Evidence of GTP-binding Protein Regulation of Phospholipase A2 Activity in Isolated Human Platelet Membranes*

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G protein regulation of human platelet membrane phospholipase A2 activity was investigated at pH 8.0 and 9.0 by studying the effects of the nonhydrolyzable GTP analogue, guanosine 5'-O-(3-thiotriphosphate) (GTPγS), and of F7/Al3+ ions on arachidonic acid (AA) release. The membrane acted as the source of the enzyme, the substrate, and the G protein. At pH 8.0, 10 and 100 μM GTPγS stimulated AA mobilization at least 6-fold. Optimum AA release conditions required 1 mM Ca2+ and 5 mM Mg2+. Nonspecific nucleotide effect was excluded since similar stimulatory effects on AA release were not observed by ATP, GTP, ADP, and NADP. Although at pH 9.0 the GTPγS-stimulated AA release was greater than at pH 8.0, it constituted only 26% of the total. At both pH values the effect of F7 (10 mM) in the presence of Al3+ (2 μM) was similar to that of GTPγS. The G protein inhibitor, guanosine 5'-O-(2-thiodiphosphate), inhibited the GTPγS-stimulated AA release by about 80% at pH 8.0 and by 100% at pH 9.0. To determine a possible contribution to AA mobilization by the phospholipase C and diacylglycerol lipase pathway, the effects of neomycin, a phospholipase C inhibitor, were investigated. 100 μM neomycin did not inhibit the GTPγS-stimulated AA release at pH 8.0 and only slightly so (17%) at pH 9.0. At pH 8.0 in the presence of Ca2+ the released fatty acids consisted mainly of arachidonic and docosahexaenoic acids (80 and 8%, respectively). GTPγS had no effect on the fatty acid profile but only on their quantity. These results provide evidence of G protein regulation of phospholipase A2 activity in isolated platelet membranes.

Experimental evidence indicates that two enzymatic pathways may be involved in AA mobilization by stimulated platelets: 1) direct mobilization of AA by PL A2-catalyzed hydrolysis of mainly phosphatidylcholine and phosphatidylethanolamine (1, 2); and 2) activation of a polyphosphoinositol-specific PLC leading to the formation of AA-rich diacylglycerol which may be further metabolized by diacylglycerol lipases to yield AA (3, 4). In view of the selectivity with which stimulated cells release AA, the apparent lack of specificity for AA by the soluble PL A2s posed a serious objection to the PL A2 pathway (5). This objection, however, seemed to have been alleviated with the isolation of a PL A2 from macrophage cell line RAW 264.7 which possessed a considerable preference for AA (6). However, the relative importance of the two pathways has yet to be resolved (5).

The mechanisms of signal transduction leading to AA mobilization are not yet well understood. There is considerable evidence in platelets of G protein regulation of PLC activity (7-10). Some evidence of G protein regulation of PL A2 activity has also been reported in platelets (11, 12) as well as in other cells (12-16). In platelets (10) and in FRTL5 thyroid cells (13), different G proteins were responsible for the regulation of the respective PLC and PL A2 activities. Although Crouch and Lapesita (17) reported that the adenylate cyclase inhibitory G protein (Gγ) was not involved in platelet PL A2 regulation, their results did not rule out the possibility of its regulation by another G protein.

We have elected to study the possibility of G protein regulation of the previously reported Ca2+-dependent PL A2 in isolated platelet membranes which required for optimum activity pH 9.5 and 10 mM Ca2+ (18). This enzyme was linked to AA mobilization in intact cells by the findings that the release of AA in isolated membranes and in thrombin- and collagen-stimulated platelets was inhibitable in a similar manner by the sulphhydril-blocking reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (19).

By means of the G protein activators, GTPγS and F7/Al3+ (20), and utilizing an assay in which the membrane acts as the source of the enzyme, the substrate (19), and the G protein, we report evidence of G protein activation of the platelet membrane PL A2.

EXPERIMENTAL PROCEDURES

Materials—All reagents were purchased from Sigma, except GTPγS and GDP/S from Boehringer Mannheim, solvents from Mallinkrodt Chemical Works, and AA and heparin from Calbiochem and from Altech Associates. Outdated human platelet concentrates were from the New York Blood Center.

