Phosphorylation-dependent Regulation of Phospholipase A$_2$ by G-proteins and Ca$^{2+}$ in HL60 Granulocytes

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We studied the regulation of arachidonic acid (AA) release by guanosine 5'-O-(3-thiotriphosphate) (GTPγS) and Ca$^{2+}$ in electropermeabilized HL60 granulocytes. Stimulation of AA release by GTPγS and Ca$^{2+}$ was mediated by phosphorylation of PLAZ (PLAz$_2$) and required the presence of MgATP (EC$_{50}$ 100–250 μM). The nucleotide effects were Ca$^{2+}$-dependent (maximal effects detected at 1 μM free cation), UTP and ATPγS, which stimulate AA release in intact HL60 granulocytes with potencies and efficiencies similar to those of ATP, were ineffective in supporting the effects of GTPγS in electropermeabilized cells. Pretreatment with pertussis toxin affected stimulation of AA release by ATP in intact cell, without altering the nucleotide effects in permeabilized cells. We observed the protein kinase C-dependent phosphorylation of PLAz in permeabilized HL60 granulocytes, together with a correlation between the effects of phorbol esters and staurosporine on this reaction and on AA release. ATP-independent activation of PLAZ by GTPγS and/or Ca$^{2+}$ was measured in subcellular fractions prepared from HL60 granulocytes. These data appear consistent with a model in which PLAz activity in resting HL60 granulocytes is subjected to an inhibitory constraint that prevents its activation by Ca$^{2+}$ and G-proteins. Removal of this constraint, either by the protein kinase C-dependent phosphorylation of the enzyme in vivo or physical disruption of the regulatory assembly (e.g. by N$_2$ cavititation), allows its activation by Ca$^{2+}$ and G-proteins.

Phospholipase A$_2$ (PLAZ)$_2$ plays a crucial role in the generation of AA and alkylether-containing lysophosphatidylcholine, which are the precursors of eicosanoids and platelet activating factor, respectively (for review, see Ref. 1). Recent studies have directly involved a pertussis toxin-sensitive G-protein in the coupling between membrane receptors and PLAz in neutrophils, HL60 granulocytes, FRTL5 thyroid cells, Swiss 3T3 fibroblasts, and platelets (2–6).

In HL60 cells (7) as well as in a variety of other cell types (e.g. Refs. 8–11), agonist-promoted PLAz-catalyzed AA release requires extracellular Ca$^{2+}$. Formation of agonist-receptor complexes in these cells triggers increases in [Ca$^{2+}$]i, (through Ca$^{2+}$ mobilization and/or influx), in addition to AA release. Prevention of the sustained elevation in [Ca$^{2+}$]i blocks agonist-stimulated AA release in several cell types including platelets, C62B gloma, and Chinese hamster ovary cells (8, 9, 11). Furthermore, a recently purified cytosolic PLAz from U937 cells undergoes substantial activation by Ca$^{2+}$ at concentrations similar to those observed in stimulated cells (12–14). Elevated intracellular Ca$^{2+}$, therefore, seems to constitute an indispensable factor in the activation of PLAz by agonists in a variety of cells. Despite these studies, however, it is still unclear whether the physiological elevation in [Ca$^{2+}$]i is sufficient to activate PLAz, as opposed to being necessary for the manifestation of receptor/G-protein-mediated effects on the enzyme activity.

The relationship between PKC and PLAz activities has also received considerable attention (e.g. Refs. 5, 6, 10–11, and 15–20). In general, short term treatment of cells with PKC activators potentiates the release of AA stimulated by Ca$^{2+}$ ionophores or agonists, while down-regulation of the kinase results in inhibition, or even complete abolishment, of AA release. There is evidence linking the differential activation of the α isozenzyme of PKC with AA release in Madin-Darby canine kidney cells (20). Although these studies undoubtedly involve PKC in the regulation of PLAz activity, little is known about the identity of the components which are modified as a consequence of the kinase activation.

In the present study we have studied the regulation of AA release in electropermeabilized HL60 granulocytes and observed that PKC-dependent phosphorylation of PLAz is associated with the activation of this enzyme by Ca$^{2+}$ and G-proteins.

