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# Receptor-stimulated Phospholipase $A_2$ Activation Is Coupled to Influx of External Calcium and Not to Mobilization of Intracellular Calcium in C62B Glioma Cells*

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**C62B rat glioma cells respond to muscarinic cholinergic stimulation with transient inositol phosphate formation and phospholipase $A_2$-dependent arachidonic acid liberation. Since phospholipase $A_2$ is a $Ca^{2+}$-sensitive enzyme, we have examined the role of the agonist-stimulated $Ca^{2+}$ response in production of the arachidonate signal. The fluorescent indicator fura-2 was used to monitor changes in cytoplasmic $Ca^{2+}$ levels ($[Ca^{2+}]_i$) of C62B cells following acetylcholine treatment. In the presence of extracellular $Ca^{2+}$, acetylcholine induces a biphasic $[Ca^{2+}]_i$ response consisting of an initial transient peak that precedes arachidonate liberation and a sustained elevation that outlasts the phospholipase $A_2$ response. The initial $[Ca^{2+}]_i$ peak is not altered by the absence of external $Ca^{2+}$ and therefore reflects intracellular $Ca^{2+}$ mobilization. The sustained elevation phase is dependent on the influx of external $Ca^{2+}$; it is lost in $Ca^{2+}$-free medium and restored on the addition of $Ca^{2+}$. Pretreating cells with phorbol dibutyrate substantially inhibits acetylcholine-stimulated inositol phosphate formation and the peak $[Ca^{2+}]_i$ response without affecting the sustained elevation in $[Ca^{2+}]_i$. This suggests that the release of internal $Ca^{2+}$ stores by inositol 1,4,5-trisphosphate can be blocked without interfering with $Ca^{2+}$ influx. Pretreatment with phorbol also fails to affect acetylcholine-stimulated arachidonate liberation, demonstrating that phospholipase $A_2$ activation does not require normal intracellular $Ca^{2+}$ release. Stimulated arachidonate accumulation is totally inhibited in $Ca^{2+}$-free medium and restored by the subsequent addition of $Ca^{2+}$. Pretreatment with verapamil, a voltage-dependent $Ca^{2+}$ channel inhibitor, also blocks both the sustained $[Ca^{2+}]_i$ elevation and arachidonate liberation without altering peak intracellular $Ca^{2+}$ release. We conclude that the influx of extracellular $Ca^{2+}$ is tightly coupled to phospholipase $A_2$ activation, whereas large changes in $[Ca^{2+}]_i$ due to mobilization of internal $Ca^{2+}$ stores are neither sufficient nor necessary for acetylcholine-stimulated phospholipase $A_2$ activation.**

A wide variety of tissues respond to hormone and neurotransmitter binding with stimulated phospholipase C-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$)[1] (reviewed in 1-3). $PIP_2$ cleavage produces inositol 1,4,5-trisphosphate (1,4,5-$IP_3$), which increases cytoplasmic calcium levels ($[Ca^{2+}]_i$) by binding to specific intracellular receptors and mobilizing sequestered $Ca^{2+}$ stores (4), and diacylglycerol, which activates protein kinase C (5). An influx of extracellular $Ca^{2+}$ is also stimulated by one or more possible mechanisms (3, 6, 7).

In many cases, agonist-stimulated $PIP_2$ hydrolysis is accompanied by phospholipase $A_2$ activation, with consequent liberation of free arachidonic acid from cellular phospholipid and generation of eicosanoid second messengers (2). Although phospholipase C is generally believed to be under the direct control of one or more guanine nucleotide-binding proteins (G proteins) (8, 9), the mechanism for phospholipase $A_2$ regulation is less certain. G proteins may play a role in phospholipase $A_2$ control (10-12), but many other cellular factors are capable of modulating the enzyme's activity as well, including $Ca^{2+}$ (13, 14), diacylglycerol (15), protein kinase C (16-19), inhibitory proteins (lipocortins) (20, 21), and $Na^+/H^+$ exhange (22). The physiological significance of all these (not mutually exclusive) factors is unclear, however.

Cytoplasmic $Ca^{2+}$ levels have long been proposed as a primary *in vivo* regulator of phospholipase $A_2$ activity (1, 13, 14). Purified phospholipase $A_2$ enzymes generally show a positive correlation between *in vitro* activity and $Ca^{2+}$ concentrations above basal physiological $[Ca^{2+}]_i$ (23-26), and at least one isolated enzyme shows complete activation by $Ca^{2+}$ over a stimulated *in vivo* $[Ca^{2+}]_i$ range (24). Further evidence has come from studies using $Ca^{2+}$ ionophores or detergents to manipulate $[Ca^{2+}]_i$ in "intact" cells; the use of millimolar external $Ca^{2+}$ with ionophores produces phospholipase $A_2$ activation in many cell types (2, 17, 27, 28), including C62B cells (29). Arachidonate liberation has been observed in glomerular mesangial cells and platelets with controlled increases in $[Ca^{2+}]_i$ over the physiological agonist-stimulated range (19, 30).

Previous work in this laboratory has demonstrated that C62B rat glioma cells respond to muscarinic stimulation with rapid inositol phosphate formation followed by a transient accumulation of arachidonate peaking at 60-90 s (29, 31). In

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[1] The abbreviations used are: $PIP_2$, phosphatidylinositol 4,5-bisphosphate; BME, basal medium Eagle's; $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; G protein, guanine nucleotide-binding protein; HBSS, Hanks' balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; 1,4,5-$IP_3$, inositol 1,4,5-trisphosphate; PDBu, phorbol 12,13-dibutyrate.

this system, arachidonate liberation occurs preferentially via phospholipase $A_2$ degradation of phosphatidylinositol (31). The present study was conducted to determine the relative roles of intracellular $Ca^{2+}$ mobilization and extracellular $Ca^{2+}$ influx in cholinergically stimulated phospholipase $A_2$ activation in these cells.

