# A Requirement for Membrane-associated Phospholipase $A_2$ in Platelet Cytotoxicity Activated by Receptors for Immunoglobulin G and Complement

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## Summary

Platelets are potent antibody- and complement-dependent cytotoxic effector cells. We showed previously that a single platelet can lyse a target cell sensitized with immunoglobulin G (IgG) and complement components up to C3 (C∿3b denotes the target cell–bound fragment of complement up to C3; the precise nature of the bound C3 fragment has not been established), and that the complete cytotoxic system capable of specific recognition and lysis resides in platelet membranes. To define the components of platelet membranes required for cytotoxicity, a set of inhibitors of phospholipase $A_2$ ($PLA_2$) that act by different chemical mechanisms was tested. The lytic reaction is blocked at appropriate concentrations of bromophenacylbromide, mepacrine, and manoalide. When platelets are treated with bromophenacylbromide, inhibition of cytolytic activity and that of $PLA_2$ enzymatic activity occur in parallel. Platelets release arachidonate when incubated with target cells bearing IgG and C∿3b, confirming that FcγR and complement receptor trigger both $PLA_2$ action and efficient lysis. Inhibition by thimerosal of a reverse reaction, i.e., reacylation catalyzed by acyltransferase, causes increased target cell lysis, presumably by increasing the products of $PLA_2$ action. Platelet cytotoxicity is increased when platelets are pretreated with some products of $PLA_2$: exogenous lysophospholipids and not free arachidonic acid increase cytotoxicity. Electron microscopy suggests that platelets and target cells may fuse, possibly as a result of the formation of lysophospholipids which are well-known membrane fusogens. Fixation with paraformaldehyde does not affect platelet cytotoxicity, suggesting that the complete cytotoxic system resides as a preformed complex in platelet membranes. The results indicate that platelet membrane–associated $PLA_2$, together with receptors for Fc and complement, are required for platelet cytotoxicity.

Although small in size and lacking nuclei, platelets are potent cytotoxic effector cells present in numbers and total surface area that are vast in comparison with other blood-borne immune effectors (1, 2). Both mouse and human platelets bear receptors for the constant domains of IgG molecules (FcγR) and CR (3–6). Platelets are antibody-dependent tumoricidal effectors of the immune system in vivo (7–9). They also have been shown to lyse IgE-coated schistosomulae (10) and *Brugia malayi* microfilariae (11). In the absence of specific antibody, platelets can lyse *Toxoplasma gondii* (12) and tumor cells (13) in vitro. In these antibody-independent processes, the activation of phospholipase $A_2$ ($PLA_2$)[1] and the involvement of arachidonic acid metabolites have been implicated.

An experimental system facilitating study of platelet-mediated cytotoxicity in vitro was developed by Soper et al. (1). Antibody-coated sheep erythrocytes were efficiently lysed in platelet-rich plasma. We subsequently showed that washed platelets specifically lyse target cells coated with IgG2a, IgG2b, or IgG3 antibody together with complement C∿3b. The lysis is a single hit phenomenon, as a single platelet can lyse a target cell. The complete system capable of recognition and lysis is localized in platelet membranes (2). The cytotoxic system includes Fc receptors (FcγIIaR, FcγIIbR, and FcγIIIR), a still unidentified CR that is not CR1 (14), and unidentified lytic effector molecules. Our previous experiments showed that some mechanisms of cytotoxicity used by other cells, such as the generation of toxic oxygen metabolites, release of proteases, or formation of active granular cytolysin, are not involved in platelet-mediated, antibody-dependent cytotoxicity (2).

We became interested in elucidating the role of membrane lipids and proteins in the lytic reaction, since the complete

[1] *Abbreviations used in this paper:* BAP, bromoacetophenone; BPB, bromophenacylbromide; EMTS, ethyl mercurithiosalicylate; PAF, platelet-activating factor; $PLA_2$, phospholipase $A_2$.


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cytotoxic system resides in platelet membranes (2). As mentioned above, $PLA_2$ activation has been implicated in the mechanism of cytotoxicity by platelets in the absence of antibody (12, 13). Furthermore, several reports have indicated that ligation of Fc$\gamma$2bR is correlated with $PLA_2$ activation in human B cells and mouse macrophages (15–17). Fc$\epsilon$R and some Fc$\gamma$R share common subunits as part of their multichain structures (18), and activation of $PLA_2$ has been demonstrated upon Fc$\epsilon$R ligation in mast cells and basophils (19). It should be noted that primary sequences of Fc receptors purified to date do not encode a known lipase (20), but in some cases bear homology with the acetylcholine receptor, consistent with a demonstrated ionophore activity upon FcR ligation (20, 21). Activation of $PLA_2$ has also been proposed to occur in cytotoxicity mediated by NK cells (22–24). We therefore sought to establish a direct association between $PLA_2$ activation and the cytotoxicity induced by platelet receptors for IgG and complement.

## Materials and Methods

*Reagents and Buffers.* All reagents and buffers were prepared or purchased as previously described in detail (2), except where noted. Bromophenacylbromide (BPB), bromoacetophenone (BAP), and ethyl mercurithiosalicylate (EMTS; thimerosal) were purchased from Sigma Chemical Co. (St. Louis, MO); mepacrine from ICN Biomedicals (Costa Mesa, CA); and manoalide was kindly provided by Marianne S. de Carvalho and Robert S. Jacobs (University of California at Santa Barbara).

*Platelets, Target Cells, and Cytotoxicity Assay.* 5–8-wk-old AKR/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and platelets and C5-deficient plasma were isolated as previously described in detail (2), except as noted below. We used acid citrate dextrose anticoagulation buffer in collecting mouse blood in order to improve the yield of platelets. Plasma was collected in a heparinized syringe, 15 U in 150 $\mu$l saline per ml blood. SRBC (designated as E) were obtained from a local dealer at 2-wk intervals, washed and loaded with $^{51}$Cr (Amersham Corp., Arlington Heights, IL), sensitized with rabbit polyclonal anti-SRBC antiserum and mouse C$\sim$3b (where indicated; denoted as EAC$\sim$3b), and stored at 4°C in veronal buffered saline before lytic assay as described (2). Cytotoxic activity by platelets was determined by measuring Cr release over a 2-h incubation at 37°C, unless otherwise indicated (2). As noted, each data point represents the mean of several replicate experiments, and error bars indicate the sample SD.

