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Phospholipase A2-Dependent and -Independent Pathways of Arachidonate Release from Vascular Smooth Muscle Cells

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ABSTRACT. [Arg^8]vasopressin (AVP), through its V_1 receptor coupled to GTP-binding proteins, and aluminum fluoride (AlF_4^-), which directly activates GTP-binding proteins, induced the release of [^{3}H]arachidonate from prelabeled A_7r_5 vascular smooth muscle-like cells. Using fura-2-loaded cells, we observed that the release induced by AVP occurred concurrently with calcium (Ca^{2+}) mobilization from internal stores and entry of external Ca^{2+}, whereas AlF_4^--dependent arachidonate release was much slower and was not accompanied by intracellular Ca^{2+} mobilization. Arachidonate transfer from phosphatidylcholine to phosphatidylethanolamine was an early event for both agonists, but phosphatidylinositol hydrolysis was an early event for AVP-stimulated cells and a late event for cells triggered with AlF_4^-. In addition, phospholipase inhibitors had no effect on arachidonate release induced by AlF_4^-. We investigated the enzymatic pathways involved in the releases of arachidonate, which occur in such different ways. Phospholipase A_2 activities were assayed in a cell-free system with various substrates, which made it possible to differentiate between cytosolic, secretory and Ca^{2+}-independent phospholipases A_2. THe specific activities were in the order alkenyl-AA-GPE > acyl-AA-GPE > acyl-AA-GPC in the presence of Ca^{2+}. No significant activity was observed in the presence of Ca^{2+}chelators and when dipalmitoyl–glycerophosphocholine was used as a substrate. Phospholipase A_2 activities did not change in homogenates from stimulated cells related to control cells. However, phospholipase A_2 activity increased in membrane fractions from AVP-stimulated cells. Imunodetected phosphorylated and unphosphorylated forms of cytosolic phospholipase A_2 (cPLA$_2$) also clearly increased in the membrane fractions of AVP-stimulated cells, and only the unphosphorylated form of cPLA$_2$ was present in AlF_4^--triggered cells. We conclude that phospholipase C and translocation of cPLA$_2$ can account for arachidonate release with AVP stimulation, whereas neither phospholipase C nor any phospholipase A_2 activity appears to be implicated in AlF_4^--dependent arachidonate release. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:327–337, 1997.

KEY WORDS. arachidonic acid; cytosolic phospholipase A_2; $[Ca^{2+}]_i$; phospholipid hydrolysis; vascular smooth muscle cells; vasopressin; aluminum fluoride.

Proliferation of VSMC† plays a crucial role in the pathogenesis of hypertension and atherosclerosis [1]. AA and/or its cyclooxygenase or lipoxygenase metabolites have recently been shown to modulate the expression of early growth-responsive genes in fibroblasts [2] and in VSMC [3] and to activate mitogen-activated protein kinases [4]. Very low levels of free AA in resting cells are maintained by rapid incorporation and remodeling in cellular lipids [5, 6], and AA has to be released from phospholipid stores to be transformed into biologically active metabolites. AA release from inflammatory cells in response to receptor stimulation occurs concurrently to an increase in intracellular calcium, through Ins(1,4,5)P_3 formation and Ca^{2+} channel opening [7]. Therefore, Ca^{2+}-dependent PLA$_2$s were thought to be involved in AA release. Two well-described PLA$_2$s are candidates: the 85-kDa cPLA$_2$ and the 14-kDa sPLA$_2$. THe cPLA$_2$ is activated with phosphorylation by MAP kinases [8, 9] and translocated to membranes by micromolar Ca^{2+} concentrations [10–12]. GTP-binding proteins would be involved in the coupling of cPLA$_2$ to the receptor [13]. The sPLA$_2$ requires a millimolar Ca^{2+} concentration to be active [14]. The cPLA$_2$ is specific for AA-containing phospholipids [10, 15–16], whereas sPLA$_2$ prefers phosphatidylethanolamine as a substrate with no fatty

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† *Abbreviations:* VSMC, vascular smooth muscle cells; AVP, [Arg^8]vasopressin; AA, arachidonic acid; $[Ca^{2+}]i$, cytosolic-free calcium concentration; PLA$_2$, phospholipase A_2; cPLA$_2$, cytosolic phospholipase A_2; sPLA$_2$, secretory phospholipase A_2; iPLA$_2$, Ca^{2+}-independent phospholipase A_2; GPE, glycerophosphoethanolamine; GPC, glycerophosphocholine; AlF_4^-, aluminum fluoride; [^{3}H]AA, ^{3}H] arachidonic acid; alkenyl-[^{3}H]AA-GPE, 1-alkenyl-2-[^{3}H]arachidonyl-GPE; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; DTT, dithiothreitol; TBS, Tris buffered saline; Mops, 3-[N-morpholino]propane-sulfonic acid; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; DPPC, dipalmitoylphosphatidylcholine.

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acid specificity [14]. Both phospholipases are involved in AA release in a number of cell systems [17–23], thus strengthening the idea of an absolute requirement of calcium for AA release.

However, treatment of endothelial cells with the GTP-binding protein activator AlF_4^- resulted in the release of a large pool of AA that did not depend on the presence of extracellular Ca^{2+} or on the mobilization of intracellular Ca^{2+} [24, 25]. In other respects, Ca^{2+}-$iPLA_2$ has been described in VSM-like cells [26] and macrophage-like cells [27]. In A_{10} VSM-like cells, involvement of $iPLA_2$ in AA release induced by AVP was deduced from inhibition of AA release by using a specific $iPLA_2$ inhibitor [26]. The AA release triggered by AVP from VSMC also implicates multiple signaling pathways such as phosphatidylinositol and phosphatidylcholine phospholipases C [28–30], GTP-binding proteins [30], mitogen-activated protein kinases [31], phospholipase D [31, 32] and ionic channels [33].

To investigate Ca^{2+}-dependent and -independent pathways of AA release, we used the A_7r_5 VSMC line that we triggered with AVP or AlF_4^-. The differences observed for the receptor-dependent agonist and the pharmacological tool in the kinetics and calcium dependence of AA release and in phospholipid hydrolysis suggested the implication of different enzymatic pathways. We demonstrate that Ca^{2+}-dependent translocation of $cPLA_2$ could be responsible, at least in part, for the AVP-induced AA release, whereas the AA release induced by AlF_4^- is independent of Ca^{2+} and does not involve any PLA_2 activity.