EXPERIMENTAL PROCEDURES

Materials—ATP, mepacrine, fMLP, PMA, arachidonic acid, TWEEN-20, Nonidet P-40, sodium orthovanadate, BSA, and the insulin-transferrin-sodium selenite supplement for culture media were obtained from Sigma. GTPγS, ATPγS, UTP, CTP, AMP-P(NH)$_2$, BtγAMP, and staurosporine were purchased from Boehringer Mannheim 2 kinase); BSA, bovine serum albumin; HBSS, Hank's balanced salts solution; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; G-proteins, heterotrimeric guanine nucleotide-binding regulatory proteins.
heim, [5,6,8,9,11,12,14,15-3H]Arachidonic acid and 125I-goat anti-rabbit IgG antiserum were obtained from Du Pont-New England Nuclear. [γ-32P]ATP was purchased from ICN Radiochemicals. IMDM was from GIBCO. Bovine calf serum was from HyClone Laboratories. Staphylococcus aureus protein A-coated cells (Pansorbin Cells, standard-ized) and normal rabbit serum were purchased from Calbiochem. The rabbit antiserum against recombinant human U937 cell cPLA₂ was kindly provided by Drs. L.-L. Lin and J. L. Knopf (Genetics Institute).

Culture and Differentiation of HL60 Cells—HL60 cells were cultured and differentiated into a neutrophil-like phenotype, as described previously (7). Briefly, cells were routinely grown in IMDM supplemented with 25 mM HEPES, pH 7.4, 100 mM NaCl, 0.8 mg/ml BSA, 100 mM glucose, and 31 mg/ml streptomycin (IMDM with defined supplements) to a density of about 0.8 x 10⁶ cells/ml. Differentiation into a neutrophil-like phenotype was then induced by culturing the cells for 72 h in the presence of 0.5 mM Br-CAM.

Labelling of Cellular Phospholipids with [3H]AA—At the end of differentiation, HL60 cells were harvested and resuspended in the IMDM with defined supplements (see previous paragraph) at a concentration of 3.5 x 10⁶ cells/ml and labeled at 37°C for 1.5 h with 1 μCi/ml of [3H]AA (specific activity = 76 Ci/mmol, 13 nm final concentration). In some cases, cells were treated with PMA and/or staurosporine (final concentration 100 nM) and, in other cases, cells were treated by incubation at 4°C for 40 min. The samples were centrifuged for 10 min at 4°C, the supernatants being removed. The pellets were washed three times with 50 mM Tris-HCl, pH 7.5, 350 mM NaCl, 10 mM LiCl, and subjected to electrophoresis as described (7). The presence or absence of 1 μM staurosporine added to the regular differentiation medium (see above), immediately before start of the experiments. Cells were then washed twice with glucose-free HBSS and incubated with 5 μM 32p-aminocyanic A and 6 mM 2-deoxy-D-glucose for 5 min at 37°C, to reduce the levels of endogenous ATP (25). Cells were then washed in EPP supplemented with 2 μM peptatin, 2 μM leupeptin, 200 μM PMFSF, 10 mM NaF, 4 mM NaVO₄, 0.92 mM CaCl₂ (resulting in 1 μM free Ca²⁺), and 3 mM MgCl₂ (EPB plus protease and phosphatase inhibitors: "EPB + PPI"). The washed pellets were re-suspended in EPP + PPI further supplemented with 4 μM DIFP and incubated on ice for 30 min. Cells were centrifuged again, re-suspended in EPP + PPI, and subjected to electrophoresis as described above. Following electrophoresis, aliquots representing 4 x 10⁶ cells were diluted in a final volume of 1 ml of EPP + PPI containing 60 μCi of [γ-32P]ATP (specific activity 4,500 Ci/mmol, final concentration = 13.2 nM), 50 μM GTP·γ·S, with and without 100 nM 125I-anti-Rabbit IgG antiserum and incubated for 20 min at 4°C. After further incubation at 37°C for 10 min, cells were diluted 30-fold with ice-cold stop solution (as described for AA release assay) supplemented with protease and phosphatase inhibitors, and pelleted by centrifugation. Pellets were extracted by resuspending in 300 μl of 150 mM NaCl and 0.1% Nonidet P-40. Extraction buffer containing 50 μg of 50% SDS, 1 mM Nonident P-40, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 4 mM NaN₃, 2 μm peptatin, 2 μg/ml leupeptin, 200 μM PMSF, and 200 μM DIFP (protease inhibitors added freshly), followed by incubation at 4°C for 40 min. The samples were centrifuged for 10 min at 16,000 x g and 4°C, and the supernatants (cell extracts) saved for the immunoprecipitation procedures.