*$PLA_2$ Assay.* $PLA_2$ activity was measured essentially as described (25). Briefly, for each sample, 0.25 $\mu$Ci [arachidonyl-1-$^{14}$C] L-$\alpha$-1-palmitoyl-2-arachidonyl phosphatidylchline (52.8 mCi/mmol; Dupont New England Nuclear, Boston, MA) was dried under a stream of nitrogen, and suspended in 2 $\mu$l DMSO followed by 98 $\mu$l assay buffer (100 mM Tris pH 9.0, 5 mM $CaCl_2$, 1 mg/ml fatty acid–free BSA; Sigma Immunochemicals) by vortexing and brief sonication in a bath sonicator. This mixture was added to $5 \times 10^7$ platelets or frozen and thawed platelets suspended in an equal volume of assay buffer. The reaction proceeded at 37°C for 60 min and was stopped by freezing samples at −70°C. Subsequently, thawed samples were extracted in chloroform and methanol (26) and samples in organic phase were spotted on predeveloped silica gel thin layer chromatography plates (prescored HL uniplates; Analtech Inc., Newark, DE). Plates were developed in 120:80:10:4 chloroform/methanol/ammonium hydroxide/water. After autoradiographic exposure (X-Omat film; Eastman Kodak, Rochester, NY) to localize spots, silica plates were scraped, and the conversion of phosphatidylcholine by $PLA_2$ to free arachidonic acid was determined by scintillation counting.

*Arachidonic Acid Release Assay.* 25 $\mu$Ci of [5, 6, 8, 9, 11, 12, 14, 15-$^3$H] arachidonic acid (1 mCi/ml solution in toluene; Amersham Corp.) was dried under nitrogen, suspended in 10 $\mu$l ethanol, added to 1 ml platelets at $2 \times 10^8$/ml, and incubated 90 min at 37°C to allow metabolic incorporation into platelet phospholipids. Platelets were centrifuged at 13,000 *g* for 15 min at 4°C, resuspended and washed again, and diluted to $4 \times 10^7$/ml. Incorporation was 20–40%. Labeled platelets, 25 $\mu$l, were added to a chilled multiwell plate with 25 $\mu$l target cells per well and shaken at 37°C for 2 h as described (2). Samples were diluted fourfold with cold buffer, centrifuged at 6,900 *g* for 15 min at 4°C, and both total radioactivity and that in the supernatant were determined to calculate percent release. Results are presented as percent increases in arachidonic acid released from platelets incubated with sensitized target cells (EAC$\sim$3b), compared with unsensitized control target cells (E); percent increase = 100 × [(percent release sample − percent release control/(percent release control)]. As a control, we determined the amount of arachidonic acid released from platelets incubated with no target cells; this was 11% of incorporated arachidonic acid. The release from platelets incubated at the highest ratio of unsensitized target cells (E) to platelets (0.5:1) was 9%.

*Lipid Loading.* A concentrated solution of lysophospholipid in ethanol was diluted 100-fold with a suspension of platelets at $2 \times 10^7$/ml at 4°C while vortexing, and incubated 60 min on ice to minimize metabolic conversion of loaded lipids. Platelets were centrifuged at 13,000 *g* for 15 min at 4°C, supernatants were decanted, and platelets were resuspended to original volumes with cold Tyrode's-gel-EDTA before addition to target cells. Cytolysis was measured by Cr release over 60 min at 37°C as described (2). Lysophosphatidylethanolamine, lysophosphatidylcholine, lysophosphatidic acid, lysophosphatidylserine, lysophosphatidylinositol, and free arachidonic acid were purchased from Serdary Research Laboratories (London, Ontario, Canada); platelet activating factor (PAF) and lyso-PAF were from Fluka Chemical Corp. (Ronkonkoma, NY).

*Electron Microscopy.* Mixtures of platelets and target cells were incubated for various periods, centrifuged, fixed in cacodylate buffer containing 4% glutaraldehyde, and processed for electron microscopy, as described (2).

*Fixation of Platelets.* Platelets were incubated with indicated concentrations of paraformaldehyde in Tyrode's buffer for 15 min at 6°C. Fixative was removed by passing platelets over a Sepharose CL-2B column (Pharmacia LKB Biotechnology, Piscataway, NJ; 27), followed by centrifugation as an additional washing step. The platelets were resuspended with no observable clumping in Tyrode's-gel-EDTA.

*Serotonin Release Assay.* [$^3$H]Serotonin (29.5 Ci/mmol, $\sim$30 pmol/$\mu$l; Dupont New England Nuclear) was added to platelets at $2.2 \times 10^8$/ml to a final concentration of 1 pmol/$\mu$l, incubated at 37°C for 30 min, centrifuged at 13,000 *g* for 10 min, and washed twice in Tyrode's buffer. Aliquots of platelets were fixed in 5% (wt/vol) paraformaldehyde and washed as described above. Loaded platelets were incubated at $1.5 \times 10^8$/ml with thrombin, A23187, target cells or buffer (as indicated) for 15 min, centrifuged at 13,000 *g* for 10 min, and radioactivity in supernatants and pellets (solubilized in Triton X-100) was determined by scintillation counting (28). Similar results were obtained in three separate experiments.

## Results

*$PLA_2$ Inhibitors Block Platelet Cytotoxicity.* Several compounds have been shown to inhibit $PLA_2$ activity by differ-

