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Cholinergic Stimulation of Arachidonic Acid and Phosphatidic Acid Metabolism in C62B Glioma Cells*

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Glioma C62B cells were incubated for 18 h with [1-^{14}C]arachidonic acid. Most (80%) of the added [1-^{14}C] arachidonic acid was taken into the intracellular pool; less than 1% of the intracellular [1-^{14}C]arachidonic acid remained unesterified; the rest was present in glycerophospholipids. Acetylcholine stimulation of the prelabeled cells resulted in the rapid accumulation of free [1-^{14}C]arachidonic acid, presumably liberated by hydrolysis from phospholipids. Labeled unesterified [1-^{14}C]arachidonic acid peaked by 90 s and returned to basal levels by 5 min. Paralleling the transient increase of unesterified [1-^{14}C]arachidonic acid were increases in level of radioactivity in an unidentified lipoxygenase metabolite of arachidonic acid and of radioactive phosphatidic acid. The release of arachidonic acid induced by acetylcholine or carbachol was blocked by muscarinic but not nicotinic receptor antagonists; adrenergic or histaminergic receptor agonists were ineffective at stimulating arachidonic acid liberation. In contrast to the transient effects of stimulation with cholinergic agonists, stimulation with the divalent cation ionophore A23187 resulted in a linear increase in the accumulation of liberated arachidonic acid for at least 1 h. Furthermore, the pattern of metabolites synthesized from arachidonic acid in response to ionophore stimulation was more complex than that observed following cholinergic stimulation and included also several metabolites derived from cyclooxygenase activity. We conclude that muscarinic receptor agonists rapidly induce specific changes in arachidonic acid and phosphatidic acid metabolism in a glioma cell line and suggest that similar responses may occur in glial cells and play a physiologically significant role in neural metabolism.

The importance of arachidonic acid metabolites as intermediates in the transmission of signals has been established in a number of cellular systems. Although definitive evidence concerning the role of arachidonic acid metabolites is less for neural systems than it is for other tissues, it appears that prostaglandins PGE_2,[1] PGD_2, and $PGF_{2\alpha}$ may act to modulate neurotransmission both in the central (Bergstrom *et al.*, 1973; Hillier and Templeton, 1980; Hayaishi, 1983) and peripheral (Hedqvist, 1973; Brody and Kadowitz, 1974; Hemker and Aiken, 1980) nervous system. Furthermore, in several neural preparations a variety of stimuli, including application of neurotransmitter receptor agonists (Lunt and Rowe, 1971; Mallorga *et al.*, 1980; Snider *et al.*, 1984), have been observed to cause the liberation of arachidonic acid from esterified pools and result in the formation of arachidonic acid metabolites (for review see Wolfe, 1982). In mixed cell preparations of neural tissue, it is difficult to relate the presence of a particular eicosanoid to a specific cell class. However, the physiological relevance of arachidonic acid metabolism by neurons is suggested by the observations that purified synaptosomal preparations have been shown to respond to depolarization with altered arachidonic acid metabolism (Bradford *et al.*, 1983). Similar approaches have not yet been attempted using homogenous preparations of cells of glial origin.

A number of recent studies indicate that glial cells may exhibit neurotransmitter receptors. The demonstration that cultures of both primary glial cells (McCarthy and de Vellis, 1978; van Calker *et al.*, 1983; Rougon *et al.*, 1983) and glial cell lines (Clark and Perkins, 1971, Bottenstein and de Vellis, 1978) respond to neurotransmitter stimulation with alterations in cyclic nucleotide metabolism suggests the possibility that, *in vivo*, glial cells may respond to neurotransmitters released by neurons. The present study was conducted to test the hypothesis that, in addition to changes in cyclic nucleotide levels, cells of glial origin respond to neurotransmitters with alterations in arachidonic acid metabolism. We have demonstrated that cholinergic stimulation of a rat glioma cell line, C62B, induces the liberation of arachidonic acid and the accumulation of phosphatidic acid and a lipoxygenase metabolite of arachidonic acid.

EXPERIMENTAL PROCEDURES

Materials

Basal medium Eagle's (BME) and fetal calf serum were purchased from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY). [1-^{14}C]arachidonic acid (58 mCi/mmol) was purchased from Amersham Corp. Prostaglandin standards and phospholipase A_2 (from *naja naja* venom), esculetin, nordihydroguaiaretic acid, and indomethacin were obtained from Sigma. LK6DF and LK5D thin-layer chromatography (TLC) plates were purchased from Whatman (Clifton, NJ). Standard for 5-HETE was generously provided by Dr. H. White (The Wellcome Research Laboratories, Research Triangle Park, NC). Standards for HHT and 12-HETE were prepared from platelets as previously reported (Lapetina and Cuatrecasas, 1979). All other materials were reagent grade.

