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Acetylcholine Stimulates Selective Liberation and Re-esterification of Arachidonate and Accumulation of Inositol Phosphates and Glycerophosphoinositol in C62B Glioma Cells*

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Glioma C62B cells, incubated for 18 h with either an unsaturated (arachidonate or oleate) or saturated (palmitate or stearate) radioactive fatty acid, incorporated label into most species of cellular glycerolipids. Treatment of prelabeled C62B cells with 1 mM acetylcholine (ACh) resulted in an accumulation of radioactive phosphatidate irrespective of which fatty acid was used as a label. However, only in cells prelabeled with unsaturated fatty acids were increases in radioactive fatty acids observed. When exogenous radioactive arachidonate was added to C62B cells in the presence of 1 mM ACh, there was a rapid, selective, and transiently enhanced incorporation of label (several times the control) into phosphatidylinositol (PI). The ACh-enhanced incorporation into PI was not preceded by enhanced incorporation of label into *sn*-1,2-diacylglycerol or phosphatidate but was followed by an increased labeling of polyphosphoinositides. Similarly, incorporation of oleate into PI was enhanced by ACh. In contrast, ACh did not enhance the incorporation of label into any glycerolipids when saturated fatty acids were used. C62B cells, incubated with [2-^{3}H]inositol for 18 h selectively incorporated label into phosphoinositides. Stimulation of [2-^{3}H]inositol-labeled cells with 1 mM ACh in the presence of 25 mM LiCl resulted in a rapid accumulation of radioactive inositol phosphates (mono-, bis-, and trisphosphates) and glycerophosphoinositol. The accumulation of inositol trisphosphates preceded that of inositol monophosphate and glycerophosphoinositol, while the accumulation of glycerophosphoinositol paralleled the time required for the ACh-stimulated esterification of arachidonate. These results suggest that ACh stimulates activation of a phospholipase C in C62B cells and release of 1,4,5-inositol trisphosphate. There is subsequent activation of phospholipase A_2, which in turn liberates arachidonate from PI. The resulting lyso PI is either rapidly reesterified with unsaturated fatty acid to resynthesize PI, or further deacylated to yield glycerophosphoinositol.

A variety of stimuli initiate the cleavage of arachidonate from glycerolipids; two primary mechanisms of liberation have been proposed. One pathway involves the activation of a calcium-regulated phospholipase A_2 that acts on membrane phospholipids to yield fatty acids and lysophospholipids (1–5). An alternative pathway involves the action of phospholipase C on phosphoinositides to yield inositol phosphates and *sn*-1,2-diacylglycerol. The *sn*-1,2-diacylglycerol may then be metabolized sequentially by di- and monoacyl glycerol lipases to yield free fatty acids and glycerol (6–8).

Stimulated liberation of arachidonate has also been shown to occur in neural systems (9–17). This is often thought of primarily in connection with neurons, although glial cells contribute substantially to the composition of the central nervous system (possibly outnumbering neurons 10 to 1; 18). Their potential contribution to neurotransmitter-stimulated liberation of arachidonate has only recently been noted. Neurotransmitter-stimulated activation of phospholipase C has been demonstrated to occur in cultured glial cells (13, 19) and cells of glial origin (12, 20). Moreover, it has been shown that certain neurotransmitters stimulate the liberation of arachidonate from membrane phospholipids of glioma cells (10, 12) and cultured astroglia (13). (The term "liberation" is used here to indicate cleavage of the covalent bond so as to avoid confusion with "release" which often suggests extracellular accumulation).

In the present work we utilize cells of glial origin to examine in parallel the metabolic pathways responsible for acetylcholine- (ACh)[1] stimulated cleavage of arachidonate from membrane phospholipids and the metabolism of inositol phospholipids. Our data suggest that although cholinergic stimulation of phosphodiesteratic degradation of phosphoinositides precedes that of arachidonate liberation the two enzymes involved do not act sequentially on the same precursor substrate. Furthermore, there is an ACh-stimulated reacylation of liberated acyl groups. Thus, the accumulation of free arachidonate is a transient event, compatible with responses characteristic of neural tissues.

EXPERIMENTAL PROCEDURES

Materials

Basal medium Eagle's (BME) and fetal calf serum were purchased from Gibco. [1-^{14}C]Arachidonate (58 mCi/mmol), [1-^{14}C]stearate (60

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[1] The abbreviations used are: ACh, acetylcholine; BME, basal medium Eagle's; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; PI, phosphatidylinositol and corresponding 1-alkenyl and 1-alkyl species; PE, phosphatidylethanolamine and corresponding 1-alkenyl and 1-alkyl species; PC, phosphatidylcholine and corresponding 1-alkenyl and 1-alkyl species; PS, phosphatidylserine and corresponding 1-alkenyl and 1-alkyl species.

mCi/mmol), [9,10-^{3}H]palmitate (590 mCi/mmol), [1-^{14}C]oleate (60 mCi/mmol), *myo*-[2-^{3}H]inositol (14.2 Ci/mmol), [2-^{3}H]inositol 1,4,5-trisphosphate, and [U-^{14}C]inositol phosphatidylinositol were purchased from Amersham Corp. [*Methyl*-^{3}H]choline (80.0 Ci/mmol) and [1-^{3}H]ethanolamine (8.7 Ci/mmol) were purchased from Du Pont-New England Nuclear and Amersham Corp., respectively.