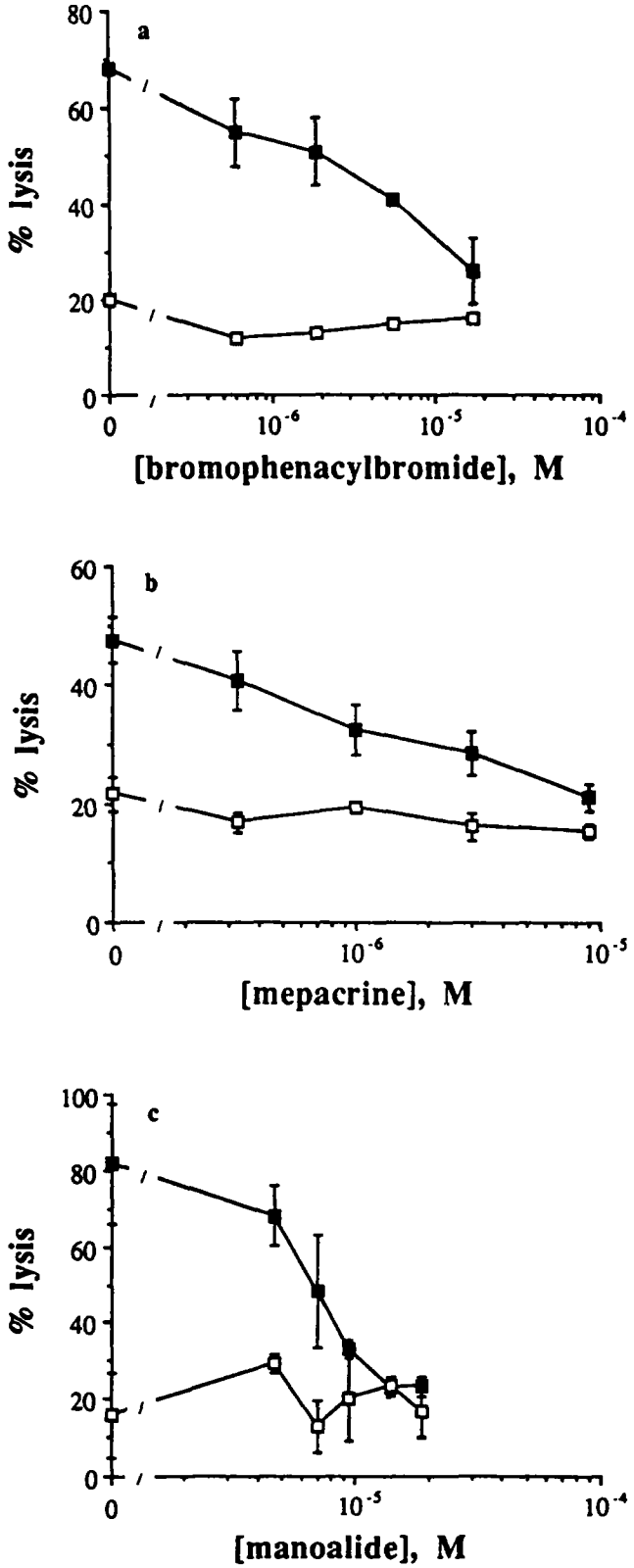


**Figure 1.** Inhibition of lysis by $PLA_2$ inhibitors. Each point represents the mean of four experiments, and error bars indicate the sample SD. (*a*) Platelets at 7 × $10^7$/ml were incubated with various concentrations of BPB (■) for 90 min at 25°C. Target cells (EAC↷3b) were added and lysis was assayed as described (2). As a control, BPB without platelets was added to target cells (□). Similar results were obtained with BAP, a chemical analog of BPB (see Fig. 2). (*b*) The effects of mepacrine were determined as in (*a*), except that platelets at 6 × $10^7$/ml were pretreated for 60 min before assay for cytotoxicity (■). As a control, mepacrine without platelets was added to target cells (□). (*c*) Platelets at 5 × $10^8$/ml were treated with the indicated concentrations of manoalide for 30 min at 37°C. The platelets were diluted 10-fold, added to target cells, and lysis was assayed (■). A a control, manoalide without platelets was added to target cells (□).

ent chemical mechanisms (29). BPB irreversibly inhibits $PLA_2$ by forming a covalent bond to a histidyl residue in the active site (30). Mepacrine is believed to prevent hydrolysis of phospholipid substrates by perturbing the substrate-enzyme interface (31). Manoalide is a relatively new $PLA_2$ inhibitor isolated from sponges that covalently modifies selective lysine residues, inactivating $PLA_2$ from a variety of sources (32). Although most careful biochemical experiments to date involve inhibition of purified enzymes, correlations between in vitro inhibitor efficacy and in vivo functions have also been established (33, 34). We tested the effect of these various $PLA_2$ inhibitors on the lytic reaction, first by preincubating intact platelets with the compounds. As shown in Fig. 1, BPB, mepacrine, and manoalide all inhibit platelet cytotoxicity. BAP, a chemical analog of BPB with a similar mode of action, also inhibits platelet lysis (see Fig. 2).

Each of the compounds tested has been shown previously to have pleiotrophic effects. BPB ($ID_{50}$ = 30–100 μM) and mepacrine ($ID_{50}$ = 0.1–0.3 mM) have been documented to inhibit phosphatidyl inositol–specific phospholipase C, and BPB also antagonizes diglyceride lipase (35, 36). Manoalide may also inhibit calcium mobilization and phospholipase C (37, 38). However, each of these compounds respectively inhibits platelet cytotoxicity in the appropriate concentration ranges documented to be necessary for $PLA_2$ inhibition (Fig. 1; 22, 29, 33, 34).

Inhibition of platelet cytotoxicity is greatest with freshly isolated platelets. We found that platelets stored for several weeks at 4°C consistently and specifically lysed target cells, but that the lytic reaction with stored platelets generally was not as efficiently inhibited as described above (data not shown). We speculate that the products of $PLA_2$ may spontaneously accumulate with prolonged storage at 4°C, so that inhibitors would fail to block cytotoxicity since the required product(s) would already have been formed.

*$PLA_2$ Inhibitors Act on Platelets and Not Target Cells.* Destruction of target cells by some effector cells has been shown to include a programmed series of reactions leading to lysis of the target cells, which presumably could include activation of target cell $PLA_2$. Sheep erythrocyte membranes have shown to possess $PLA_2$ activity (39). To exclude the possibility that the $PLA_2$ activity involved in platelet cytotoxicity is localized in target cells, we preincubated either platelets or target cells with $PLA_2$ inhibitors, BPB or BAP. The treated cells were washed and the corresponding untreated target cells or platelets were added to assess lytic function. As shown in Fig. 2, pretreatment of platelets results in irreversible inhibition of cytotoxicity. Pretreatment of target cells at similar concentrations followed by washing results in virtually no inhibition of lysis, thereby showing that $PLA_2$ in-

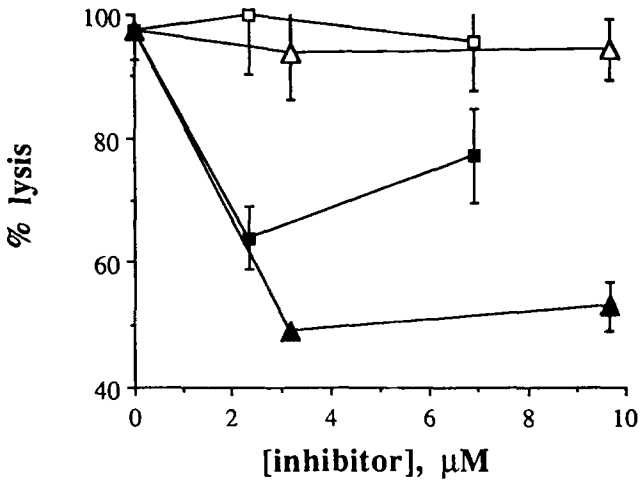


**Figure 2.** $PLA_2$ inhibitors act on platelets and not target cells. Various concentrations of BPB (*squares*) or BAP (*triangles*) were added to platelets (*solid symbols*) or target cells (EAC∿3b; *open symbols*) for 2-h pretreatment at 37°C. The pretreated platelets or target cells were washed. Untreated platelets or target cells were added to pretreated target cells or platelets, respectively, and lytic activity was determined. Each point represents the mean of four experiments, and error bars indicate SD.

hibitors act irreversibly on platelets and not on target cells. Although target cells contain $PLA_2$ activity (39), the $PLA_2$ activity relevant in platelet cytotoxicity is found in platelets.