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[1] The abbreviations used are: PGE_2, prostaglandin E_2; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGD_2, prostaglandin D_2; BME, basal medium Eagle's; 5-HETE, 5-hydroxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; HHT, 12-hydroxy-5,8,10-hepadecatrienoic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TLC, thin-layer chromatography.

Methods

Cell Culture Conditions—The culture medium for C62B cells consisted of BME supplemented with 5% (v/v) fetal calf serum, 1 mM glucose, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were grown in 20 ml of this culture medium in 75 cm^2 Corning culture flasks in a humidified environment of 5% CO_2, 95% air at 37 °C. The medium was changed every 3–4 days until the cells reached confluency, at which time the medium was changed every second day.

[1-^{14}C]Arachidonate Labeling of C62B Cells—Confluent cultures of cells were harvested by incubating cells for 5 min with 0.05% trypsin in BME and mechanically dislodging cells from the culture dish surface. Trypsinization of harvested cells was stopped by addition of an equal volume of culture medium containing fetal calf serum. The cells were centrifuged for 5 min at 100 × *g*, and the pellet was resuspended in fresh complete medium (3.5×10^5 cells/ml). Portions (1 ml) of the cell suspension were seeded into sterile 20-ml glass scintillation vials. The culture vials were incubated as indicated above for 24 h. The incubation medium was removed by aspiration and the cells labeled by incubation for 18 h in 0.75 ml of fresh culture medium containing 0.2 μCi [1-^{14}C]arachidonic acid/vial. Prior to treatment of cells with receptor agonists, the [1-^{14}C]arachidonic acid-containing culture medium was removed, and the cultures were rinsed three times with 1 ml of pH 7.2 BME containing 30 mM Hepes. The final rinse was removed, 0.70 ml of the same medium was added, and the vials were gased with 5% CO_2, 95% air and placed in a 37 °C water bath.

Effects of Inhibitors on Arachidonate Metabolism—Homogenates of C62B (3.0×10^6 cells/ml) were prepared in 30 mM Hepes-buffered BME. Portions (200 μl) of the cell homogenate were added to culture tubes containing arachidonic acid metabolism inhibitors in 5 μl of ethanol:H_2O (1:1) and incubated for 5 min at room temperature. Control incubations contained homogenate and vehicle alone. The ionophore A23187 (2.5 μM) and [1-^{14}C]arachidonic acid (0.2 μCi) were added (addition of the ionophore resulted in maximal stimulation of synthesis of all arachidonic acid metabolites so that inhibition could be most easily quantitated). The incubation was continued for 5 min at 37 °C. Controls, containing homogenate and vehicle only, were placed in a boiling water bath for 5 min prior to addition of [1-^{14}C] arachidonic acid to measure nonenzymatic conversion of arachidonic acid to metabolites. Lipids were extracted and separated as outlined below.

Treatment with Neurotransmitter Receptor Ligands—Solutions of receptor ligands were prepared in Hepes-buffered BME (the rinse medium) immediately prior to use; the concentrations were adjusted so that addition of 50-μl aliquots to the cultures gave the desired final concentration. The cultures were then incubated at 37 °C for the times indicated in the figure legends. In experiments where acetylcholine was used as the receptor agonist, the final incubation medium contained eserine (100 μM) to inhibit acetylcholine esterase. Acetylcholine receptor antagonists were added to the final incubation medium 5 min prior to treatment of cultures with receptor agonists.

