Cytosolic phospholipase A_2 is coupled to muscarinic receptors in the human astrocytoma cell line 1321N1: characterization of the transducing mechanism

Yolanda BAYON*, Marita HERNANDEZ*, Andrés ALONSO*, Lucía NUÑEZ*, Javier GARCIA-SANCHO*, Christina LESLIE†, Mariano SANCHEZ CRESPO*‡ and María Luisa NIETO*

*Instituto de Biología y Genética Molecular, Universidad de Valladolid-CSIC, 47005-Valladolid, Spain, and †Division of Basic Science, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206, U.S.A.

The cholinergic agonist carbachol induced the release of arachidonic acid in the 1321N1 astrocytoma cell line, and this was blocked by atropine, suggesting the involvement of muscarinic receptors. To assess the mechanisms of signalling involved in the response to carbachol, a set of compounds characterized by eliciting responses through different mechanisms was tested. A combination of 4β-phorbol 12β-myristate 13α-acetate and thapsigargin, an inhibitor of endomembrane Ca^{2+}-ATPase that induces a prolonged elevation of cytosolic Ca^{2+} concentration, induced an optimal response, suggesting at first glance that both protein kinase C (PKC) and Ca^{2+} mobilization were involved in the response. This was consistent with the observation that carbachol elicited Ca^{2+} mobilization and PKC-dependent phosphorylation of cytosolic phospholipase A_2 (cPLA_2; phosphatide *sn*-2-acylhydrolase, EC 3.1.1.4) as measured by a decrease in electrophoretic mobility. Nevertheless, the release of arachidonate induced by carbachol was unaltered in media containing decreased concentrations of Ca^{2+} or in the presence of neomycin, a potent inhibitor of phospholipase C which blocks phosphoinositide turnover and Ca^{2+} mobilization. Guanosine 5′-[γ-thio]triphosphate added to the cell-free homogenate induced both [^{3}H]arachidonate release and cPLA_2 translocation to the cell membrane fraction in the absence of Ca^{2+}, thus suggesting the existence of an alternative mechanism of cPLA_2 translocation dependent on G-proteins and independent of Ca^{2+} mobilization. From the combination of experiments utilizing biochemical and immunological tools the involvement of cPLA_2 was ascertained. In summary, these data indicate the existence in the astrocytoma cell line 1321N1 of a pathway involving the cPLA_2 which couples the release of arachidonate to the occupancy of receptors for a neurotransmitter, requires PKC activity and G-proteins and might operate in the absence of Ca^{2+} mobilization.

INTRODUCTION

Phospholipase A_2 (PLA_2)-catalysed reactions occur in the nervous system in response to different stimuli, and it has been suggested that there is an association between the reduction of this activity and some pathophysiological conditions, including changes in Alzheimer's-disease brains [1]. However, in spite of the recent molecular characterization of the main types of PLA_2, there has been a limited number of studies directed to relate the cell type(s) involved in arachidonate metabolism with the type(s) of PLA_2 expressed in the nervous system. This seems of interest in view of the general agreement that the recently cloned and sequenced 85 kDa cytosolic PLA_2 (cPLA_2) is thought to play a central role in the release of arachidonic acid (AA) triggered by hormones and growth factors [2–6]. Current views regarding the regulation of cPLA_2 stress the requirement for two independent events, namely Ca^{2+}-dependent translocation to the cell membrane [4] and activation by phosphorylation of Ser^{505} by members of the mitogen-activated protein kinase (MAP kinase) family [7]. Stephenson et al. [8] have shown that the expression of cPLA_2 is restricted to astrocytes of the grey matter. This appears relevant in view of current data showing that astrocytes are important in controlling the ionic and chemical environment of neurons. This regulation seems to be mediated, at least in part, by direct interactions of neurotransmitters, growth factors, and cytokines with glial receptors, which lead to different responses, including elevation of $[Ca^{2+}]_i$ and changes in membrane potential [9,10]. AA release in primary cultures of astrocytes has been observed in response to metabotropic glutamatergic receptors [11], and P_{2Y}-purinergic receptors [12]. It is also known that muscarinic acetylcholine receptors (mAchRs) are expressed in astrocytes [13], and activation of these receptors may be coupled to AA release in other cell types [14–16]. Generation of PLA_2 products by astrocytes stimulated by neurotransmitters could be of functional importance for the generation of diffusible messengers that could reach presynaptic terminals and play a physiological role in long-term potentiation [17].

The 1321N1 astrocytoma cell line has been widely studied in terms of both cytoplasmic and nuclear responses to the occupancy of receptors, since it displays thrombin and muscarinic acetylcholine M_3 receptors [18]. The pattern of responses elicited by ligand binding in these cells includes Ca^{2+} mobilization and phosphoinositide hydrolysis [19,20] induction of AP-1 transcriptional activity [21,22] and hydrolysis of choline-phosphoacylglycerols via phospholipase D (PLD) [23,24]. However, the hydrolysis of arachidonoyl-containing phosphoacylglycerols, the type(s) of enzyme(s) involved, and its coupling to hormonal receptors have not been studied. Here we show that

Abbreviations used: AA, arachidonic acid; A23187, calcium ionophore A23187; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; (c)PLA_2, (cytosolic) phospholipase A_2; G-protein, guanine nucleotide-binding protein; GTP[γS], guanosine 5′-[γ-thio]triphosphate; GTP[βS], guanosine 5′-[β-thio]triphosphate; mAchR, muscarinic acetylcholine receptor; MAP kinase, mitogen-activated protein kinase; PKC, protein kinase C; PL, phospholipase; PMA, 4β-phorbol 12β-myristate 13α-acetate; PTX, pertussis toxin

‡ To whom correspondence should be addressed.

carbachol induces AA release, and this is consistent with coupling to activation of $cPLA_2$. Interestingly, AA release could be dissociated under some circumstances from an increase in $[Ca^{2+}]_i$, suggesting alternative mechanisms for $cPLA_2$ activation linked to interaction with G-proteins.

EXPERIMENTAL

Materials

Thapsigargin was from Calbiochem, La Jolla, CA, U.S.A. Fura-2/AM was purchased from Molecular Probes, OR, U.S.A. 4β-Phorbol 12β-myristate 13α-acetate (PMA) and *Pertussis* toxin were from Sigma Chemical Co., St. Louis, MO, U.S.A. Atropine and carbachol were from Aldrich Quimica, Madrid, Spain.