Immunoprecipitation, Electrophoresis, and Autoradiography of Phosphorylated cPLA₂—Experiments were carried out using a modification of the procedure described by Carlin and Knowles (30). The first step consisted of "preclearing" the extracts from proteins in buffer containing 0.1% Triton X-100, and irrelevant IgG antiserum and/or the protein A-coated Pansorsbin cells. To this end, 200-μl aliquots of 10% Pansorbin suspension (pretreated with 3% BSA) were combined with 20 μl of normal rabbit serum and incubated on ice for 2 h, followed by centrifugation. The Pansorbin cell pellets bearing antibodies unrelated to PLAz were washed with extraction buffer containing 50 ng of cPLA₂ standard and incubated on ice for 2 h, followed by centrifugation (16,000 x g, 10 min at 4°C). The supernatants (precleared extracts) obtained after two cycles of preclearing were subsequently treated with 2 μl of the rabbit anti-cPLAZ antiserum and incubated on ice for 2 h. The antigen-antibody complexes thus formed were used to resuspend BSA-pretreated Pansorbin pellets (equivalent to 200 μl of 10% Pansorbin) and incubated on ice for 2 h, followed by centrifugation (12,000 x g, 2 min at 4°C). The pellets, containing the immune complexes bound to protein A, were washed three times with 50 mM Tris·HCl pH 7.5, 350 mM NaCl,
5 mM EDTA, 0.5% Nonidet P-40, and supplemented with protease inhibitors, and once using this solution without Nonidet P-40. The washed pellets were resuspended in Laemmli buffer, boiled for 10 min, centrifuged at 16,000 x g for 10 min at room temperature, and the supernatants subsequently subjected to SDS-PAGE (10% acrylamide). At the end of electrophoresis, the gels were dried and exposed at −70°C using Kodak XAR films and intensifying screens.

**Western Blotting of cPLA₂**—The position of cPLA₂ on the autoradiograms was determined by subjecting either cPLA₂ standards (50 ng) or unlabeled neutrophil-like HL60 cells (3 x 10⁶ cells) to the extraction (in 200 μl of extraction buffer), pre-clearing, immunoprecipitation, washes, and PAGE procedures described above (in parallel with the purinergic receptors) using the electroporated cells incubated with [γ-³²P] ATP. Samples were transferred to nitrocellulose (pore size = 0.45 μm) by semi-dry electrophoresis in Towbin transfer buffer consisting of 192 mM glycine, 25 mM Tris, and 20% methanol (27), supplemented with 0.02% sodium azide. Non-specific binding sites in the blots were blocked first with 1% Tween-20 in PBS (25 mM Na₂HPO₄, pH 7.4, 150 mM NaCl, and 0.02% sodium azide) for 30 min and, subsequently, using a 3% solution of BSA in PBS for 1 h at room temperature. The blocked filters were incubated first with a 1:1000 dilution of rabbit anti-cPLA₂ in 3% BSA in PBS for 1 h at room temperature, followed by washing and reaction for 2 h with 0.5 μCi/ml ³²P-I-got anti-rabbit IgG antiserum in a 3% solution of BSA in PBS. The membranes were subjected to repeated washings both, between incubations with primary and secondary antisera, and after reaction with the secondary antiserum. Washing steps were carried out at room temperature using first 1% Tween-20 in PBS (three times, 5 min each) and then PBS (two times, 5 min each). Blots were subsequently air-dried and exposed as described above. Bands in addition to cPLA₂ were visible in all of the immunoreciproitated samples, corresponding to the recognition of rabbit IgG heavy and light chains (approximately 55 and 25 kDa, respectively) by the iodinated goat anti-rabbit IgG antiserum during Western blotting.

**Preparation of EGTA-Bivalent Cation Buffers**—Calculation of free Ca²⁺ and Mg²⁺ in EGTA-containing buffers was performed using the FREECA computer program (28).

**Presentation of Data**—The release of [³H]arachidonic acid was expressed as the percentage of the total radioactivity incorporated into intact or electroporated cells (or their subcellular fractions). Data are representative of results obtained in two to four independent experiments.

**RESULTS**

**Requirement of ATP for GTPyS and Ca²⁺-promoted AA Release in Electropermeabilized HL60 Granulocytes**—As shown in Fig. 1A GTPyS did not exert significant effects on AA release in electroporereabilized HL60 granulocytes included in the absence of ATP. However, when 1 mM ATP was included, addition of GTPyS resulted in a significant, Ca²⁺-dependent, stimulation of AA release. Maximal activation by GTPyS was detected at about 1 μM Ca²⁺, consistent with [Ca²⁺] levels in agonist-stimulated HL60 cells. The ATP-dependent effect of GTPyS was concentration-dependent, displaying an EC₅₀ of approximately 2 μM (Fig. 1B). Addition of 80–300 μM GTPyS resulted in the progressive inhibition of the maximal stimulatory effects detected at 10–50 μM nucleotide. It is also worth noting that addition of Ca²⁺ (up to 10 μM) did not elicit AA release in the absence of ATP. The effect of ATP was concentration dependent, displaying an EC₅₀ of approximately 0.25 mM, regardless of the presence or absence of GTPyS (Fig. 2A). Mg²⁺ was also required to support the effect of ATP as shown in Fig. 2B. Maximal stimulation and EC₅₀ were detected at 0.75 and 0.1 mM added MgCl₂ (corresponding to 0.6 and 0.085 mM Mg²⁺, respectively, under the assay conditions).