Lipid Analysis—Lipid extracts were prepared as previously reported (Lapetina and Cuatrecasas, 1979). Incubations were stopped by addition of 2.8 ml of chloroform:methanol (1:2) (and placing the vial in a sonicating bath for 1 min to separate the tissue from the glass). The sonicated cell samples 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 was removed and evaporated to dryness under a stream of nitrogen at room temperature. The resulting sample residue was dissolved in 50 μl of chloroform, and 20-μl portions were applied to Silica Gel LK6DF TLC plates. Arachidonic acid and its metabolites were separated from esterified lipids, using a solvent consisting of the upper phase obtained after phase separation of a mixture of ethyl acetate:isooctane:acetic acid:H_2O (93:47:20:100). Arachidonic acid and its metabolites were separated with the following R_F values in this system: arachidonic acid, 0.77; HHT, 0.60; 5-HETE, 0.57; PGD_2, 0.31; PGE_2, 0.21; 6-keto-$PGF_{1\alpha}$/$PGF_{2\alpha}$, 0.12; phosphatidic acid, 0.05. Separation of $PGF_{2\alpha}$ from 6-keto-$PGF_{1\alpha}$ was achieved using the same solvents in the ratio of 90:50:20:100 (Tansik *et al.*, 1978) with LK5D TLC plates. The resultant R_F values for $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were 0.16 and 0.11, respectively. A second system, petroleum ether:diethyl ether:acetic acid (50:50:1) was used to better resolve 5-HETE from HHT and arachidonic acid. In this system, R_F values were arachidonic acid, 0.66; HHT, 0.33; and 5-HETE, 0.24.

Separation of phospholipids was performed on Silica Gel LK5D TLC plates developed in chloroform:methanol:petroleum ether:acetic acid:boric acid in the ratio 40:20:30:10:1.8 (v/v/v/v/w) (Gilfillan *et al.*, 1983). The [1-^{14}C]arachidonyl lipids were visualized by autoradiography and tentatively identified by co-chromatography with standards detected with iodine vapor. Quantification of radioactivity incorporated into lipids was determined by scraping regions of the TLC plates and liquid scintillation counting.

Hydrolysis of Phospholipids by Phospholipase A_2— Pooled lipid extracts from control cultures labeled with [1-^{14}C]arachidonic acid were dried under nitrogen. The residues were dissolved in 0.75 ml of diethyl ether:methanol (98:2), and 50 μl of phospholipase A_2 (1 mg/ml in water) and 50 μl of pH 7.2 Hanks' balanced salt solution were added. The samples were vigorously mixed and incubated with gentle shaking overnight at room temperature. Hydrolysis of phospholipids was quantiated by isolation of esterified radioactive fatty acid by TLC and determination of radioactivity as noted above. The radioactivity in cellular phospholipids was almost completely (more than 95%) converted to unesterified arachidonic acid by phospholipase A_2 treatment. Control samples incubated in the absence of enzyme released less than 3% of the radioactivity from phospholipids.

RESULTS

Incorporation of Arachidonic Acid into C62B Cells—When C62B glioma cells were incubated for 18 h in the presence of [1-^{14}C]arachidonic acid, 80 ± 1.5% ($n = 14$) of the radioactivity added was present in the cellular pool (not removed by rinsing the cultures). Of that radioactivity incorporated into cells, most (84–94%) was present in phospholipids and was esterified in the 2-position. Some [1-^{14}C]arachidonic acid (5–15%) was esterified in neutral lipids, while less than 0.5% remained as free arachidonic acid. The radioactivity that was not cell-associated and remained in (or was released into) the incubation medium was primarily free arachidonic acid (65%), while about 30% was present as the lipoxygenase metabolite 5-HETE. Several minor radioactive bands were also resolved on the chromatographic plates of the culture medium lipids. However, these were also present in similar amounts in medium incubated for 18 h in the absence of cells.

Cholinergic Stimulation of Arachidonic Acid Metabolism—Treatment of prelabeled C62B cells with 1 mM acetylcholine increased the liberation of radioactive arachidonic acid from esterified cellular pools and the accumulation of radioactive arachidonic acid metabolite and radioactive phosphatidic acid (Figs. 1 and 2). Measurable accumulation of unesterified [1-^{14}C]arachidonic acid occurred within 15 s of exposure to acetylcholine; the accumulation was maximal by 60–90 s, and levels of unesterified radioactive arachidonic acid returned to base-line within 5 min. The return to basal levels was not the result of hydrolysis and eventual depletion of acetylcholine from the treatment medium, as the hydrolysis of acetylcholine was presumably inhibited by eserine. Furthermore, the same time course of radioactive arachidonic acid accumulation was observed when the nonhydrolyzable cholinergic receptor agonist, carbachol, was used. At the peak of arachidonic acid accumulation, approximately 4% of the total radioactivity incorporated into glycerolipids had been liberated. The amount of [1-^{14}C]arachidonic acid accumulating in response to cholinergic stimulation was variable at the 90-s time point by a factor of 2 from experiment to experiment. This might be due to uncontrolled variables which shifted the time course of the peak response; inspection of Fig. 3 reveals that a 15-s difference in sampling time (or combination of variables such as batch to batch cell differences resulting in similar variability) would result in such data scatter. Radioactive phosphatidic acid rose in parallel with arachidonic acid accumulation for 60 s and remained at an elevated level for over 15 min, long after the pool of unesterified radioactive lipids (arachi-