MATERIALS AND METHODS

Materials

[^{3}H]AA (200 Ci/mmol), 1-stearoyl-2-[1-^{14}C]arachidonyl-phosphatidylcholine (54 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]palmitoyl-phosphatidylcholine (59 mCi/mmol) and 1-acyl-2 [1-^{14}C]arachidonyl-phosphatidylethanolamine (55 mCi/mmol) were purchased from Amersham (Buckinghamshire, UK); 1-alkenyl-2-[^{3}H]arachidonyl-phosphatidylethanolamine (83 mCi/mmol) was purified from U_{937} cells labeled with [^{3}H]AA. Briefly, 50×10^6 cells were incubated with 110 μCi [^{3}H]AA for 8 hr. The cells were then scraped in cold PBS, and the lipids were extracted by the procedure of Bligh and Dyer [34]. During this period of time, 90% of the initial AA were incorporated in cell lipids and 32% were esterified in ethanolamine-containing phospholipids. The lipid extract was then treated with phospholipase A_1 from Rhizopus Arrhizus (5UI/μmol) to remove the diacyl phospholipids [35]. After extraction, the remaining ether-phosphatidylethanolamine pool was purified on a silicic acid column. The column was washed first with chloroform/methanol/NH_4OH (20%) (90:10:0.02, v/v/v) [36], and the ether-phosphatidylethanolamine fraction was eluted with chloroform/methanol/NH_4OH (60:10:0.02, v/v/v). The substrate was free from any compound acylated on the *sn*1 position of the glycerol but contained approximately 20% 1-alkyl-2-[^{3}H]AA-GPE as determined by treatment with HCl fumes, the remaining compound being alkenyl-[^{3}H]AA-GPE. The specific activity of 1-alkyl- plus alkenyl-[^{3}H]AA-GPE compounds, as determined from phosphorus assay [37] and radioactivity counting, was 83 mCi/mmol.

AVP, NaF, $AlCl_3$, ATP, BAPTA, EGTA, PMSF, DTT, leupeptine, bacitracine, benzamidine, Mops, quinacrine, neomycin, fatty-acid-free BSA, unlabeled phospholipids and AA were obtained from Sigma. Fura-2/AM was obtained from Molecular Probes (Eugene, OR, USA). Lipase from *Rhizopus arrhizus* was obtained from Boehringer-Mannheim (Mannheim, Germany). DMEM, PBS, FCS, glutamin, penicillin, streptomycin and trypsin/EDTA were purchased from Gibco (Grand Island, NY, USA). Silica gel 60 HPTLC was purchased from Merck (Darmstadt, Germany). Silica gel TLC and nitrocellulose membranes were obtained from Schleicher and Schuell (Dassel, Germany). Rabbit polyclonal anti $cPLA_2$ antibody was obtained from Santa-Cruz Biotechnology Inc. (Santa-Cruz, CA, USA) and anti-rabbit IgG (H+L) horseradish peroxidase conjugated was obtained from Biorad (Hercules, CA, USA).

Cell Culture and [^{3}H]AA Release

Rat aortic A_7r_5 smooth muscle cells were obtained from the European Collection of Animal Cell Cultures (ECACC; Salisbury, UK). The cells were grown at 37°C in DMEM supplemented with 10% (v/v) FCS, 2 mM glutamin, 100 U/mL penicillin and 100 μg/mL streptomycin (DMEM/FCS) under a 5% CO_2 atmosphere. A_7r_5 cells were subcultured every 7 days using trypsin/EDTA in 75-cm^2 flasks. For experiments, cells between passages 4–30 were seeded into 35- or 60-mm dishes and used at confluence.

To measure AA release, A_7r_5 cells grown in 6-well plates (10^5 cells/well), were incubated with [^{3}H]AA (0.2 μCi/well) for 1 hr in 1 mL of DMEM/FCS. We determined that approximately 30–40% of [^{3}H]AA was incorporated in cells during the 60-min incubation period. The medium was removed and nonincorporated [^{3}H]AA was determined. Cells were washed twice with 1 mL of DMEM/FCS to eliminate free [^{3}H]AA. Prelabeled cells were then incubated with inhibitors or/and stimulated by the agonists AVP (1 μM) or AlF_4^- (5 mM NaF and 10 μM $AlCl_3$) at 37°C for different periods of time in 2 mL of DMEM/FCS. Supernatants were removed and released radioactivity was measured by counting in Ready Safe scintillation mixture on an LS-6000-SC counter (Beckman Instruments, Inc., Palo Alto, CA, USA) and expressed as a percentage related to initial incorporation. The noncytotoxicity of 2 hr exposures to AlF_4^- (5 mM NaF and 10 μM $AlCl_3$) was verified by the neutral red method [38].

Measurement of Intracellular Calcium

$[Ca^{2+}]_i$ was monitored using fura-2 as a fluorescent Ca^{2+} indicator. Confluent monolayers of A_7r_5 cells attached to a glass coverslip were loaded with fura-2 by incubating them

with 2–4 μM fura-2-acetoxymethylester for 90–120 min at room temperature in control medium containing 140 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes pH 7.4 and 10 mM glucose. Loaded cells were washed three times and then incubated with the same buffer for an additional 20 min to hydrolyze completely the entrapped ester. The slides were then placed in the heated (30°C) compartment of the spectrofluorimeter. $[Ca^{2+}]_i$ was measured under magnetic stirring in a fluorescence spectrophotometer constructed by Cairn Research Ltd. (Newnham, Sittingbourne, Kent, UK), which allows simultaneous excitation of fluorescence at 340 and 380 nm. Fluorescence emission was set at 530 nm. Fluorescence readings were integrated at every 1-sec period, and $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence excited at 340 and 380 nm. During the experiment, the solution bathing the cells (flow rate of 2 mL/min) was changed by perfusing fresh solution into the bottom of the cuvette while aspirating continuously from just above the coverslip [39].

To follow Mn^{2+} entry and quenching of fura-2 fluorescence, cells were superfused with Ca^{2+}-free buffer containing 200 μM $MnCl_2$. Fluorescence was followed at 530 nm and cells excited at 360 nm, a wavelength insensitive to changes in $[Ca^{2+}]_i$ [39].

[^{3}H]AA Repartition in Phospholipids

Following incubation with radiolabel, stimulation with agonists and removal of the supernatants to quantify the arachidonic acid release, cells were scraped in 1 ml of cold methanol and 800 μL of water. Lipids were extracted as previously described and spotted on HPTLC plates. The first mobile phase consisted of chloroform/methanol/acetic acid/water (75:45:12:6, v/v/v/v) for 25 min and the second phase of petroleum ether (40–65°C)/diethyl ether/acetic acid (70:30:1, v/v/v) for 15 min, both in the same dimension. The first dimension separated phosphatidylcholine, phosphatidylinositol/phosphatidylserine and phosphatidylethanolamine and the second separated fatty acids, diglycerides and triglycerides. Lipids were visualized by autoradiography and identified by their comigration with standards by staining with iodine vapor. The bands were scraped from the plates and counted for radioactivity.