1-Stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H]arachidonoyl-*sn*-glycero-3-phosphocholine (132 Ci/mmol), and [^{3}H]AA (100 Ci/mmol) were from du Pont–New England Nuclear. Polyclonal antibody to $cPLA_2$ was prepared as previously described [25]. The specific inhibitor of protein kinase C (PKC), RO 31-8220 [26], was provided by Dr. T. J. Hallam, Roche Research Centre, Welwyn Garden City, Herts., U.K.

Assay of PLA_2 activity

Cytosolic PLA_2 activity was measured in the cytosolic fraction from cell homogenates as described [2]. The medium was removed and 1321N1 cells were washed with ice-cold buffer containing 2 mM EGTA. The cells were then scraped into a medium containing 10 mM Hepes, 2 mM EGTA, 0.34 M sucrose, 10 % (v/v) glycerol, 10 μg/ml leupeptin, and 1 mM PMSF, pH 7.4; and then sonicated in an ice bath for 10 s. The homogenate was centrifuged at 100000 ***g*** for 60 min, and the supernatant was used for PLA_2 activity assay using sonicated dispersions of phospholipid substrate. The reaction was carried out in a volume of 100 μl, containing 30 μM 1-stearoyl-2-[^{3}H]arachidonoyl-phosphocholine ($\approx$ 50000 d.p.m./assay), 9 mM dioleoylglycerol, 150 mM NaCl, 5 mM $CaCl_2$, 1 mg/ml BSA and 50 mM Tris, pH 7.4. The incubation was initiated by the addition of the 100000 ***g*** supernatant ($\approx$ 25 μg of protein) and maintained in a water bath at 37 °C for 30 min. The reaction was terminated by the addition of 1 ml of chloroform/acidified methanol, followed by Bligh and Dyer extraction [27]. The unchanged substrate and the product were separated by TLC in the system benzene/hexane/diethyl ether (50:45:4, by vol.). The fractions containing [^{3}H]AA and the substrate were scraped from the plate and the amount of radioactivity in each was measured using liquid-scintillation spectrometry.

Type II secreted PLA_2 was assayed in the 100000 ***g*** membrane and cytosol fractions by incubation with $\approx$ 5000 d.p.m. of [^{14}C]oleate-labelled autoclaved *E. coli*, containing 10–20 nmol of phospholipid [28]. Briefly, the assay medium contained 0.1 M Tris/HCl, 1 mg/ml fatty-acid-free BSA and 0.5 mM $CaCl_2$, pH 7.4. The reaction was allowed to proceed for 30 min and was terminated by the addition of 0.04 ml of ice-cold 2 M HCl and 0.02 ml of 10 % BSA, followed by centrifugation for 5 min at 13000 rev./min (10000 ***g***) in an Eppendorf microcentrifuge. The radioactivity released into the supernatant was assayed by liquid-scintillation counting.

Cell culture and metabolic labelling with [^{3}H]AA

1321N1 astrocytoma cells were cultured in DMEM containing 5 % fetal-calf serum at 37 °C in an atmosphere containing 5 % CO_2. Labelling with [^{3}H]AA acid was performed in cells in monolayer at a concentration of $\approx 5 \times 10^6$ cells/35 mm dish that had been deprived of fetal-calf serum for 16 h to render them quiescent. After testing several protocols, labelling was carried out for 2 h after quiescing in the presence of 0.3 μCi of [^{3}H]AA in 1 ml of medium. After labelling, cells were washed four or five times with medium at 37 °C containing 0.25 % essentially fatty-acid-free BSA, and finally allowed to equilibrate at 37 °C before addition of stimuli. The release of [^{3}H]AA was measured in 200 μl aliquots of culture medium, omitting Bligh and Dyer extraction and TLC separation, since initial experiments showed the absence of significant radioactivity other than that associated to non-esterified AA. Experiments were carried out with triplicate samples.

Measurements of the cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured as previously described [29]. Briefly, cells attached to polylysine-coated glass coverslips were incubated with 4 mM fura-2/AM for 60 min at room temperature in a solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1.8 mM glucose, 10 mM Hepes, pH 7.4. The coverslips were then mounted in the chamber of a fluorescence spectrophotometer (Cairn Research Ltd., Newnham, Sittingbourne, Kent, U.K) that allowed rapid alternation (30–300 Hz) of excitation light of up to six different wavelengths. Fluorescence emission was measured at 530 nm and integrated at 1 s intervals. $[Ca^{2+}]_i$ was computed from the ratio of the fluorescence excited at 340 and 380 nm [30]. The system allowed rapid exchange of perfusing solutions. The temperature was 36 °C.

Permeabilization of cells

Permeabilization to allow the entry of guanosine 5′-[γ-thio]triphosphate (GTP[γS]) in [^{3}H]AA-labelled 1321N1 cells, was performed in cells attached to polylysine-coated glass coverslips to prevent the detachment that is elicited by the elimination of Ca^{2+} from the culture medium. The cells were labelled under standard conditions, and then the DMEM medium was replaced with an intracellular buffer containing KCl (110 mM), NaCl (10 mM), KH_2PO_4 (1 mM), Hepes (20 mM), $MgCl_2$ (4 mM) and EGTA (1 mM). Saponin (20 μg/ml) was added to the buffer [31], and after 10 min the medium was changed to DMEM with 0.1 mM Ca^{2+} and 100 μM of either GTP[γS] or guanosine 5′-[β-thio]triphosphate (GTP[βS]). After 15 min the culture medium was removed to assay the release of [^{3}H]AA.

Immunoblotting

Cell lysates from preconfluent 1321N1 cells deprived of calf serum were loaded on to an SDS/10 %-polyacrylamide gel and transferred to poly(vinylidene difluoride) membrane (Immobilon P; Millipore Corp., Bedford, MA, U.S.A.) using a liquid-transfer module from CBS Scientific Company Inc., Del Mar, CA, U.S.A. The membranes were blocked with dry milk for 2 h, washed with Tris-buffered saline and used for immunoblotting using a rabbit polyclonal anti-$cPLA_2$ antibody. Detection was performed using the Amersham ECL system. Densitometric scanning of the blots was performed using equipment from pdi-Pharmacia with software of the series Discovery 3.0.

