2-^{3}H]glycerol was used for these and other studies, because of the possibility of a tritium isotope effect. Bowley *et al.* [14] reported that mitochondrial sn-glycerol-3-phosphate oxidase discriminated against [2-^{3}H]glycerol. This enzyme was over eight times more active with [1-^{14}C]glycerol-3-phosphate than with [2-^{3}H]-glycerol-3-phosphate.

Incorporation in vivo *of* [1,3-^{3}*H*]*glycerol and* [^{32}*P*]*orthophosphate into lipids of whole brain.* Three- and six-week-old control and treated rats were lightly anesthetized with ether. A small hole was made in the skull with a 27 gauge needle at a position approximately 4 mm posterior and 4 mm lateral to the bregma in the right hemisphere. Rats were injected at a depth of 4 mm from the skull surface with 20 μl of isotonic saline containing either 10 μCi of [1,3-^{3}H]glycerol (10 μCi/μmole) or 20 μCi or 50 μCi of carrier free [^{32}P]orthophosphate ($^{32}P_i$) (Amersham Corp). The rats injected with [^{3}H]glycerol were killed after 1 hr and those injected with $^{32}P_i$ were killed at various times between 1 and 18 hr after injection. Whole brains were homogenized in chloroform–methanol as before, and the recovered lipids were fractionated by the two TLC systems described above. The radioactive content of each lipid fraction was measured by scintillation counting, and the results were expressed as dpm or cpm incorporated/g brain (wet weight).

Assay of enzyme activities. The effects *in vitro* of U18666A upon the activity of key enzymes in glycerolipid synthesis were measured using preparations from liver and brain. The enzymes studied were glycerol-3-phosphate acyltransferase plus monoacylglycerol-3-phosphate acyltransferase, phosphatidate phosphohydrolase, phosphatidate cytidylyltransferase and diacylglycerol acyltransferase.

Glycerol-3-phosphate acyltransferase. The acylation of glycerol-3-phosphate by microsomes from liver and brain of adult Sprague–Dawley rats was measured essentially as described by Lamb and Fallon [15]. A reaction mixture (0.25 ml) containing 2.1 mM dithiothreitol, 126 mM palmitoyl CoA, 98 mM sodium phosphate buffer (pH 6.5), 1.25 mg fatty acid-poor bovine serum albumin and 0.84 mM [U-^{14}C]glycerol-3-phosphate (0.1 μCi) was preincubated for 5 min at 37° with 0.05 ml of 20% PG (propylene glycol)–water (v/v) containing U18666A at a concentration of 0, 7×10^{-3} M, 3.5×10^{-3} M or 7×10^{-4} M. The reaction was started by adding 0.2 mg of liver or brain microsomal protein in 0.05 ml of phosphate buffer (pH 6.5). Microsomes were prepared according to Fallon and Lamb [16]. After a 10-min incubation, the reaction was stopped by adding 0.1 ml of 6 N HCl followed by 4.5 ml of the extraction solvent of Hajra *et al.* [13]. The recovered chloroform layer was washed twice with the upper phase mixture of Brindley and Bowley [4] with the proportion of glycerol to glycerol-3-phosphate reversed. Monoacylglycerolphosphate and diacylglycerolphosphate were separated from each other and from other lipids by TLC on silica gel G plates using a solvent of chloroform–methanol–3.5 M NH_4OH–H_2O (50:40:8:2, by vol.). Mono-oleoylglycerol-3-phosphate (Serdary Research Laboratories, London, Ontario) (100 μg) and phosphatidic acid, from egg yolk (100 μg), was added as carrier to each sample prior to chromatography. The distribution of the radiolabel among the various gel zones recovered from plates was measured by scintillation counting.

Over 95% of the radiolabel incorporated into total lipids by both liver and brain microsomes was recovered from monoacylglycerolphosphate plus diacylglycerolphosphate (phosphatidate). However, the radiolabel was unexpectedly found mainly in phosphatidic acid. Lamb and Fallon [17] explain that phosphatidate is the major phospholipid product formed in this reaction when whole tissue homogenates rather than washed microsomes are used. However, the microsomes used in our studies were prepared by their procedure [16]. Lands and Hart [18] also identified diacylglycerol-3-phosphate as the main product formed when liver microsomes were incubated with glycerol-3-phosphate and [^{14}C]oleic acid. We, therefore, expresssed glycerol-3-phosphate acyltransferase activity as nmoles of monoacylglycerolphosphate plus diacylglycerolphosphate formed per mg microsomal protein per min. The reaction was linear for at least 10 min.