Preparation of Subcellular Fractions

Cells in 75-cm^2 flasks were stimulated with AVP (1 μM for 10 min) or AlF_4^- (5 mM NaF and 10 μM $AlCl_3$ for 120 min) in 5 mL of DMEM/FCS at 37°C. The stimulation was stopped by aspirating media and adding 2 mL trypsin/EDTA. The cells were diluted in DMEM/FCS and then washed twice with PBS. Cell pellets were suspended in 500 μL of buffer containing 25 mM Tris, 125 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, pH 9.6 and protease inhibitors (0.5 mM PMSF, 50 μM leupeptine, 50 μg/mL benzamidine, 50 μg/mL bacitracine). Cells were lysed by N_2 cavitation (600 psi, 20 min, 4°C) in the presence of 2 mM $CaCl_2$ or 2 mM EGTA. Nucleus and intact cells were removed by centrifugation at 600*g* for 10 min at 4°C. Supernatants were further diluted v/v with Hepes buffer (4.2 mM Hepes, 137 mM NaCl, 2.6 mM KCl, 1 mg/mL glucose, pH 7.4) and centrifuged at 100,000*g* for 60 min at 4°C. Cytosols were aliquoted and stored at −80°C. Membrane pellets were resuspended in Hepes buffer, sonicated 2 × 10 sec at step 6 in MSE probe sonicator and stored at −80°C until used for PLA_2 assays. Proteins were determined according to Bradford [40], using BSA as standard.

Measurements of PLA_2 Activities

To differentiate between various cytosolic, secretory and Ca^{2+}-independent PLA_2 activities, we used different substrates and assays. Hydrolysis of three arachidonate-labeled substrates, 1-alkenyl-2[^{3}H]AA-GPE, 1-acyl-2-[^{14}C]AA-GPE and 1-acyl-2-[^{14}C]AA-GPC, was assayed using mixed micelles composed of labeled substrate, egg yolk phosphatidylcholine and Triton X-100 [41]. Briefly, the radiolabeled substrate was mixed with egg yolk phosphatidylcholine in a ratio of 3:1 and evaporated to dryness. The residues were resuspended in Mops buffer (0.1 M, pH 7.4) containing Triton X-100 and sonicated twice for 15 sec. The reaction mixture contained 2 μM sonicated phospholipids, 200 μM Triton X-100, 0.25 mg/mL fatty-acid-free BSA, 2 mM DTT, 5 mM $CaCl_2$ and 2–5 μg protein in a total volume of 0.25 mL of Mops buffer containing 20% glycerol. In some experiments devoted to $sPLA_2$, concentration of DTT was 0–10 mM. In other experiments designed to assay $iPLA_2$, $CaCl_2$ was omitted and incubations were performed in the presence of 2 mM EGTA. Following incubation at 37°C for 30 min, the reaction was stopped by adding 960 μL chloroform/methanol (1:2, v/v), and the lipids were extracted by the procedure of Bligh and Dyer [34]. The lipidic extract was separated on TLC plates developed in the solvent system petroleum ether (40–65°C)/diethyl ether/acetic acid (70:30:1, v/v/v) for 15 min and exposed to I_2 vapor. AA (10 μg) was added as a carrier. The spots corresponding to AA and phospholipids were scraped and counted for radioactivity.

Phospholipase A_2 assay using DPPC was performed as described by Ackerman *et al.* [42]. The incubation medium contained 400 μM Triton X-100, 10 or 100 μM DPPC and 2 × 10^5 dpm 1-palmitoyl-2-[^{14}C]palmitoyl-GPC, 0.1 M Hepes pH 7.5, 5 mM EDTA, 0.8 mM ATP in 250 μL final volume. The substrate was prepared by resuspending the dried phospholipids in 100 μL Hepes buffer containing Triton X-100 and EDTA. Mixed micelles were formed by heating (above 40°C) and sonication with an MSE probe sonicator. The incubation was performed at 40°C for 60 min. The reaction was stopped by adding chloroform/methanol and subsequently processed as for the previously described assays.

Immunoblotting Analysis

Control or stimulated cells (7.5 × 10^5 cells) were washed with 3 × 10 mL of ice-cold PBS, scraped in 300 μL of 25

mM Hepes buffer pH 7.4 containing 5 mM EDTA, 50 mM NaF, 100 μM sodium orthovanadate, 1 mM PMSF and 10 μg/mL leupeptine (lysis buffer) and homogenized by passing repeatedly through a 25-G needle. Cytosol and membrane fractions were separated by centrifugation at 100,000g and 4°C, and pellets were resuspended in 300 μL of lysis buffer. Cell extracts (1.25×10^5 cells equivalent per lane) were boiled for 5 min in Laemmli buffer [43], and proteins were resolved by a 7.5% SDS-PAGE. Following electrophoresis, gels were equilibrated for 10 min in transfer buffer [25 mM Tris, 192 mM glycine and 20% (v/v) methanol]. Proteins were transferred onto 0.2-μm nitrocellulose membranes for 2 hr at 200 mA, using a semidry transfer unit (Hoefer, San Francisco, CA, USA), and membranes were stained with Ponceau S to verify efficient and equal transfer of protein samples. The membranes were then blocked overnight with 3% (w/v) BSA in TBS containing 50 mM Tris pH 7.2, 150 mM NaCl and 0.1% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20). After washing with TBS, membranes were probed with rabbit polyclonal antibody to $cPLA_2$ (diluted 1:50) for 2 hr. After washing with TBS, membranes were treated with peroxydase-conjugated goat anti-rabbit secondary antibody (diluted 1:1000) for 1 hr, and peroxydase activity was detected by using enhanced chemiluminescence reagents (Amersham Corp.). Human $cPLA_2$ was overexpressed using the baculovirus-insect cell expression system [44]. Cytosol from infected cells was used as standard.

RESULTS

Both AVP and AlF_4^- were potent activators of AA release from A_7r_5 cells incubated in the presence of FCS. Preliminary experiments showed that maximum release was observed with 1 μM AVP from cells prelabeled with [^{3}H]AA for 1 hr. Exposure to AlF_4^- involved 5 mM NaF and 10 μM $AlCl_3$. The time course of AA release was completely different depending on the agonist (Fig. 1). The AVP response was immediate. The release of AA increased very rapidly during the first 3 min and more slowly up to 30 min, when it represented 10% of total lipid labeling. In contrast, a 30-min latent period was required after AlF_4^- addition. The release of AA was then linear up to 120 min when it represented 20% of lipid labeling.