**The Permissive Effect of ATP Is Unrelated to Occupancy of Purinergic Receptors**—The permissive effect of ATP on GTPyS-elicited AA release does not represent the interaction of ATP with membrane-bound purinergic receptors, since the EC₅₀ corresponding to the nucleotide effect depicted in Fig. 2A is approximately two orders of magnitude higher than that obtained when measuring ATP-induced AA release in intact HL60 cells (7). The results presented in Fig. 3 support this conclusion. Pretreatment of cells with pertussis toxin impaired agonist-promoted AA release in intact differentiated HL60 cells (total inhibition was observed for the stimulation driven by fMLP) but had minor consequences on the effects of ATP and GTPyS in electroporereabilized cells. Further evidence supporting the action of ATP through an intracellular pathway was obtained when studying the effects of ATP⁺ and UTP. These nucleotides, which show potentials

**Fig. 1.** ATP-dependent stimulation of AA release by GTPyS and Ca²⁺ in electropermeabilized HL60 granulocytes. A, AA release in electropermeabilized cells was assayed in the presence of different concentrations of free Ca²⁺. Experiments were carried out in EPB containing 3 mM MgCl₂, 1 mM EGTA, and the concentrations of CaCl₂ required to obtain the indicated values of free Ca²⁺ (as calculated using the FREECA program). Closed and open symbols represent the inclusion or exclusion of 50 μM GTPyS in the assay, respectively. Triangles and circles represent the presence or absence of 1 mM ATP, respectively. B, concentration-response curve for GTPyS in the presence (closed circles) or absence (open circles) of 1 mM ATP. Assays were carried out in EPB containing 1 mM EGTA, 0.925 mM CaCl₂, and 3 mM MgCl₂. The concentration of MgATP⁺ was 1 mM, throughout all the range of concentration of GTPyS tested (closed circles); under this condition (1 mM ATP and increasing GTPyS), free Mg²⁺ decreased from 2 to 1.76 mM at 0.3 mM added GTPyS. Calculated free Ca²⁺ ranged from 0.98 to 0.96 μM and from 1.08 to 1.05 μM in the curves derived in the presence or absence of 1 mM ATP, respectively. For details see “Experimental Procedures.” CTRL, control.
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Fig. 2. Concentration-response curves for the effects of ATP and Mg\(^{2+}\) on AA release in electropermeabilized HL60 granulocytes. A, AA release was assayed in EPB buffer containing 1 mM EGTA, 0.92 mM CaCl\(_2\), 3 mM MgCl\(_2\), and increasing concentrations of ATP in the presence (closed symbols) or absence (open symbols) of 100 \(\mu\)M GTP\(_{Y}\).S. The concentration of free Ca\(^{2+}\) and Mg\(^{2+}\) ranged from 1.0 to 0.8 \(\mu\)M and 2.87 to 1.1 mM, respectively, under either condition (plus or minus GTP\(_{Y}\).S). Maximal effects were obtained at 960 \(\mu\)M MgATP\(^{2-}\). The highest concentration of MgATP\(^{2-}\)-assayed in the experiment was 1.82 mM. B, AA release was assayed in EPB containing 1 mM EGTA, 1 mM ATP, 0.935 mM CaCl\(_2\), and increasing concentrations of MgCl\(_2\) with (closed symbols) or without (open symbols) 100 \(\mu\)M GTP\(_{Y}\).S. Free Ca\(^{2+}\) ranged from 0.8 to 1 \(\mu\)M under either condition. Maximal effects were detected at 0.75 mM added MgCl\(_2\) which results in a calculated value of 0.6 mM MgATP\(^{2-}\); the calculated EC\(_{50}\) value for MgATP\(^{2-}\) was approximately 100 \(\mu\)M. Increasing total Mg\(^{2+}\) from 3 mM (last value shown in the figure) to 6 mM (which implies an increase in free Mg\(^{2+}\) from 2.04 to 5 mM) resulted in a significant reduction in AA release (not shown).

and efficacies identical to those of ATP in stimulating AA release in intact HL60 cells (7), could not replace ATP in supporting the action of GTP\(_{Y}\).S and Ca\(^{2+}\) (Fig. 4). Furthermore, other nucleotides that are poor substrates for protein kinases, such as AMP(NH)P (29), did not support the effects of GTP\(_{Y}\).S and Ca\(^{2+}\) on AA release (Fig. 4). These results, together with the high EC\(_{50}\) values observed for the ATP-permissive effect (Fig. 2A) and the Mg\(^{2+}\) requirement (Fig. 2B), suggest that phosphorylation of certain factors may be required for the stimulatory effects of GTP\(_{Y}\).S and Ca\(^{2+}\) on AA release.