## EXPERIMENTAL PROCEDURES

### *Materials*

Basal medium Eagle's (BME) was purchased from GIBCO. Fetal calf serum was from Flow Laboratories (McLean, VA). Fura-2/AM and ionomycin were obtained from Calbiochem. [1-$^{14}$C]Arachidonic acid (58 mCi/mmol) and *myo*-[2-$^{3}$H]inositol (18.7 Ci/mmol) were from Amersham Corp. Acetylcholine, phorbol 12,13-dibutyrate (PDBu), and verapamil were from Sigma. The Partisil 10 SAX anion exchange high performance liquid chromatography (HPLC) column (4.5 × 25 cm) and Silica Gel LK6D thin layer chromatography (TLC) plates were purchased from Whatman (Maidstone, United Kingdom). All other materials were reagent grade.

### *Methods*

*Cell Culture*—C62B cells were cultured in BME supplemented with 5% fetal calf serum, 1 mM glucose, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified environment of 5% $CO_2$ and 95% air at 37 °C. The cells were grown to confluence in 75-cm$^2$ Corning culture flasks and harvested by trypsinization as described previously (29); the only modification was the use of a calcium-free medium (11 mM glucose, 20 mM HEPES, 10.2 mM trisodium citrate, 4 mM potassium chloride, 110 mM sodium chloride, pH 7.2) for harvesting.

*Cytoplasmic $Ca^{2+}$ Measurement*—Aliquots of harvested cells were allowed to settle onto sterile glass coverslips in 100-mm Corning tissue culture dishes, and the cells were grown to confluence over 2–10 days in supplemented BME as described above. Coverslips were mounted into a chamber and washed three times with unsupplemented BME, and the attached cells were incubated with 5 μM fura-2/AM in BME for 17 min at 37 °C. The cells were washed three times with Hanks' balanced salt solution (HBSS, containing 137 mM sodium chloride, 5.4 mM potassium chloride, 0.8 mM magnesium sulfate, 0.4 mM sodium phosphate dibasic, 0.4 mM potassium phosphate monobasic, 5.6 mM glucose, 1.3 mM calcium chloride, pH 7.2) prior to measurement of fura-2 fluorescence. Fura-2 loading under these conditions produced a diffuse fluorescence with no visible intracellular localization of the dye.

Epifluorescence microscopy (32) was used to monitor changes in the fura-2 fluorescence of the C62B cell monolayer. The chamber was mounted on an inverted Nikon Diaphot-TMD microscope, and a Fluor 40 × objective was used to examine 12–25 cells at a given time. Fura-2 fluorescence was recorded at 1-s intervals using a dual excitation wavelength spectrofluorometer (Spex Industries, Edison, NJ) with excitation at 340 and 380 nm and emission at 500 nm. All experiments were performed in either the $Ca^{2+}/Mg^{2+}$-containing HBSS described above or in HBSS without added $Ca^{2+}$ or $Mg^{2+}$ and with 0.5 mM EGTA. Experiments were conducted at room temperature since the $Ca^{2+}$ response was found to be identical at room temperature and 37 °C, but the higher temperature greatly accelerated dye leakage. Cells were treated with acetylcholine (2.75 mM) or drugs by the addition of 4–10 × concentrates to obtain the desired final concentration. PDBu and verapamil were added as ethanolic solutions of HBSS 20–30 min prior to acetylcholine stimulation; solvent concentrations, which never exceeded 0.5%, had no effect on the fura-2-monitored $Ca^{2+}$ response. After stimulation, the cells were rinsed free of agonist by 10–12 media changes and were given 20 min to recover prior to restimulation. Media changes and additions of agonist or drugs were made without an interruption in recording. In some experiments, the initial acetylcholine-stimulated $Ca^{2+}$ response varied slightly from all subsequent control responses, perhaps due to incomplete dye hydrolysis or a transient buffering effect (32, 33); in such cases, the initial response was discarded.

The free intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) was calculated from the ratio of the fluorescence intensities at the two excitation wavelengths as described by Grynkiewicz *et al.* (34) using a $K_d$ of 224 nM for the fura-2·$Ca^{2+}$ complex. The minimum and maximum ratios were determined for the cells at the end of each experiment using 10 μM ionomycin; $R_{min}$ was obtained by equilibration with 2.5 mM EGTA in a depolarizing medium (130 mM potassium chloride, 20 mM sodium chloride, 10 mM HEPES, 1 mM magnesium chloride, pH 7.2), and $R_{max}$ was then found by the readdition of $Ca^{2+}$ in the same medium until the fura-2 signal reached saturation. The excitation spectrum of the cellular fura-2 under these conditions was similar to that of free fura-2 in the same solutions. Background autofluorescence, determined by the subsequent addition of 1 mM manganese sulfate to the permeabilized cells, was subtracted from the 340 and 380 nm traces prior to calculating $[Ca^{2+}]_i$.

*Labeling Cells with [$^{14}$C]Arachidonic Acid and [$^{3}$H]Inositol*—Sterile glass scintillation vials were exposed to 1.5 ml of poly-D-lysine (10 μg/ml) for 20 min and allowed to air dry. C62B cells were seeded into the vials as described previously (29). The cultures were then incubated for 18–24 h, the medium was removed by aspiration, and cells were labeled by incubation for 24 h in 0.75 ml of fresh medium containing 0.2 μCi of [1-$^{14}$C]arachidonic acid. In some cases, 25 μCi of *myo*-[2-$^{3}$H]inositol was also included (31).

*Assays for Arachidonic Acid Liberation and Inositol Phosphate Formation*—These assays were conducted following protocols described previously (29, 31). Briefly, the labeling medium was removed, the cultures were rinsed, fresh medium was added, and the cells were placed in a 37 °C water bath. BME containing 30 mM HEPES, pH 7.2, was used for arachidonate experiments, whereas double-label experiments were performed using $Ca^{2+}/Mg^{2+}$-containing HBSS (15 mM HEPES, pH 7.2) with 25 mM lithium chloride. Drugs were added in 50-μl aliquots to give the desired final concentrations in a total volume of 0.75 ml. PDBu and verapamil were made up in the appropriate medium and added as described above; solvent concentrations used had no effect on stimulated arachidonate liberation or inositol phosphate formation. Incubations were carried out in the 37 °C water bath for the times indicated and were terminated by the addition of 2.8 ml of chloroform/methanol/hydrochloric acid (v/v/v, 100:200:2) followed by agitation in a sonicating water bath. Cell extracts were transferred to centrifuge tubes, 0.9 ml of water and 0.9 ml of chloroform were added, and the tubes were agitated on a Vortex shaker. The tubes were kept overnight at 0 °C, and the phases were separated by centrifugation.