Phosphatidate phosphohydrolase. The activity of microsomal and soluble phosphatidate phosphohydrolase was determined by measuring release of inorganic phosphorus (P_i) from phosphatidic acid (dipalmitoylglycerol-3-phosphate) in an aqueous dispersion [19]. A uniform suspension of phosphatidic acid (sodium salt) in water was produced by sonication (Branson-Sonifier-Cell-Disrupter-200, Smith–Kline Co., Danbury, CT). The incubation mixture was prepared immediately before each experiment and contained 1.5 mM phosphatidic acid, 0.5 mM dithiothreitol, 2 mM $MgCl_2$ and 180 mM Tris–acetate buffer (pH 7.4). Microsomes and soluble supernatant fraction were prepared from brain and liver of 4- to 8-week-old rats [20]. U18666A was added in 20–100 μl of 33% PG–water to give final

concentrations of between 0 and 1×10^{-3} M in a total reaction mixture volume of 2.0 ml. The reaction was started by adding 1 mg of microsomal or supernatant protein in 100 μl. After incubation for 30 min at 37°, the reaction was stopped by addition of 2 ml of 12% (w/v) trichloroacetic acid. Following removal of the precipitated protein by centrifugation, the P_i liberated from phosphatidic acid was measured by colorimetric assay [21]. Enzyme activity was expressed as μmoles of P_i liberated per mg protein per min. In the absence of added drug, the reaction was linear for at least 30 min.

Phosphatidate cytidylyltransferase. The activity of microsomal (liver and brain) phosphatidate cytidylyltransferase was measured according to Sturton and Brindley [5]. Microsomes were prepared from liver and brain of adult rats by the procedure of Mangiapane *et al.* [22]. A 0.45-ml mixture containing 110 mM Tris buffer (pH 7.4), 2.2 mM phosphatidate, from egg yolk (Sigma Chemical Co., St. Louis, MO), 1.1 mM [^{3}H]CTP (2 μCi/μmole), 5.5 mM ATP, 1.1 mM dithiothreitol, 3 mg of fatty acid-poor bovine serum albumin, 0.5 mg of microsomal protein and U18666A at a concentration of 0, 1.1, 2.78, 8.3 or 11.1 mM was preincubated for 5 min at 37°. The reaction was started by adding 0.05 ml of 200 mM $MgCl_2$, allowed to proceed for 10 min, and stopped by adding 0.1 ml of 6 N HCl followed immediately with 4.5 ml of chloroform–methanol (1:2, v/v) [13]. The recovered chloroform layer was washed twice with upper phase mixture [4]. After adding 100 μg of CDP-dioleoylglycerol (Serdary Research Labs) to each sample as carrier, the total lipids were separated by TLC on plates of silica gel 60 (Merck) using a solvent of chloroform–methanol–acetic acid–water (25:14:2:4, by vol.). The CDP-diacylglycerol spot separated clear of all other major lipids. It accounted for more than 95% of the radiolabel recovered from the total lipids of both the liver and brain microsomes. The results were expressed as the nmoles of CDP-diacylglycerol formed per mg of microsomal protein per min. The reaction was linear over the reaction period studied.

Diacylglycerol acyltransferase. The activity of diacylglycerol acyltransferase was measured in the presence of a 1,2-dioleoylglycerol by the method of Jamdar *et al.* [23] except that the diacylglycerol was dissolved in ethanol to a concentration of 20 mM as recommended by Coleman and Bell [24]. The standard assay volume was 1 ml and it contained 50 mM Tris buffer (pH 7.4), 1.2 mM 1,2-dioleoylglycerol, 0.25 mM dithiothreitol, 8 mM $MgCl_2$, 0.08 mM palmitoyl CoA containing 0.1 μCi of [1-^{14}C]palmitoyl CoA (1.25 μCi/μmole) and 1.25 mg/ml of fatty acid-poor albumin. U18666A was added in 20–100 μl of 33% PG to give final concentrations of between 0 and 1×10^{-3} M. The reaction was started by addition of 100 μg of liver or brain microsomal protein. The systems were incubated for 10 min at 37° for liver and 5 min for brain. The reaction was stopped by adding the lipid extracting solvent of Hajra *et al.* [13]. Total lipids were recovered and subjected to single-dimensional TLC on silica gel G plates using a solvent of hexane–glacial acetic acid–diethyl ether (73:2:25, by vol.). Carrier triacylglycerol (300 μg) was added to each sample prior to chromatography. The gel zone containing the triacylglycerol (located after brief exposure to iodine vapor) was recovered, and the ^{14}C-content was measured directly by scintillation counting. Enzyme activity was expressed as nmoles of palmitoyl CoA incorporated into triacylglycerol per mg protein per min. The reaction rates were linear over the incubation periods used.