Dual Role of PKC in the Regulation of AA Release—Numerous studies have provided evidence for the involvement of PKC in the regulation of AA release in a variety of cell types (5, 6, 10, 11, 15–20). The role of PKC in the ATP-dependent stimulation of AA release triggered by Ca\(^{2+}\) and GTP\(_{Y}\).S was investigated by measuring the effects of PMA on AA release in intact and permeabilized cells. Fig. 5A shows the time course and concentration dependence (inset) of the effect of PMA on agonist-promoted AA release in intact cells. Preincubation of cells for 5–10 min with 100 nM PMA blunted agonist-stimulated AA release. The maximal effect could be obtained at 10 nM PMA, after a 1-h incubation (inset). On the contrary, stimulation of AA release by AlF\(_3\) or Ca\(^{2+}\) ionophore A23187, agents that bypass receptor occupancy,
This enzyme blocks agonist-promoted (but not A1F- or Ca2+ of PMA in permeabilized cells could be partially reversed by the pretreatment of cells with 1 mM EGTA, 0.925 mM CaCl2 (1 mM free Ca2+), and 1 mM of the indicated nucleotides with (filled bars) or without 50 nM GTPyS. The calculated concentration of free Ca2+ was 1 μM, regardless of the presence or absence of 1 mM nucleoside triphosphates. No changes in free Ca2+ can be expected when using the other compounds, in view of the lower affinity for metals displayed by the nucleoside diphosphates and monophosphates. □, control; □, 30 nM GTPyS.

was not attenuated by pretreatment with 100 nM PMA (Fig. 5B). In fact, both AIF2- and A23187-stimulated AA release were significantly potentiated by such pretreatment. These differential effects of PMA on agonist and Ca2+ ionophore-induced release of AA resemble those reported in human platelets (15) and rabbit neutrophils (16).

Pretreatment of HL60 granulocytes for 10 min with 100 nM PMA resulted in a significant increase in ATP and Ca2+ dependent release of AA measured after electroporation (Fig. 6). Addition of GTPyS resulted in further stimulation of AA release in permeabilized cells treated with PMA. The effects of PMA in permeabilized cells could be partially reversed by the pretreatment of cells with 1 μM staurosporine, a PKC-selective inhibitor which interacts with its catalytic domain. Staurosporine, per se, completely abolished the ATP-dependent AA release stimulated by GTPyS and Ca2+ in permeabilized cells (Fig. 6). Addition of 1 μM staurosporine also resulted in inhibition of agonist-stimulated AA release in intact cells (data not shown). These results suggest a model of dual regulation of AA release by PKC: on one hand, activation of this enzyme blocks agonist-promoted (but not AIF2- or Ca2+ ionophore-dependent) release of AA, consistent with an impairment of the interaction between agonist-receptor complexes and G-proteins. On the other, PKC activation appears to be required for G-protein- and Ca2+-driven release, as measured in electropermeabilized cells.

Ca2+ and GTPyS Induce AA Release in HL60 Cell Homogenates and Isolated Plasma Membranes in an ATP-independent Manner—Ca2+, per se, could induce a substantive release of AA in HL60 granulocyte cavitates (Fig. 7A). Maximal effect of Ca2+ was obtained at about 1 μM. Addition of 50 nM GTPyS resulted in a 2-fold increase in the efficacy of AA release. The effect of GTPyS in cell homogenates was also Ca2+-dependent, as measured in the presence of ATP in electropermeabilized cells. However, in contrast with the results obtained with that system, ATP was not required to support the effects of Ca2+ and GTPyS on the release of AA in the cell homogenates. Addition of ATP actually resulted in a small, but reproducible, inhibition of GTPyS-stimulated AA release in cell homogenates. The ATP-independent effects were also detected when measuring AA release by a combination of plasma membrane and cytosolic fractions derived from HL60 granulocytes (Fig. 7B). The requirement of cytosol was explained by the presence of cPLA2 in this fraction, as measured in immunoblotting experiments using an anti-cPLA2 antiserum (results not shown). Taking into account that the cavitation and homogenization procedures were carried in Ca2+-free buffers containing 1 mM EGTA, this finding was consistent with the Ca2+-dependent translocation of PLA2 activity from membranes to cytosol observed in RAW 264.7 (30) and U937 cells (14, 31). A similar requirement of a combination of plasma membranes and cytosol was also observed when measuring

**Fig. 4.** Nucleotide specificity in the regulation of AA release by GTPyS in electropermeabilized HL60 granulocytes. AA release was assayed in EPB containing 3 mM total MgCl2, 1 mM EGTA, 0.925 mM CaCl2 (1 mM free Ca2+), and 1 mM of the indicated nucleotides with (filled bars) or without 50 nM GTPyS. The calculated concentration of free Ca2+ was 1 μM, regardless of the presence or absence of 1 mM nucleoside triphosphates. No changes in free Ca2+ can be expected when using the other compounds, in view of the lower affinity for metals displayed by the nucleoside diphosphates and monophosphates. □, control; □, 30 nM GTPyS.

