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 [2-3H]inositol for 18 h selectively incorporated label into phosphoinositides. Stimulation of [2-3H]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 A2, 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 that acts on membrane phospholipids to yield fatty acids and lysophospholipids (1–3). 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)-stimulated cleavage of arachidonate from membrane phospholipids and the metabolism of inositol phospholipids. Our data suggest that although cholinergic stimulation of phosphodiesterase 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. [1-14C]Arachidonate (58 mCi/mmol), [1-14C]stearate (60

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mCi/mmol; [3H]palmitate (560 mCi/mmol), [1-14C]oleate (60 mCi/mmol), myo-[2-3H]inositol (14.2 Ci/mmol), [2-3H]inositol 1,4,5-trisphosphate, and [U-3H]inositol phosphatidylinositol were purchased from DuPont New England Nuclear and Amersham Corp., respectively. Acetylcholine, phospholipid standards, and phospholipase A2 (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 L-glutamine, 250 units/ml of penicillin, and 250 mg/ml of streptomycin. Cells were grown in 15 ml of this medium in 75-cm² Corning culture flasks in a humidified environment of 5% CO₂ and 95% air at 37 °C. The medium was changed every 3 days.

Prelabeling of Cells with Radioactive Fatty Acids, and myo-[2-3H] Inositol, [methyl-3H]Choline, and [1-13C]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 changed every 3 days. The cells were labeled by incubation for 18 h in 0.75 ml of fresh culture medium containing 125 or 5 µCi/ml of radioactive fatty 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 fresh BME containing [2-3H]myo-inositol (25 µCi/vial), [methyl-3H]choline (3 µCi/vial), or [1-13C]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 2.5 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 pmol 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 agitaion 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 (RF = 0.85), diglycerides (RF = 0.75), and fatty acids (RF = 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/isoctane/water (v/v/v, 93:47:120). Use of this system in combination with nonacidic extraction (see above) prevented acid hydrolysis of plasmalogens. Phospholipids were extracted from other lipids in a solvent consisting of the upper phase of a mixture of ethyl acetate/isoctane/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 conjugating 1-alkenyl and 1-alkyl species) had RF values as follows: phosphatidylcholine (PC), 0.12; phosphatidylethanolamine (PE), 0.17; phosphatidylserine (PS), 0.24; phosphatidate, 0.30; phosphatidylethanolamine (PE), 0.36. Polyphosphoinositides were resolved using acid extracts of cell lipids and TLC on oxalate-coated LK6D TLC plates in a system of chloroform/methanol, 4 N HCl, water, and isoctane (90:10:10). Phosphatidylcholine 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 plasmalogens by exposure to concentrated hydrochloric acid vapor, and separation of lysophospholipids generated from phospholipids in the second dimension with chloroform/methanol/acetic acid/water (v/v/v, 78:13:5:7:5). 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-3H]Choline-labeled lipids and [1-13C]ethanolamine-labeled lipids were resolved on LK6D TLC plates in a system of chloroform/methanol/water (v/v/v, 65:35:5) 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 water. The samples were then either subjected to Dowex AG1-X8 microprocessor-controlled pre-pump gradient mixer with a 3-step linear gradient of water and 1 mM 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.

Hydrolysis of Phospholipids by Phospholipase A2—Phospholipase A2 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 pre-pump gradient mixer with a 3-step linear gradient of water and 1 mM 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 methylation of HCl hydrolysis of [1-3H]inositol-labeled cell lipid extracts (25). Cyclic inositol monophosphate was prepared by cyclization of [U-3H]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 [1-14C]arachidonate or from [1-14C]oleate was 3-4 fold enriched in PI relative to the percentage PI composition of the cells. In contrast, oleate and palmitate preferentially labeled PC.

Plasmalogens Composition of C62B Cells—The incorporation
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 co-migrated 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 plasmalogens (18 ± 6% and 6 ± 2%, n = 4, respectively).

The presence of inositol plasmalogens was further substantiated using butanol-extracted [2-3H]inositol-labeled cells, indicating that 14 ± 2% (n = 5) of the [3H]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 A₂. 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 A₂-mediated hydrolysis). In contrast, 75 ± 4% (n = 3) of the radioactivity of stearate remained incorporated after phospholipase A₂ treatment, suggestive of a primary localization in the sn-1 position. The radioactivity incorporated from olate and palmitate remaining as glycerolipid following phospholipase A₂ treatment (28 ± 4% and 53 ± 8%, respectively, n = 3) was intermediate to that for arachidonate and stearate.

Fatty Acid Liberation from Prelabelled Cells—Acetylcholine-stimulated Liberation of Fatty Acid from Prelabelled Cells—When C62B cells were prelabelled 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 olate, were there statistically significant and substantial increases (3.2- and 2.3-fold of the respective control levels, Table II).