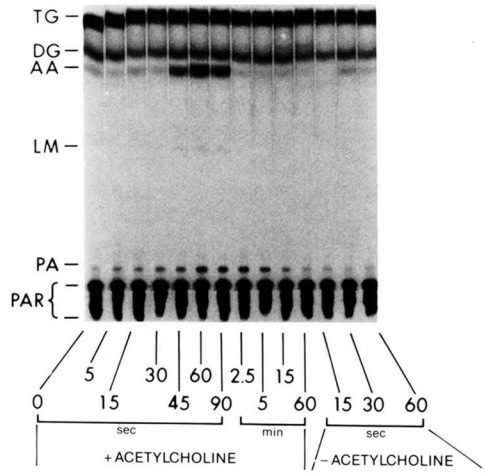

FIG. 1. **Autoradiograph of arachidonic acid and its metabolites following cholinergic stimulation of C62B cells.** Lipid extracts were prepared and lipids separated as indicated under "Methods." Cultures of C62B cells were incubated in the presence of 1.0 mM acetylcholine for the times indicated; controls were incubated in the absence of neurotransmitter. The abbreviations used in the figure are: *AA*, arachidonic acid; *LM*, lipoxygenase metabolite; *PA*, phosphatidic acid; *TG*, triglyceride, *DG*, 1,2-diacylglycerol, *PAR*, preabsorbent region (containing primarily phospholipids which did not migrate in the solvent system used).

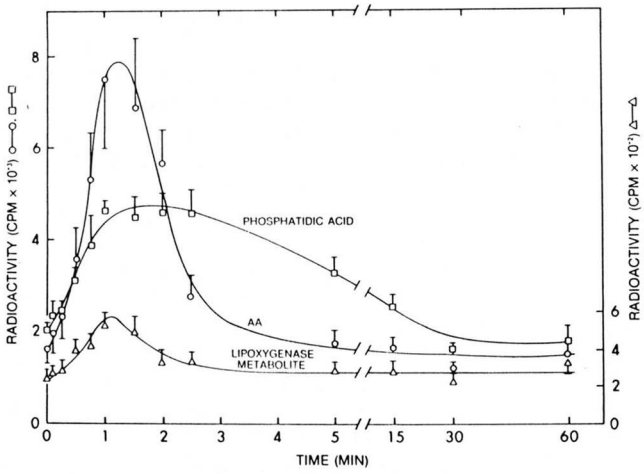

FIG. 2. **Time course of accumulation of liberated radioactive arachidonic acid, phosphatidic acid, and lipoxygenase metabolite during cholinergic stimulation.** Cultures of C62B cells were incubated in the presence of 1.0 mM acetylcholine for the times indicated (arachidonic acid, ○; phosphatidic acid, □; lipoxygenase metabolite, △). Cultures incubated in the absence of acetylcholine for various periods of time fall within the S.E. of the time zero controls and are thus not included. Each value is the mean ± S.E./culture of three to five separate experiments, each performed in duplicate.

donic acid metabolite and arachidonic acid) had returned to base-line.

The stimulation of [1-^{14}C]arachidonic acid liberation by acetylcholine was detectable at concentrations of agonists as low as 5 μM (Fig. 3, note the presence of 100 μM eserine to inhibit acetylcholinesterase). The response was maximal at approximately 1.0 mM acetylcholine. Carbachol, while not as potent as acetylcholine, had a similar concentration-dependent effect (Fig. 3 and Table I). The concentration response curves for cholinergic stimulation (levels of metabolite at 1 min as a function of the concentration of cholinergic agonist) were nearly identical for arachidonic acid and phosphatidic

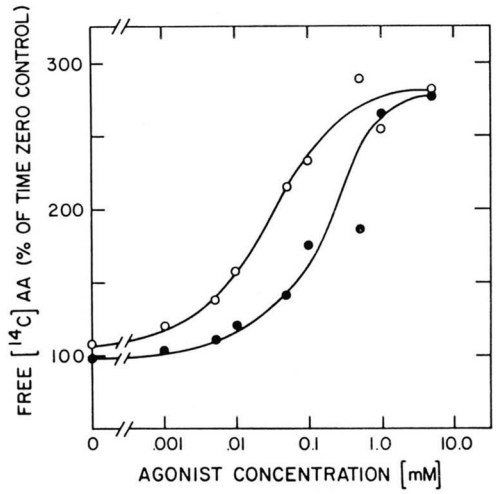

FIG. 3. **Liberation of ratioactive arachidonic acid as a function of cholinergic receptor agonist concentration.** Prelabeled C62B cells were incubated in the absence or presence of acetylcholine (○) or carbachol (●) for 1 min. Each value represents the mean of two or three experiments each performed in duplicate. Results are expressed as the percentage of unesterified radioactive arachidonic acid present in control cultures (not treated with agonists) at time zero.