Acetylcholine, phospholipid standards, and phospholipase A_2 (from *naja naja* venom) were obtained from Sigma. LK6D thin-layer chromatography (TLC) plates were purchased from Whatman. All other materials were reagent grade.

Methods

Cell Culture Conditions—The culture medium for C62B cells consisted of BME supplemented with 5% (vol/vol) fetal calf serum, 1 mM glucose, 2 mM L-glutamine, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. Cells were grown in 15 ml of this medium in 75-cm^2 Corning culture flasks in a humidified environment of 5% CO_2 and 95% air at 37 °C. The medium was changed every 3 days.

Prelabeling of Cells with Radioactive Fatty Acids, and myo-[2-^{3}H] Inositol, [methyl-^{3}H]Choline, and [1-^{3}H]Ethanolamine—Confluent cell cultures were harvested and seeded into sterile glass scintillation vials as previously described (12). The cultures were incubated for 24 h prior to addition of radioactive fatty acids or inositol. The culture medium was removed by aspiration, and the cells were labeled by incubation for 18 h in 0.75 ml of fresh culture medium containing either radioactive arachidonate, oleate, stearate (0.25 μCi/vial), or palmitate (2.0 μCi/vial). This protocol resulted in 90.5 ± 2.6, 82.2 ± 1.1, 74.3 ± 6.3, and 41.3 ± 1.7 (%X ± S.E., n = 6), respectively, of the radioactivity incorporated in the cellular lipid pool (not removed by rinsing the cultures). In instances where metabolism of phosphoinositides, phosphatidylcholine, or phosphatidylethanolamine were to be examined, cultures were incubated for 18 h with culture medium containing [2-^{3}H]*myo*-inositol (25 μCi/vial), [*methyl*-^{3}H]choline (3 μCi/vial), or [1-^{3}H]ethanolamine (3 μCi/vial), respectively.

Assay for Accumulation of Fatty Acids, Inositol Phosphates, and Glycerophosphoinositol—Following labeling of cultures, the medium containing unincorporated precursor was removed. The cultures were then rinsed three times with BME, the medium replaced with fresh BME, pH 7.2, containing 30 mM Hepes, and the culture vials placed in a 37 °C water bath. In the inositol-labeling experiments, the final wash and incubation was with Hanks' balanced salt solution buffered at pH 7.2 with 15 mM Hepes. Lithium chloride (25 mM) was added to inhibit inositol phosphate phosphatase.

Solutions of ACh and eserine were made as previously reported (12). The cultures were incubated at 37 °C for the times indicated. The reactions were stopped and the extracts prepared by addition of 2.8 ml of either chloroform/methanol (1:2), chloroform/methanol/hydrochloric acid (v/v/v, 100:200:2), or butanol (21) as noted in the text.

Assay for Incorporation (Esterification) of Fatty Acids—Cell cultures were prepared as above with the omission of radioactive fatty acid in the culture medium. Following replacement of the culture medium with buffered BME, assay for incorporation of radioactive fatty acid was initiated by addition of 4–5 nmol of radioactive fatty acid per culture in the presence or absence of ACh. The incorporation of fatty acid into glycerolipids was terminated as above.

Lipid Analysis—Cell extracts were transferred to centrifuge tubes and 0.9 ml of chloroform and 0.9 ml of water were added. Following agitation on a Vortex shaker, the phases were separated by centrifugation and the organic phase removed and evaporated to dryness under a stream of nitrogen. The resulting sample residues were dissolved in 50 μl of chloroform/methanol (1:1) and portions applied to TLC plates.

Triglycerides (R_F = 0.85), diglycerides (R_F = 0.73), and fatty acids (R_F = 0.55) were separated from phospholipids (origin) on LK6D TLC plates using a solvent consisting of the upper phase obtained after phase separation of a mixture of ethyl acetate/isooctane/water (v/v/v, 93:47:120). Use of this system in combination with nonacidic extraction (see above) prevented acid hydrolysis of plasmalogens. Phosphatidate was separated from other lipids in a solvent consisting of the upper phase of a mixture of ethyl acetate/isooctane/acetic acid/water (v/v/v/v, 93:47:20:100) as previously reported (12).

Separation of phospholipids was performed on Silica Gel LK6D TLC plates developed in chloroform/ethanol/water/triethylamine (v/v/v/v, 30:34:8:35) (22). In this system the major diacyl-phospholipids (and the corresponding comigrating 1-alkenyl and 1-alkyl species) had R_F values as follows: phosphatidylcholine (PC), 0.12; phosphatidylserine (PS), 0.17; phosphatidylinositol (PI), 0.24; phosphatidate, 0.30; phosphatidylethanolamine (PE), 0.36. Polyphosphoinositides were resolved using acid extracts of cell lipids and TLC on oxylate-coated LK6D TLC plates in a system of chloroform/methanol; 4 N ammonium hydroxide (v/v/v, 45:35:10). Plasmalogen composition of glycerophospholipids was determined by two-dimensional TLC (23). The protocol involves separation of phospholipids in the first dimension with chloroform/methanol/ammonium hydroxide (v/v/v, 65:25:4), hydrolysis of the acid labile vinyl ether bond of plasmalogen by exposure to concentrated hydrochloric acid vapor, and separation of lysophospholipids generated from phospholipids in the second dimension in chloroform/methanol/acetone/acetic acid/water (v/v/v/v/v, 75:15:30:15:7.5). In experiments where glycerolipids were to be examined without hydrolysis of plasmalogens, either an aqueous butanol or neutral chloroform/methanol extraction was used (21). [*methyl*-^{3}H]Choline-labeled lipids and [1-^{3}H]ethanolamine-labeled lipids were resolved on LK6D TLC plates in a system of chloroform/methanol/water (v/v/v, 65:35:6) or chloroform/methanol/water (v/v/v, 65:25:4), respectively. Relative mass distribution of phospholipids was determined by spraying TLC plates with 50% sulfuric acid in ethanol and heating at 180 °C to visualize lipids by charring. Quantitation was performed using soft laser densitometry (Zenith SL-504-XL), and data are presented as a percentage of the total phospholipid (this was determined to be 22 ± 2.7 μg of phospholipid/culture (mean ± S.E., n = 4)).