To determine the Ca^{2+} dependence of AA release from A_7r_5 cells, we used EGTA to chelate extracellular calcium and/or BAPTA to chelate intracellular calcium. The results indicate that the presence of 2 mM EGTA and/or 10 μM BAPTA decreased AA release induced by AVP in DMEM/FCS by 70–80% (Fig. 2). These data suggest that an influx of extracellular calcium and calcium release from endogenous pools are required for AA release by AVP. Figure 2 also shows that chelation of intracellular calcium with 10 μM BAPTA did not affect AlF_4^--induced AA release, whereas chelation of extracellular calcium by EGTA reduced AA release by more than 50%. These results suggest

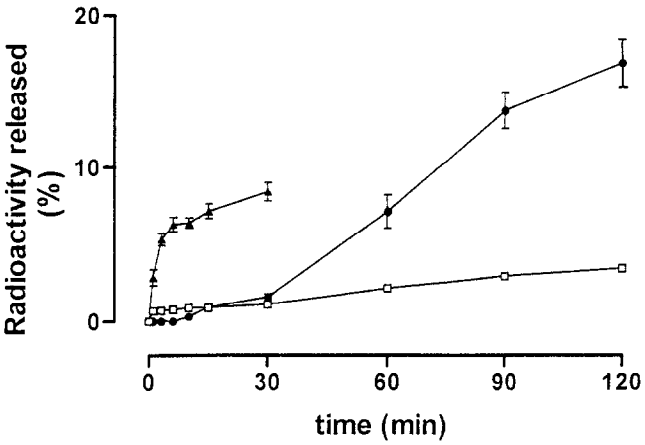

FIG. 1. Time course of AVP- and AlF_4^--stimulated AA release from A_7r_5 cells. Cells prelabeled with 0.2 μCi of [^{3}H]AA for 60 min were treated as described in Materials and Methods in DMEM/FCS control medium (open squares), with 1 μM AVP (triangles) or with AlF_4^- (5 mM NaF and 10 μM $AlCl_3$; circles). Data represent mean values ± SEM of five determinations.

that almost half of the AlF_4^--induced AA release involved a calcium-independent pathway and did not depend on a rise in intracellular calcium.

The cytosolic Ca^{2+} levels observed after stimulation with AVP or AlF_4^- confirmed the results obtained with the calcium chelators. A_7r_5 cells were loaded with the calcium-sensitive dye fura-2, and the fluorescent signal at 340 and 380 nm was monitored over time by using single cells.

When cells were stimulated with 1 μM AVP in the

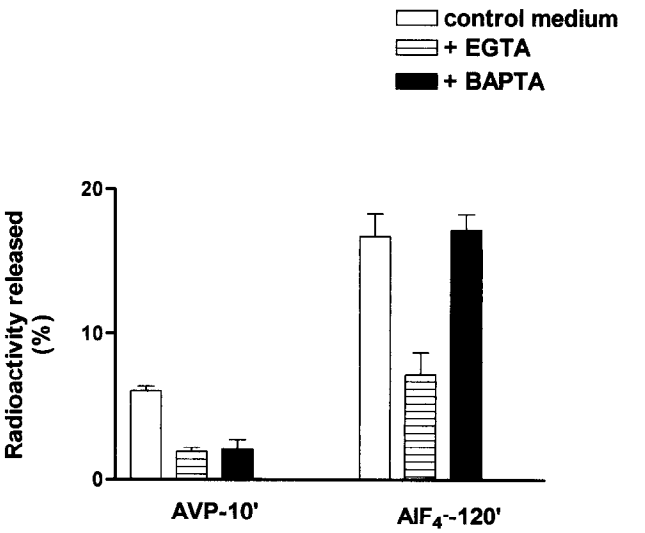

FIG. 2. Effects of extracellular and intracellular Ca^{2+} chelations on AVP- and AlF_4^--stimulated AA release. [^{3}H]AA prelabeled A_7r_5 cells were stimulated with AVP (1 μM) or AlF_4^- (5 mM NaF and 10 μM $AlCl_3$) for the indicated periods of time in DMEM/FCS control medium or in DMEM/FCS containing 2 mM EGTA; 10 μM BAPTA was preincubated with cells for 10 min prior to addition of the agonists. Data represent the percent of [^{3}H] radioactivity release and are mean values ± SD of two determinations.

absence of extracellular calcium (0 Ca^{2+}), we observed a very sharp increase in $[Ca^{2+}]i$, reaching a maximum between 600 and 800 nM and returning to baseline level within 40–60 sec after stimulation (Fig. 3A). When the same experiment was done in the presence of 1 mM extracellular calcium, $[Ca^{2+}]_i$ reached 1 μM and the peak became wider, perhaps corresponding to an entry of extracellular calcium. To confirm this hypothesis, a parallel experiment was carried out to monitor the influx of Mn^{2+}, which enters cells via the same channels as Ca^{2+} [39]. When Mn^{2+} enters the cell, it combines with fura-2 and quenches its fluorescence. Figure 3C shows a continuous decrease in the fura-2 fluorescent signal at 360 nm when cells were stimulated with AVP in the presence of 100 μM $MnCl_2$. These data suggest that AVP induced an influx of extracellular calcium by opening calcium channels in the plasma membranes. Both intracellular calcium mobilization and extracellular calcium entry then occurred in smooth muscle cells stimulated with AVP.

When cells were triggered with AlF_4^-, there was very little effect on $[Ca^{2+}]_i$ (Fig. 3B). The slight increase in fluorescence observed after AlF_4^- addition could correspond to a slight influx of extracellular calcium and thereby account for the effect of EGTA on AA release.