Solutions and Buffers—Buffers in the PL A2 assays were: at pH 8.0, 0.133 M Tris, 0.067 M KCl, and 0.533 M bovine serum albumin (essentially fatty acid free); at pH 9.0 and 9.5, 0.133 M glycine was substituted for Tris. The concentrations of other reagents used in the assay are reported under "Results." Membrane Preparation—Within 3 days following expiration, platelet concentrates were washed and disrupted by nitrogen decompression as previously described (19). Briefly, the homogenate was layered on a 30% (w/v) sucrose cushion and ultracentrifuged in an SW 50.1 rotor at 300,000 x g for 60 min. The membranes, from buffer-sucrose interface, were washed and collected by ultracentrifugation in a type 65 fixed angle rotor at 217,000 x g for 2 h. The membranes, were stored at -80 °C and used within 6 months.

PL A2 Assay—PL A2 activity was measured by the extent of AA release as previously described (19) except 40-60 μl of membrane suspension containing 0.5-0.5 mg of protein were added to the incubation solution containing 300 μl of the assay buffer and any additional reagents indicated under "Results." The final reaction volume was 400 μl. Following incubation, the suspension was acidified, and the free fatty acids were extracted with ethyl acetate, methylated with diazomethane, and analyzed on a Varian 4600 gas-liquid chromato-

21466
were done in duplicate.

**RESULTS**

To determine possible G protein regulation of the platelet membrane PLA2, the effects on AA mobilization of both the nonhydrolyzable GTP analogue, GTPγS, and of F-/Al³⁺ were investigated (Table I). At pH 8.0 in the presence of 1 mM Ca²⁺ and 10 mM Mg²⁺, both GTPγS and F-/Al³⁺ stimulated the membrane PLA2 activity 6-fold. 10 mM Ca²⁺ alone had an effect on AA release comparable with that of 100 μM GTPγS at a 10-fold lower Ca²⁺ concentration. Under those conditions the GTPγS effect was not observed (results not shown). At pH 9.0 (Table I) the GTPγS- and the non-GTPγS-stimulated AA release increased markedly. However, the fraction attributable to GTPγS activation was substantially reduced. The effects of F-/Al³⁺ were similar to those of GTPγS. In the presence of 10 mM EDTA and no added Ca²⁺ and Mg²⁺, AA release was very low at pH 8.0 and 9.5 and no GTPγS stimulation was observed. We also found that 2 mM GSH increased GTPγS-stimulated AA mobilization by about 16% but had no effect on control values (results not shown).

Time courses of the reactions at pH 8.0 and 9.0 are shown in Fig. 1. The increase in GTPγS-stimulated AA accumulation was linear during the first hour of a 3-h incubation period. Approximately 80% of the AA was released during the first hour of incubation at pH 8.0 and 67% at pH 9.0. Because the ratio of the GTPγS-stimulated AA release to that obtained under control conditions was about the same, valid comparisons could be made between the results obtained from 1- or 3-h incubation periods.

The possibility of a nonspecific nucleotide effect on the PLA2 activity was also investigated. The effects of ATP, GTP, ADP, and NADP were compared with those of GTPγS (Table II). No significant difference in AA mobilization was observed when GTPγS concentration was reduced from 100 to 10 μM. AA mobilization was not stimulated either by 10 or 100 μM ADP or NADP, or by 10 μM GTP or ATP. However, 100 μM ATP appeared to stimulate AA release about 3-fold.

Events mediated by G proteins are known to be inhibitable by nonhydrolyzable GDP analogs (14, 20). The effects of GDPβS on GTPγS-stimulated PLA2 activity are shown in Table III. 524 μM GDPβS inhibited 100 μM GTPγS-stimulated AA release by about 80% at pH 8.0 and by 100% at pH 9.0 but had no significant effect on the control values.

Reports of the presence in platelets of a membrane-bound PLC (7, 9) raised the possibility that we might be observing the results of sequential actions of a G protein-activated PLC followed by a diacylglycerol lipase. We therefore used neomycin, a PLC inhibitor (10), to determine a possible contribution to the AA mobilization by the PLC/diacylglycerol lipase pathway. At pH 8.0, 0.1 mM neomycin appeared to have no significant inhibitory effect. However, 1.0 mM neomycin inhibited GTPγS-stimulated AA release by about 37% (Fig.