*Correlation between Inhibition of $PLA_2$ Enzymatic Activity and Inhibition of Cytotoxicity.* We sought to strengthen the hypothesis that $PLA_2$ activity is required in platelet cytotoxicity, by determining whether or not BPB affects both enzymatic activity and lytic function, in the same concentration range and with parallel dose–response curves (33). We initially determined the amount of $PLA_2$ activity in various platelet preparations by measuring the formation of arachidonic acid by platelet extracts incubated with [$^{14}$C-arachidonyl]-L-α-1-palmitoyl-2-arachidonyl-phosphatidylcholine according to the method of Kramer et al. (25). As expected, the measured $PLA_2$ activity increased linearly with increasing platelet concentration, with intact platelets possessing more total enzymatic activity than isolated platelet membranes, even though both preparations are effective in lysing target cells (2). Furthermore, both platelet cytotoxicity and $PLA_2$ activity, as measured by formation of free fatty acid, increase in parallel with increasing platelet doses (data not shown).

To establish that BPB inhibits platelet cytotoxicity by inhibiting $PLA_2$ activity, we compared inhibition curves for the two effects. As shown in Fig. 3 *a*, the cytotoxicity by intact platelets treated with BPB and then washed, and by platelet membranes treated with BPB and washed, are inhibited to the same degree. The results indicate substantial inhibition of cytotoxicity caused by platelet membranes, as was the case with BPB-treated washed or unwashed intact platelets (Figs. 1 and 2).

We determined $PLA_2$ activity of these treated platelets or platelet membranes as described above. As shown in Fig. 3 *b*, increasing doses of BPB progressively diminish $PLA_2$ activity as measured by separation of products by thin layer chromatography followed by radioautography. The correlation between the inhibition of cytotoxic activity and of $PLA_2$ activity by BPB is shown for frozen and thawed platelet membranes in Fig. 3 *c*, and for intact platelets in Fig. 3 *d*. Both total enzymatic activity and cytotoxic function are inhibited over comparable concentration ranges, and the dose–inhibition curves are approximately parallel over a 5–20-fold concentration range, in particular for the isolated platelet membranes (Fig. 3 *c*). It is possible that the decreased total $PLA_2$ activity in the partially purified membranes compared with that in intact platelets is more directly relevant to the cytotoxic reaction and more easily accessible to inhibition by exogenous BPB, accounting for the good correlation between inhibition of membrane $PLA_2$ activity and of cytotoxicity (Fig. 3, *b* and *c*).

*Free Arachidonic Acid, a Product of $PLA_2$ Action, Is Formed and Released by Platelets during the Lytic Reaction.* Given the above results with $PLA_2$ inhibitors, we sought to obtain more direct evidence indicating platelet $PLA_2$ activation triggered by EAC∿3b target cells. Thus platelets were loaded with radiolabeled arachidonic acid and then incubated with various target cells. Supernatants were collected and the amount of released arachidonic acid determined as shown in Fig. 4. In comparison to the amount of arachidonic acid formed and released by platelets incubated with unsensitized E target cells, platelets incubated with EAC∿3b released almost twice as much free fatty acid. The results indicate that platelet $PLA_2$ is activated by EAC∿3b target cells.

Previously, a relationship has been described between FcεR ligation and $PLA_2$ activation. Furthermore, $PLA_2$ activity and Fcγ2bR have been copurified (15, 16, 19). To determine the relative role of receptors for antibody (FcR) in activating $PLA_2$, we determined whether or not target cells sensitized with antibody alone (EA) would also trigger arachidonic acid release by platelets. As shown in Fig. 4, platelets do not release more arachidonic acid when incubated with EA as compared with E target cells. Similar results were obtained when target cells were sensitized with monoclonal IgG2a or IgG2b anti-SRBC antibodies. Both IgG2a and IgG2b were individually effective in triggering arachidonate release by platelets only when target cells were also sensitized with complement (data not shown). In only 2 out of 16 determinations using target cells sensitized with monoclonal or polyclonal anti-SRBC antibodies, platelet arachidonate release was in fact increased, in contrast to the usual case described above (Fig. 4). We have previously shown that neither ligation of FcR alone, nor of CR alone, is sufficient to trigger efficient target cell lysis. Simultaneous ligation of FcR and CR is required for platelet cytotoxicity (2). The present results suggest that ligation of receptors not only for Fc but also for complement is required, to trigger both efficient $PLA_2$ activity and cytotoxicity (Fig. 4).

*Inhibition of Lysophospholipid Reacylation Increases Platelet Cytotoxicity.* EMTS (thimerosal) has been shown to inhibit acyl CoA:lysophosphatide acyltransferase, which catalyzes the formation of phospholipids from lysophospholipids and free fatty acids (40). We reasoned that inhibition of platelet acyltrans-