RESULTS

Brain phospholipid levels. By about 3 weeks of age, the concentrations of all phospholipids examined in the brains of U18666A-treated rats were decreased by about 30–40% as compared with the concentrations in brains of age-matched controls (Table 1). The brain weights of control and treated rats were 1.54 ± 0.02 g and 1.47 ± 0.02 g respectively. The concentrations of phospholipids in brains of treated rats were still decreased by 20–30% at 6

Table 1. Effect of treatment with U18666A on whole brain levels of individual phospholipids*

	Phospholipid (mg/g brain, wet wt)			
	18–22 Days of age		42 Days of age	
Phospholipid†	Control (N = 10)	Treated (N = 10)	Control (N = 4)	Treated (N = 4)
PC	14.92 ± 0.54	10.87 ± 0.67‡	15.32 ± 0.39	12.13 ± 0.22‡
EPG	12.96 ± 0.46	9.55 ± 0.58‡	15.56 ± 0.38	12.60 ± 0.40‡
PS	4.72 ± 0.19	2.73 ± 0.28‡	6.35 ± 0.10	4.53 ± 0.30‡
PI	1.49 ± 0.07	0.90 ± 0.09‡	2.38 ± 0.09	1.54 ± 0.13‡
DPG	0.82 ± 0.06	0.54 ± 0.08‡	1.16 ± 0.03	0.93 ± 0.08‡
SPH	1.66 ± 0.10	1.02 ± 0.09‡	2.43 ± 0.34	1.99 ± 0.11

* Rats were injected (s.c.) with 10 mg/kg of U18666A beginning at one day after birth and injected every fourth day thereafter. Age-matched controls received no injections. Values are the mean ± S.E.M. for the indicated number (N) of rats.

† PC = phosphatidylcholine, EPG = ethanolamine phosphoglycerides, PS = phosphatidylserine, PI = phosphatidylinositol, DPG = diphosphatidylglycerol, and SPH = sphingomyelin. These phospholipids together accounted for about 97% of the total lipid phosphorus in rat whole brain.

‡ P of difference from age-matched control < 0.05 (Student's *t*-test).

Table 2. Incorporation of intracerebrally injected [1,3-^{3}H]glycerol into phospholipids by brain of control and U18666A-treated rats*

	Tritium incorporated (dpm × 10^{-3}/g brain)			
	22 Days of age		42 Days of age	
Fraction	Control	Treated	Control	Treated
PC	340 ± 11	178 ± 14†	227 ± 8	152 ± 14†
EPG	143 ± 2	81 ± 3†	102 ± 2	63 ± 8†
PS	19 ± 2	12 ± 1†	17 ± 2	8 ± 1†
PI	83 ± 8	51 ± 1†	82 ± 6	49 ± 5†
PA	15 ± 1	9 ± 0†	12 ± 3	8 ± 1
DPG	14 ± 1	9 ± 1†	5 ± 0	3 ± 0†
DG	35 ± 0	21 ± 1†	25 ± 2	16 ± 1†
TG	55 ± 2	34 ± 1†	45 ± 2	27 ± 2†

* Control rats and treated rats (24 hr after last dose of U18666A; 10 mg/kg, s.c., every fourth day beginning at one day of age) were lightly anesthetized with ether and injected intracerebrally with 20 μCi of [1,3-^{3}H]glycerol (10 μCi/μmole) in 20 μl of saline. Animals were killed 1 hr after injection; total brain lipid was isolated and fractionated as described in the text. Values are the mean ± S.E.M. of four rats in each group. Abbreviations are as described in the footnotes of Table 1; in addition, PA = phosphatidic acid, DG = diacylglycerol and TG = triacylglycerol.

† $P < 0.05$ from control value.

weeks of age; however, the decrease in the sphingomyelin level was not statistically significant. Brain weights were 1.65 ± 0.06 g (control) and 1.76 ± 0.06 g (treated) at this age. No significant differences were seen in the relative composition of phospholipids from brains of control and treated animals at either age. In earlier work we observed similar decreases in all phospholipids, except phosphatidylinositol (PI), in subcellular fractions from brains of U18666A-treated rats [3]. Phospholipid concentrations were expressed relative to brain protein levels in this earlier study [3]. We also observed before that treatment with U18666A had no effect on brain levels of total sterols but markedly increased the concentration of polysialogangliosides [3].

Effects of treatment with U18666A on incorporation in vivo of [1,3-^{3}H]glycerol and $^{32}P_i$ into cerebral lipids. When [1,3-^{3}H]glycerol was injected intracerebrally into 3- and 6-week-old treated rats, incorporation of tritium into all glycerolipids of brain was decreased by 30–40% relative to controls at 1 hr following injection (Table 2).