TABLE I

Effects of cholinergic receptor agonists and antagonists on [^{14}C] arachidonate liberation from C62B glioma cells

Cultures of C62B cells were prelabeled with [1-^{14}C]arachidonic acid (AA) and treated with receptor agonists and antagonists as indicated ("Methods"). Incubations were stopped after 1 min and lipids prepared as indicated ("Methods").

Treatment	Free [^{14}C]AA
	cpm/sample + S.E. (n = 4)
Buffer	2062 ± 98
Acetylcholine	
1×10^{-5} M	3150 ± 183
1×10^{-4} M	4582 ± 230
1×10^{-3} M	5550 ± 147
Acetylcholine	
5×10^{-5} M	4820 ± 250
5×10^{-5} M plus atropine, 1×10^{-9} M	3950 ± 425
5×10^{-5} M plus atropine, 1×10^{-8} M	2752 ± 120
5×10^{-5} M plus atropine, 1×10^{-7} M	1927 ± 128
5×10^{-5} M plus *d*-tubocurarine 1×10^{-6} M	5927 ± 532
Carbachol	
1×10^{-5} M	1935 ± 150
1×10^{-4} M	2785 ± 273
1×10^{-3} M	5678 ± 782
Oxotremorine	
1×10^{-5} M	2160 ± 180
1×10^{-4} M	2115 ± 290
1×10^{-3} M	2002 ± 188
Pilocarpine	
1×10^{-5} M	1995 ± 158
1×10^{-4} M	1930 ± 68
1×10^{-3} M	1852 ± 32

acid accumulation (data not shown). Although the accumulation of the unidentified metabolite was low, 10% of that of arachidonic acid, the maximal accumulation of the metabolite also occurred at 1.0 mM acetylcholine.

The cholinergic stimulation of the arachidonic acid liberation response was specific for muscarinic receptor activation (Table I). Low concentrations of the muscarinic receptor antagonist, atropine (1–10 nM) inhibited the acetylcholine stimulation of arachidonic acid accumulation. Incubation with

a nicotinic receptor antagonist *d*-tubocurarine (1.0 μM), however, failed to prevent the acetylcholine-stimulated response. It was of interest that while carbachol and acetylcholine stimulated the liberation of radioactive arachidonic acid at low concentrations, the muscarinic receptor agonists pilocarpine and oxotremorine were ineffective promoters of this process, even at concentrations as high as 1.0 mM. The possibility of an adrenergic response was tested since α- and β-adrenergic receptor agonists have been shown to stimulate arachidonic acid release in some cells (Leslie, 1976; Levine and Moskowitz, 1979), and C62B cells exhibit both α- and β-adrenergic receptors (Bottenstein and de Vellis, 1978). Neither norepinephrine, an agonist for both α- and β-receptors, nor the β-selective receptor agonist, isoproterenol, increased the liberation of arachidonic acid (the assays were conducted at 1 and 15 min time points over the 0.1–25 μM range). Histamine (1 μM–1 mM) likewise failed to increase arachidonic acid metabolism in C62B cells.

The metabolic pathway involved in production of the unidentified arachidonic acid metabolite accumulating as the result of cholinergic stimulation was identified by use of inhibitors of the cyclooxygenase and/or lipoxygenase metabolic pathways (Table II). Drugs which inhibited both pathways (BW755 and nordihydroguaiaretic acid; Blackwell and Flower, 1978; Hamberg, 1976; Higgs *et al.*, 1979) blocked the synthesis of the cyclooxygenase metabolites (6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, and PGE_2) as well as of the lipoxygenase metabolite 5-HETE and of the unidentified metabolite (LM of Fig. 1, Table II). Esculetin (Sekiya *et al.*, 1982; Neichi *et al.*, 1983) and baicalein (Sekiya and Okuda, 1982), presumed selective lipoxygenase inhibitors, also blocked the synthesis of 5-HETE and the unidentified metabolite, but actually increased the synthesis of cyclooxygenase metabolites of arachidonic acid. In contrast, indomethacin (Flower, 1974) at low concentrations decreased the synthesis of the cyclooxygenase metabolites, but not 5-HETE or the unidentified metabolite. These results suggest that the unidentified arachidonic acid metabolite is a lipoxygenase metabolite.