**Table I**

**Effects of GTPγS and F-/Al³⁺ on arachidonic acid accumulation at pH 8.0 and 9.0**

Isolated platelet membranes were incubated with shaking at 37 °C for 3 h. All buffers contained 1 mM Ca²⁺ and 10 mM Mg²⁺ except Ca²⁺ and Mg²⁺ were omitted from the EDTA experiments and Mg²⁺ was added to the membrane PLA2 activity. The concentrations of the reagents were: GTPγS, 100 μM; F-/Al³⁺, 10 mM NaF, 2 μM Al₃⁺. All experiments were done in duplicate except the EDTA experiment was done at a different time.

| Additions               | pH 8.0 | pH 9.0 |
|-------------------------|--------|--------|
| GTPγS                   | 11.4 ± 0.3 | 71.1 ± 1.7 |
| F-/Al³⁺                 | 11.0 ± 0.6 | 74.4 ± 1.9 |
| Control                 | 1.8 ± 0.3 | 58.2 ± 1.1 |
| Ca²⁺, 10 mM             | 12.2 ± 0.3 |                |
| EDTA, 10 mM             | 2.3 ± 0.4 | 1.8 ± 0.6 |
| GTPγS + EDTA, 10 mM     | 1.1 ± 0.2 | 3.5 ± 0.3* |

*These observations were determined at pH 9.5.

![Fig. 1. Time course of arachidonic acid accumulation in the presence and absence of GTPγS at pH 8.0 and 9.0.](image-url)
Effects of nucleotides on arachidonic acid mobilization at pH 8.0

Isolated platelet membranes were incubated with shaking at 37 °C for 1 h. All buffers contained 1.9 mM GSH, 0.1 mM Ca^{2+}, and 5.0 mM Mg^{2+}. All buffers and quantitation of the released AA are described under "Experimental Procedures." Values are mean ± S.D. from duplicate observations. Estimated membrane proteins per each determination = 300 μg.

| Additions         | AA/observation nmol/observation | pH 8.0 | pH 9.0 |
|------------------|--------------------------------|--------|--------|
| EDTA, 10 mM      | 0.5 ± 0.1                      |        |        |
| Control          | 0.8 ± 0.6                      |        |        |
| GTPγS, 100 μM    | 7.6 ± 0.2*                     |        |        |
| GTPγS, 10 μM     | 6.5 ± 0.6*                     |        |        |
| ATP, 100 μM      | 2.2 ± 0.4*                     |        |        |
| ATP, 10 μM       | 0.8 ± 0.1*                     |        |        |
| GDP, 100 μM      | 0.2 ± 0.2*                     |        |        |
| GDP, 10 μM       | 0.1 ± 0.1*                     |        |        |
| NADP, 100 μM     | 0.5 ± 0.1*                     |        |        |
| NADP, 10 μM      | 0.4 ± 0.2*                     |        |        |

* No significant difference between these observations, p > 0.05.
* No significant difference between these observations, p < 0.05.

Effect of GDPβS on GTPγS-stimulated arachidonic acid mobilization

Isolated platelet membranes were incubated with shaking at 37 °C for 1 h. All buffers contained 1.9 mM GSH, 0.1 mM Ca^{2+}, and 5.0 mM Mg^{2+} and where indicated 100 μM GTPγS and 524 μM GDPβS. GDPβS was added to the membranes just prior to GTPγS addition. All buffers and quantitation of AA are described under "Experimental Procedures." Values are mean ± S.D. from duplicate observations. Estimated membrane proteins per each observation = 430 μg.

| Additions                 | Arachidonic acid nmol/observation | pH 8.0 | pH 9.0 |
|--------------------------|----------------------------------|--------|--------|
| GTPγS                    | 6.0 ± 0.6                        | 19.6 ± 1.1 |
| GTPγS + GDPβS            | 2.0 ± 0.3*                      | 9.2 ± 1.7 |
| GDPβS                    | 1.1 ± 0.3*                      | 11.2 ± 0.9 |
| Control                  | 0.7 ± 0.3*                      | 11.7 ± 1.0 |

* No significant difference compared with the control, p > 0.05.
* No significant difference compared with control, p < 0.05.