The locations of labeled species separated by TLC were visualized by autoradiography using Kodak XAR film. In the case of tritium-labeled samples, TLC plates were sprayed with EN^{3}HANCE (Du Pont) prior to autoradiography. Glycerolipid identity was determined by comparison with standards visualized by exposure to iodine. Quantification of radioactivity was determined by scraping regions of the TLC plates into scintillation vials followed by liquid scintillation spectrometry.

Hydrolysis of Phospholipids by Phospholipase A_2—Phospholipase A_2 hydrolysis of phospholipids was as previously described (12). Hydrolysis of phospholipids was quantitated by isolation of esterified radioactive fatty acid by TLC and determination of radioactivity as noted above.

Separation of Inositol Phosphates and of Glycerophosphoinositol—The aqueous phase of cell extracts prepared from inositol-labeled cells were dried under nitrogen to 1 ml then diluted to 20 ml with water. The samples were then either subjected to Dowex AG1-X8 anion exchange chromatography (4) or HPLC analysis using a modification of the method of Irvine and co-workers (24). The modification entailed the use of a microprocessor-controlled prepump gradient mixer with a 3-step linear gradient of water and 1 M ammonium formate buffered to pH 3.7 with phosphoric acid (100% water, 0–20 min; 100% water to 45% ammonium formate, 20–40 min; and 45% ammonium formate to 85% ammonium formate, 40–70 min). The samples were chromatographed at a flow rate of 1 ml/min, and 70 fractions (1 ml each) were collected. The radioactivity present in the eluent was quantitated by liquid scintillation spectrometry. Inositol phosphates were identified by coelution with standards. Glycerophosphoinositol was prepared by methylamine hydrolysis of [^{3}H]inositol-labeled cell lipid extracts (25). Cyclic inositol monophosphate was prepared by cyclization of [U-^{14}C]inositol monophosphate (26).

RESULTS

Distribution of Label From Incorporated Fatty Acids

Distribution among Glycerolipid Species—C62B glioma cells were incubated for 18 h in the presence of the various fatty acids, and the incorporation of radioactivity into glycerophospholipids was determined (Table I). This distribution did not correspond to the glycerophospholipid relative mass composition of the cells. Label from [1-^{14}C]arachidonate or from [1-^{14}C]stearate was 3- to 4-fold enriched in PI relative to the percentage PI composition of the cells. In contrast, oleate and palmitate preferentially labeled PC.

Plasmalogen Composition of C62B Cells—The incorporation

TABLE I

Distribution of radioactive free fatty acids incorporated into glycerolipids

Each value is the mean of six determinations.

	Phospholipid composition[a]	Arachidonate	Oleate	Stearate	Palmitate
			cpm × 10^{-2}/culture + S.E.		
PC	56.5 ± 4.4	1180 ± 40 (36.5)[b]	2463 ± 26 (74.6)	1126 ± 49 (48.4)	2814 ± 39 (72.6)
PE	38 ± 4.0	989 ± 18 (30.6)	368 ± 27 (11.1)	372 ± 30 (16.0)	248 ± 29 (6.4)
PI	3.5 ± 1.9	598 ± 26 (18.4)	100 ± 3.8 (3.0)	373 ± 32 (16.0)	193 ± 7.8 (5.0)
PS	5.2 ± 1.6	155 ± 6.1 (4.8)	128 ± 13 (3.9)	273 ± 31 (11.7)	110 ± 3.1 (2.8)
PA[c]	<1.0	32 ± 1.3 (1.0)	28 ± 1.7 (0.7)	30 ± 1.1 (1.3)	28 ± 1.9 (0.7)
Neutral lipids		279 ± 27 (8.6)	222 ± 25 (6.7)	151 ± 16 (6.5)	484 ± 7.5 (12.5)

[a] Values are the mean of four determinations and are presented as the percentage of total phospholipid.
[b] Numbers in parenthesis represent the percentage of radioactivity incorporated into the individual glycerolipids.
[c] PA, phosphatidate.

of labeled fatty acid into plasmalogens was determined in a separate series of experiments. Following 18 h of incubation with radioactive arachidonate, much of the label which comigrated with PC and PE was present as plasmalogen (19 ± 3% and 71 ± 2%, $n = 4$, respectively). Radioactivity in lipids isolated from stearate-labeled cells comigrating with PC and PE also included plasmalogens (5 ± 0.3% and 26 ± 3%, $n = 4$, respectively). This was determined by weak acid hydrolysis of lipid classes separated by TLC after extraction of phospholipids by nonacidic extraction procedures (weak acid causes cleavage of the vinyl ether bond of plasmalogens to release the aldehyde). Radioactivity of arachidonate present in plasmalogens was primarily in the lysophospholipid generated by such treatment, whereas radioactivity incorporated from stearate was released from the phospholipid. Interestingly, when cells were labeled with radioactive arachidonate or stearate, a significant amount of radioactivity comigrating with PI was present as plasmalogen (18 ± 6% and 6 ± 2%, $n = 4$, respectively).