**Fig. 5.** Effects of pretreatment with PMA on AA release promoted by various agonists in intact HL60 granulocytes. A, time course and concentration-response curve (inset) corresponding to the effects of pretreatment of cells with PMA on 2 μM fMLP- or 15 μM ATP-promoted AA release. Cells were pretreated with 100 nM PMA for various times during labeling with [3H]AA. AA release was assayed in the absence or presence of various agonists in HBSS as described under "Experimental Procedures." The inset shows the effects of pretreatment with increasing concentrations of PMA (for 1 h) on the release of AA stimulated by 2 μM fMLP. B, effects of PMA on the time courses of A1F2- or A23187-stimulated AA release. Cells were preincubated at 37 °C for 10 min in the presence or absence of 100 nM PMA in HBSS. Assay was started by adding either vehicle, 5 μM A23187 or 10 mM NaF plus 10 μM ATP-promoted AA release. Samples were collected in indicated times and diluted 15-fold with ice-cold stop solution. Time courses corresponding to control (open squares) and PMA-treated cells (closed squares) were almost superimposable. Values were corrected by subtracting the release corresponding to zero time. For details, see "Experimental Procedures." □, control.
Effects on AA release in permeabilized cells (Fig. 6), PMA and staurosporine significantly enhanced and inhibited, respectively, the phosphorylation of PLA2. The effect of PMA was clearly evidenced by the phosphorylation of cPLA2 in HL60 granulocytes. Furthermore, in good agreement with their effects on AA release in permeabilized cells (Fig. 6), PMA and staurosporine significantly enhanced and inhibited, respectively, the phosphorylation of PLA2 in this cell type (7).

The involvement of PLA2 in AA release in this cell type (7). Mepacrine also inhibited AA release driven by ATP, Ca2+ and GTPγS in electropermeabilized HL60 cells (Fig. 8A). More importantly, pretreatment of cytosol and plasma membranes with specific antibodies directed against a cPLA2 clone from U937 cells (12, 14) significantly attenuated the basal as well as GTPγS- and Ca2+-stimulated AA release (Fig. 8B). A similar treatment, using membranes from HL60 cells that were prelabeled with [3H]inositol, did not result in inhibition of inositol phosphate release driven by receptors (chemotactic or purinergic), GTPγS or high Ca2+ (Fig. 8C). These results confirmed that release of AA in this system is mediated by PLA2.

**Phosphorylation of PLA2 in Electropermeabilized HL60 Granulocytes**—The data presented so far suggested the hypothesis that PLA2 is normally under a certain inhibitory constraint which prevents its activation by Ca2+ and G-proteins and that phosphorylation by a PKC-dependent mechanism, or physical disruption (e.g. by N2 cavitation), can release this constraint. To investigate the locus of this regulation, we examined the phosphorylation of cPLA2 by carrying out immunoprecipitations of extracts derived from HL60 granulocytes (32).

The Release of AA in Electropermeabilized HL60 Cells and Subcellular Fractions Is Mediated Through PLA2—We previously showed that mepacrine, a widely used PLA2 inhibitor, abolishes agonist-stimulated AA release, but not phospholipase D activity, in intact HL60 cells. This effect suggested the involvement of PLA2 in AA release in this cell type (7). Mepacrine also inhibited AA release driven by ATP, Ca2+ and GTPγS in electropermeabilized HL60 cells (Fig. 8A). More importantly, pretreatment of cytosol and plasma membranes with specific antibodies directed against a cPLA2 clone from U937 cells (12, 14) significantly attenuated the basal as well as GTPγS- and Ca2+-stimulated AA release (Fig. 8B). A similar treatment, using membranes from HL60 cells that were prelabeled with [3H]inositol, did not result in inhibition of inositol phosphate release driven by receptors (chemotactic or purinergic), GTPγS or high Ca2+ (Fig. 8C). These results confirmed that release of AA in this system is mediated by PLA2.