Ionophore Stimulation of Arachidonic Acid Liberation and Metabolism—The divalent cation ionophore, A23187 (2.5 μM), stimulated the liberation of arachidonic acid from C62B cellular glycerolipids in a time-dependent manner (Fig. 4). There were, however, several significant differences between the response to cholinergic stimulation and that to ionophore

TABLE II

Inhibition of C62B cell arachidonate metabolism

Treatment	Metabolite			
	PGE_2	6-Keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$	5-HETE	LM[a]
	% of control[b]			
NDGA,[c] 20 μM	0 ± 1	0 ± 3	0 ± 3	16 ± 6
BW755, 100 μM	2 ± 2	0 ± 5	0 ± 1	0 ± 2
Baicalein, 50 μM	155 ± 23	172 ± 32	2 ± 1	7 ± 4
Esculetin, 100 μM	230 ± 38	266 ± 31	18 ± 6	27 ± 8
Indomethacin, 1.0 μM	8 ± 4.0	9 ± 5	80 ± 14	73 ± 5
Indomethacin, 0.1 μM	39 ± 10	51 ± 12	105 ± 22	115 ± 10

[a] LM, lipoxygenase metabolite.

[b] Values, expressed as the percentage of drug vehicle-treated homogenates corrected for nonenzymatic metabolism of arachidonic acid, are the mean ± S.E. for three experiments performed in duplicate. The median net production by control homogenates (ionophore in the absence of inhibitors) was: PGE_2, 3242; 6-keto-$PGF_{1\alpha}$/$PGF_{2\alpha}$, 1507; 5-HETE, 2027; and lipoxygenase metabolite, 2467 cpm respectively. These values are corrected for nonenzymatic production of metabolites by heat-inactivated homogenates, these values ranged from 335 to 580 cpm.

[c] NDGA, nordihydroguaiaretic acid.

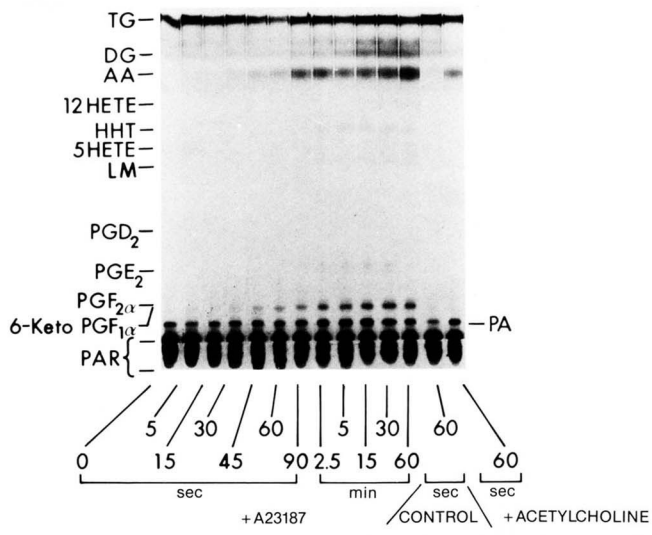

FIG. 4. **Autoradiograph of arachidonic acid and its metabolites at various times following application of the ionophore A23187.** Cultures of prelabeled C62B cells were incubated in the presence of 2.5 μM A23187 for times indicated. Controls included cultures incubated for 1 min without the ionophore or with 1.0 mM acetylcholine substituted for A23187. Abbreviations are as in Fig. 1.

A23187 treatment. Most marked was the observation that, while arachidonic acid accumulation in response to acetylcholine stimulation peaked at 60–90 s and then fell rapidly, the levels of radioactive arachidonic acid liberated in response to ionophore continued to rise during the entire course of the incubation (Fig. 4 and Table III). Second, although following acetylcholine stimulation there was only one major radioactive eicosanoid accumulated, a lipoxygenase metabolite, the ionopore additionally induced the formation of HHT, PGE_2, PGD_2, 6-keto-$PGF_{1\alpha}$, and $PGF_{2\alpha}$. During a 1-min incubation with A23187, unesterified arachidonic acid, HHT, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, and a lipoxygenase metabolite accumulated; with a longer incubation, the accumulation of PGE_2, PGD_2, 5-HETE, and 12-HETE was also detectable. Finally, whereas during cholinergic stimulation the accumulation of radioactive arachidonic acid and phosphatidic acid occurred nearly simultaneously, in ionophore-stimulated cells the accumulation of unesterified [1-^{14}C]arachidonic acid consistently preceded the increase in radioactive phosphatidic acid (Fig. 4 and Table III).