The presence of inositol plasmalogen was further substantiated using butanol-extracted [2-^{3}H]inositol-labeled cells, indicating that 14 ± 2% ($n = 5$) of the [^{3}H]inositol-labeled PI was present as plasmalogen. The extreme lability of plasmalogens upon exposure to acid dictates that caution be used in interpreting data concerning the lysophospholipid content of cells where acidic lipid extraction procedures or acidic solvent systems for separation of phospholipid classes have been used. Such treatment could substantially elevate "basal" levels of lysophospholipids and mask potential agonist-induced changes in cellular lysophospholipid content (see lyso PI determination below).

Distribution of Label with Regard to Positional Specificity—The distribution of radioactive fatty acids esterified at the *sn*-2 position of the total glycerolipids was determined by their susceptibility to release by phospholipase A_2. This treatment substantially decreased the radioactivity of arachidonate present in glycerolipids leaving only 13 ± 3% ($n = 3$) incorporated (of the remaining covalently linked radioactive arachidonate, 41% was present as neutral lipids and not susceptible to phospholipase A_2-mediated hydrolysis). In contrast, 75 ± 4% ($n = 3$) of the radioactivity of stearate remained incorporated after phospholipase A_2 treatment, suggestive of a primary localization in the *sn*-1 position. The radioactivity incorporated from oleate and palmitate remaining as glycerolipid following phospholipase A_2 treatment (28 ± 4% and 53 ± 8%, respectively, $n = 3$) was intermediate to that for arachidonate and stearate.

TABLE II

Acetylcholine-stimulated accumulation of radioactive free fatty acids and radioactive phosphatidates

Free fatty acid and phosphatidate accumulations were determined at 90 s and 2 min post ACh treatment, respectively. Each value represents the mean of two experiments performed in triplicate. Asterisks (*) indicate statistically significant differences ($p < 0.01$) from untreated control.

ACh (1 mM)		Fatty acids	Phosphatidate
	1 mM	*cpm culture ± S.E.*	
Arachidonate	−	1178 ± 119	929 ± 88
	+	3746 ± 278*	3161 ± 186*
Oleate	−	1048 ± 78	1124 ± 38
	+	2487 ± 212*	2882 ± 139*
Palmitate	−	4199 ± 208	1310 ± 106
	+	4466 ± 309	2940 ± 201*
Stearate	−	3442 ± 246	1021 ± 77
	+	4219 ± 431	2662 ± 387*

Fatty Acid Liberation from Prelabeled Cells

Acetylcholine-stimulated Liberation of Fatty Acid from Prelabeled Cells—When C62B cells were prelabeled with any of the fatty acids examined, treatment with 1 mM ACh increased the accumulation of radioactive phosphatidate (Table II) (a concentration previously shown to give maximal response, Ref. 12). Arachidonyl-labeled phosphatidate showed the greatest (3.4-fold) elevation following a 2-min stimulation with ACh (time of maximal stimulated accumulation). Stimulated accumulation of phosphatidate labeled with the other fatty acids ranged from 2.2 to 2.6 times control values. The accumulation of radioactive phosphatidate, irrespective of the fatty acid used, suggest that the phospholipase C activity stimulated by ACh hydrolyzes phosphoinositides without regard for fatty acid composition.

When the ACh-stimulated accumulation of radioactive fatty acids was examined, only in the case of the unsaturated fatty acids, arachidonate and oleate, were there statistically significant and substantial increases (3.2- and 2.3-fold of the respective control levels, Table II).

Esterification of Fatty Acids into Glycerolipids

Acetylcholine-stimulated Esterification of Arachidonate into Glycerolipids—We had previously demonstrated that, even in

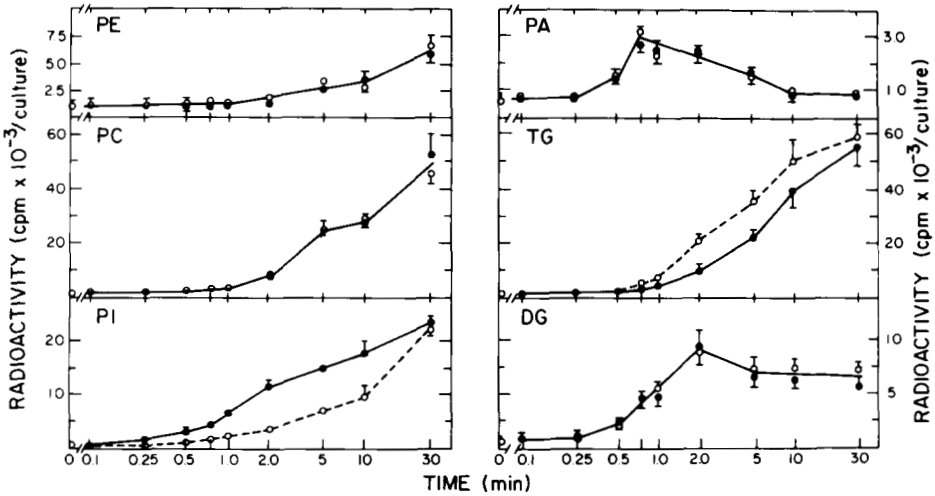

FIG. 1. **Time course of [1-^{14}C]arachidonate incorporation into glycerolipids.** Cultures of C62B cells were incubated for the times indicated with exogenous [1-^{14}C]arachidonate in the presence (●) or absence (○) of 1 mM ACh. Each data point is the mean ± S.E. of 3–6 experiments performed in duplicate. Additional abbreviations used in this figure are: *DG*, diacylglycerol; *PA*, phosphatidate; *TG*, triacylglycerol.