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The Release of AA in Electropermeabilized HL60 Cells and Subcellular Fractions Is Mediated Through PLA2—We previously showed that mepacrine, a widely used PLA2 inhibitor, abolishes agonist-stimulated AA release, but not phospholipase D activity, in intact HL60 cells. This effect suggested the involvement of PLA2 in AA release in this cell type (7). Mepacrine also inhibited AA release driven by ATP, Ca2+ and GTPγS in electropermeabilized HL60 cells (Fig. 8A). More importantly, pretreatment of cytosol and plasma membranes with specific antibodies directed against a cPLA2 clone from U937 cells (12, 14) significantly attenuated the basal as well as GTPγS- and Ca2+-stimulated AA release (Fig. 8B). A similar treatment, using membranes from HL60 cells that were prelabeled with [3H]inositol, did not result in inhibition of inositol phosphate release driven by receptors (chemotactic or purinergic), GTPγS or high Ca2+ (Fig. 8C). These results confirmed that release of AA in this system is mediated by PLA2.
FIG. 8. Inhibition of AA release by mepacrine in electropermeabilized HL60 granulocytes (A) and by preincubation of plasma membrane and cytosolic fractions with anti-cPLA$_2$ antiserum (B). A, cells were preincubated with (triangles) or without (circles) 50 µM mepacrine at 37 °C for 10 min before electropermeabilization and AA release assay. AA release was assayed in the presence of 1 mM ATP, 3 mM MgCl$_2$, 1 mM EGTA, and various concentrations of CaCl$_2$ (to generate the indicated calculated concentrations of free Ca$^{2+}$) with (closed symbols) or without (open symbols) 50 µM GTPyS. B, plasma membrane and cytosolic fractions were isolated as described under "Experimental Procedures." The combined fractions were incubated in the absence of added serum (empty bars) or in the presence of 1.5% dilution of normal rabbit serum (filled bars) or rabbit anti-cPLA$_2$ antiserum (hatched bars) for 80 min at 4 °C before assay. AA release was measured in the presence of 3 mM total MgCl$_2$ and the other indicated conditions. C, Phosphoinositide-specific phospholipase C activity in membranes (20,000 x g pellet) prepared from nitrogen cavitated of HL60 granulocytes prelabeled with [3H]inositol. Membranes were preincubated for 80 min under the same conditions explained in B (same symbols are used), followed by assay of inositol phosphate release driven by receptor occupancy (UTP or fMLP plus GTP), receptor-independent G protein activation (GTPyS), or 1 mM Ca$^{2+}$. Release of inositol phosphates was expressed as percentage of total incorporated counts. For details see "Experimental Procedures." CTRL, control.

Fig. 9. Phosphorylation of cPLA$_2$ in electropermeabilized HL60 granulocytes. A, phosphorylation of cPLA$_2$. Electropermeabilized cells were incubated with [$\gamma$-32P]-ATP in the presence or absence of 100 nM PMA on ice for 20 min, followed by further incubation for 10 min at 37 °C. For treatment with staurosporine (ST), cells were preincubated with 1 µM of this compound for 1 h at 37 °C, before electropermeabilization. Samples were extracted, immunoprecipitated with anti-cPLA$_2$ antiserum, and processed as indicated under "Experimental Procedures." PM+ST indicates the effect of the combined treatment of cells with PMA and staurosporine, as described above. B, Western blotting of purified recombinant U937 cPLA$_2$ (15), either directly applied to the gel or previously subjected to immunoprecipitation (cPLA$_2$ standard) and of immunoprecipitated cPLA$_2$ extracted from HL60 granulocytes (differ. HL60). See "Experimental Procedures" for details.

DISCUSSION

In the present report we have studied the mechanism underlying the ATP-dependent stimulation of AA release by GTPyS and Ca$^{2+}$ in electropermeabilized HL60 granulocytes. The electropermeabilization technique used in the present study has many advantages over other procedures widely utilized to introduce substances into living cells (e.g. detergent permeabilization) (33, 34). The relatively small pores induced by electric fields permits the introduction of small (Mr $<$ 1000) intracellular substances but prevents the loss or dilution of enzymes and relevant regulatory proteins, problems which are frequently encountered in detergent-induced permeabilization. More importantly, the interaction of the detergents with the cholesterol groups present in the plasma membrane results in disruption of the lipid microenvironment. On the contrary, the effects of electric fields are only localized on cell sites oriented at 0 and 180° with respect to the direction of the field, leaving other regions of the plasma membrane intact (33). This is relevant in view of the importance of normal lipid microenvironments in the molecular assembly of phospholipases, accessory regulatory proteins, and plasma membrane activation (GTPyS), or 1 mM Ca$^{2+}$. Release of inositol phosphates was expressed as percentage of total incorporated counts. For details see "Experimental Procedures." CTRL, control.
The release of AA in the systems used in the present study is mediated by PLA2, as evidenced by the inhibitory effects of mepacrine and the anti-PLA2 antiserum (Fig. 8, A and B). The effects of GTPγS on AA release can be at least partially attributed to the direct interaction of a G-protein with PLA2, as opposed to a mechanism whereby G-protein-dependent activation of phospholipase C results in the generation of diacylglycerol and Ca2+-mobilizing inositol phosphates, activation of PKC, and subsequent events leading to the phosphorylation and activation of PLA2. This conclusion is supported by the experiments using cell homogenates and isolated membranes, where ATP was not required to support AA release by GTPγS (Fig. 7, A and B). The fact that GTPγS could further enhance AA release in the presence of a saturating concentration of PMA (100 nM) also supports a direct interaction of a G-protein with PLA2 (Fig. 6). In agreement with this notion is the demonstration of the differential regulation of phospholipase C and PLA2 activity by G-proteins in HL60 granulocytes (2, 46).