DISCUSSION

Our data demonstrate that in C62B glioma cells, the release of arachidonic acid and formation of phosphatidic acid in response to cholinergic agonists is rapid, transient, dependent upon the activation of a specific class of muscarinic receptors, and leads to selective production of certain eicosanoids. The importance of these results lies in their implication for the physiological significance of arachidonic acid metabolism in glial responses to neurotransmitters.

The time course of the response suggests that glia are capable of a rapid response to neuronal activity and could act quickly to affect the extracellular environment of neurons. The time course of this modulation would not be sufficiently rapid to affect the neuronal signal which initiated the glial response, but could result in an altered neuronal response to subsequent stimulation. In other systems, it has been shown that certain physiological processes are coupled to rapid arachidonic acid release and metabolism, *e.g.* secretion of insulin by endocrine islet cells (Dunlop and Larkins, 1984), platelet secretion and aggregation (Lapetina and Cuatrecasas, 1979;

TABLE III

Liberation of arachidonic acid and formation of arachidonate metabolites following stimulation of C62B glioma cells with acetylcholine and the ionophore A23187

Cultures of C62B cells prelabeled with [1-^{14}C]arachidonic acid (AA) were treated with acetylcholine (Ach; 1 mM) or the ionophore A23187 (2.5 μM) for the times indicated. Control cultures were incubated for 1 min in the absence of acetylcholine or ionophore. Lipids were extracted and prepared as detailed under "Methods." Values, expressed in cpm/culture, are the mean ± S.E. for five or more cultures.

Products	Treatment				
	Control	1 min Ach	15 min Ach	1 min	15 min A23187
AA	1285 ± 107	3015 ± 308	1835 ± 300	2755 ± 390	4020 ± 643
6-Keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$[a]	660 ± 135	667 ± 93	645 ± 113	1825 ± 297	2050 ± 412
PGE_2	202 ± 60	200 ± 15	258 ± 30	253 ± 30	360 ± 42
PGD_2	123 ± 15	120 ± 15	145 ± 13	160 ± 19	290 ± 15
LM[b]	223 ± 15	470 ± 52	320 ± 63	417 ± 48	490 ± 47
5-HETE	302 ± 13	282 ± 18	402 ± 88	385 ± 20	585 ± 60
HHT	175 ± 13	180 ± 16	290 ± 68	342 ± 27	618 ± 75
12-HETE	300 ± 35	252 ± 43	368 ± 43	365 ± 42	865 ± 73
PA[c]	3255 ± 270	5465 ± 627	4390 ± 578	3877 ± 462	6577 ± 885

[a] Resolution of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ into separate bands (see "Methods") indicated that after a 15-min exposure to ionophore, 76 and 24% of the radioactivity co-chromatographed with $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, respectively.

[b] LM, lipoxygenase metabolite.

[c] PA, phosphatic acid.

Lapetina, 1982), and neutrophil activation (Cockcroft *et al.*, 1981).

The transitory nature of the arachidonic acid liberation and phosphatidic acid formation following exposure to cholinergic agonists is as might be expected of a physiologically significant response to neurotransmitter release by neurons. The mechanism of this transient accumulation might initially involve a liberation of arachidonic acid from phospholipids via the rapid activation of a phospholipase A_2 (Bills *et al.*, 1976; Billah *et al.*, 1981, Lapetina, 1983) or possibly diglyceride and monoglyceride lipases (Bell *et al.*, 1979; Rittenhouse-Simmons, 1979, Prescott and Majerus, 1983). This would be followed by a rapid re-acylation of the free arachidonic acid (Walsh *et al.*, 1983). The similar time course for accumulation of the unidentified lipoxygenase metabolite suggests that this product is rapidly made from liberated arachidonic acid, although the possibility that it is rapidly released independently from a pre-existing esterified store has not been eliminated. It would then be either rapidly degraded or removed by a re-acylation reaction similar to that reported to occur for some other lipoxygenase products (Stenson and Parker, 1979; Walsh *et al.*, 1981; Bonser *et al.*, 1981).