2. At pH 9.0, 0.1 mM neomycin inhibited GTPγS-stimulated AA mobilization by about 17%.

Studies of the effects of Ca^{2+} and Mg^{2+} on GTPγS-stimulated AA mobilization at pH 8.0 are shown in Fig. 3. In the presence of 5 mM Mg^{2+}, GTPγS-stimulated AA mobilization exhibited a sharp maximum at 1.0 mM Ca^{2+}. No significant GTPγS effect was observed in the absence of added Ca^{2+} or in the presence of 1.0 mM EGTA. At a constant 1.0 mM Ca^{2+} concentration, the GTPγS-stimulated AA release was maximal at 5.0 mM Mg^{2+}. Although omission of Mg^{2+} from the incubation buffer had no inhibitory effect on the GTPγS-stimulated AA release, Mg^{2+} was required for optimum effect (Fig. 3). AA release of the control experiments was low and varied minimally with Ca^{2+} and Mg^{2+} concentrations.

At pH 8.0, the released fatty acids consisted mostly of arachidonic (80%) and docosahexaenoic (22;6, 8%) acids. No significant variation in the fatty acid profile was observed in the presence or absence of GTPγS and neomycin or when Ca^{2+} concentration was varied between 0.05 and 1 mM.

DISCUSSION

The previously demonstrated PLA2 activity in isolated platelet membranes required high pH and high Ca^{2+} concentration (pH 9.5, 10 mM Ca^{2+}) for optimum activity (18). In view of such nonphysiological activating conditions and the recently emerging evidence of G protein regulation of a number of PLA2s (10–16), we investigated the existence of a similar regulating mechanism in platelet membranes. The membranes acted as the source of PLA2, the enzyme substrate, and a regulatory G protein. In this assay the use of labeled exogenous phospholipids was avoided and all of the released AA was quantitated. In view of reports that PLA2 activities depend on the nature and physical state of the substrates (21, 22) the preservation of the substrate-enzyme relationship in the isolated membranes seemed most desirable. At pH 8.0, our results were consistent with G protein activation of PLA2 (20). The enzyme was activated by both GTPγS and by F~-/Al3+ ions (Table I). Activation by GTPγS was specific. None of the other nucleotides used, with an apparent exception of 100 μM ATP, seemed to have any PLA2 stimulatory effect (Table II). GTPγS activation of the PLA2 was significantly inhibited by GDPβS (Table III). The lack of inhibition of AA release by 0.1 mM neomycin (Fig. 2) as well as the reported (9) low pH optimum (6.5–7.0) of the membrane-bound PLC argued against the possibility that AA was released by the PLC/diacylglycerol lipase pathway. In platelets, the reported stimulation of AA release by neomycin at concentrations greater than 0.1 mM indicated still another function of this reagent (11). Therefore, in our experiments, the partial inhibition of the GTPγS stimulation of AA release by 1 mM neomycin may be due to effects other than on PLC. The above results provided evidence that at pH 8.0, G protein-activated PLA2 was responsible for the released AA.

We found at pH 8.0 a disproportional release of arachidonic (80%) and docosahexaenoic acids (8%) to their reported concentrations in membrane phospholipids (29% and less than 2%, respectively) (23). Docosahexaenoic acid was reported to be almost exclusively acylated in phosphatidylinethanolamine which contained also 48% of all of the platelet membrane AA. These results are indicative of an enzymatic process where at least part of the AA would be expected to be mobilized from phosphatidylinethanolamine. The findings that GTPγS in the

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presence of Ca\(^{2+}\) affected only the quantity of the released fatty acids without altering their relative concentrations and that high Ca\(^{2+}\) concentration activated the membrane PLA\(_2\) to the same extent as G protein effectors at lower Ca\(^{2+}\) concentrations (9).

At pH 9.0, two apparently independent Ca\(^{2+}\)-requiring mechanisms contributed to AA mobilization: 1) G protein-mediated and 2) high pH-facilitated (Table I). As at pH 8.0, PLA\(_2\) activity was responsible for the G protein-mediated fraction of the released AA. AA release was activated by GTP\(_\gamma\)S and by F\(^-\)/Al\(^{3+}\). The GTP\(_\gamma\)S-stimulated AA release was inhibitable by GDP\(_\beta\)S (Table III) but only moderately so (17%) by 100 \(\mu\)M neomycin. The high pH-facilitated AA release had an absolute requirement for Ca\(^{2+}\) indicating an enzymatic nature of the mechanism. There is some experimental evidence that negatively charged substances introduced into the substrate environment increased substrate availability and thereby increased PLA\(_2\) activity (6, 21). At pH 9.0 an increase in the membrane negative charges may thus effect PLA\(_2\) activity. Whether in our experiments the pH-facilitated and the G protein-activated AA release was due to the activation of the same enzyme remains to be elucidated.

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