RESULTS

$cPLA_2$ accounts for PLA_2 activity in 1321N1 cells

Cytosol from 1321N1 cells contained PLA_2 activity which hydrolysed AA, whereas no hydrolysis was detectable when the substrate was [^{14}C]oleate-labelled autoclaved *Escherichia coli*,

Table 1 PLA_2 activity in 1321N1 cells is inhibited by anti-$cPLA_2$ antibody

Cells were incubated in the presence of 0.1% DMSO (control), 1 μM A23187 and 32 nM PMA and 0.5 mM carbachol for 30 min at 37 °C, and then homogenized. The supernatant obtained after centrifugation of cell homogenates at 100 000 ***g*** was used for the assay of enzyme activity using [^{3}H]AA-labelled substrate as described in the Experimental section. A value of 100% corresponds to a PLA_2 activity of 0.411 pmol of AA/min per mg of protein. In the experiments shown in the respective columns, either rabbit anti-$cPLA_2$ or rabbit preimmune serum were added and incubated for 1 h at 4 °C before PLA_2 assay. Data represent mean ± S.E.M. for six experiments or means ± S.E.M. for three experiments in the presence of antibodies or preimmune serum. $^*P < 0.05$ as compared with the control.

	PLA_2 activity (% of control)		
Treatment	No addition	Anti-$cPLA_2$ antibody	Pre-immune serum
Control	100 ± 16	3 ± 0	93 ± 21
PMA + A23187	163 ± 19	–	–
Carbachol	188 ± 22	4 ± 0	186 ± 14

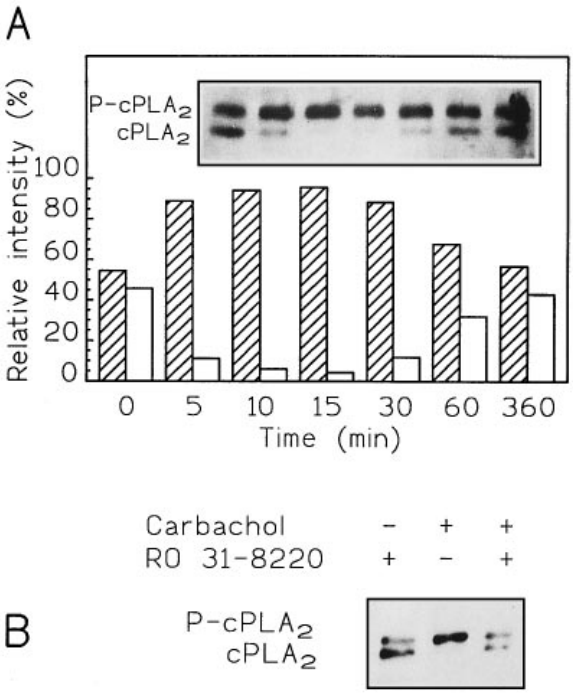

Figure 1 Immunochemical identification of $cPLA_2$

(**A**) 1321N1 cells were stimulated with 0.5 mM carbachol for the times indicated and then lysed in appropriate buffer. A protein sample of about 100 μg was loaded in each lane and then resolved by SDS/PAGE to separate the phosphorylated (P-$cPLA_2$, hatched columns) from the non-phosphorylated form ($cPLA_2$, open columns). The proteins were electrophoretically transferred to poly(vinylidene difluoride) membranes for immunoblotting with anti-$cPLA_2$ antibody. Quantitative analysis of the protein was obtained by densitometric scanning and is expressed as percentage of total protein distributed in each band. (**B**) Cells were incubated for 30 min in the presence of 5 μM RO 31-8220 or vehicle and then stimulated with carbachol for 15 min. At the end of this period samples were taken for immunoblotting with anti-$cPLA_2$ antibody.

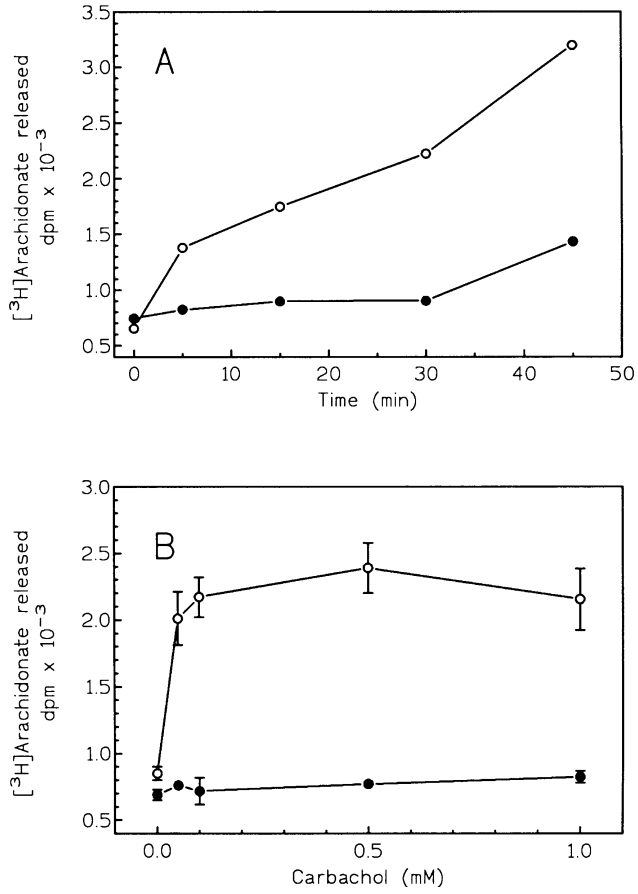

Figure 2 Release of [^{3}H]AA in response to carbachol and blockade by atropine

(**A**) 1321N1 cells were incubated in the presence of 0.5 mM carbachol (○) or vehicle (●) for different times. Data represent mean ± S.E.M. for three experiments. (**B**) 1321N1 cells were incubated with different concentrations of carbachol for 30 min in the absence (○) or presence of 10 μM atropine (●). Data represent mean ± S.E.M. for three independent experiments.

and the membrane fraction was used for the assay (not shown). This is a strong argument against the involvement of type II PLA_2. PLA_2 activity in cytosols from cells treated with stimuli consistently showed a near-twofold increase of the specific activity (Table 1). A complete blockade of the PLA_2 activity was produced by incubating cytosol from stimulated 1321N1 cells with polyclonal anti-$cPLA_2$ for 1 h at 4 °C. The activation of $cPLA_2$ in the astrocytoma cells was also evaluated by determining whether cell stimulation resulted in a retardation of its mobility in SDS/polyacrylamide gels. This gel shift is characteristic of $cPLA_2$ that has been phosphorylated by MAP kinase [7]. In resting cells, 54% of the immunoreactive protein appeared as the retarded phosphorylated band form, whereas addition of carbachol induced a time-dependent increase of the phosphorylated enzyme, which remained for at least 60 min (Figure 1A).