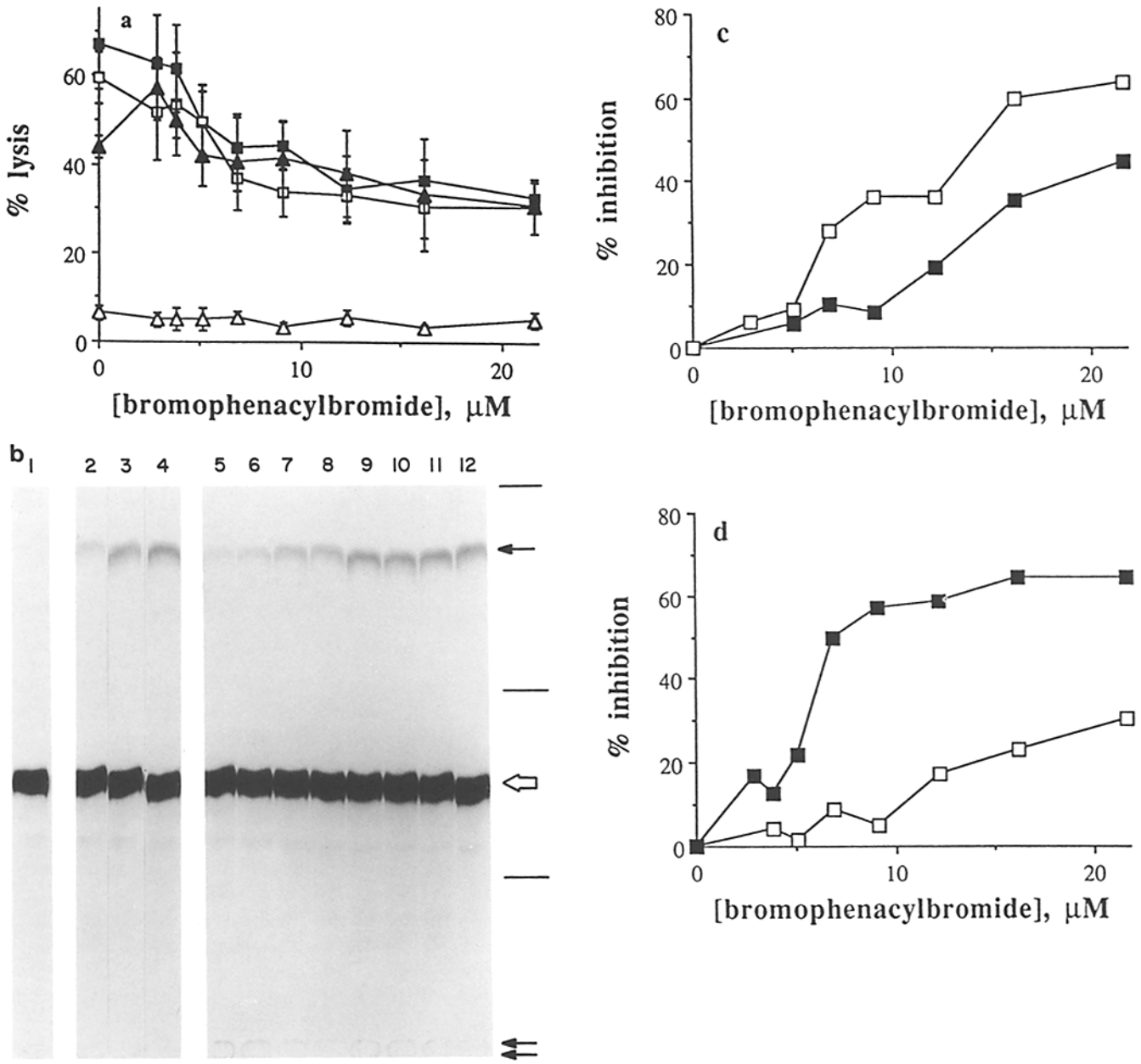


**Figure 3.** Correlation between inhibition of platelet $PLA_2$ activity and inhibition of cytotoxicity. (*a*) The relationship between cytotoxicity and $PLA_2$ inhibitor concentration was determined as in Fig. 1 under different assay conditions. Platelets ($5 \times 10^8$/ml) were pretreated with various concentrations of BPB in DMSO for 30 min at 37°C, and then assayed at $2.5 \times 10^7$/ml with target cells. The various lytic assay conditions are defined as follows: platelets added directly to EAC∼3b target cells (■); washed and then added to target cells (□); frozen and thawed for three cycles, washed, and then added to target cells (▲); or added directly to unsensitized E target cells (△). Lysis was then assayed. Results of the lytic assay are shown as the mean of five replicate experiments, and error bars indicate the sample SD. (*b*) An autoradiograph of radiolabeled phospholipids separated by thin layer chromatography, after inhibition of platelet membrane $PLA_2$ by various doses of BPB. (*Arrows*) Relative migration of substrate (phosphatidylcholine, *large open arrow*), product (arachidonic acid, *single solid arrow*), and origin (*double arrows*). Lanes are designated as follows: *1*, no platelets; *2–4*, control platelets without BPB, at 0.25, 0.5, or 1× final cell concentration; *5–12*, platelet membranes pretreated with BPB and then washed; *5*, 22 μM BPB; *6*, 16 μM; 7, 12 μM; *8*, 9 μM; *9*, 7 μM; *10*, 5 μM; *11*, 4 μM; *12*, 3 μM. (*c*) Comparison of inhibition by BPB of platelet membrane cytotoxicity (■) and $PLA_2$ activity (□). The $PLA_2$ activity of platelet membranes, obtained by three cycles of freezing and thawing (2) and treated with various concentrations of BPB, was determined as in Fig. 3 *b*, followed by scraping of the silica gel (between single lines as depicted on autoradiograph), and quantitation of radioactivity by scintillation counting. We defined 100% inhibition to occur when no platelets were added. (*d*) Comparison of inhibition by BPB of intact platelet cytotoxicity (■) and $PLA_2$ activity (□), determined as shown in (*b*) but using intact platelets.

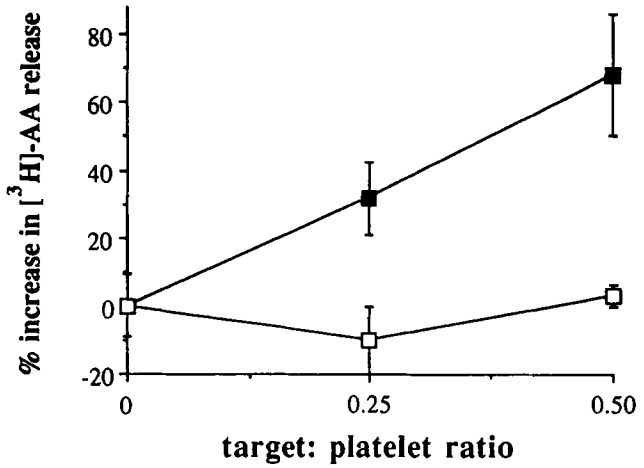


**Figure 4.** Arachidonic acid is released by radiolabeled platelets incubated with EAC∿3b but not E or EA target cells. Platelets were loaded with tritium-labeled arachidonic acid for 60 min at 37°C, washed, and incubated with EAC∿3b target cells (■) or EA target cells (□) for 60 min at 37°C. Samples were centrifuged at 6,900 *g* for 15 min, and the amount of radioactive arachidonic acid in sample supernatants and pellets was determined by scintillation counting. Results are presented as the percent increase in arachidonate release over that triggered by unsensitized E target cells. Each point represents the mean of five replicates, and error bars indicate the sample SD.