Following intracerebral injection of $^{32}P_i$ into 3-week-old rats, radiolabel was rapidly incorporated into PI such that 1 hr following injection PI contained more than 60% of the total ^{32}P in phospholipids of brain (Fig. 1). Incorporation of ^{32}P at this time would appear to reflect turnover of the phosphorus moiety of phospholipids rather than *de novo* synthesis. The preferential labeling of PI is consistent with the well established rapid turnover of cellular PI [25]. Incorporation into PI plateaued at about 4 hr after injection while incorporation into the quantitatively major phospholipids of brain (PC, EPG and PS, Table 1) continued to increase at an essentially linear rate for at least 18 hr (Fig. 1). Appearance of ^{32}P in brain phospholipid at these later times likely reflects entry of phosphorus into new phospholipid molecules by *de novo* synthesis, that is via glycerol-3-phosphate or dihydroxyacetone phosphate.

The ^{32}P-labeling of brain phospholipids was examined in control and treated rats at both 1 and 4 hr after intracerebral injection of $^{32}P_i$. At 1 hr after injection of $^{32}P_i$, no significant differences were seen

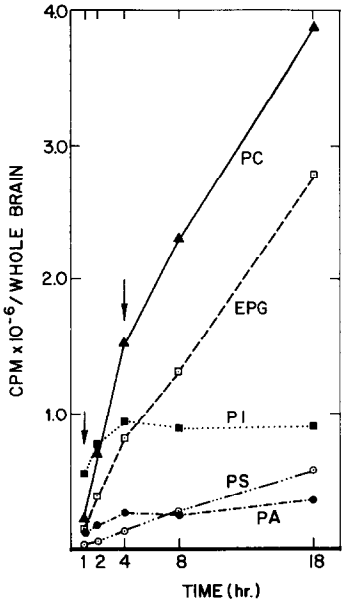

Fig. 1. Time course of incorporation of $^{32}P_i$ into phospholipids of whole brain. Twenty-one-day-old rats were intracerebrally injected with 50 μCi of $^{32}P_i$ (carrier free) in 20 μl of isotonic saline. Animals were killed at various times, and the whole brain was homogenized in chloroform–methanol (2 : 1, v/v). The extracted phospholipids were separated by two-dimensional TLC; the individual lipids were recovered and assayed for $^{32}P_i$ content. The values at each time point are the average incorporation into the brain of two rats. Arrows indicate the incorporation patterns at 1 and 4 hr after injection. Abbreviations are the same as in the legends to Tables 1 and 2.

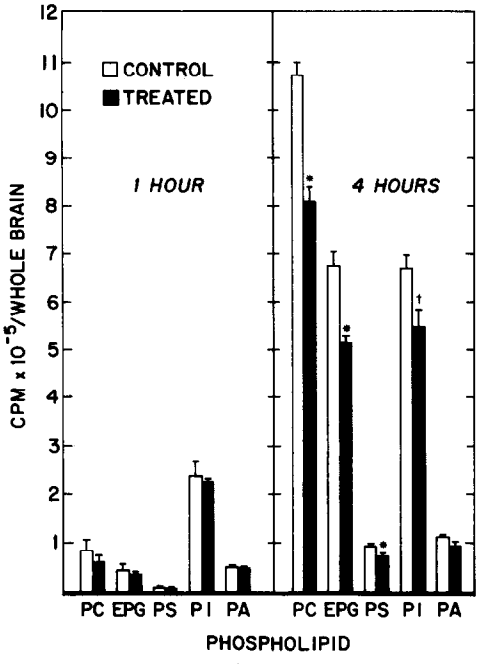

Fig. 2. Effect of treatment with U18666A on incorporation of $^{32}P_i$ into phospholipids of whole brain. Eighteen- to 22-day-old control rats and U18666A-treated rats were intracerebrally injected with 20 μCi of $^{32}P_i$ (carrier free) in 20 μl of isotonic saline. Animals were killed at 1 and 4 hr after injection. Brain lipids were extracted and fractionated by TLC. Each value is the mean ± S.E.M. (bars) of the incorporation into brain phospholipids of three control and three treated rats at both times. Key: (*) P of difference from control < 0.05 (Student's *t*-test). (†) P of difference from control ≃ 0.05.

between control and treated rats in the amount of radiolabel incorporated into any of the phospholipids examined (Fig. 2). Thus, treatment with U18666A would appear to have little effect on phospholipid turnover. However, at 4 hr after injection, incorporation into PC, EPG and PS was decreased significantly. This observation appears to reflect inhibition of *de novo* synthesis of phospholipid as does the inhibition of incorporation of [^{3}H]glycerol measured at 1 hr after intracerebral injection.