Separation of lipids was performed by evaporating the organic phases under a stream of nitrogen, redissolving the residues in 50 μl of chloroform, and applying 20-μl portions to LK6D TLC plates. Arachidonic acid was separated from esterified lipids using a solvent system consisting of the upper phase of a mixture of ethyl acetate/isooctane/acetic acid/water (v/v/v/v, 93:47:20:100) as reported previously (29). Radiolabeled species were visualized by autoradiography using Kodak XAR film and quantitated by scraping portions of the silica gel into scintillation vials followed by liquid scintillation spectrometry.

Inositol phosphates were separated in [$^{3}$H]inositol-labeling experiments by anion exchange chromatography using HPLC or Dowex AG 1-X8 resin. For HPLC analysis (35), the aqueous phases were evaporated to 1 ml under a stream of nitrogen, diluted to 4 ml with water, and applied to a Partisil 10 SAX analytical HPLC column. The column was washed with 20 ml of water to remove free inositol, and inositol phosphates were eluted using a 70-min linear gradient of 0–1.5 M ammonium formate, pH 3.7, with a flow rate of 1 ml/min and 1-ml fractions collected. The radioactivity present in the eluent was quantitated by liquid scintillation spectrometry. Peaks of radioactivity were identified by coelution with radiolabeled standards. Dowex chromatography (36) was carried out using an ammonium formate step gradient as described previously (37).

*Statistical Analysis*—The data are presented as the mean ± S.E. of the indicated number of experiments. Statistical differences between control and treated values were analyzed using a *t* test for $Ca^{2+}$ experiments and a one-way ANOVA with Tukey's Studentized range test for arachidonate studies.

## RESULTS

*Acetylcholine Stimulation Produces a Biphasic Response in Cytoplasmic Calcium Levels*—Previous work in this laboratory has demonstrated that acetylcholine stimulation of muscarinic receptors produces phospholipase C activation and 1,4,5-$IP_3$ formation in C62B cells (31). The fluorescent $Ca^{2+}$ dye fura-2 (34) was used to characterize associated changes in $[Ca^{2+}]_i$ (Fig. 1). Acetylcholine produces a biphasic $[Ca^{2+}]_i$ response in the presence of external $Ca^{2+}$. Basal $[Ca^{2+}]_i$, 26 ± 4 nM, increases to a peak value of 420 ± 54 nM ($n$ = 19)

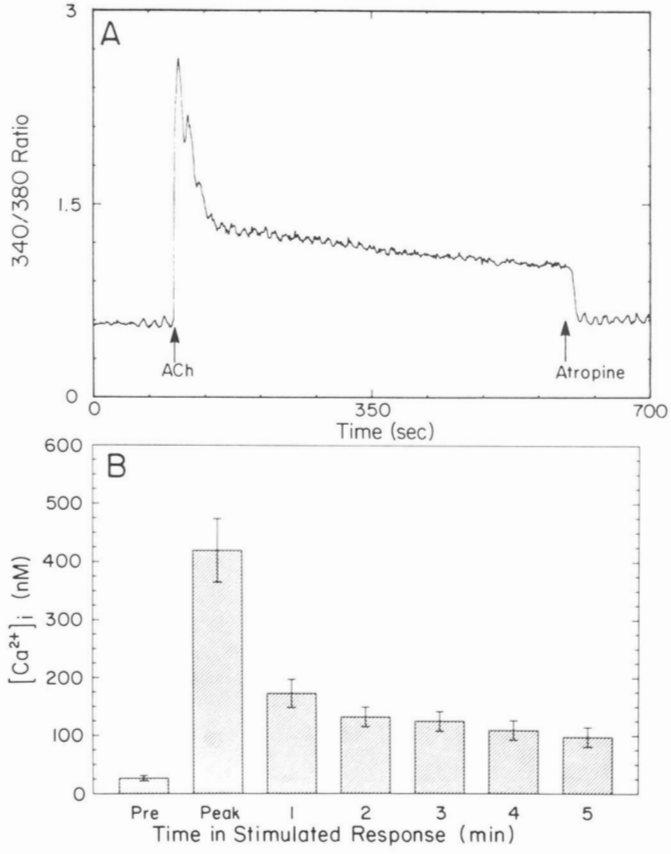


FIG. 1. **Acetylcholine-stimulated changes in fura-2 fluorescence and $[Ca^{2+}]_i$ in the presence of 1.3 mM external $Ca^{2+}$.** C62B cells loaded with fura-2 were stimulated with 2.75 mM acetylcholine (*ACh*), and the changes in fluorescence were recorded using a dual excitation wavelength spectrofluorometer with excitation at 340 and 380 nm, as described under "Methods." *Panel A* shows the ratio of the fluorescence intensities at these two wavelengths in a typical experiment after the addition of agonist and 10 $\mu$M atropine at the times indicated. In *panel B*, calculated $[Ca^{2+}]_i$ values from multiple experiments are shown at various points in the stimulated response, either prior to acetylcholine addition (*pre*), at the peak ratio intensity (*peak*), or at 1, 2, 3, 4, or 5 min after acetylcholine addition. Data shown are the average ± S.E. of mean control responses in 17–19 separate experiments.