Labeled AA was rapidly incorporated into cell lipids and approximately 30–40% of initial radioactivity was esterified after 60 min. Following this period, more than 70% of esterified [^{3}H]AA was recovered in phosphatidylcholine and phosphatidylinositol, whereas approximately 20% was esterified in phosphatidylethanolamine (Fig. 4). Under control conditions, [^{3}H]AA was slowly redistributed from phosphatidylcholine to phosphatidylethanolamine. When cells were stimulated by 1 μM AVP, we observed a rapid hydrolysis of phosphatidylinositol, which paralleled the re-

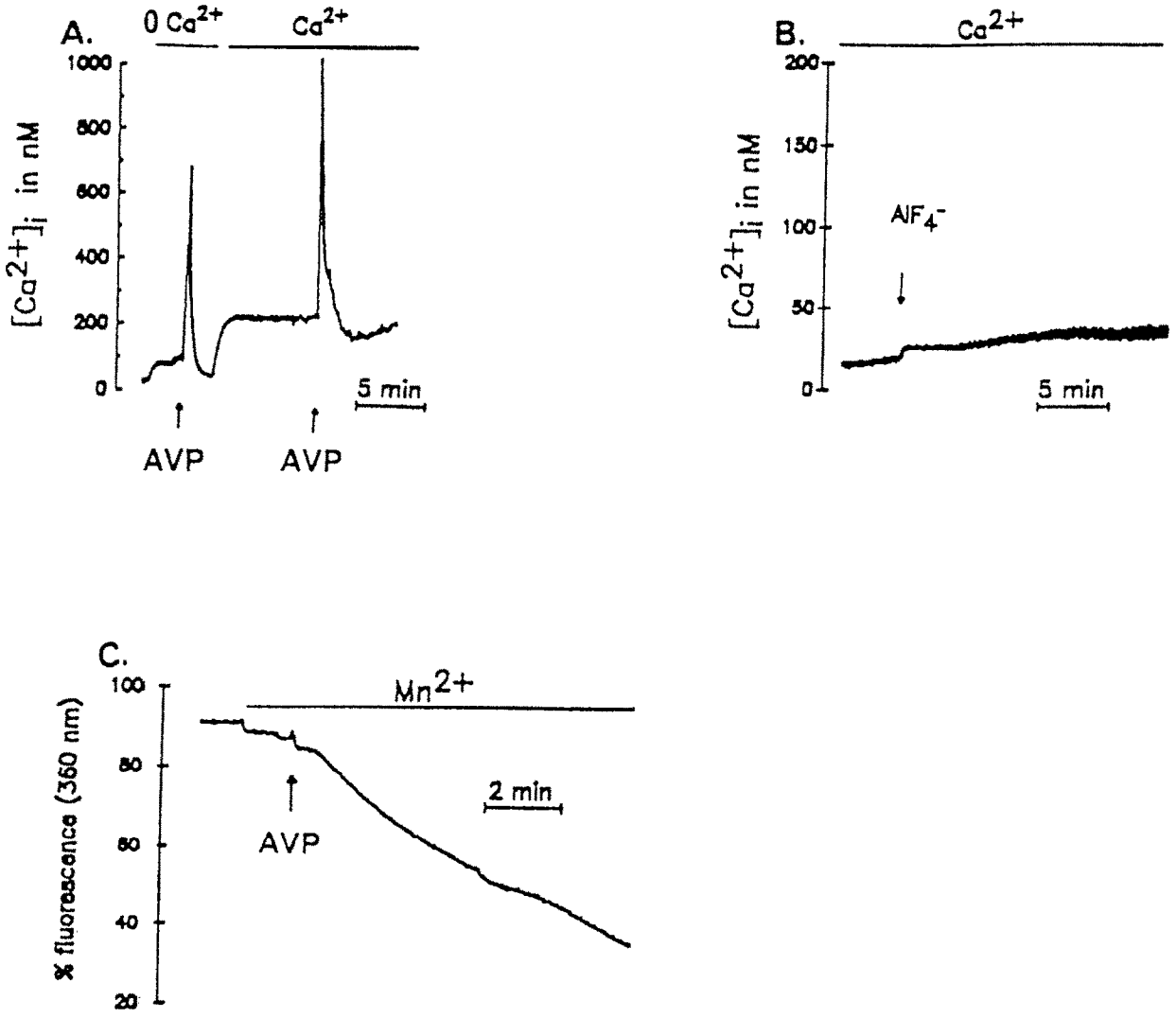

FIG. 3. Effects of AVP and AlF_4^- on $[Ca^{2+}]_i$ in A_7r_5 cells. Cells were grown to confluence on a glass coverslip, loaded with 2–4 μM fura-2/AM for 90–120 min at room temperature and placed in a cuvette. Cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) were monitored continuously as buffer was perfused through a cuvette at 2 mL/min and 37°C. (A) AVP (1 μM) or (B) AlF_4^- (5 mM NaF and 10 μM $AlCl_3$) was added to perfusion in medium containing 1 mM $CaCl_2$ as indicated. (C) 100 μM $MnCl_2$ was added to cells before stimulation by AVP. These traces represent five experiments.

lease of AA in the medium, followed by a large transfer of [^{3}H]AA to phosphatidylethanolamine. In response to AlF_4^-, a large transfer of AA from phosphatidylcholine to phosphatidylethanolamine, largely preceding the release of arachidonate, was observed. Thereafter, the release of [^{3}H]AA seemed to be correlated with hydrolysis of phosphatidylinositol (Fig. 4).

The mechanisms by which either AVP or AlF_4^- liberate AA from A_7r_5 VSMC appear to be completely different because we observed different time courses, calcium dependencies and phospholipid hydrolysis. We then investigated the enzymes that could be responsible for AA release induced by AVP or AlF_4^-.

Preincubation of cells with quinacrine or neomycin, widely used as inhibitors of PLA_2 and phospholipase C, respectively, reduced the AVP-stimulated AA release by approximately 50%, whereas these compounds did not modify the AA release induced by AlF_4^- (Fig. 5). Because neomycin binds to phosphatidylinositol mono- and biphosphates, thus preventing their hydrolysis by phospholipase C, its absence of effect in AlF_4^--triggered cells suggests that phospholipase C is not involved in phosphatidylinositol hydrolysis following AlF_4^- addition. The mechanism of action of quinacrine is not clearly elucidated, but this compound inhibits AA release in a number of cell systems. Again, it had no effect in AlF_4^--triggered cells, suggesting that classical PLA_2 was not implicated.

Low-molecular-mass $sPLA_2$ [45] and high-molecular-mass $cPLA_2$ [46] have been described in rat VSMC. In addition, the presence of $iPLA_2$ has been deduced from the inhibitory effect of a bromo*enol*lactone, a specific $iPLA_2$ inhibitor, on agonist-induced AA release from A_{10} cells, a rat VSMC clone [26]. It was then of interest to determine which P.LA_2s were present in our A_7r_5 cell line. $sPLA_2$ is sensitive to reducing agents [14] and specific for diacyl-GPE hydrolysis over diacyl-GPC [14], whereas $cPLA_2$ is specific for arachidonate-containing phospholipids [10, 15, 16]. The $iPLA_2$ would preferentially hydrolyze plasmalogen phospholipids [47]. Therefore, we investigated PLA_2 activities in a cell-free system using three different arachidonate-