Effects of U18666A on incorporation in vitro *of [1,3-^{3}H]glycerol into glycerolipids by brain.* When U18666A was added to slices of rat brain at a concentration of 10^{-4} M, incorporation of [1,3-^{3}H]glycerol into triacylglycerols was decreased by about 40% while incorporation into phosphatidic acid, phosphatidylinositol and diacylglycerol increased from about 50 to 100% (Fig. 3). Incorporation into other glycerolipids was unchanged. In the presence of 0.5×10^{-3} M U18666A, the appearance of radiolabel in the zwitterion phospholipids (PC, EPG and PS) and into triacylglycerol was decreased greatly, while incorporation into the acidic phospholipids (PA, PI and DPG) and diacylglycerol was unchanged from control. In the presence of the highest concentration studied, 10^{-3} M, U18666A almost totally blocked incorporation of [^{3}H]glycerol into all glycerolipids.

Effect in vitro *of U18666A on the activity of enzymes of glycerolipid synthesis.* U18666A is a potent inhibitior of several key enzymes in glycerolipid synthesis. At a concentration of 0.6 to 0.7 mM, it produced 50% inhibition of the microsomal acyltransferase enzymes which convert glycerol-3-phos-

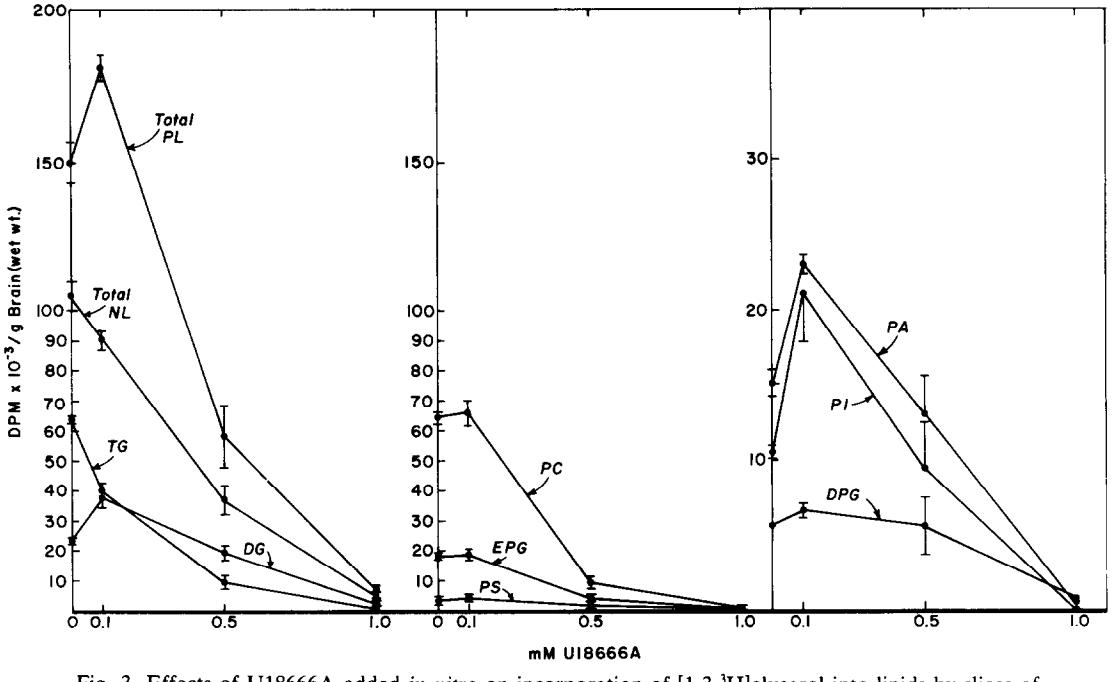

Fig. 3. Effects of U18666A added *in vitro* on incorporation of [1,3-^{3}H]glycerol into lipids by slices of rat brain. Slices of whole brain from untreated rats were incubated for 30 min in Krebs phosphate buffer (pH 7.4) containing 20 μCi of [1,3-^{3}H]glycerol (80 μCi/μmole) and various concentrations of U18666A. Total lipids were extracted by the method of Hajra *et al.* [13]. Neutral lipids were separated by single-dimensional TLC on plates coated with silica gel G, and phospholipids were separated by two-dimensional TLC on plates coated with silica gel H. Values are the mean ± S.E.M. (bars) of three incubations at each concentration. Abbreviations are the same as in the legends to Tables 1 and 2; in addition, NL = neutral lipid and PL = phospholipid.