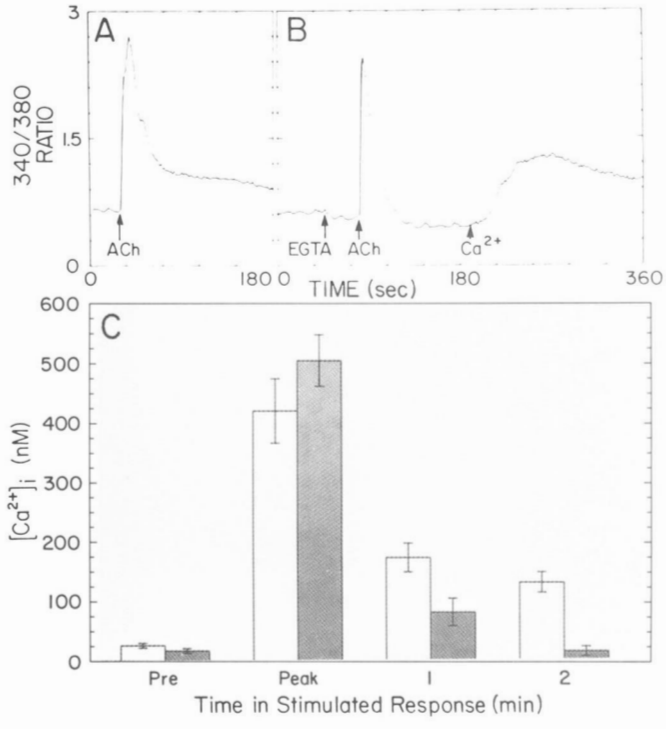


FIG. 2. **Effects of removing external $Ca^{2+}$ on the acetylcholine-stimulated $[Ca^{2+}]_i$ response.** Fura-2-loaded C62B cells were stimulated with acetylcholine (*ACh*) in the presence of 1.3 mM external $Ca^{2+}$ (*panel A*), rinsed, and allowed to recover for 20 min. The same cells were then switched to $Ca^{2+}$-free medium with 0.5 mM EGTA, restimulated with acetylcholine, finally switched back to medium with 1.3 mM $Ca^{2+}$ and acetylcholine at the times indicated (*panel B*). $[Ca^{2+}]_i$ values calculated from multiple experiments are shown in *panel C* for various times in the stimulated response as described in Fig. 1; the response in $Ca^{2+}$-free medium (*hatched bars*) is contrasted with previous data from Fig. 1 on the response in 1.3 mM external $Ca^{2+}$ (*blank bars*). Data in $Ca^{2+}$-free media are averages ± S.E. of mean responses in five separate experiments.

within 10 s of agonist addition, consistent with the previously determined time course of 1,4,5-$IP_3$ formation (31). A rapid drop in $[Ca^{2+}]_i$, which lasts about 1 min, is followed by a more gradual decline to a sustained plateau of approximately 100 nM, which can last at least 20 min in the continued presence of agonist (not shown). Addition of the muscarinic receptor antagonist atropine at any time rapidly returns $[Ca^{2+}]_i$ to basal levels (Fig. 1). Acetylcholine was used at a saturating concentration of 2.75 mM to facilitate comparison of results with previous biochemical studies (29, 31, 37, 38). The identical dose-response curves for phospholipase C and phospholipase $A_2$ activation rule out the use of agonist concentration for separating the two responses (29).

*Removing Extracellular $Ca^{2+}$ Blocks the Second Phase of the $[Ca^{2+}]_i$ Response without Affecting the Initial Peak*—Acetylcholine-stimulated changes in $[Ca^{2+}]_i$ were examined in $Ca^{2+}$-free medium to dissect the contributions of intracellular and extracellular $Ca^{2+}$ pools. $Ca^{2+}$-containing medium was aspirated away from fura-2-loaded C62B cells and replaced with $Ca^{2+}$-free medium with 0.5 mM EGTA; agonist was then added within 30 s (Fig. 2). This produced a slight lowering of basal $[Ca^{2+}]_i$ but failed to affect the initial peak $[Ca^{2+}]_i$ response. Stimulated $[Ca^{2+}]_i$ levels returned to basal values within 2 min of acetylcholine addition. Replacing the $Ca^{2+}$-free medium with the original medium containing 1.3 mM $Ca^{2+}$ restored the sustained $[Ca^{2+}]_i$ plateau (Fig. 2; 3 min after readdition $[Ca^{2+}]_i$ = 220 ± 32 nM, $n$ = 5). These results suggest that mobilization of intracellular $Ca^{2+}$ stores accounts for the initial peak $[Ca^{2+}]_i$ response, whereas the sustained elevation in $[Ca^{2+}]_i$ is dependent on extracellular $Ca^{2+}$ influx. The two events appear to overlap to some extent; stimulated $[Ca^{2+}]_i$ levels at 1 min in $Ca^{2+}$-free medium are significantly elevated over basal values and significantly lower than in the presence of external $Ca^{2+}$ ($p < .05$).

*PDBu Pretreatment Inhibits Acetylcholine-stimulated Inositol Phosphate Formation and Intracellular $Ca^{2+}$ Release without Blocking $Ca^{2+}$ Influx or Arachidonate Liberation*—We have demonstrated previously that PDBu pretreatment effectively inhibits acetylcholine-stimulated inositol phosphate formation in C62B cells (37). We therefore tested the effects of PDBu on the stimulated $[Ca^{2+}]_i$ response in cells loaded with fura-2. Pretreatment with 200 nM PDBu for 20 min inhibited the initial peak increase in $[Ca^{2+}]_i$ ($p < 0.02$) without substantially affecting the sustained plateau levels (Fig. 3). Peak increases in $[Ca^{2+}]_i$ were inhibited by 83 ± 4% ($n$ = 5) in $Ca^{2+}$-free medium and 67 ± 11% ($n$ = 6) in 1.3 mM external $Ca^{2+}$. The time to peak response was also delayed by PDBu pretreatment (Fig. 3), going from 6 ± 1 to 19 ± 3 s in $Ca^{2+}$-free medium ($n$ = 5) and from 10 ± 1 to 18 ± 5 s in the presence of external $Ca^{2+}$ ($n$ = 6). Identical PDBu pretreatments on [$^{14}$C]arachidonate-labeled cells had no effect on