TABLE II
Acetylcholine-stimulated accumulation of radioactive free fatty acids and radioactive phosphatidate

| Fatty Acids | Phosphatidate |
|------------|--------------|
| ACh (1 mM) |              |
|            | 1 mM         | Phosphatidate |  | cm culture ± S.E. |
| Arachidonate | - | 1178 ± 119 | 929 ± 88 |
| + | 3746 ± 278* | 3161 ± 186* |
| Olate | - | 1048 ± 78 | 1124 ± 38 |
| + | 2487 ± 212* | 2882 ± 139* |
| Palmitate | - | 4199 ± 308 | 1310 ± 106 |
| + | 4466 ± 309 | 2940 ± 201* |
| Stearate | - | 3442 ± 246 | 1021 ± 77 |
| + | 4219 ± 431 | 2682 ± 387* |

respectively, n = 3) was intermediate to that for arachidonate and stearate.

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

| Phospholipid composition* | Arachidonate | Olate | Stearate | Palmitate |
|--------------------------|--------------|-------|----------|-----------|
| PC | 56.5 ± 4.4 | 1180 ± 40 (36.5)* | 75.0 ± 5.0 | 70.0 ± 6.0 |
| PE | 38 ± 4.0 | 949 ± 18 (30.0) | 35.0 ± 2.0 | 15.0 ± 2.0 |
| PI | 3.5 ± 1.9 | 598 ± 26 (18.4) | 2.0 ± 0.2 | 1.0 ± 0.2 |
| PS | 5.2 ± 1.6 | 155 ± 6.1 (4.8) | 1.0 ± 0.2 | 0.5 ± 0.2 |
| PA' | <1.0 | 32 ± 1.3 (1.0) | 0.5 ± 0.2 | 0.2 ± 0.2 |
| Neutral lipids | 279 ± 27 (8.3) | 222 ± 25 (6.7) | 151 ± 16 (6.5) | 484 ± 7.5 (12.5) |

* Values are the mean of four determinations and are presented as the percentage of total phospholipid.

Numbers in parenthesis represent the percentage of radioactivity incorporated into the individual glycerolipids.

PA, phosphatidate.
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 [1-14C]arachidonate into glycerolipids (Fig. 1). In the absence of ACh, following a short delay period, the added [1-14C]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 [1-14C]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 μM) was added simultaneously with the [1-
14C]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 [1-14C]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 polypehosphoinositides (Fig. 2). The data indicate that the ACh-enhanced incorporation of [1-14C]arachidonate into polypehosphoinositides 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 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
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**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 [2-3H]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 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 stimulates 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-stimulated cultures treated 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 lipids determined. Each value is the mean ± S.E. 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 significantly vary for the time or treatment variables noted.

| Treatment       | Lyso-PI          | Lyso-PE          | Lyso-PC          |
|-----------------|------------------|------------------|------------------|
| Control 90 s    | 8,620 ± 372      | 19,688 ± 1,213   | 5,462 ± 345      |
| ACh (1 mM) 90 s | 11,470 ± 556     | 20,249 ± 866     | 5,888 ± 517      |
| ACh (5 µM) 90 s | 14,614* ± 1,051  | 21,429 ± 1,025   | 6,564 ± 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)   | 13,571* ± 785    | 23,878* ± 717    | 7,310 ± 684      |

*Fig. 3. Time course of ACh-stimulated accumulation of inositol phosphates and glycerophosphoinositol. Cultures of C62B cells labeled with [2-3H]inositol were incubated in the presence (solid line) or absence (broken line) of 1 mM ACh for the times indicated. Inositol trisphosphate (IP3), inositol bisphosphate (IP2), and inositol monophosphate (IP1) 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-IP3, inositol 1,4,5-trisphosphate; 1,3,4-IP2, inositol 1,3,4-trisphosphate.

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
ionophore indicates that glycerophosphoinositol production is independent of phospholipase C activation and supports the involvement of calcium-regulated phospholipase A₂ 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 A₂. Phospholipase A₂ 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).

FIG. 5. Effect of ACh, and of the ionophore A23187, on the accumulation of glycerophosphoinositol and inositol phosphates. Cultures of C62B cells prelabeled with [2-³H]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.

![Diagram](attachment:image.png)

**FIG. 6. Schematic representation of ACh-stimulated phosphoinositide metabolism. Bold arrows indicate major metabolic pathways. ACh receptor occupation activates phospholipase C with production of Ca²⁺ mobilizing inositol 1,4,5-trisphosphate. Elevation of cellular Ca²⁺ activates phospholipase A₂ 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; Phospholipase A₂, phospholipase A₃; Phospholipase C; DC, diacylglycerol; I, inositol.
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arachidonate metabolites (43) can activate protein kinase C (Ca<sup>2+</sup>/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 arachidonate metabolites (43) can activate protein kinase C (44) and metabolites of arachidonate (45) elevate intracellular Ca<sup>2+</sup> (46, 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 astrocytes in culture indicate that they, like C62B12 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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