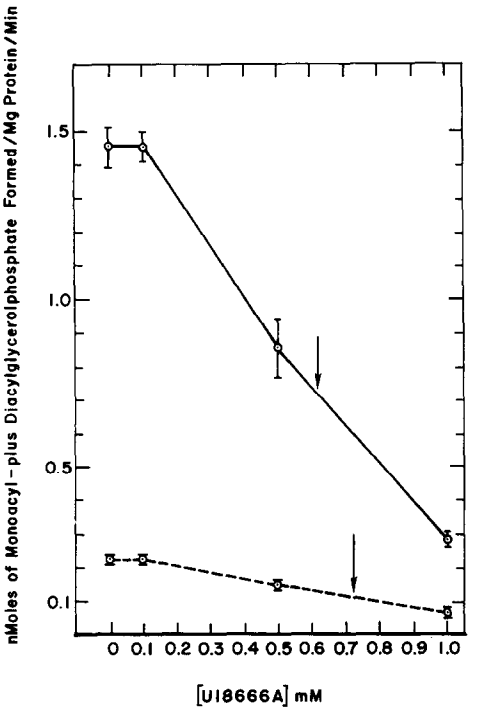

Fig. 4. Effects of U18666A on the activities of glycerol-3-phosphate acyltransferases of liver (——) and brain (----). Microsomes from rat liver and brain were incubated for 10 min in the presence of 0.6 mM [U-^{14}C]glycerol-3-phosphate (0.5 μCi/μmole) and 90 μM palmitoyl CoA. See the text for details of the assay. Enzyme activity is expressed as the nmoles of monoacylglycerolphosphate plus diacylglycerolphosphate formed per mg of microsomal protein per min. Each value is the mean ± S.E.M. (bars) of three separate incubations. Arrows indicate the approximate concentration of U18666A which produced 50% inhibition.

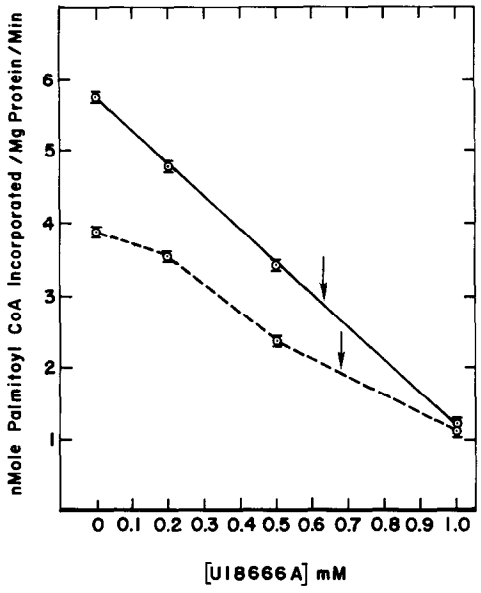

Fig. 5. Effects of U18666A on the activities of diacylglycerol acyltransferase of liver (——) and brain (----). Microsomes from rat liver and brain were incubated for 10 and 5 min, respectively, in the presence of 0.08 mM [1-^{14}C]palmitoyl CoA (1.25 μCi/μmole) and 1.2 mM 1,2-dioleoylglycerol. See the text for details of the assay. Enzyme activity is expressed as the nmoles of palmitoyl CoA incorporated into triacylglycerol per mg of microsomal protein per min. Each value is the mean ± S.E.M. (bars) of three separate incubations. Arrows indicate the approximate concentration of U18666A which produced 50% inhibition.

phate to phosphatidic acid (Fig. 4) and which convert diacylglycerol to triacylglycerol (Fig. 5). The acyltransferase enzymes of the brain microsomes were considerably less active than those of liver; however, the enzymes from both tissues were similarly sensitive to inhibition by U18666A. Of the studied enzymes, the soluble form of phosphatidate phosphohydrolase was most sensitive to inhibition by U18666A. A 50% decrease in the activity of both the brain and liver enzyme was recorded at about 0.4 mM (Fig. 6). The microsomal form of this enzyme from brain and liver was 50% inhibited at drug concentrations of between 0.7 and 1.0 mM (Fig. 6). In contrast to the other enzymes examined, U18666A poorly inhibited phosphatidate cytidylyltransferase. Fifty percent inhibition of this microsomal enzyme from brain and liver was not seen until the concentration of the drug reached about 2 mM (Fig. 7). The activity of both the phosphatidate phosphohydrolase enzymes and of phosphatidate cytidylyltransferase was considerably greater in the brain preparations than in those from liver (Figs. 6 and 7).

DISCUSSION

When U18666A was administered at the same dose used in the present study, a chronic seizure state was induced in the rat [1, 2]. The epileptiform condition was accompanied by major and sustained decreases in the concentration of brain phospholipids [3]. This decrease in phospholipids was probably not due to a nonspecific toxic effect of the drug, since animals treated with U18666A well tolerated the drug as judged by weight gain and general appearance. Also, at the dose used in the present study, U18666A had little effect on brain wet weights or on the concentration of brain total sterols [3]. Rather, the results of the current study indicate that the decrease in brain phospholipids by this agent is likely due to inhibition of phospholipid synthesis.