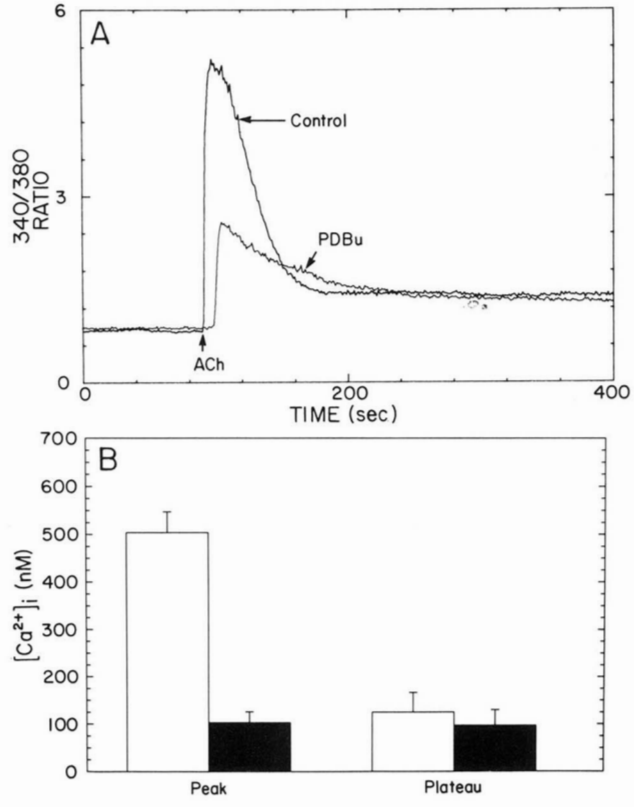


FIG. 3. **Effects of phorbol dibutyrate pretreatment on the acetylcholine-stimulated $[Ca^{2+}]_i$ response.** *Panel A*, in a typical experiment, C62B cells loaded with fura-2 were stimulated with acetylcholine (*ACh*), washed, treated with 200 nM PDBu for 20 min, and then restimulated with acetylcholine. The two responses have been superimposed with the same time for agonist addition as indicated. *Panel B*, calculated $[Ca^{2+}]_i$ values from multiple experiments are shown at the peak of the response in $Ca^{2+}$-free medium (*peak*) and at 5 min after acetylcholine addition in 1.3 mM external $Ca^{2+}$ (*plateau*), in the presence (*solid bars*) or absence (*blank bars*) of PDBu pretreatment. The data are means ± S.E. of control and treated responses from the same cells in five or six separate experiments in each type of medium.

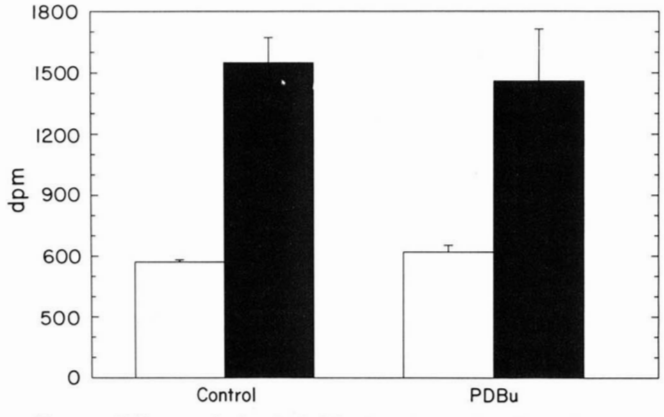


FIG. 4. **Effects of phorbol dibutyrate pretreatment on arachidonic acid liberation.** C62B cell cultures prelabeled with [$^{14}$C] arachidonate were incubated for 20 min in the presence or absence of 200 nM PDBu and then treated with acetylcholine (*solid bars*) or buffer (*blank bars*) for 90 s. Unesterified [$^{14}$C]arachidonic acid was assayed by TLC as described under "Methods." Each data value, presented as radioactivity/lane on the TLC plate, is the mean ± S.E. of three experiments performed in duplicate or triplicate.

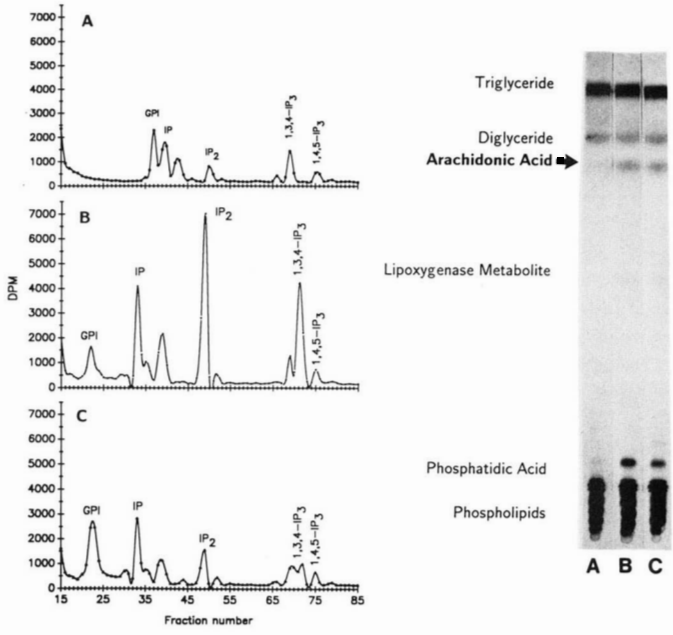


FIG. 5. **Effects of phorbol dibutyrate pretreatment on inositol phosphate formation and arachidonate liberation in double-labeled C62B cells.** Cell cultures were prelabeled with [$^{14}$C] arachidonate and [$^3$H]inositol for 24 h and then treated with either buffer for 90 s (*sample A*), acetylcholine for 90 s (*sample B*), or 200 nM phorbol dibutyrate for 20 min followed by acetylcholine for 90 s (*sample C*). The cells were extracted, inositol phosphates in the aqueous phases were separated by HPLC, and arachidonic acid in the organic phases was separated from other lipids by TLC as described under "Methods." HPLC traces (*left*) and TLC autoradiographs (*right*) are shown for each sample. Similar results were obtained with two other sets of samples. *GPI*, glycerophosphoinositol; *IP*, inositol phosphate; *$IP_2$*, inositol bisphosphate.