the presence of continuous cholinergic stimulation, the elevated levels of unesterified radioactive arachidonate returned to control levels within 5 min (12). Our observations suggested the hypothesis that the rapid return to basal levels resulted from an ACh-stimulated re-esterification of the arachidonate (or metabolites of arachidonate) into cellular glycerolipids.

The above hypothesis was tested by examining the effect of ACh on the esterification of exogenous [1-^{14}C]arachidonate into glycerolipids (Fig. 1). In the absence of ACh, following a short delay period, the added [1-^{14}C]arachidonate was rapidly incorporated into neutral lipids and phospholipids. Phosphatidate and *sn*-1,2-diacylglycerol labeled most rapidly, with transient accumulations which peaked by 2 min. After initial delays of 1–2 min PC, PE, PI, and triacylglycerol showed increased incorporation of radioactivity that continued for over 30 min. The largest incorporation of [1-^{14}C]arachidonate during the 30-min labeling period was shown by PC and triacylglycerol, accounting for over 74% of the total radioactivity incorporated. Little label was incorporated into PS during this time (data not shown).

When ACh (1 mM) was added simultaneously with the [1-^{14}C]arachidonate, the total incorporation of radioactivity into phospholipids was increased by 48 ± 6% at 2 min (sum of data from different panels, Fig. 1). This was largely accounted for by a 3-fold increased incorporation into phosphoinositides (Fig. 1). ACh had little effect on the incorporation of arachidonate into PC, PE, *sn*-1,2-diacylglycerol, or phosphatidate. Incorporation into triacylglycerol was decreased by half at 2 min (Fig. 1). This may reflect increased competition for acyl-CoA, diversion to PI synthesis rather than triacylglycerol synthesis. The ACh-enhanced esterification of exogenous arachidonate into PI closely parallels the time course for liberation and re-esterification of endogenous [1-^{14}C]arachidonate previously reported (12).

Because the enhanced incorporation of arachidonate into phospholipids was selective for PI, we examined the incorporation of exogenous radioactive arachidonate into polyphosphoinositides (Fig. 2). The data indicate that the ACh-enhanced incorporation of [1-^{14}C]arachidonate into polyphosphoinositides followed the appearance of label in PI. This temporal sequence is compatible with sequential phosphorylation of the radioactive arachidonyl-PI to form phosphatidylinositol mono- and bisphosphates.

Acetylcholine-enhanced Esterification of Other Fatty Acids into Glycerolipids—In addition to the time course studies with arachidonate (Fig. 1), the effects of ACh on the esterification of oleate, stearate, and palmitate were examined at a single

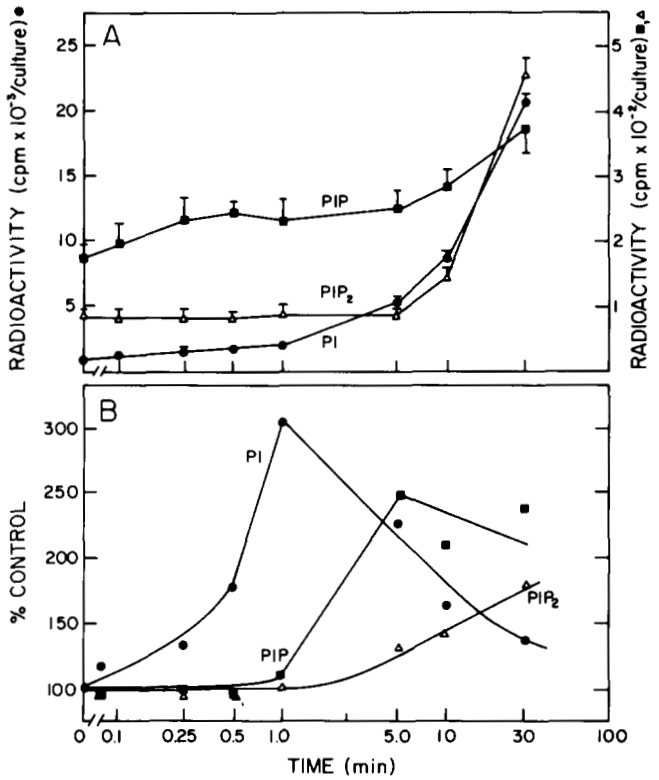

FIG. 2. **Time course of acetylcholine-stimulated incorporation of [1-^{14}C]arachidonate into phosphoinositides.** Cultures of C62B cells were treated as in Fig. 1 and phosphoinositides separated by TLC. Incorporation of radioactivity into PI (●), phosphatidylinositol-monophosphate (PIP; ■), and phosphatidylinositol-bisphosphate (PIP_2; △) was determined. *Panel A*, incorporation of [1-^{14}C] arachidonate under control conditions. Each value is the mean ± S.E. cpm/culture from two experiments performed in triplicate ($n = 6$). *Panel B*, the data for ACh-stimulated (1 mM ACh) cultures are plotted as a percentage of label incorporated into control cultures incubated for the equivalent times in the absence of ACh. Each value is the average of two experiments, each performed in triplicate. The S.E. for the stimulated cultures were less than 15% of the corresponding means and similar to those of the control cultures.

time point (10 min). The only statistically significant difference ($p < 0.01$, $n = 6$) was the enhanced incorporation of radioactive oleate into a single phospholipid, PI. ACh did not increase the incorporation of the saturated fatty acids (stearate or palmitate) into any glycerolipids. These results suggest that there is a selective effect of ACh on the incorporation of unsaturated fatty acids, which are preferentially esterified at

the *sn*-2 position of phospholipids. Moreover, the increased esterification of unsaturated fatty acids is specific for PI.