Compounds similar to U18666A, i.e. other amphiphilic cationic drugs, have been shown to be potent inhibitors of phospholipid synthesis perhaps through inhibition of phosphatidate phosphohydrolase, an important enzyme in glycerolipid synthesis [4–6]. Drugs of this type are thought to inhibit phosphatidate phosphohydrolase by decreasing the availability of phosphatidic acid to the enzyme as a result of interaction between the negatively charged phosphatidate and the positively charged drug [26]. Pappu and Hauser [6] suggested that the ability of propranolol and similar amphipathic cationic drugs to

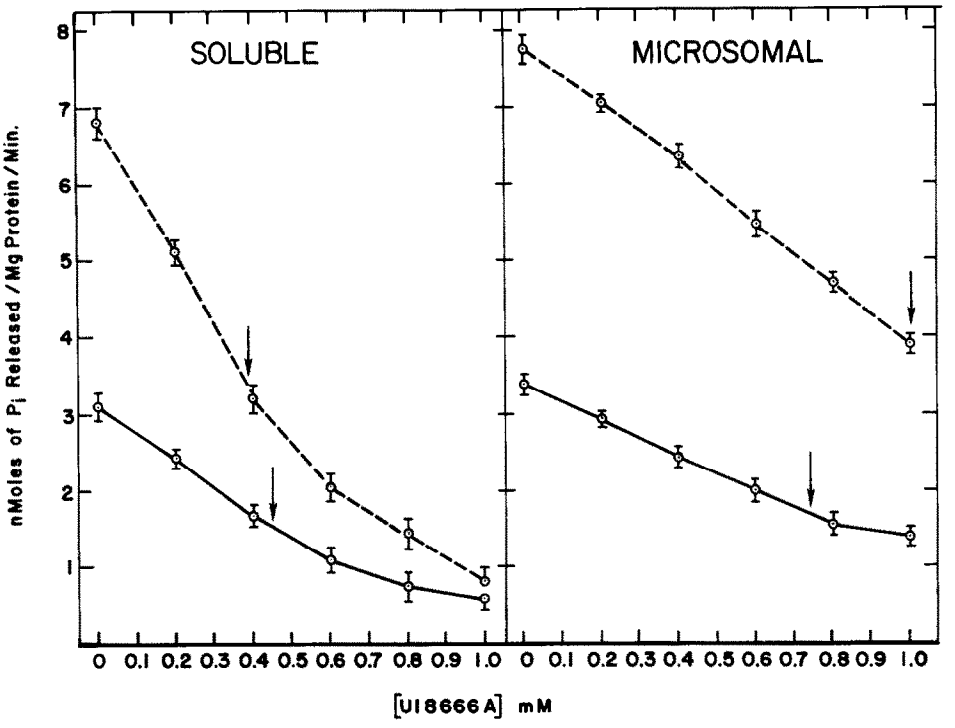

Fig. 6. Effect of U18666A on phosphatidate phosphohydrolase activities of liver (——) and brain (– – – –). Microsomes and soluble supernatant fraction from homogenates of rat liver and brain were incubated for 30 min with an aqueous dispersion of phosphatidic acid. See the text for details of the assay. Enzyme activities are expressed as the nmoles of inorganic phosphorus (P_i) liberated from phosphatidic acid per mg of protein per min. Each value is the mean ± S.E.M. (bars) of four incubations. Arrows indicate the approximate concentration of U18666A which produced 50% inhibition.

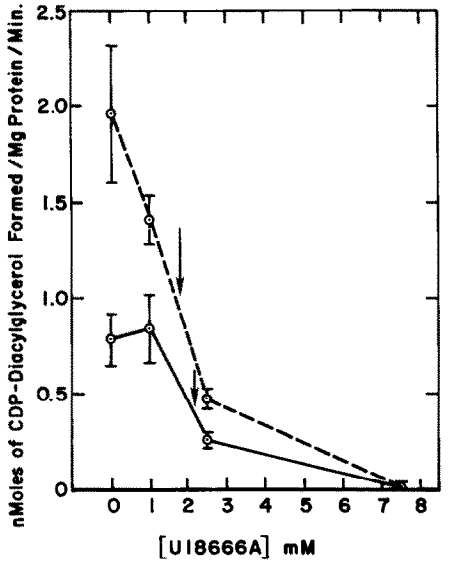

Fig. 7. Effect of U18666A on the activities of phosphatidate cytidylyltransferase of liver (——) and brain (– – – –). Microsomes from rat liver and brain were incubated for 10 min in the presence of 1.0 mM [^{3}H]CTP (2 μCi/μmole) and 2.0 mM phosphatidic acid. See the text for details of the assay. Enzyme activity is expressed as the nmoles of CDP-diacylglycerol formed per mg of microsomal protein per min. Each value is the mean ± S.E.M. (bars) of three incubations. Arrows indicate the approximate concentration of U18666A which produced 50% inhibition.

inhibit incorporation *in vitro* of [^{3}H]glycerol and $^{32}P_i$ into rat cerebral cortex was due to inhibition of phosphatidate phosphohydrolase.