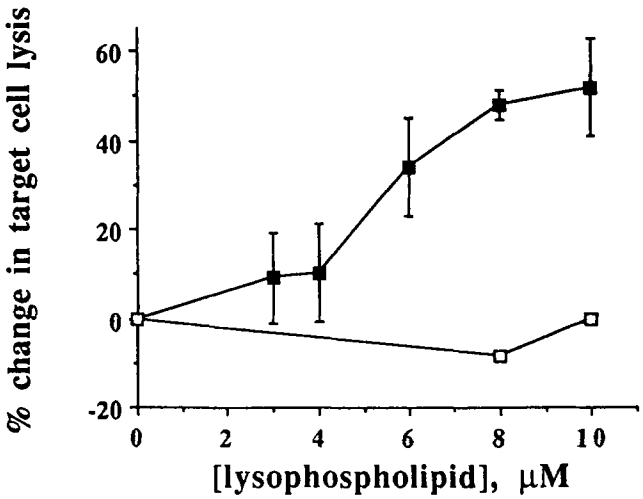


**Figure 6.** Platelet cytotoxicity is increased by exogenous lyso-PAF. Platelets at 2 × $10^7$/ml were preincubated on ice for 60 min with indicated doses of exogenous lyso-PAF, washed, and then tested for lytic activity (■). As a control, buffer without platelets was also tested (□). With no added lyso-PAF, lysis of EAC∿3b by platelets was 44%, and lysis of E was 6%. We calculated changes in lysis due to exogenous lyso-PAF as defined in Materials and Methods, as for arachidonic acid release. Each point represents the mean of five replicates, and error bars indicate the sample SD.

ferase activity would trap the products of $PLA_2$, and therefore enhance pellet cytotoxicity. The effect of treatment with EMTS is shown in Fig. 5. The results show that platelet cytotoxicity is specifically and substantially increased when EMTS is added at appropriate concentrations (40).

*Exogenous Lysophospholipids, but Not Free Arachidonic Acid, Increase Platelet Cytotoxicity.* To elucidate which of the products of $PLA_2$ activation is (or are) responsible for cytotoxicity, we pretreated platelets with various products, i.e., exogenous lysophospholipids and/or arachidonic acid, before assay for target cell lysis. As shown in Fig. 6, platelets that have been pretreated with lyso-PAF, and then washed, more potently lyse EAC∿3b target cells than did untreated platelets. Neither pretreated nor untreated platelets lyse unsensitized E target cells (Fig. 6). Based on these results, we compared the enhancement of lysis caused by various other lysophospholipids preincubated with platelets at 6 μM followed by washing. Exogenous lysophosphatidycholine is most effective, followed by lyso-PAF (Fig. 6), lysophosphatidylinositol, lysophosphatidylserine, and lysophosphatidylethanolamine. Exogenous lysophosphatidic acid and PAF are not active under these conditions in increasing platelet cytotoxicity.

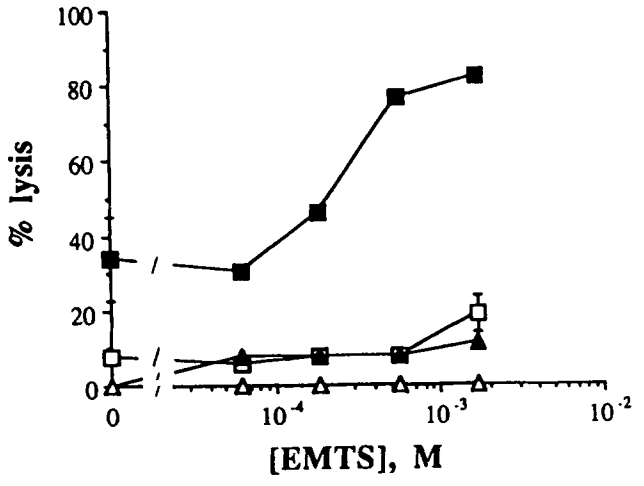


**Figure 5.** Platelet cytotoxicity is increased by an inhibitor of acyl transferase. Various doses of EMTS were added to platelets at 5 × $10^7$/ml (■) or buffer (□), EAC∿3b target cells were added, and lytic activity was assayed. To establish further the specificity of this drug's effect, we added unsensitized target cells (E) to platelets at 5 × $10^7$/ml (▲) or buffer (△). Each point represents mean of four experiments, and error bars indicate sample SD.

In contrast to the results with lysophospholipids, lysis is not specifically increased when arachidonic acid is added. Arachidonic acid is most likely the predominant fatty acid that is released upon $PLA_2$ activation in stimulated cells (41, 42). Thus although arachidonic acid is specifically released as one of the products of $PLA_2$ activation in platelets (Fig. 4), when added alone it does not increase the lytic reaction. Furthermore, inhibitors of cyclooxygenases or lipoxygenases, modifiers of arachidonic acid that are active in many other intercellular processes, have had no consistent effect on platelet cytotoxicity (43; data not shown).

Although we have shown that platelet cytotoxicity is increased when platelets are pretreated with lysophospholipids, and that platelets release arachidonic acid upon interaction with EAC∿3b target cells, labeling experiments have not revealed which or how much lysophospholipid is formed in