Phosphoinositide Metabolism Stimulated by Acetylcholine

ACh-stimulated Increase in Lyso-PI—The selective enhanced incorporation of exogenous unsaturated fatty acids into PI and the lack of ACh-enhanced esterification of exogenous fatty acids into 1,2-diacylglycerol or phosphatidic acid suggested to us that the increased incorporation occurred via the esterification of a lyso-PI. Therefore, we attempted to determine if lyso-PI was formed upon stimulation of cells with ACh. C62B cells, prelabeled for 18 h with [2-^{3}H]inositol, were stimulated for 90 s with ACh or the calcium ionophore A23187 and the lipid extracts analyzed (Table III). We detected small but significant ($p < 0.01$) accumulations of radioactive lyso-PI for both ACh and A23187 treatment (30 and 70% above control, respectively). The accumulation in response to ACh was transient; after a 5-min stimulation with ACh, lyso-PI returned to basal levels. In contrast, after treatment with A23187 for 5 min radioactive lyso-PI levels remained elevated. The ACh-stimulated increase in lyso-PI was not statistically significant when acidic extraction procedures were used presumably because of the elevated basal lyso-PI levels resulting from plasmalogen degradation (see data in plasmalogen content section). The effects of ACh and ionophore on the accumulation of lyso-PE and lyso-PC were also examined (Table III). No statistically significant increases were observed with 90-s exposure to either agent, however, A23187 did increase accumulation of lyso-PE after 5 min.

Determination of ACh-stimulated Accumulation of Inositol Phosphates and Glycerophosphoinositol—ACh stimulated a rapid (within 5 s) and transient (peaking within 1–5 min) accumulation of inositol trisphosphates (Fig. 3). The early accumulation of inositol trisphosphates was followed by slower accumulations of inositol bisphosphate and glycerophosphoinositol. The accumulation of inositol monophosphate occurred more slowly and was accompanied by a return to basal levels of inositol bis- and trisphosphates. The accumulation of glycerophosphoinositol remained elevated and paralleled in time the ACh-enhanced esterification of arachidonate (*cf.* Fig. 1).

Analysis by HPLC of the water-soluble components from ACh-treated cultures allowed us to examine independently the accumulation of inositol 1,4,5- and 1,3,4-trisphosphates (Fig. 4) and indicated that ACh caused a rapid accumulation of inositol 1,4,5-trisphosphate (this compound is known to mobilize intracellular calcium, Ref. 27).

Treatment of C62B cells with ionophore A23187 likewise resulted in increased glycerophosphoinositol accumulation (Fig. 5) but unlike ACh, failed to elevate inositol phosphates. This uncoupling of the accumulation of these products by the

TABLE III

Acetylcholine- and ionophore A23187-stimulated accumulation of radioactive lysophospholipids

Cultures preincubated with radioactive inositol, ethanolamine, or choline "Methods" were treated with acetylcholine or A23187 for the times indicated. The lipids were extracted, the lipid classes separated, and the radioactivity present in the lysophospholipids determined. Each value is the mean from duplicate determinations from 6–9 cultures. Asterisk (*) indicates statistically significant difference ($p < 0.01$) from equivalent time control. Total radioactivity (cpm ± S.E.) at the 90-s time point present as PI + lyso-PI, PE + lyso-PE, and PC + lyso-PC was 247,377 ± 12,067, 366,262 ± 15,921, and 471,592 ± 17,077 and did not vary significantly for the time or treatment variables noted.

Treatment	Lyso-PI	Lyso-PE	Lyso-PC
	cpm/culture + S.E.		
Control 90 s	8,620 ± 372	19,589 ± 1,213	5,462 ± 345
ACh (1 mM) 90 s	11,470* ± 555	20,248 ± 860	5,889 ± 517
A23187 (5 μM) 90 s	14,614* ± 1,051	21,429 ± 1,025	6,664 ± 681
Control 5 min	9,360 ± 383	18,977 ± 904	6,508 ± 623
ACh (1 mM) 5 min	9,531 ± 483	17,751 ± 1,012	5,993 ± 637
A23187 (5 μM) 5 min	13,571* ± 785	23,978* ± 717	7,310 ± 684