basal or acetylcholine-stimulated arachidonate liberation (Fig. 4, $p > 0.5$). As a control, C62B cells were double labeled with [$^{14}$C]arachidonate and [$^3$H]inositol to allow assessment of inositol phosphate formation and arachidonate liberation in the same samples. PDBu pretreatment was found to inhibit agonist-stimulated inositol phosphate formation without altering arachidonate liberation (Fig. 5). Total inositol phosphate formation was inhibited by 81 ± 3% ($n = 3$) under these conditions.

*$Ca^{2+}$ Influx Is Tightly Coupled to Acetylcholine-stimulated Arachidonate Liberation*—[$^{14}$C]Arachidonate labeling was used with the $Ca^{2+}$-free medium protocol described above to examine the role of extracellular $Ca^{2+}$ in phospholipase $A_2$ activation. In the presence of external $Ca^{2+}$, arachidonate accumulation peaks 60–90 s after acetylcholine addition and returns to basal values by 3 min (29). Switching C62B cells to $Ca^{2+}$-free medium with 0.5 mM EGTA 30 s prior to agonist addition completely blocks stimulated arachidonate accumulation (Fig, 6, $p < 0.001$, $n = 7$ experiments performed in triplicate). If the inhibited cells are then switched back to medium containing 1.3 mM $Ca^{2+}$ to restore $Ca^{2+}$ influx (Fig. 2), cholinergic arachidonate liberation is also recovered (Fig. 6). Arachidonate accumulation at 3 min under these conditions was not significantly different from control 90-s stimulated levels ($p > 0.5$, $n = 4$ experiments performed in triplicate). Media switches alone had no effect on basal arachidonate levels.

*Verapamil Pretreatment Inhibits Acetylcholine-stimulated $Ca^{2+}$ Influx and Arachidonate Liberation without Affecting Intracellular $Ca^{2+}$ Release*—Verapamil, a voltage-dependent $Ca^{2+}$ channel blocker (39, 40), was used to test further the

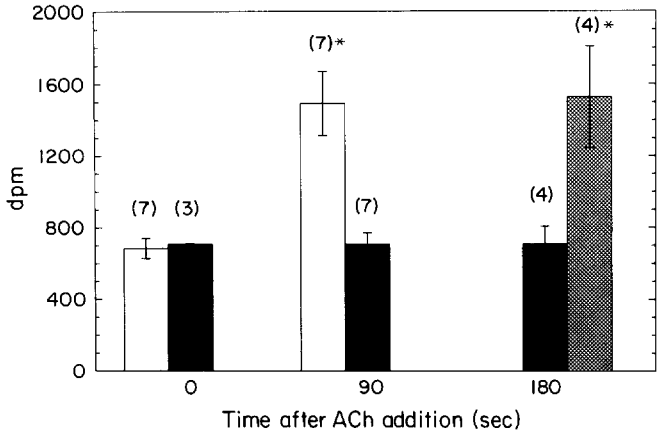


FIG. 6. **Effects of external $Ca^{2+}$ on acetylcholine-stimulated arachidonic acid liberation.** Cultures of C62B cells prelabeled with [$^{14}$C]arachidonate were incubated with acetylcholine (*ACh*) for the times indicated above in medium with 1.3 mM external $Ca^{2+}$ (*blank bars*), $Ca^{2+}$-free medium with 0.5 mM EGTA (*solid bars*), or $Ca^{2+}$-free medium for 90 s followed by a switch with 1.3 mM external $Ca^{2+}$ (*hatched bar*). Data for unesterified arachidonate are presented as in Fig. 4. Each value is the mean ± S.E. of three to seven experiments performed in triplicate, with the number of experiments given in *parentheses*. Values indicated by *asterisks* are significantly different from all others at $p < 0.001$. The *error bars*, which are shown for the raw data, are smaller when the values are normalized within each experiment.

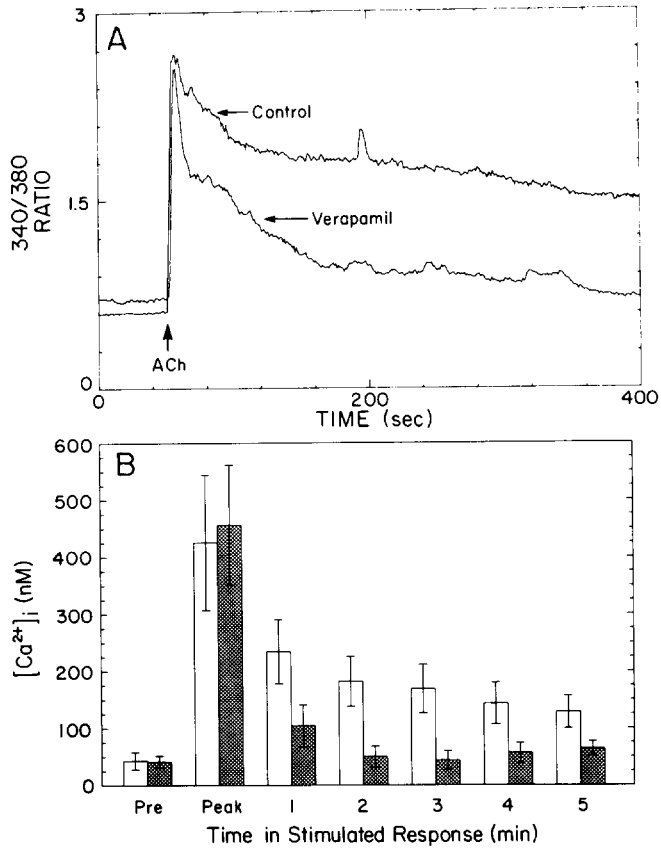


FIG. 7. **Effects of verapamil pretreatment on the acetylcholine-stimulated $[Ca^{2+}]_i$ response.** *Panel A*, fura-2-loaded C62B cells were stimulated with acetylcholine (*ACh*) in the presence of 1.3 mM external $Ca^{2+}$, rinsed, treated with 250 μM verapamil for 21.5 min, and restimulated with acetylcholine in the same medium. The two responses are superimposed with the same time for agonist addition as indicated. *Panel B*, $[Ca^{2+}]_i$ values from multiple experiments are shown for responses before (*blank bars*) and after (*hatched bars*) verapamil treatment. Data are presented as in Fig. 1; the values are means ± S.E. of control and treated responses from the same cells in four separate experiments.