Release of [^{3}H]AA by 1321N1 astrocytoma cells: involvement of PKC-related mechanisms

Carbachol induced a time- and dose-dependent release of [^{3}H]AA into the culture medium which reached a plateau after 30–45 min. As the spontaneous release appeared to increase at 45 min, we selected an incubation of 30 min to obtain an optimal signal-to-noise ratio (Figure 2A). [^{3}H]AA release was completely blocked by 10 μM atropine (Figure 2B), which indicates involvement of mAchRs. Stimuli such as PMA, ionophore A23187 (A23187) and thapsigargin which produce AA release in other cell types [32–35] were also tested alone or in combination in 1321N1 cells.

Table 2 Effect of different additions on [^{3}H]AA release by 1321N1 astrocytoma cells

Cells were incubated for 30 min in the presence of 0.5 mM carbachol, 32 nM PMA, 1 μM A23187, 200 nM thapsigargin or a combination of stimuli. Results represent means ± S.E.M. for seven to nine independent experiments in triplicate. *Indicates $P < 0.05$ as compared with the control; §$P < 0.05$ as compared with the effect of both carbachol and PMA + A23187.

Stimulus	[^{3}H]AA released (d.p.m.)
Control	867 ± 12
Carbachol	2356 ± 119*
PMA	885 ± 140
A23187	911 ± 23
Thapsigargin	1238 ± 147
PMA + A23187	1990 ± 124*
PMA + thapsigargin	2752 ± 121*§

Table 3 Effect of PKC down-regulation on [^{3}H]AA release induced by carbachol and AlF_4^-

Cells were incubated with 1 mM PMA or vehicle for 18 h prior to the addition of 0.5 mM carbachol or AlF_4^-. [^{3}H]AA release was assayed 30 min after addition of the stimuli. AlF_4^- was obtained by addition of 10 mM NaF and 20 μM $AlCl_3$. *Indicates $P < 0.05$ as compared with the results obtained in the absence of PMA. §$P < 0.05$ as compared with the effect of either compound alone. Data represent mean ± S.E.M. for eight experiments in triplicate.

	[^{3}H]AA released (d.p.m.)	
Stimulus	−PMA	+PMA
Control	815 ± 11	873 ± 13
AlF_4^-	2016 ± 144	2187 ± 152
Carbachol	2236 ± 157	825 ± 17*
AlF_4^- + carbachol	3029 ± 178§	2238 ± 219

As shown in Table 2, A23187, thapsigargin and PMA, when tested alone had no (or minimal) effect on AA release. PMA induced AA release only when used in combination with agonists which promote Ca^{+2} mobilization, the combination of PMA and thapsigargin being more active than PMA together with A23187. Release of [^{3}H]AA by thapsigargin and PMA was only observed in the presence of extracellular Ca^{2+}, which is at variance with the results obtained with carbachol (see below). Long-term treatment with PMA, which has been found to down-regulate PKC in this cell [23], produced a complete blockade of [^{3}H]AA release (Table 3), whereas it did not influence the effect of AlF_4^-. Moreover, the specific PKC inhibitor RO 31-8220 blocked the bandshift of $cPLA_2$ produced by carbachol (Figure 1B). Taken together, these data suggest that both PKC activation and Ca^{2+} mobilization are signalling mechanisms that should operate in concert to produce [^{3}H]AA release, although they are not conclusive about the participation of these mechanisms in response to all the stimuli.

Mobilization of Ca^{2+} in 1321N1 astrocytoma cells

Both carbachol and thapsigargin produced an increase of $[Ca^{2+}]_i$ which was composed of a steep rise followed by a sustained plateau (Figures 3A and 3C). These two phases are likely to correspond to the release of Ca^{2+} from the intracellular Ca^{2+} stores followed by entry through a capacitative Ca^{2+} pathway in the plasma membrane, which is activated on emptying the Ca^{2+} stores, and has been reported in 1321N1 astrocytoma cells [36]. The effect of carbachol was prevented by treatment with PMA for 2–5 min (Figure 3B). Pretreatment with PMA had little or no

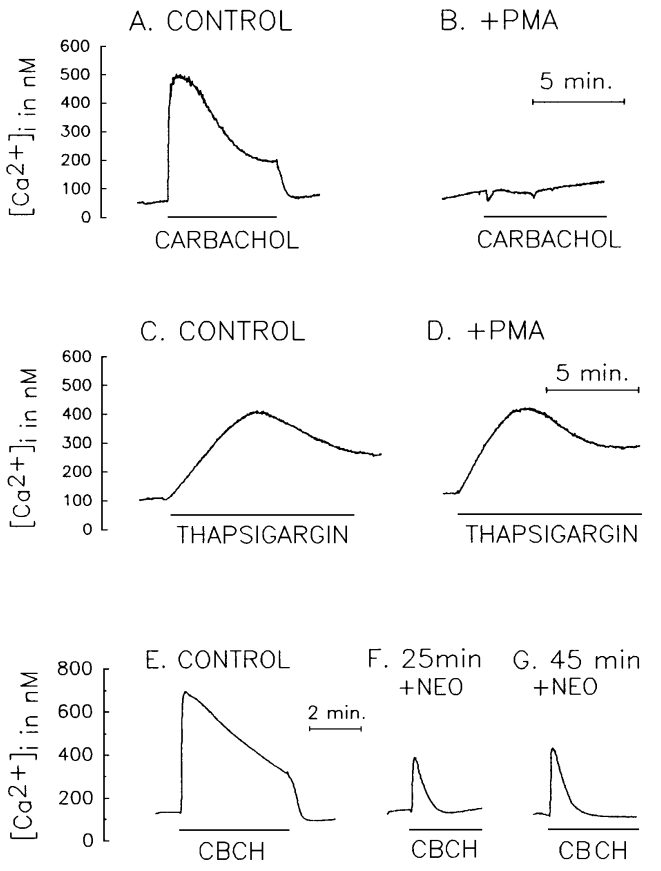

Figure 3 Effects of carbachol (CBCH, 0.5 mM) and thapsigargin (200 nM) on $[Ca^{2+}]_i$: blocking by treatment with PMA and neomycin

In the panels on the left, cells were stimulated with the stimuli as indicated. In the panels on the right, cells from the same batch were treated either with 32 nM PMA for 5 min (**B** and **D**) or with 1 mM neomycin for 25–45 min (NEO, **F** and **G**) prior to the addition of the stimuli. Each experiment is representative of three similar ones. (**A**), (**C**) and (**E**) are controls.