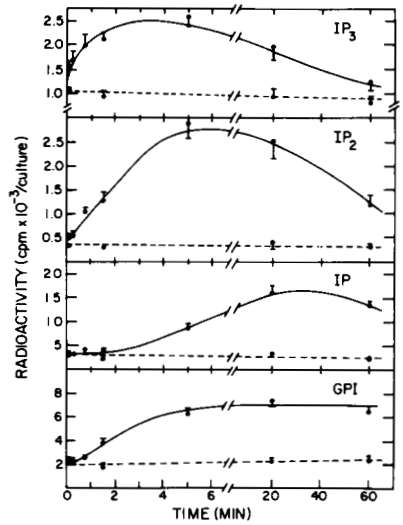

FIG. 3. **Time course of Ach-stimulated accumulation of inositol phosphates and glycerophosphoinositol.** Cultures of C62B cells labeled with [2-^{3}H]inositol were incubated in the presence (*solid line*) or absence (*broken line*) of 1 mM ACh for the times indicated. Inositol trisphosphate (IP_3), inositol bisphosphate (IP_2), inositol monophosphate (*IP*), and glycerophosphoinositol (*GPI*) present in the aqueous fraction of cell extracts were separated by Dowex anion exchange chromatography "Methods". The data are presented as radioactivity/culture present in the relevant fraction. Each value is the mean ± S.E. ($n = 6$, two experiments in triplicate).

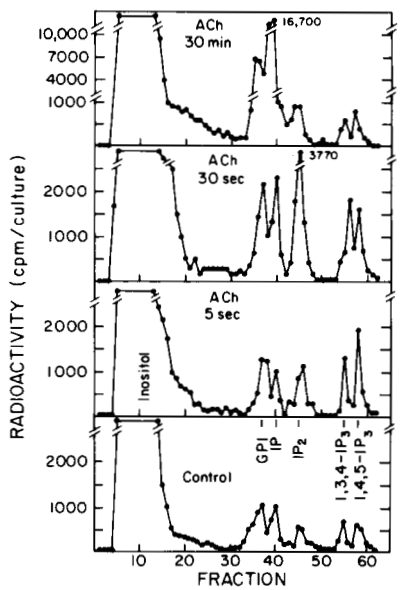

FIG. 4. **HPLC analysis of inositol phosphates accumulating after ACh stimulation (1 mM ACh) of C62B cells.** Aqueous phase of C62B cell extracts prepared as in Fig. 3 were subjected to HPLC separation "Methods". Data from a representative experiment are shown for control (no ACh), 5-s ACh stimulation, 30-s ACh stimulation, and 30-min ACh stimulation. In addition to the abbreviations used in Fig. 3: 1,4,5-IP_3, inositol 1,4,5-trisphosphate, 1,3,4-IP_3, inositol 1,3,4-trisphosphate.

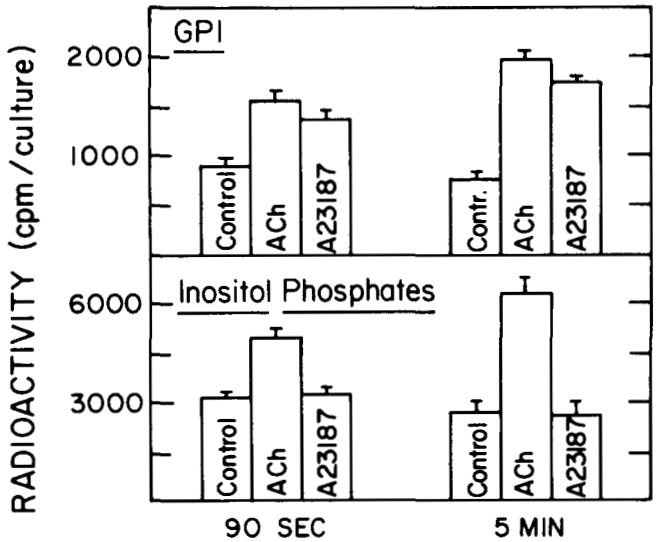

FIG. 5. **Effect of ACh, and of the ionophore A23187, on the accumulation of glycerophosphoinositol and inositol phosphates.** Cultures of C62B cells prelabeled with [2-^{3}H]inositol were treated with 1 mM ACh or 5 μM A23187 for the times indicated. Glycerophosphoinositol was separated from pooled inositol phosphates (includes inositol mono-, bis-, and trisphosphates). Each value (presented as radioactivity/culture) is the mean ± S.E. of two experiments performed in triplicate.

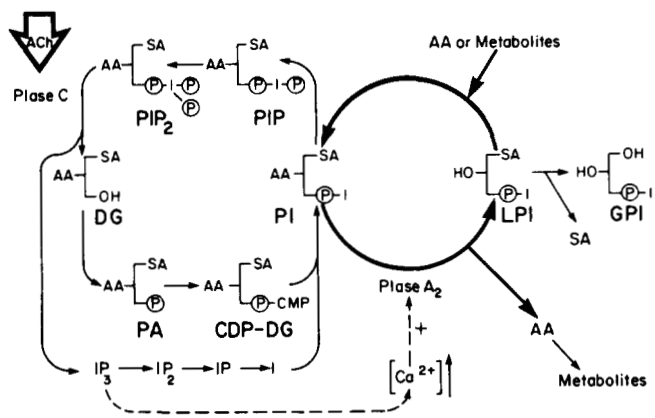

FIG. 6. **Schematic representation of ACh-stimulated phosphoinositide metabolism.** *Bold arrows* indicate major metabolic pathways. ACh receptor occupation activates phospholipase C with production of Ca^{2+} mobilizing inositol 1,4,5-trisphosphate. Elevation of cellular Ca^{2+} activates phospholipase A_2 which selectively liberates unsaturated fatty acids from PI generating LPI. LPI is either further deacylated to glycerophosphoinositol or subsequently reacylated with unsaturated fatty acids (arachidonate or metabolites of arachidonate) to yield PI. The PI may then be successively phosphorylated to yield the polyphosphoinositides or may be reutilized in the release of arachidonate. Abbreviations used in addition to those in previous figures are: *AA*, arachidonate; *SA*, stearate; *CDP-DG*, CDP-diacylglycerol; *LPI*, lysophosphatidylinositol; *Plase A$_2$*, phospholipase A_2; *Plase C*, phospholipase C; *DG*, diacylglycerol; *I*, inositol.

ionophore indicates that glycerophosphoinositol production is independent of phospholipase C activation and supports the involvement of calcium-regulated phospholipase A_2 activity.