relationship between $Ca^{2+}$ influx and phospholipase $A_2$ activation. Pretreatment of fura-2 loaded C62B cells with 250 μM verapamil for 20–30 min inhibited the influx-dependent plateau phase of the acetylcholine-stimulated $[Ca^{2+}]_i$ response ($p < 0.05$) without altering initial peak $[Ca^{2+}]_i$ levels (Fig. 7). The resulting $[Ca^{2+}]_i$ response resembles that obtained in the absence of extracellular $Ca^{2+}$, with a return to basal levels within 2 min of agonist addition (Figs. 7*B* and 2*C*). Pretreatment of [$^{14}$C]arachidonate-labeled cells under similar conditions produced a corresponding inhibition of acetylcholine-stimulated arachidonate liberation (Fig. 8, $p < 0.001$).

## DISCUSSION

*Acetylcholine Produces a Biphasic $[Ca^{2+}]_i$ Response*—Muscarinic stimulation of C62B rat glioma cells produces a biphasic $[Ca^{2+}]_i$ response, a rapid peak in $[Ca^{2+}]_i$ followed by a sustained elevation, similar to that seen in other systems with receptors coupled to phospholipase C (3, 41). The two phases of the response have been linked to the mobilization of intracellular $Ca^{2+}$ stores and the opening of plasma membrane $Ca^{2+}$ channels, respectively (3, 6), and our data support this interpretation. A temporal comparison of acetylcholine-stimulated changes in $[Ca^{2+}]_i$ and arachidonate accumulation does not support the idea that phospholipase $A_2$ activity in C62B cells is regulated solely by changes in overall cytoplasmic $Ca^{2+}$ levels. Although $[Ca^{2+}]_i$ peaks within 10 s of agonist stimulation and then declines to a sustained plateau level after about 1 min (Fig. 1), arachidonate accumulation has been shown to increase slowly over the first min and peak at 60–90 s (29). Furthermore, $[Ca^{2+}]_i$ remains elevated in the continued presence of agonist and external $Ca^{2+}$, whereas phospholipase $A_2$ activation is a transient event under these conditions with arachidonate levels returning to baseline by 3 min (29).

*Phorbol Dibutyrate Inhibits the Intracellular $Ca^{2+}$ Response without Blocking $Ca^{2+}$ Influx or Phospholipase $A_2$ Activation*—Internal $Ca^{2+}$ release is believed to result from the binding of 1,4,5-$IP_3$ to specific intracellular receptors (3, 4, 7, 42), and the time course of the intracellular $Ca^{2+}$ response in C62B

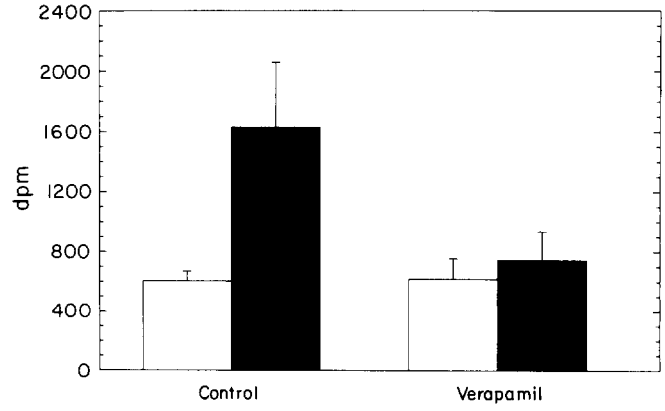


FIG. 8. **Effects of verapamil pretreatment on arachidonic acid liberation.** C62B cell cultures prelabeled with [$^{14}$C]arachidonate were incubated for 20–30 min in the presence or absence of 250 μM verapamil and then treated with acetylcholine (*solid bars*) or buffer (*blank bars*) for 90 s. Unesterified arachidonate was assayed, and the data are presented as in Fig. 4; each value is the mean ± S.E. of three experiments performed in triplicate.

cells (Fig. 2) is consistent with changes in 1,4,5-$IP_3$ levels determined previously (31). PDBu is an effective inhibitor of acetylcholine-stimulated inositol phosphate formation in C62B cells (37), and we therefore used PDBu to test the relationship among inositol phosphate formation, intracellular $Ca^{2+}$ release, and phospholipase $A_2$ activation. As in other systems (*e.g.* 43, 44), pretreatment with PDBu substantially inhibited the peak stimulated $[Ca^{2+}]_i$ response in C62B cells (Fig. 3). This result is probably a direct result of decreased 1,4,5-$IP_3$ formation since submaximal doses of agonist or 1,4,5-$IP_3$ have been shown to produce corresponding submaximal increases in $[Ca^{2+}]_i$ and only fractional release of accessible internal $Ca^{2+}$ stores (42). It is also possible, however, that the phorbol ester may be affecting $Ca^{2+}$ release at the level of the 1,4,5-$IP_3$-operated channel (45). The second phase of the $[Ca^{2+}]_i$ response was not substantially affected by PDBu, suggesting that $Ca^{2+}$ influx is far less sensitive than intracellular $Ca^{2+}$ release to stimulated changes in inositol phosphate levels. This result may reflect regulation of $Ca^{2+}$ influx by protein kinase C or G proteins (6, 46–48) rather than inositol phosphate metabolites (3, 7). PDBu pretreatment also had no effect on stimulated arachidonate liberation under conditions that depressed inositol phosphate formation and internal $Ca^{2+}$ release (Figs. 4 and 5), demonstrating that normal 1,4,5-$IP_3$ production and $[Ca^{2+}]_i$ increases are not required for phospholipase $A_2$ activation in the presence of normal $Ca^{2+}$ influx. We cannot rule out the possibility, however, that a small amount of intracellular $Ca^{2+}$ release or a sustained elevation in $[Ca^{2+}]_i$ is required for phospholipase $A_2$ activation. Although PDBu has been found to increase basal and stimulated arachidonate liberation in other systems (17, 18), it failed to alter these parameters significantly in C62B cells.