effect on the Ca^{2+} mobilization induced by thapsigargin (Figure 3D). The above results would be consistent with a role for $[Ca^{2+}]_i$ increase on the stimulation of AA release produced by carbachol, as in parallel experiments this was completely blocked by a 2 min treatment with PMA (310 ± 15% of control versus 98 ± 2% of control produced by carbachol in the presence of PMA, $n = 5$). However, several conditions allowed dissociation between the $[Ca^{2+}]_i$ increase and the AA release in response to carbachol. Thus preincubation of the cells for 20–40 min with neomycin, a blocker of PLC [37], inhibited the $[Ca^{2+}]_i$ increase induced by carbachol (Figure 3E), but had little effect on [^{3}H]AA release (Table 4). In four similar experiments the $[Ca^{2+}]_i$ increase induced by carbachol (estimated as the mean area under the $[Ca^{2+}]_i$ peak) was inhibited by 85 ± 5% (mean ± S.E.M.) when an integration period of 90 s following stimulation with the agonist was chosen and by 93 ± 3% using a 3 min integration period. The initial $[Ca^{2+}]_i$ rise was inhibited less than the plateau by neomycin (50–60%; Figures 3E–3G). This rise could be due to a transient Ca^{2+} influx elicited by carbachol not sensitive to neomycin. Consistently, stimulation by carbachol in a medium containing 0.1 mM Ca^{2+} and neomicyn completely blocked Ca^{2+} transient (results not shown). As should be expected, neomycin did not prevent the $[Ca^{2+}]_i$ increase induced by thapsigargin (results not shown). Modifications of the external Ca^{2+} concentrations (from

Table 4 [^{3}H]AA release induced by carbachol in media with a defined Ca^{2+} concentration and in the presence of either neomycin or thapsigargin

1321N1 cells were incubated for 15 min in the standard medium containing 1.8 mM $CaCl_2$ or in medium supplemented with EDTA to obtain the indicated Ca^{2+} concentrations. At the end of this period, the agonists were added and the release of [^{3}H]AA assessed 30 min thereafter. §1321N1 cells were preincubated for 20 min with either drug before the addition of carbachol or vehicle. Data are expressed as percentage of control and represent mean ± S.E.M. for three to seven independent experiments in triplicate.

	[^{3}H]AA released (% of control)	
Addition	Carbachol	Control
1.8 mM Ca^{2+}	315 ± 9	100 ± 8
0.5 mM Ca^{2+}	283 ± 12	100 ± 3
0.1 mM Ca^{2+}	284 ± 23	100 ± 4
1 mM Neomycin§ (in 1.8 mM Ca^{2+})	288 ± 15	100 ± 3
1 mM Neomycin (in 0.1 mM Ca^{2+})	291 ± 19	100 ± 6
200 nM Thapsigargin§ (in 1.8 mM Ca^{2+})	366 ± 21	100 ± 5
200 nM Thapsigargin (in 0.1 mM Ca^{2+})	325 ± 18	100 ± 7

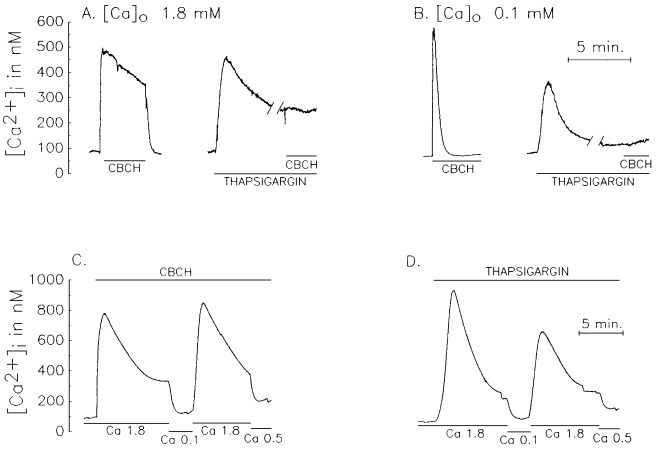

Figure 4 Effects of the extracellular Ca^{2+} concentration on the increase of $[Ca^{2+}]_i$ induced by carbachol or thapsigargin

The Ca^{2+} concentration in the incubation medium was 1.8 mM in (**A**) and 0.1 mM in (**B**). A part of the traces has been removed for convenience (-/ /-), the real time start of the treatment with thapsigargin (200 nM) to the addition of carbachol (CBCH, 0.5 mM) being 10 min. In (**C**) and (**D**) the external Ca^{2+} concentration (0.1–1.8 mM) was changed as indicated, the agonist concentration remaining constant. Experiments representative of two to four similar ones.

0.1 to 1.8 mM) produced substantial modifications of the effect of carbachol on $[Ca^{2+}]_i$ (compare A and B in Figure 4), but not on [^{3}H]AA release (Table 4). The size of the initial $[Ca^{2+}]_i$ peak was little modified, as would be expected if it is mainly due to the release of stored Ca^{2+}. In contrast, the $[Ca^{2+}]_i$ plateau that followed this rapid peak was very much decreased with the lower external Ca^{2+} concentration, as it would be expected if this plateau is due to increased Ca^{2+} entry. This interpretation is confirmed by the experiments with thapsigargin, where the $[Ca^{2+}]_i$ plateau has to be interpreted as being due to stimulation of capacitative Ca^{2+} entry. The second traces in Figures 4(A) and 4(B) show that the height of this plateau was strongly dependent on the external Ca^{2+} in thapsigargin-treated cells. The experiments shown in Figures 4(C) and 4(D) allow a more precise quantification of this point. In these experiments the cells were treated with either carbachol (Figure 4C) or thapsigargin (Figure 4D) and the external Ca^{2+} concentration was adjusted (0.1, 0.5 or 1.8 mM) to modify the steady-state $[Ca^{2+}]_i$ levels at the plateau phase. These would correspond to the $[Ca^{2+}]_i$ concentration reached at the plateau phase with different extracellular $[Ca^{2+}]$ concentrations, but eliminate the plate-to-plate variation. These steady-state concentrations were 120, 205 and 340 nM for 0.1, 0.5 and 1.8 mM external $[Ca^{2+}]$ respectively, in carbachol-treated cells (Figure 4C) and 85, 150 and 240 nM in thapsigargin-treated cells (Figure 4D). The resting $[Ca^{2+}]$ in these experiments was 70–80 nM. Finally, it is also shown that addition of carbachol to thapsigargin-treated cells suspended in either high- (Figure 4A) or low-Ca^{2+} (Figure 4B) medium did not produce further changes of $[Ca^{2+}]_i$, indicating that carbachol is unable to activate Ca^{2+}-entry pathways other than the capacitative one, which was already maximally activated because of the complete emptying of the intracellular Ca^{2+} stores induced by thapsigargin. Note that, again, these results allow dissociation of the effects of carbachol on $[Ca^{2+}]_i$ and on AA release, as the last was little dependent on the external Ca^{2+} concentration and was further increased by the agonist in thapsigargin-treated cells. The experiments directed to assess the effect of the combined addition of carbachol and thapsigargin shown in Table 4 as percentage of controls to normalize the results from different experimental conditions represent as actual values a release of 1198 ± 163 d.p.m. of [^{3}H]AA by thapsigargin, 2731 ± 77 d.p.m. by carbachol, and 4384 ± 252 d.p.m. by the combination of stimuli, thus suggesting an additive effect, which would indicate that these stimuli do not share the same signalling pathways.