DISCUSSION

Pathway for Arachidonate Release and Reacylation—ACh stimulates the liberation of arachidonate and the subsequent reesterification of arachidonate (or metabolites of arachidonate) in C62B cells. We suggest this involves the pathway depicted in Fig. 6. In this scheme ACh occupation of a muscarinic receptor on C62B cells results in activation of phospholipase C with subsequent hydrolysis of phosphoinositides and production of inositol phosphates (including the calcium mobilizing inositol 1,4,5-trisphosphate) and 1,2-diacylglycerol. The 1,2-diacylglycerol formed is rapidly phosphorylated to form phosphatidate and utilized in the resynthesis of PI. The accumulation of inositol 1,4,5-trisphosphate (27), the degradation of polyphosphoinositides (28), or the formation of other products of phosphoinositide metabolism (29, 30) in turn may elevate intracellular calcium, possibly resulting in the activation of the calcium regulated phospholipase A_2. Phospholipase A_2 then selectively liberates unsaturated fatty acids present in the *sn*-2 position of PI to yield primarily arachidonate and lyso-PI. The lyso-PI formed is either rapidly reacylated with unsaturated fatty acids (or metabolites of unsaturated fatty acids) or is further deacylated to yield glycerophosphoinositol. Because we detect little accumulation of stearate under our experimental conditions, it is suggested that the re-esterification pathway predominates in C62B cells (there may be some stimulation of stearate release, but it is low compared to arachidonate, Table II).

The failure to observe ACh-enhanced esterification of arachidonate into *sn*-1,2-diacylglycerol or phosphatidate, coupled with the selectivity for esterification into PI, suggests that the increased incorporation of radioactive arachidonate into PI is not the result of *de novo* phospholipid synthesis, but instead occurs via a stimulation of a deacylation-reacylation pathway. The selectivity for unsaturated fatty acid release and esterification further support our hypothesis that the enhanced incorporation is not due to phospholipase C-dependent catabolism of PI and subsequent release of both the *sn*-1 and *sn*-2 fatty acids from diacylglycerol. Were this later pathway involved we would expect saturated and unsaturated fatty acids to demonstrate nearly equivalent release and esterification.

The model presented emphasizes that the accumulation of liberated arachidonate within the cells is dependent upon the relative rates of deacylation and reacylation (31), both of which appear stimulated in C62B cells. This scheme also emphasizes that in cells of glial origin (as has been shown in some non-neural systems (32)), the liberation of arachidonate by phospholipase A_2 and phosphodiesteratic degradation of phosphoinositides can share a common precursor pool, PI. In other neural systems where agonist-stimulated phosphoinositide metabolism has been studied, it has been reported that tetraenoic species of phosphoinositides are conserved (33–35) and that elevation of lyso-PI is not detected (34, 36). Although these observations were interpreted differently by the authors, the data are not inconsistent with a transient liberation of arachidonate from PI via a phospholipase A_2 with subsequent reacylation of the lyso-PI formed. In C62B cells, and possibly in other neural systems, the accumulation of glycerophosphoinositol is stable under conditions where lyso-PI may be rapidly further metabolized (reacylated or further deacylated). Glycerophosphoinositol may thus provide a more sensitive and readily monitored indicator of fatty acid liberation from phosphoinositides than does lyso-PI.

Possible Significance of the Transient Liberation of Arachidonate—The release and accumulation of arachidonate in response to physiologically significant stimuli has been well characterized in many systems (14, 37–40). The transient nature of the accumulation of arachidonate following exposure to ACh noted in the present study (it is rapidly reversed by re-esterification) might be of particular relevance with regard to the short term responses characterized by neural systems. It has been shown that arachidonate (41, 42) and

arachidonate metabolites (43) can activate protein kinase C (Ca^{2+}/phospholipid-dependent enzyme) independent of phospholipid requirements. It is also noteworthy that arachidonate (44) and metabolites of arachidonate (45) elevate intracellular calcium, in some instances this effect may be additive with the elevation caused by inositol 1,4,5-trisphosphate (44). Thus, free arachidonate may contribute to the regulation of calcium-dependent and phosphorylation-dependent events within the cell. Finally, as ACh stimulates a transient accumulation of a lipoxygenase metabolite (12), the lipoxygenase metabolite may also be rapidly esterified (46), and may serve to modulate membrane function as has been shown with alteration of phospholipid fatty acid composition (47, 48).

Our supposition that these findings obtained with a glial cell line are of physiological relevance (glial-neuronal interactions *in vivo*) remains to be tested. Observations with astroglia in culture indicate that they, like C62B cells, respond to putative neurotransmitter receptor agonists with arachidonate liberation (13) and phosphoinositide metabolism (13, 19). Our results are compatible with a growing body of literature, which suggests that glial cells may contribute substantially to neurotransmitter-stimulated phospholipid metabolism in central nervous system tissues.

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