These results are consistent with a model where PLA2 is normally under an inhibitory constraint that prevents its activation by Ca2+ and G-proteins and that phosphorylation of PLA2 by a PKC-dependent mechanism, or physical disruption (e.g. N2 cavitation) of the molecular assembly of the enzyme system, can release this constraint. We have also excluded the possibility that the N2 cavitation procedure may cause the phosphorylation of the PLA2 (as an explanation for the ATP-independent effects measured in homogenates and subcellular fractions). This was investigated by comparing the levels of phosphorylation attained in permeabilized cells suspended in solutions containing [32P]ATP and subjected to either N2 cavitation or to the incubation conditions used in the experiments shown in Figs. 9 and 10 (results not shown).
The above mentioned model can interpret the stimulatory effects brought about by PKC activation, and the inhibitory effects of its down-regulation, on AA release in various cell types. In view of the close correlation between the effects of activators and inhibitors of PKC on AA release in electropermeabilized HL60 cells (Fig. 6) and their effects on the phosphorylation of PLAr (Fig. 9), it is unlikely that phosphorylation of additional factors (such as lipocortins, Ref. 47) represents the only mechanism responsible for the release of the inhibitory constraint. In this context, Lin et al. (45) have recently observed that agonists and phorbol esters increase serine phosphorylation of cytosolic PLAr in Chinese hamster ovary cells and RAT-2 fibroblasts. The phosphorylation of PLAr in HL60 granulocytes may be directly catalyzed by PKC (as observed in vitro for a cytosolic PLAr purified from rat mesangial cells) or, alternatively, by a protein kinase or protein kinase cascade dependent on PKC activity. A likely candidate for a PKC-dependent pathway that may lead to PLAr phosphorylation is the MAPK kinase/MAPK cascade. This possibility is based on the reported involvement of PKC in the phosphorylation and activation of MAPK by mitogenic agonists (48, 49), the serine/threonine phosphorylation-dependent activation of MAPK kinase (50), and on in vitro experiments showing the direct phosphorylation (and activation) of purified forms of PLAr by MAPK.23

Cockcroft et al. (2) observed that depletion of endogenous ATP completely abolished the stimulation of PLAr-mediated AA release by agonists in HL60 granulocytes and neutrophils. This result could be explained by the inability of the PKC-dependent pathway to phosphorylate PLAr in the absence of ATP. Interestingly, Lu et al. (25) reported that under conditions similar to those in the present study, GTPyS-stimulated superoxide production was dependent on ATP and could be inhibited by staurosporine. In view of the stimulation of superoxide anion production by AA (51, 52), it seems reasonable to propose that the ATP dependence of GTPyS-stimulated superoxide anion generation reported by Lu et al. is linked to the PKC-dependent phosphorylation of PLAr reported here.

In summary, we have investigated the mechanism for the regulation of PLAr by G-proteins and Ca++. We propose that PLAr is maintained in situ under an inhibitory constraint that prevents its activation by G-proteins and Ca++. This constraint can be released by PKC-dependent phosphorylation of PLAr. The role of Ca++ in the regulation of PLAr-catalyzed AA release is apparently 3-fold. First, it is involved in the activation of PKC and, hence, subsequent phosphorylation of PLAr. Second, once the inhibitory constraint is released, Ca++ per se becomes directly stimulatory to PLAr, as evidenced by the ability of Ca++ to stimulate PLAr in cell homogenates and isolated membranes (Fig. 7) and its ability to increase the activity of purified PLAr from rat mesangial (53) and U937 (12-14) cells in vitro. Third, Ca++ is required for the activation of PLAr by G-proteins after the inhibitory constraint of PLAr is released (Figs. 1, 6, and 7). The present study therefore suggests that the activation of PLAr by G-protein-coupled receptors in intact cells requires the parallel activation of phospholipase C, with generation of diacylglycerol and Ca++. This leads to the phosphorylation of PLAr and its activation by G-proteins (Fig. 9). 24. Xie, M., and Dubyak, G. R. (1991) J. Biol. Chem. 266, 25974-25979.
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