Involvement of G-proteins

Since G-proteins are involved in the regulation of AA release in different cell types [38–42], 1321N1 cells were incubated with AlF_4^-, which is an activator of heterotrimeric G-proteins. This treatment induced [^{3}H]AA release of a magnitude not significantly different from that elicited by carbachol (Table 3). When AlF_4^- was used in combination with carbachol, the release of [^{3}H]AA reached a mean value of 3029 ± 178 d.p.m., which is higher than that elicited by each agonist, but is below the value of 3432 that could be expected if both stimuli were additive. This suggests that a portion of the effect of carbachol is independent of the mechanism triggered by AlF_4^-. Further evidence of the possible involvement of G-proteins was obtained in homogenates of [^{3}H]AA-labelled cells. Addition of GTP[γS] to the cell-free system in the presence of 5 mM EGTA increased the release of [^{3}H]AA in a way similar to that observed after addition of $CaCl_2$ at the concentration required to attain 0.5 mM free Ca^{2+} (Figure 5A). Preincubation of the cell-free homogenate with anti-$cPLA_2$ antibody prior to the addition of either $CaCl_2$ or GTP[γS] inhibited [^{3}H]AA release (closed columns in Figure 5A). Translocation of $cPLA_2$ to the membrane was studied by immunoblotting on the fractions obtained by centrifugation for 1 h at 105000 ***g***. Again, both Ca^{2+} and GTP[γS] increased the amount of $cPLA_2$ associated with the 105000 ***g*** pellet, but not the fraction of enzyme that is phosphorylated (Figure 5B), suggesting that either mechanism might mediate $cPLA_2$ translocation in intact cell signalling without promoting phosphorylation. Addition of 100 μM GTP[γS] to [^{3}H]AA-labelled 1321N1 cells permeabilized with saponin induced a release of [^{3}H]AA of 143 ± 11 % ($n = 5$) of that observed when GTP[βS] was used as control nucleotide, which, in spite of the artificial conditions required for these experiments, also points to the ability of G-proteins to trigger [^{3}H]AA release. Attempts to characterize the G-proteins involved in coupling mAchR to $cPLA_2$ activation were performed by treatment with 100 ng/ml pertussis toxin (PTX). Under these

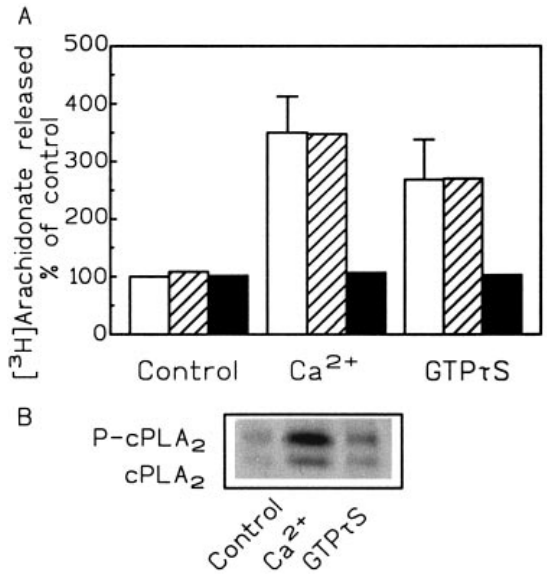

Figure 5 Effect of GTP[γS] and Ca^{2+} on [^{3}H]AA release and $cPLA_2$ translocation in the cell-free system

(**A**) Cells were labelled under standard conditions and then disrupted by sonication in medium containing 50 mM Tris, 1 mM PMSF, 5 mM EGTA, 100 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM sodium orthovanadate, pH 7.4. The homogenate was then supplemented with 3 mM $MgCl_2$ and either 100 μM GTP[γS] or $CaCl_2$ to obtain a final free Ca^{2+} concentration of 0.5 mM. The [^{3}H]AA released was assayed 30 min after the additions. Data represent means $\pm$ S.E.M. for three experiments in duplicate. In the experiment shown by the diagonal bars rabbit preimmune serum (2.8 mg of protein) was added to the cell-free homogenate and incubated for 1 hour at 4 °C prior to the addition of either $CaCl_2$ or GTP[γS]. Closed columns show the effect of rabbit anti-$cPLA_2$ antibody under the same conditions. (**B**) Cells not labeled with [^{3}H]AA were treated as above. After addition of either $CaCl_2$ or GTP[γS], the cell homogenate was centrifuged at 10000 ***g*** for 10 min to remove unbroken cells. The supernatant was collected and then ultracentrifuged at 105000 ***g*** for 1 h. The cell pellet was taken and resolved by SDS/10%-PAGE with prolonged electrophoresis to separate the phosphorylated (P-$cPLA_2$) from the non-phosphorylated form ($cPLA_2$). The proteins were electrophoretically transferred to poly(vinylidene difluoride) membranes for immunoblotting with anti-$cPLA_2$ antibody. Lanes were loaded with 50 μg of protein as indicated.

conditions neither phosphorylation of $cPLA_2$ by carbachol (not shown) nor AA release were inhibited ($297 \pm 9\%$ of control in the absence of PTX versus $303 \pm 5\%$ in the presence of PTX, $n = 3$).