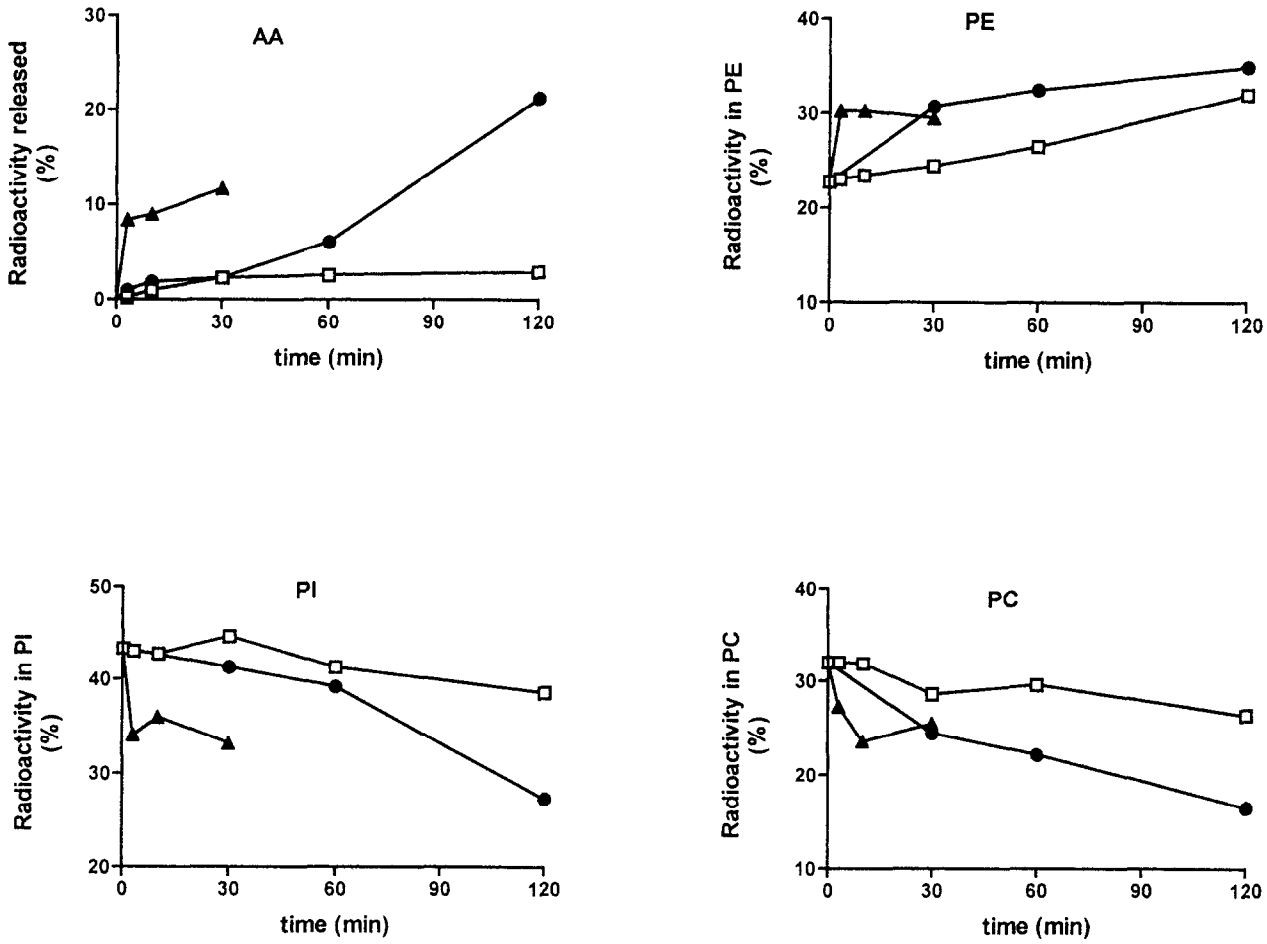

FIG. 4. Time course of [^{3}H]AA release and movements in the major lipid classes of A_7r_5 cells. Cells were incubated in DMEM/FCS in presence of 0.2 μCi of [^{3}H]AA for 1 hr. After washing, the prelabeled cells were incubated in DMEM/FCS (open squares), DMEM/FCS plus AVP (triangles) or AlF_4^- (circles) for the indicated periods of time. Radioactivity released in supernatant was counted and cells were extracted for lipid HPTLC analysis as described in Materials and Methods. The bands were identified by standard comigration, stained by I_2 vapor, scraped and counted for radioactivity. (PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine). The data are expressed as a percentage of total radioactivity and represent three independent experiments.

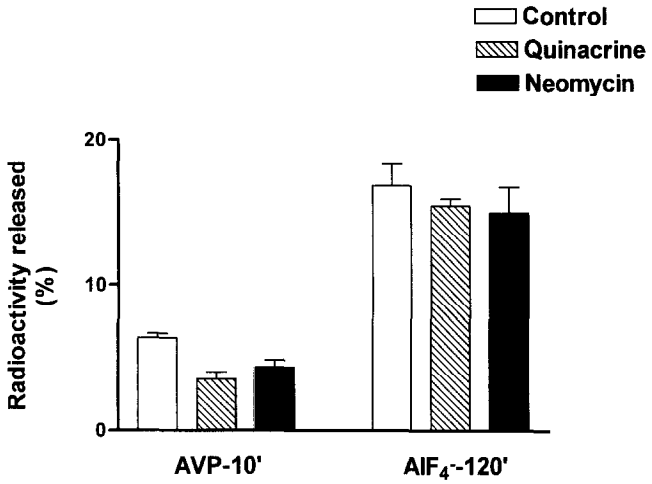

FIG. 5. Effects of phospholipase inhibitors on AVP- and AlF_4^--stimulated AA release. Cells prelabeled with [^{3}H]AA were treated by 5 μM quinacrine or 2 mM neomycin for 10 min prior to addition of the agonists in DMEM/FCS control medium. Release of [^{3}H]AA was performed as described in Materials and Methods. Values are the means ± SEM of two experiments.

labeled substrates:diacyl-GPE, alkenyl-acyl-GPE and diacyl-GPC. To minimize the effects due to different physical conformations of the various substrates, mixed micelles containing egg phosphatidylcholine and Triton X-100 with labeled substrate were always used.