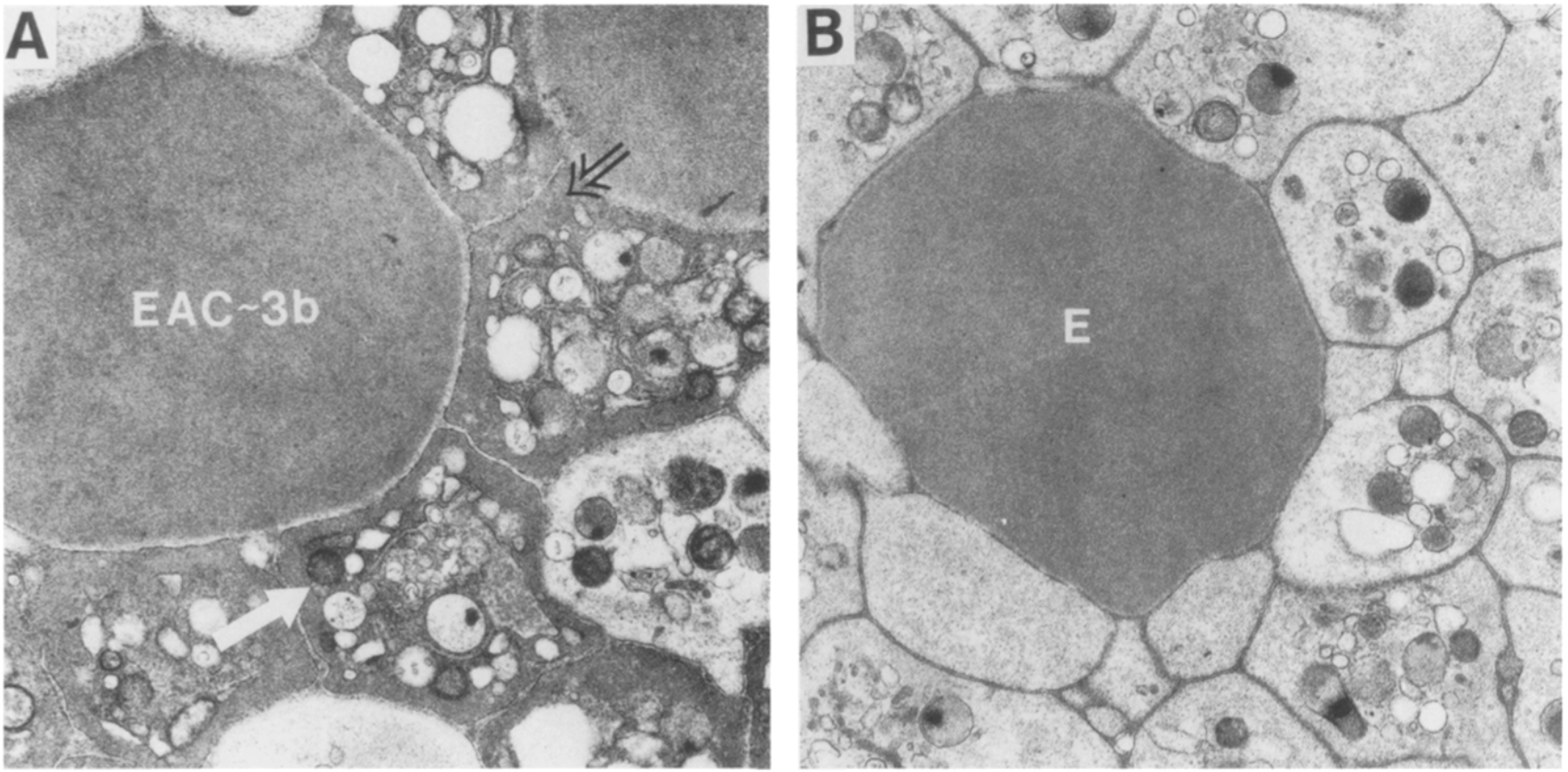


**Figure 7.** Ultrastructural analysis of the lytic interaction between platelets and target cells. (*A*) Platelets with EAC~3b after 50 min incubation. (*EAC~3b*) An antibody and complement-sensitized target cell surrounded by platelets, some of which have increased cytoplasmic electron density (*black arrow*) similar to that of the target cell. Platelet granules remain morphologically intact in platelets even as the lytic reaction proceeds (*white arrow*). (*B*) Platelets with unsensitized E after 130 min incubation. (*E*) An unsensitized target cell. ×6,700.

the course of the lytic reaction. It is possible that small local changes in the concentration of lysophospholipids triggered upon ligation of FcR and CR in platelet membranes are sufficient to cause target cell lysis, and that these changes were not detected. Lysophospholipids might be more labile than free fatty acids that are released, i.e., lysophospholipids may be metabolized before extraction and analysis of platelet lipids (44, 45).

*Ultrastructural Analysis of the Lytic Reaction.* Lysophospholipids are amphoteric molecules with well-known inherent lytic properties, and have been studied widely as fusogens (46). These molecules have been used in constructing hybridomas and in permeabilizing cells to facilitate incorporation of exogenous material (47–49).

To obtain ultrastructural evidence that membrane fusion may occur during the lytic reaction, mixtures of platelets and EAC∿3b or E target cells coincubated for various periods were examined by transmission electron microscopy. The results are shown in Fig. 7. As shown in Fig. 7 *A*, when platelets are incubated with EAC∿3b target cells, changes in platelet morphology are visualized after only 50 min incubation. The delineation between platelet and target cell membranes is increasingly difficult to resolve as the lytic reaction proceeds. The platelet cytoplasm has become more electron dense and attained a density similar to that of the target cell contents (predominantly hemoglobin), possibly suggesting that target cell hemoglobin has leaked into platelets. By contrast, platelets incubated with unsensitized target cells (E) do not become more electron dense even after 130 min (Fig. 7 *B*).

Leakage of $^{51}$Cr-modified hemoglobin into the supernatant is measured in the lytic assay (2). Samples were taken at 0, 50, 90, and 130 min for electron microscopy and for determination of cell lysis by $^{51}$Cr release. The lysis of EAC∿3b is 11, 14, 69, and 70%, respectively. The lysis of control E by platelets is <16% during the entire 130-min period. Thus, electron-dense platelets become apparent before there is significant release of hemoglobin into the supernatant (Fig. 7 *A*). Less than 1% of platelets incubated with unsensitized E target cells are electron dense (Fig. 7 *B*). The results suggest that before target cell lysis occurs, a platelet-to-target cell communication may be established.

It is notable that platelet granules remain morphologically intact in platelets even after substantial target cell lysis has occurred (Fig. 7 *A*, and data not shown). In the following section, we present additional biochemical evidence that degranulation is not required and does not occur during the cytotoxic reaction.

*Effect of Fixation on Platelet Degranulation and Lysis.* We showed previously that platelets lyse target cells bearing appropriate Fc and complement components most efficiently when these ligands are closely apposed. The results indicated that platelet receptors for IgG and complement must exist in close physical proximity, perhaps in a physical complex (2).

As shown in Fig. 8, platelets can be fixed in up to 10% (wt/vol) paraformaldehyde at 6°C for 15 min without loss of specific recognition or cytotoxic function. These fixation conditions are sufficient to inhibit lateral diffusion of integral membrane proteins in other mammalian cells as measured by immunofluorescence localization (50). Presumably, platelet FcR, CR, and $PLA_2$ lie in close physical proximity

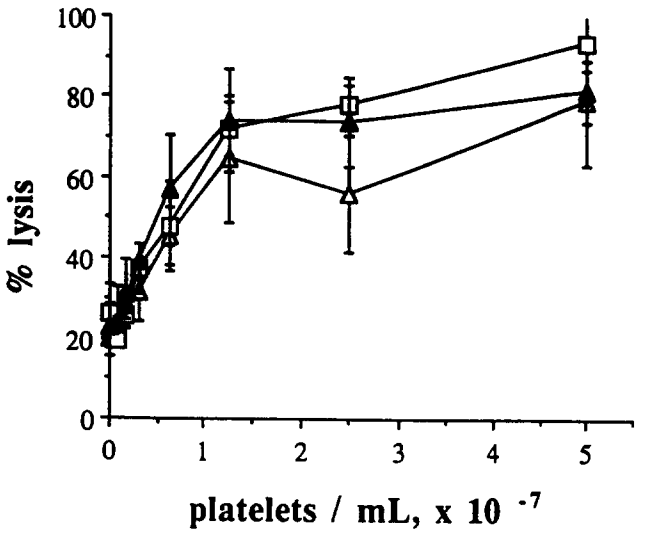


**Figure 8.** Effect of paraformaldehyde fixation on platelet-mediated lysis. Platelets were treated with 10% wt/vol (△), 5% (□), or no (▲) paraformaldehyde at 8 × $10^7$ cells/ml in Tyrode's solution for 15 min at 6°C, washed, EAC∿3b target cells were added, and target cell lysis was determined. Each point represents the mean of four experiments, and error bars indicate sample SD. The lysis of unsensitized target cells, E, by unfixed or fixed platelets was <10%.