DISCUSSION

The experiments presented here show the activation of $cPLA_2$ to be responsible for the mAchR-induced AA release in the 1321N1 astrocytoma cell line. This conclusion is based on: (i) the phosphorylation of this enzyme by carbachol, (ii) the blockade of this activity by anti-$cPLA_2$ antibody and (iii) the absence of type-II PLA_2 activity in 1321N1 cells. Activation of PKC and Ca^{2+} mobilization are major mechanisms regulating $cPLA_2$ by promoting phosphorylation and translocation, but there is no evidence that PKC directly phosphorylates $cPLA_2$ *in vivo*. Rather, PKC activation triggers a kinase cascade that leads to $cPLA_2$ phosphorylation at Ser^{505} by MAP kinase [7]. In our system, PMA alone failed to elicit AA release, although we found $cPLA_2$ phosphorylation measured by a decrease in electrophoretic mobility (results not shown). Interestingly, thapsigargin treatment only caused a slight increase in the amount of AA released, whereas it raised $[Ca^{2+}]_i$ to the same extent as did carbachol. On the other hand, the combination of PMA and thapsigargin results in a greater-than-additive effect, suggesting that both signals should occur simultaneously to yield efficient AA release. However, these findings could not be taken as direct evidence that these mechanisms operate upon stimulation of mAchR. Our studies demonstrate an obligate role for PKC in the stimulation of the 1321N1 astrocyte $cPLA_2$. Thus neither in cells depleted of PKC by long-term PMA treatment nor in those pretreated with the PKC inhibitor RO 31-8220 is it possible to stimulate AA release. Although an enhancement of AA release induced by PMA in cells transfected with the M_1 type of mAchR [15] has been reported, we found, contrary to that report, that PMA blocks mAchR-mediated AA release. Since the PMA concentration that blocks carbachol-stimulated AA release also blocks phosphoinositide hydrolysis, Ca^{2+} mobilization [19] and thrombin-induced AA release (M. Hernandez, Y. Bayon, M. Sanchez Crespo and M. L. Nieto, unpublished work), we suggest that the effect of PMA is on a more general component than a specific receptor. In contrast, the response to AlF_3, which does not act via receptor, is not modified. Although PMA does not affect agonist binding to the receptor and in fact inhibits internalization and down-regulation of the mAchR [43], PMA might be acting through protein phosphorylation at the receptor level or very early in the transduction. We cannot rule out the possibility that, depending on the cell system and the type of mAchR expressed, the molecular mechanisms by which signals are transduced could be different. In this way, a different behaviour has been found in PLD desensitization and recovery between fibroblasts expressing M_1 type receptor [44] and astrocytes [22]. Also, whereas the dependence on external calcium of AA release in our system is not absolute, it is in A9L cells transfected with M_1 and M_3 subtype mAchRs [14]. The fact that PMA desensitizes carbachol-mediated AA release in the same way that it blocks carbachol-mediated phosphoinositide turnover does not indicate the involvement of the G_q/PLC/InsP_3/Ca^{2+} pathway in $cPLA_2$ activation, since we were able to dissociate this pathway from $cPLA_2$ stimulation. Alternatively, a PLC acting on phospholipids other than phosphatidylinositol and/or a G_q/PLD/phosphatidate/diacylglycerol pathway that has been shown in these cells to be the major one for the generation of diacylglycerol following mAchR stimulation [20,23] could lead to the activation of a Ca^{2+}-independent PKC isoform in the absence of phosphoinositide turnover. In fact, 1321N1 cells contain a Ca^{2+}-independent isoform of PKC (PKC-ϵ) that is activated by carbachol [24].

As regards Ca^{2+} mobilization, carbachol induces Ca^{2+} release from intracellular Ca^{2+} stores, thus producing an early $[Ca^{2+}]_i$ peak. This seems to be mediated by PLC activation and is blocked by neomycin. The emptying of the stores produced by the agonist activates a capacitative Ca^{2+}-entry pathway in 1321N1 cells [36], which is responsible for the sustained plateau of $[Ca^{2+}]_i$ increase and is maintained as long as the agonist is present. This sustained $[Ca^{2+}]_i$ increase may be required for some of the physiological actions of the agonist, e.g. production of platelet-activating factor and AA release in human polymorphonuclear leucocytes [45]. However, carbachol-induced [^{3}H]AA release seems not to be related to the $[Ca^{2+}]_i$ plateau as manoeuvres which reduced or abolished it did not modify [^{3}H]AA production. It could be argued that stimulation of $cPLA_2$ could happen with minimal modifications of resting $[Ca^{2+}]_i$. Of course, this possibility cannot be excluded, but the $[Ca^{2+}]_i$ increase does not seem to be the main controller in this cell type. Incubation of astrocytoma cells in a Ca^{2+}/EGTA buffer to vary external Ca^{2+} levels was without effect on carbachol-stimulated [^{3}H]AA release, whereas it did have a major effect on carbachol-mediated $[Ca^{2+}]_i$ levels, suggesting that carbachol-stimulated entry of the ion is

not required for the activation of $cPLA_2$. Moreover, depletion of intracellular Ca^{2+} with thapsigargin in a medium containing a low concentration of calcium blocked the Ca^{2+} spike produced by subsequent addition of carbachol without modifying the ability of the agonist to release [^{3}H]AA, thus suggesting that Ca^{2+} mobilization is not involved in carbachol-induced $cPLA_2$ activation. In addition, treatment with neomycin did not interfere with carbachol's ability to elicit [^{3}H]AA release, even though Ca^{2+} mobilization was clearly inhibited. Moreover, stimulation with carbachol in the presence of neomycin in a medium with a low concentration of Ca^{+2}, which completely blocks the Ca^{2+} transient, did not influence [^{3}H]AA release. These results lead us to conclude that even though stimulation of $cPLA_2$ might need minimal modification of resting $[Ca^{2+}]_i$, the Ca^{2+}-depletion experiments along with the slight release of [^{3}H]AA observed with stimuli that only increase $[Ca^{2+}]_i$, suggest that Ca^{2+} mobilization is not the main controller in ligand-stimulated $cPLA_2$ activation in 1321N1 cells.

On this basis we propose the involvement of other signalling events in the translocation of $cPLA_2$ such as G-protein-dependent mechanisms. Direct coupling of receptors to $cPLA_2$ via G-proteins has been reported in other cell systems [38,39]. Supporting this hypothesis, we found that the G-protein activator AlF_4^- resulted in a stimulation of [^{3}H]AA release and an enhancement of the release induced by carbachol. It has also been demonstrated in vascular endothelium that AlF_4^- stimulates AA release in a Ca^{2+}-independent manner [46]. To strengthen this point, we show that exposure of the cellular homogenate to the non-hydrolysable GTP analogue GTP[γS] in the presence of EGTA initiated AA accumulation and $cPLA_2$ translocation to the cell membrane, but not its phosphorylation. This agent binds to the α-subunits of the G-protein and maintains them in the activated state. A slight effect was also observed in permeabilized cells exposed to this nucleotide. Since it has been reported that G-protein-coupled receptors interact with the PTX-insensitive G-protein G_q and/or G_{11} to stimulate PLC/PLD and PKC in 1311N1 cells [22], our findings on the lack of sensitivity of $cPLA_2$ phosphorylation and AA release to PTX would agree with the notion of these G-proteins coupling to the mAchR. In summary, we have demonstrated AA release in response to carbachol in 1321N1 cells, and its dissociation from Ca^{2+} mobilization under some circumstances. In addition, the activation of G-proteins appears to initiate AA release.