Table 1 shows that in the presence of calcium the A_7r_5 lysate from control cells hydrolyzed the three substrates in the order of preferential hydrolysis:alkenyl-acyl-GPE > diacyl-GPE > diacyl-GPC. The hydrolysis of the various substrates was not inhibited by the presence of DTT in the incubation medium. The insensitivity to reducing agent and the absence of $sPLA_2$ secretion (data not shown) exclude the involvement of $sPLA_2$ in the release of AA by the A_7r_5 cell line. In the absence of calcium, hydrolysis represented less than 10% of the hydrolysis observed in Ca^{2+}-containing medium, whatever the substrate. Thus it was not possible to assess this residual activity to actual $iPLA_2$. Furthermore, when using 10 or 100 μM DPPC as substrate, we were unable to show any hydrolysis of this phospholipid, which has been described as a better substrate than arachidonate-containing phospholipids for $iPLA_2$ [27]. As a consequence, hydrolysis of the three substrates can only be attributed to Ca^{2+}-dependent cellular PLA_2. Surprisingly, the PLA_2 activities did not significantly increase when the homogenates were prepared from cells stimulated with AVP or AlF_4^- (Table 1). High-molecular-mass $cPLA_2$ has to bind membranes in a Ca^{2+}-dependent manner to become active [10–12]. Therefore, we separated membranes from cytosol of control and stimulated cells to investigate the presence and translocation of $cPLA_2$ by immunoblot analysis and by assaying the enzyme activity in membrane and cytosol fractions.

The presence of $cPLA_2$ was demonstrated by immunoblotting with $cPLA_2$ polyclonal antibody. Reduced mobility of immunodetected $cPLA_2$ has been linked to its phosphorylated form [18]. Two bands migrating at 100–110 kDa were detected in membrane fraction from control cells, with the lower band (unphosphorylated form) being the larger (Fig. 6). In contrast, the phosphorylated band was larger in membrane fraction from cells previously stimulated with AVP. The two bands were also present in cytosol from stimulated cells, although to a lesser extent, whereas a single band corresponding to unphosphorylated protein was only present in cytosol from control cells. After treatment with AlF_4^-, a weak single phosphorylated band was observed in both cytosol and membrane fractions.

Translocation of $cPLA_2$ to membranes was confirmed by assaying the activity. Figure 7 shows that when cells, stimulated or not, were cavitated in the presence of 2 mM Ca^{2+}, approximately 70% of PLA_2 activity was associated with membranes. In contrast, when control cells were cavitated in the presence of 2 mM EGTA, the majority of the PLA_2 activity was found in the cytosolic fraction. However, the PLA_2 activity recovered in the membrane fraction increased by almost 100% when cells were stimulated with

TABLE 1. Substrate specificity of PLA_2 activity (pmol/mg/min) in A_7r_5 cells

	Control	AVP for 10 min	AlF_4^- for 120 min
Acyl-[^{14}C]AA-GPC (n = 2)	294 ± 26	260 ± 32	
Acyl-[^{14}C]AA-GPE (n = 8)	439 ± 34	385 ± 47	420 ± 28
Aklenyl-[^{3}H]AA-GPE (n = 6)	626 ± 52	612 ± 81	

Homogenates from control or stimulated cells were incubated with mixed micelles formed from 2 μM of the indicated radiolabeled substrate, egg yolk phosphatidylcholine in a ratio of 3:1 and 200 μM Triton X-100 in 100 mM Mops buffer (pH 7.4) containing 20% (v/v) of glycerol and 5 mM $CaCl_2$, for 30 min at 37°C. Released fatty acids were extracted by the procedure of Bligh and Dyer [34], separated by TLC and quantified by scintillation spectrometry. Aklenyl-[^{3}H]AA-GPE was prepared from [^{3}H]AA-prelabeled U_{937} cells as described in Materials and Methods and contained approximately 20% of aklyl species.

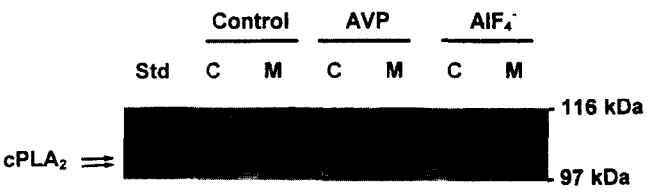

FIG. 6. Immunoblot of $cPLA_2$ in membrane and cytosol fractions of A_7r_5 cells. After triggering cells with AVP (1 μM, 10 min) or AlF_4^- (5 mM NaF and 10 μM $AlCl_3$, 120 min), cell extracts were prepared under conditions described in Materials and Methods. Proteins (from 1.25 10^5 cells/lane) were subjected to 7.5% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with rabbit polyclonal antibody to $cPLA_2$. Std, recombinant human $cPLA_2$.

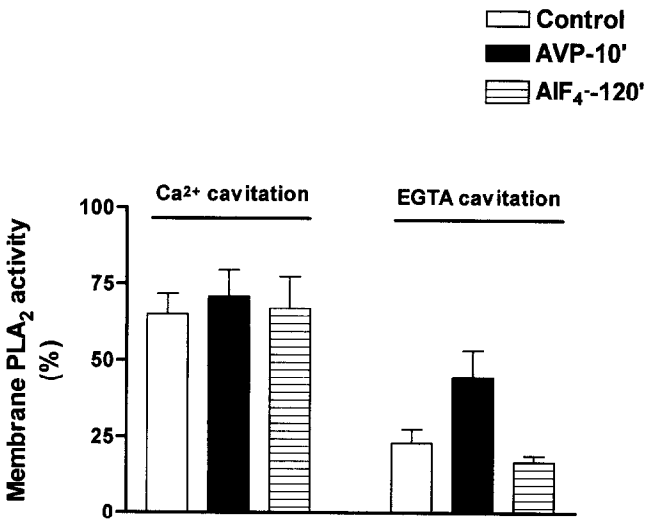

FIG. 7. AVP enhances translocation of $cPLA_2$ activity to membranes. Cytosolic and membrane fractions of control or stimulated A_7r_5 cells were prepared by N_2 cavitation in the presence of 3 mM $CaCl_2$ or 2 mM EGTA as described in Materials and Methods. Each fraction (2–5 μg of protein) was assayed with acyl-[^{14}C]AA-GPE (2 μM) mixed with egg yolk phosphatidylcholine in a ratio of 3:1 in Mops buffer (pH 7.4) containing 5 mM $CaCl_2$. The results are expressed as a percentage of the activity recovered in the membrane fraction related to total activity and are the mean ± SEM of five determinations.

AVP related to control cells, whereas AlF_4^- did not induce any PLA_2 translocation.

DISCUSSION

AVP is a potent vasoconstrictor agent that acts on VSMC through a specific V_1 receptor that is coupled to GTP-binding proteins [30]. AVP initiates a series of biochemical events that result in the formation of Ins(1,4,5)P_3 and Ca^{2+} mobilization [28–30] concurrently to AA release, which may involve PLA_2 activity. Our results show that the pharmacological tool AlF_4^-, known as GTP-binding protein activator, also releases AA from VSMC, thereby suggesting activation of PLA_2 through direct GTP-binding protein stimulation. In this paper, we demonstrate that, whereas the $cPLA_2$ is involved in the release of AA induced by AVP, the appearance of AA in response to AlF_4^- is a completely different mechanism that does not involve any PLA_2 activity.