*$Ca^{2+}$ Influx Is Tightly Coupled to Phospholipase $A_2$ Activation*—Since PDBu did not block external $Ca^{2+}$ influx or arachidonate liberation, it was possible that such influx provided the $Ca^{2+}$ required for phospholipase $A_2$ activation. This hypothesis was tested by assessing stimulated arachidonate liberation using a $Ca^{2+}$-free medium protocol that was shown to inhibit the influx-dependent second phase of the $[Ca^{2+}]_i$ response without altering peak intracellular $Ca^{2+}$ release (Fig. 2). Acetylcholine-stimulated arachidonate accumulation was totally inhibited under such conditions (Fig. 6), demonstrating that $Ca^{2+}$ influx is required for phospholipase $A_2$ activation and, additionally, showing that normal intracellular $Ca^{2+}$ release and the resulting large changes in $[Ca^{2+}]_i$ are not sufficient to support phospholipase $A_2$ activation. Readdition of external $Ca^{2+}$ to the inhibited cells restores not only $Ca^{2+}$ influx and the sustained phase of the $[Ca^{2+}]_i$ response (Fig. 2) but also stimulated arachidonate accumulation (Fig. 6), reinforcing the conclusion that $Ca^{2+}$ influx is a crucial event in phospholipase $A_2$ activation. In addition, this latter experiment suggests that $Ca^{2+}$ influx may be involved in the timing of the transient arachidonate signal; delaying $Ca^{2+}$ influx for 90 s after agonist stimulation results in arachidonate liberation at a time (3 min) when the normal response has ended (29).

Corroborative date on the importance of external $Ca^{2+}$ in phospholipase $A_2$ activation were obtained using verapamil, an inhibitor of voltage-dependent $Ca^{2+}$ channels (39, 40). Verapamil pretreatment produced a stimulated $[Ca^{2+}]_i$ response similar to that obtained with $Ca^{2+}$-free medium; the influx-dependent phase of the response was blocked without affecting the peak intracellular $Ca^{2+}$ release (Fig. 7). Once again, these alterations in the $[Ca^{2+}]_i$ response were associated with essentially quantitative inhibition of acetylcholine-stimulated arachidonate accumulation (Fig. 8). Although verapamil is assumed to be acting directly on the voltage-dependent $Ca^{2+}$ channels in these experiments (39), we cannot rule out the possibility that the dose used affects receptor-operated channels (49) or indirectly affects the voltage-sensitive channels by interfering with $Na^+$ or $K^+$ currents (40).

The present results can be explained by a model for phospholipase $A_2$ regulation in which the enzyme is functionally associated with plasma membrane $Ca^{2+}$ channels. Activation of phospholipase $A_2$ may require $Ca^{2+}$ influx in order to generate very high $Ca^{2+}$ concentrations in the microenvironment of the enzyme, as suggested for regulation of neurotransmitter release (50). Evidence exists for localized membrane-associated increases in $[Ca^{2+}]_i$ following muscarinic stimulation of rat parotid acinar cells (51), and such a hypothesis would explain why many phospholipase $A_2$ enzymes require millimolar $Ca^{2+}$ concentrations for maximal *in vitro* activation (*e.g.* 13, 25, 26). Phospholipase $A_2$ may be permanently associated with the membrane near $Ca^{2+}$ channels or may bind to the membrane as a consequence of $Ca^{2+}$ influx during activation (52). The $Ca^{2+}$ released into the cell from sequestered intracellular stores is assumed to be functionally uncoupled from the enzyme by virtue of its location.

This model is consistent with the observed phospholipase $A_2$ requirement for extracellular $Ca^{2+}$ in other systems (*e.g.* 11, 17, 18). In addition, it may help to explain the discrepancy between $[Ca^{2+}]_i$ changes and phospholipase $A_2$ activation noted with different agonists in platelets (53), and the recent demonstration of temperature-dependent dissociation of bradykinin-stimulated 1,4,5-$IP_3$ and lysophosphatidylinositol formation in endothelial cells (54). Although it does conflict with experiments showing inhibition of arachidonate liberation with 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoic acid (11, 28), an inhibitor of intracellular $Ca^{2+}$ release (55), recent studies have indicated that this compound may block phospholipase $A_2$ in a $Ca^{2+}$-independent manner (19). Work by Balsinde *et al.* (56) in human neutrophils has suggested that cell compartmentalization may play a role in phospholipase $A_2$ regulation, demonstrating preferential ionophore-induced activation of the enzyme within an undefined intracellular region. Interestingly, this same study found that plasma membrane-associated phospholipase $A_2$ used phosphatidylinositol preferentially as substrate, as does the C62B cell enzyme (31).

We would stress that this model does not preclude a coincident role for G proteins (10–12), protein kinase C (16–19), lipocortins (20, 21), $Na^+/H^+$ exchange (22), or other factors in phospholipase $A_2$ regulation. In fact, since $Ca^{2+}$ influx does not desensitize in C62B cells following acetylcholine stimulation and arachidonate liberation does, one or more of these factors may be responsible for terminating the enzyme's action in this system. Our results strongly suggest, however, that $Ca^{2+}$ influx is required for muscarinic receptor activation of phospholipase $A_2$ in these cells.

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