We assume that the formation of phosphatidic acid reflects the action of phospholipase C on the inositol phospholipids to yield 1,2-diacylglycerol and a subsequent phosphorylation by 1,2-diacylglycerol kinase to form phosphatidic acid (Lapetina and Cuatrecasas, 1979; Lapetina, 1982). The phospholipase C activity reflected by this elevation of radioactive phosphatidic acid may be directly relevant to the elevation of radioactive arachidonic acid (*e.g.* via diglyceride and monoglyceride lipases, Bell *et al.*, 1979; Rittenhouse-Simmons, 1979; Prescott and Marjerus, 1983, or a phosphatidic acid-specific phospholipase A_2, Billah *et al.*, 1981). Another possibility is that the formation of phosphatidic acid and other metabolites resulting from phospholipase C action may subserve some independent functions. In this regard, phosphatidic acid has been recognized as a Ca^{2+} ionophore (Tyson *et al.*, 1976; for review see Lapetina, 1982) and in neuroblastoma cells has been shown to stimulate calcium influx and cGMP synthesis (Ohsako and Deguchi, 1981). Muscarinic stimulation of phospholipase C is well documented in neural tissues (Berridge *et al.*, 1982; Fisher and Agranoff, 1980; Brown *et al.*, 1984) and has recently been demonstrated in astrocytoma cells (Masters et al., 1984).

The specificity of this response for muscarinic receptors was demonstrated by the ability of the muscarinic receptor antagonist, atropine, but not the nicotinic receptor antagonist, *d*-tubocurarine, to block the action of acetylcholine. Interestingly, in contrast to the potent effects of carbachol and acetylcholine, the muscarinic receptor agonists, oxotremorine and pilocarpine, failed to stimulate arachidonic acid liberation. This is consistent with similar observations in several cell types demonstrating heterogeneity of muscarinic receptors (see Birdsall and Hulme, 1983). Especially relevant is the observation that, in heart cell cultures, one class (or state) of muscarinic receptors is linked to stimulation of inositol phospholipid metabolism, while a second is linked to inhibition of adenylate cyclase (Brown and Brown, 1984). Only the latter receptor system is activated by oxotremorine and pilocarpine, while carbachol and acetylcholine activate both receptor systems.

The selective effect of cholinergic receptor activation on arachidonic acid metabolism is apparent in the specific accumulation of a specific lipoxygenase metabolite. No other lipoxygenase metabolite accumulates in response to cholinergic receptor occupation nor does there appear to be accelerated production of cyclooxygenase metabolites. (Note that there is accumulation of another lipoxygenase metabolite, 5-HETE, in the culture medium during the 18-h prelabeling period, but its formation does not appear to be regulated by cholinergic stimulation.) Relatively little is known of the action of lipoxygenase metabolites on the nervous system, although recent evidence suggests that they may serve an important role (Palmer *et al.*, 1981). Furthermore, it has been demonstrated in a murine neuroblastoma cell line, N1E-115, that elevation of cGMP levels by cholinergic stimulation is dependent upon arachidonic acid metabolism along the lipoxygenase pathway (Snider *et al.*, 1984). It is noteworthy that in other cell types, the formation of lipoxygenase metabolites of arachidonic acid is often associated with stimulation of secretory responses (Walenga *et al.*, 1980; Metz *et al.*, 1983).

The accumulation of prostaglandins within minutes of ionophore stimulation (see Fig. 3) indicates that C62B cells have the capacity to rapidly produce cyclooxygenase metabolites. The ability of these cells to produce cyclooxygenase metabo-

lites may be relevant to neuronal-glial interactions for, as indicated earlier, some prostaglandins that have been shown to modulate neurotransmission (Hedqvist, 1973). However, these metabolites are probably not involved in the cholinergic response we have studied, as only small amounts of these products are observed to accumulate in response to cholinergic stimulation of C62B cells.

The results reported above are the first demonstration of muscarinic cholinergic stimulation of arachidonic acid liberation and an associated accumulation of phosphatidic acid in cells of glial origin. This most probably reflects phospholipase A_2 and phospholipase C activation, and the products formed from these activities may provide a mechanism by which glial cells interact with neurons. Our supposition that these findings obtained with a glial cell line are relevant to glial-neuronal interactions will have to be tested using primary culture or tissue slice preparations. However, our results are compatible with a growing body of literature which suggests that neuronal neurotransmitter release may be involved in coupling glial cell metabolism to neuronal function.

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