The release of [^{3}H]AA induced by AVP was rapid up to 3 min, reaching 5–7% of the total radioactivity of cells prelabeled with [^{3}H]AA for 60 min. Using fura-2 loaded cells, we verified that AVP induced Ca^{2+} mobilization from intracellular pools. This mobilization was followed by an influx of Ca^{2+} from the extracellular medium as demonstrated by monitoring Mn^{2+} entry. The two waves of Ca^{2+} were necessary for AA release since chelators from both intracellular and extracellular Ca^{2+} largely prevented the release of labeling. Because cells were labeled for a short period of time, equilibrium labeling was not reached [48, 49], allowing us to observe transfer reactions between phospholipids. In addition to rapid phosphatidylinositol and phosphatidylcholine hydrolysis, AVP also resulted in [^{3}H]AA transfer to ethanolamine-containing phospholipids. The decrease of AA labeling in phosphatidylinositol is consistent with Ins(1,4,5)P_3 formation [30] by phospholipase C and was confirmed by the inhibition of AA release observed when cells were previously treated with neomycin, a phospholipase C inhibitor. The labeling movements among phospholipids also suggested the involvement of PLA_2. Therefore, we investigated the PLA_2 activities of our cell system.

Using a cell-free system and AA-labeled phospholipid substrates, we observed an important Ca^{2+}-dependent PLA_2 activity. We were not able to detect any PLA_2 activity in the absence of Ca^{2+} whenever we used plasmalogen phospholipids and DPPC which have been described as substrates for the $iPLA_2$ [27, 47]. This finding was surprising because $iPLA_2$ is involved in AA release induced by AVP from A_{10} VSM-like cells [26], which are very close to A_7r_5 VSM-like cells [50]. In A_{10} cells, the implication of $iPLA_2$ was deduced from the inhibitory effect on AA release of the bromo*enol*lactone compound, which is specific for $iPLA_2$ over a number of PLA_2 [51] and which covalently binds $iPLA_2$ [51]. Either the two clones contain different amounts of $iPLA_2$ or the inhibitor compound also acts on as yet unknown targets. Control or AVP-stimulated cells did not secrete any PLA_2 activity, and the activity measured in the cell-free system was insensitive to reducing agents, therefore excluding the implication of $sPLA_2$ in AA release from A_7r_5 cells.

The fact that all AA-containing substrates were hydrolyzed speaks in favor of $cPLA_2$ activity, which was described as not specific for polar head group [10, 15]. Nevertheless, under our incubation conditions using mixed micelles containing egg yolk phosphatidylcholine, there was some preferential hydrolysis of phosphatidylethanolamine over phosphatidylcholine and of alkenyl-acyl-GPE over diacyl-GPE. This preference could account for the more rapid hydrolysis of the AA-richest species since the majority of cellular AA is present in ethanolamine-containing phospholipids [5, 52]. However, the PLA_2 activity of whole homogenate was not increased following AVP stimulation, in contrast to other cell systems in which the enzyme has been implicated in AA release [8, 18]. Conversely, an increase in $cPLA_2$ was not always accompanied by an increase in AA release [53]. Because translocation of $cPLA_2$ to membranes [10–12] is necessary for the enzyme to be in contact with its natural substrate, we separated cytosol from membranes and investigated AVP-dependent translocation by Western blot analysis and by assaying the activity. The $cPLA_2$ antibody revealed that phosphorylated and unphosphorylated forms of $cPLA_2$ were present in membrane fractions of control and stimulated cells. Both forms of $cPLA_2$ clearly increased in the membrane fraction with AVP stimulation. Enhance-

ment of $cPLA_2$ binding to membranes was confirmed by $cPLA_2$ activity, whose recovery in the membrane fraction was increased by almost 100%. These data demonstrate that with AVP stimulation $cPLA_2$ was translocated to membrane, and this translocation could be responsible at least in part for the AA release in A_7r_5 cells.

In contrast, when cells were triggered with AlF_4^-, AA was released slowly and regularly. The AA release was totally independent of intracellular Ca^{2+} mobilization and depended only partly on extracellular Ca^{2+}. AlF_4^- addition induced AA transfer from choline- to ethanolamine-containing phospholipids and a delayed hydrolysis of phosphatidylinositol. Neomycin had no effect on AA release. Clearly, phospholipase C, which induces intracellular Ca^{2+} mobilization and is inhibited by neomycin, is not involved in the phosphatidylinositol hydrolysis triggered by AlF_4^-. Because AA transfer between phospholipids is induced by lysoderivatives [54], the increased transfer rate of [^{3}H]AA from phosphatidylcholine to phosphatidylethanolamine is likely to involve lysophosphatidylethanolamine formation through phosphatidylethanolamine hydrolysis by PLA_2. However, the AA release in response to AlF_4^- does not appear to implicate $cPLA_2$ because quinacrine exhibited no inhibitory effect and because $cPLA_2$, measured either by Western blot or enzyme activity was neither stimulated nor translocated to membranes. The disappearance of the unphosphorylated band could result from the ability of AlF_4^- to inhibit phosphatases [55].

Continual AA remodeling between phospholipids of resting cells [48, 49; reviewed in 5, 6] implicates basal PLA_2 activity and acyltransferase and transacylase activities. Recently, Balsinde *et al.* suggested that $iPLA_2$ was responsible for AA remodeling in macrophagelike cells [56]. We could not show such $iPLA_2$ in our VSMC. Conversely, some $cPLA_2$ was bound to membranes of resting VSMC and could account for AA remodeling and basal AA release. When cells were stimulated, translocation of the enzyme to membranes, which occurs with AVP, could result in AA release. Alternatively, because the basal $cPLA_2$ activity was not increased and even slightly decreased as observed following AlF_4^- triggering, the appearance of AA in the extracellular medium after AlF_4^- addition could be the consequence of decreased reacylation mechanisms. Preliminary experiments showing a decrease in AA incorporation into phospholipids of cells exposed to AlF_4^- support this hypothesis. Further experiments are necessary to determine the mechanisms of this impairment of AA acylation. Is depletion of ATP, as in oxidant-induced AA release from alveolar macrophages [57], involved? Our results do show that direct activation of GTP-binding proteins by AlF_4^- does not result in $cPLA_2$ activation or translocation. Rather, AlF_4^- could increase availability in free AA by preventing AA from being reincorporated.

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