The observed 20–40% decreases in the concentration of all major phospholipids in whole brain following treatment with U18666A correlated well with the extent of decreased labeling of brain phospholipids following intracerebral injection of either [1,3-^{3}H]glycerol or $^{32}P_i$ into U18666A-treated rats. The decreased formation of phospholipids could be explained by the ability of U18666A to inhibit several key enzymes in glycerolipid synthesis; these include various acyltransferases and phosphatidate phosphohydrolase. Inhibition of the acylation of glycerol-3-phosphate to phosphatidic acid could account for the observed decreased formation and concentration of all glycerolipids in brains of treated animals.

There was variable sensitivity of enzymes in glycerolipid synthesis to inhibition by U18666A. In particular, microsomal phosphatidate cytidylyltransferase was resistant to inhibition relative to phosphatidate phosphohydrolase and the acyltransferase enzymes. The dose-dependent pattern of effects of U18666A on the incorporation of [^{3}H]glycerol into glycerolipids by brain slices further illustrates the variable sensitivity of these enzymes to inhibition. The results suggest that, in brain slices, diacylglycerol acyltransferase was especially sensitive to inhibition. The increased labeling of the acidic phospholipids (PA and PI) and diacylglycerol by brain slices in the

presence of the lowest level of U18666A examined (10^{-4} M) might reflect a redirection of glycerolipid synthesis due to an accumulation of diacylglycerol. However, our observations that the formation and concentration of the zwitterion and acidic phospholipids were comparably decreased in whole brain of U18666A-treated rats suggests that inhibition of acylation of glycerolphosphate to phosphatidate could be the primary action of U18666A *in vivo*. Simultaneous inhibition by U18666A of phosphatidate phosphohydrolase and phosphatidate cytidylyltransferase to similar extents might also explain the comparable decreases in the brain levels of PC, EPG and PI. However, the results of the enzyme studies indicate that phosphatidate cytidylyltransferase is comparatively resistant to inhibition by U18666A. Differences between the patterns of inhibition by U18666A of glycerolipid synthesis observed *in vivo* and *in vitro* could reflect differences in the accessibility of the drug to the potentially inhibited enzymes or to the substrates for these enzymes. Recall that Brindley *et al.* [26] state that enzyme inhibition could result from interaction of the cationic drug with anionic substrates.

The concentration of U18666A required *in vitro* to inhibit the activity of the acyltransferase enzymes and soluble phosphatidate phosphohydrolase by 50% was in the range of $4–6 \times 10^{-4}$ M. A maximum concentration of about 10^{-5} M U18666A is achieved in whole brain of rats when the drug was given as in the present study [27]. However, since this drug localizes almost totally in brain lipids [27], which occupy about 10% of the total volume of brain, it seems possible that the local concentrations of U18666A in brain (i.e. in cerebral membranes) could reach about 1×10^{-4} M, a concentration approaching the range of that required to inhibit the *in vitro* activity of both phosphatidate phosphohydrolase and the acyltransferase enzymes.

The ability of U18666A to produce 50% inhibition of glycerolphosphate acyltransferase, phosphatidate phosphohydrolase and diacylglycerol acyltransferase all at drug concentrations of under 1 mM indicates that it is a potent inhibitor of glycerolipid synthesis. While many amphiphilic cationic drugs readily inhibit various phosphatidate phosphohydrolase activities at concentrations similar to U18666A [4, 5], and even at lower drug levels [28], none also readily inhibit acyltransferase reactions [4, 5]. U18666A could thus be a very unusual inhibitor of glycerolipid synthesis.

We observed earlier [3] that the decreased levels of brain phospholipids caused by treatment of the rat with U18666A also resulted in pronounced changes in the molar ratio of sterols to phospholipids [3], since total sterol levels in brain were not changed. For example, the ratio of sterol to phospholipid in myelin from treated rats was 40–60% higher than in myelin from age-matched controls [3]. The fluidity of myelin lipids appeared to be greatly decreased (preliminary electron spin resonance measurements, unpublished observations). We suggest that major changes in the phospholipids of neural membranes, caused by inhibition of phospholipid synthesis, could alter both the structure and function of these membranes and, therefore, these changes could be the basis of the epileptiform activity induced by U18666A.

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