This work was supported by DGICYT grants PM92-0006 and PB92-0268. Dr. Joan Heller Brown is thanked for providing the 1321N1 astrocytoma cell line.

REFERENCES

1 Gattazz, W. F., Maras, A., Cairns, N. J., Levy, R. and Forstl, H. (1995) Biol. Psychiat. **37**, 13–17
2 Leslie, C. C., Voelker, D. R., Channon, J. Y., Wall, M. M. and Zelarney, P. T. (1988) Biochim. Biophys. Acta **963**, 476–492
3 Clark, J. D., Milona, N. and Knopf, J. L. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 7708–7712
4 Kramer, R. M., Roberts, E. F., Manetta, J. and Putnam, J. E. (1991) J. Biol. Chem. **266**, 5268–5272
5 Clark, J. D., Lin, L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N. and Knopf, J. L. (1991) Cell **65**, 1043–1051
6 Sharp, J. D., White, D. L., Chiou, X. G., Goodson, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskin, J., Skatrud, P. L., Sportsman, J. R. et al. (1991) J. Biol. Chem. **266**, 14850–14853
7 Lin, L.-L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A. and Davis, R. J. (1993) Cell **72**, 269–278
8 Stephenson, D. T., Manetta, J. V., White, D. L., Chiou, X. G., Cox, L., Gitter, B., May, P. C., Sharp, J. D., Kramer, R. M. and Clemens, J. A. (1994) Brain Res. **637**, 97–115
9 Murphy, S. and Pearce, B. (1987) Neuroscience **22**, 381–394
10 Mucke, L. and Eddleston, M. (1993) FASEB J. **7**, 1226–1232
11 Stella, N., Tencé, M., Glowinski, J. and Premont, J. (1994) J. Neurosci. **14**, 568–575
12 Bruner, G. and Murphy, S. (1990) J. Neurochem. **55**, 1569–1575
13 Enkvist, K. M. O., Holopainen, I. and Akerman, K. E. O. (1989) Brain Res. **500**, 46–54
14 Conklin, B. R., Brann, M. R, Buckley, N. J., Ma, A. L., Bonner, T. I. and Axelrod, J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 8698–8702
15 Felder, C. C., Kanterman, R. Y., Ma, A. L. and Axelrod, J. A. (1989) J. Biol. Chem. **264**, 20356–20362
16 Gurwitz D., Haring R., Heldman E., Fraser C. M., Manor, D. and Fisher, A. (1994) Eur. J. Pharmacol. **267**, 21–31
17 Herrero, I., Miras Portugal, M. T. and Sánchez Prieto, J. (1992) Nature (London) **360**, 163–166
18 Wall, S. J., Yasuda, R. P., Li, M. and Wolfe, B. B. (1991) Mol. Pharmacol. **40**, 783–789
19 Orellana, S., Solski, A. and Heller Brown, J. (1985) J. Biol. Chem. **260**, 5236–5239
20 Martinson, E. A., Goldstein, D. and Heller Brown, J. (1989) J. Biol. Chem. **264**, 14748–14754
21 Trejo, J. A. and Heller Brown, J. (1991) J. Biol. Chem. **266**, 7876–7882
22 LaMorte, V. J., Kennedy, E. D., Collins, L. R., Goldstein, D., Harootunian, A. T., Heller Brown, J. and Feramisco, J. R. (1993) J. Biol. Chem. **268**, 19411–19415
23 Martinson, E. A., Trilivas, I. and Heller Brown, J. (1990) J. Biol. Chem. **265**, 22282–22287
24 Nieto, M. L., Kennedy, E., Goldstein, D. and Heller Brown, J. (1994) Mol. Pharmacol. **46**, 406–413.
25 deCarvalho, M. S., McCormack, F. X. and Leslie, C. C. (1993) Arch. Biochem. Biophys. **306**, 534–540
26 Fiztke, P. D. (1991) Biochem. Biophys. Res. Commun. **181**, 396–401
27 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol **37**, 911–917
28 Elsbach, P., Weiss, J., Franson, B., Beckerdite-Quagliata, F., Schneider, A. and Harris, L. (1979) J. Biol. Chem. **254**, 11000–11009
29 Villalobos, C., Fonteriz, R., López, M. G., García, A. G. and García-Sancho, J. (1992) FASEB J. **6**, 2742–2747
30 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
31 Jones, L. G., Goldstein, D. and Heller Brown, J. (1988) Circ. Res. **62**, 299–305
32 Channon, J. Y. and Leslie, C. C. (1990) J. Biol. Chem. **265**, 5409–5413
33 Qiu, Z.-H., deCarvalho, M. S. and Leslie, C. C. (1993) J. Biol. Chem. **268**, 24506–24513
34 Kramer, R. M., Roberts, E. F., Manetta, J., Hyslop, P. A. and Jakubowski, J. A. (1993) J. Biol. Chem. **268**, 26796–26804
35 Nemenoff, R. A., Winitz, S., Qian, N. X., Van Puten, V., Johnson, G. L. and Heasley, L. (1993) J. Biol. Chem. **268**, 1960–1964
36 Randriamampita, C. and Tsien, R. Y. (1993) Nature (London) **364**, 809–813
37 Schacht, J. (1976) J. Neurochem. **27**, 1119–1124
38 Axelrod, J., Burch, R. M. and Jelsema, C. L. (1988) Trends Neurosci. **11**, 117–123
39 Burch, R. M. (1989) Mol. Neurobiol. **3**, 155–171
40 Tsujishita, Y., Asaoka, Y. and Nishizuka, Y. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 6274–6278
41 Winitz, S., Gupta, S. K., Qian, N.-X., Heasley, L. E., Nemenoff, R. A. and Johnson, G. L. (1994) J. Biol. Chem. **269**, 1889–1895
42 Axelrod, J. (1995) Trends Neurosci. **18**, 64–65
43 Hoover, R. K. and Toews, M. L. (1990) J. Pharmacol. Exp. Ther. **253**, 185–191
44 McKenzie, F. R., Seuwen, X. and Pouysségur, J. (1992) J. Biol. Chem. **267**, 22759–22769
45 García Rodríguez, M. C., Montero, M., Alvarez, J., García-Sancho, J. and Sánchez Crespo, M. (1993) J. Biol. Chem. **268**, 24751–24757
46 Buckley, B. J. and Whorton, A. R. (1994) Biochem. J. **300**, 449–455

Received 10 September 1996/13 November 1996; accepted 3 December 1996