**Table 1.** *Lack of Platelet Degranulation after Fixation or during the Lytic Reaction*

| Treatment | [$^3$H]Serotonin release percent change (vs. control) | |
|---|---|---|
| | Platelets not fixed | Platelets fixed in 5% paraformaldehyde |
| Control | 0 | 0 |
| A23187, 10 nM | 10 | −3 |
| A23187, 100 μM | 93 | −3 |
| Thrombin, 100 nM | 7 | −3 |
| Thrombin, 10 μM | 93 | −2 |
| E target cells | 3 | −6 |
| EAC∿3b target cells | 0 | −6 |

Lack of platelet degranulation during the lytic reaction or after fixation. Platelets at 2 × $10^8$/ml were preloaded with [$^3$H]serotonin, washed, incubated in 5 or 0% paraformaldehyde for 15 min at 6°C, and washed again. Calcium ionophore A23187, thrombin, or target cells were added before incubation for 15 min at 25°C. Reaction mixtures were centrifuged and radioactivity in the supernatants and pellets was measured. Spontaneous serotonin release was 22% from unfixed and 32% from fixed platelets, respectively. Similar results were obtained in three independent trials.

before interaction with target cells, perhaps in a preformed complex associated with platelet membranes that is unperturbed even under relatively stringent fixation conditions. This is consistent with previous observations that FcR and $PLA_2$ copurify from human and mouse cells, that FcR and CR cocap, and that FcR exists in an aggregated state in situ in other cell types (15–16, 51, 52).

We previously showed that isolated platelet membranes, obtained by freezing and thawing or by sonicating platelets, are sufficient to cause efficient and specific target cell lysis (2). We were interested, however, in determining whether or not platelet degranulation is required for cytotoxicity, since $PLA_2$ activation has been correlated with degranulation (53). Therefore, platelets were preloaded with radiolabeled serotonin, washed, and then incubated with various target cells. As a control, the radiolabeled platelets were treated with two known secretagogues, thrombin and A23187. As shown in Table 1, serotonin is released in the presence of thrombin or A23187, indicating degranulation of platelet-dense granules. However, platelets do not release serotonin when incubated with E or EAC∿3b target cells, showing that degranulation does not occur during the cytotoxic reaction (Table 1). As an additional control, we verified that serotonin loading does not affect platelet cytotoxicity (data not shown). When platelets are fixed with 5% paraformaldehyde, no serotonin release occurs in the presence of thrombin or A23187 (28), even though target cell lysis is unaffected (Fig. 8). These results further substantiate our previous evidence that the complete cytotoxic system is localized in platelet membranes (2), probably as a preformed complex.

## Discussion

By using a set of $PLA_2$ inhibitors and correlating biochemical activity with function, we have shown that $PLA_2$ is required for platelet cytotoxicity. This is further corroborated by the enhancement of lysis observed in the presence of an inhibitor of acyltransferase, which catalyzes a reaction opposing that of $PLA_2$. Additional support for the activation of $PLA_2$ comes from the concentration-dependent release of arachidonic acid by platelets when they are incubated with appropriate EAC∿3b but not E or EA target cells. We were not able to detect concomitant formation of lysophospholipids upon interaction with target cells, possibly because of the lability of these lytic molecules (44, 45). This possibility is consistent with the fact that unsensitized bystander cells are not lysed even while lysis of appropriately sensitized target cells is underway (2). However, the fact that platelets pretreated with lysophospholipids but not arachidonic acid lyse target cells more efficiently suggests that lysophospholipids are the active lytic molecules generated during the lytic reaction. It is interesting that schistosomula have been shown to release lysophosphatidylcholine in lysing adherent host RBC, although the rate of release is small (54).

We previously demonstrated that efficient target cell lysis is triggered by membrane-bound IgG (complement-fixing subclasses IgG2a, IgG2b, and IgG3) and complement components C∿3b (2). The fact that target cells were lysed when sensitized with IgG and C∿3b, but not with IgM and C∿3b followed by IgG, suggests that bound IgG and C∿3b must be in close physical proximity for the lytic reaction to occur. The evidence that fixed platelets can lyse sensitized target cells (Fig. 8) extends the idea that the complete cytotoxic system resides in platelet membranes, suggesting that receptors for Fc and complement and membrane-associated $PLA_2$ may exist as preformed complexes in platelet membranes.

It is not clear at this time whether ligation of FcγR and CR directly activates a closely associated $PLA_2$, or if there exists an intermediate factor required for activation of the lipase. Evidence in favor of both possibilities has been obtained using other cells. Suzuki et al. (15, 16) found that $PLA_2$ activity and FcγR copurify from B cells and macrophages. Narasimhan et al. (19) showed that activation of a G protein intermediate is required for $PLA_2$ activation by FcεR in basophils. Recent evidence suggests that other lipase activities may also be triggered by platelet Fc receptor ligation (55).

We previously showed that platelet cytotoxicity occurs efficiently in a plasma-free system, where platelets are washed and incubated in Tyrode's buffer containing gelatin and 4 mM EDTA (2). It is notable that most of the known $PLA_2$ isozymes require calcium (56), although exceptions have been described (57). The incubation buffer used in this system at pH ∿7.4 contains at least micromolar amounts of free calcium, despite added EDTA (57, 58; and Symer, D. E., unpublished observations). Therefore, the presence of EDTA in this experimental system does not contradict the proposed involvement of a membrane-associated $PLA_2$ in the lytic reaction.

Progress in molecular biology has facilitated elucidation of the structure and localization of Fc receptors and complement receptors, and now there is evidence that distinct receptors utilizing common receptor subunits and signaling pathways exist in disparate cells of different species (18, 20, 59). Similarities in the inhibition of NK cell–mediated cytotoxicity by $PLA_2$ inhibitors suggest that a similar mechanism of cytotoxicity, caused by lysophospholipid generation, may exist in those cells as well (22–24).

If lysophospholipids are formed as a result of contact with sensitized target cells, and if these lytic molecules behave as fusogens in vivo, as observed in vitro (48) and as suggested by electron microscopy of the platelet lytic reaction (Fig. 7), then it may be possible to deliver cytotoxic drugs to antibody-coated target cells by preloading the platelet cytoplasm. Lysophospholipids have been used to mediate fusion of liposomes with intact cells (48, 60). It may also be possible to increase antibody-dependent cytotoxicity by increasing the products of platelet membrane–associated $PLA_2$ activity using various exogenous inhibitors (Fig. 5). Further elucidation of the mechanism of antibody-dependent platelet cytotoxicity will facilitate some of these goals.

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