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Ionophore A23187- and thrombin-induced platelet aggregation: Independence from cycloxygenase products

(phospholipase/arachidonate/thromboxanes/phosphatidate/eicosatetraynoic acid)

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ABSTRACT **Stimulation of platelets labeled with [^{14}C]-arachidonate by ionophore A23187 or thrombin produces rapid degradation of specific membrane phospholipids. This is also reflected by the release of [^{14}C]arachidonate, which is immediately transformed into products of the cycloxygenase and lipoxygenase enzyme systems, and by increased labeling of phosphatidic acid. Arachidonate metabolism can be effectively prevented by preincubation with indomethacin and eicosatetraynoic acid, but platelet aggregation induced by ionophore A23187 or thrombin is not blocked under these conditions. Nevertheless, in the virtually total absence of metabolism of arachidonate, platelet aggregation still occurs concomitantly with phospholipid breakdown and with increased labeling of phosphatidic acid. Increased levels of cyclic AMP block both phospholipase activation and aggregation induced by ionophore A23187 and thrombin. These data suggest that some early consequence of phospholipase activation, independent of a metabolic product of arachidonate but possibly related to the production of phosphatidic acid, may play a central, causative role in mediating platelet aggregation.**

Metabolism of arachidonic acid by the cycloxygenase enzyme system leads to production of the active cyclic endoperoxides, prostaglandins G_2 and H_2 (PGG_2 and PGH_2), and of thromboxane A_2 (TXA_2). Although these products are capable of inducing platelet aggregation (1–5), their relevance in mediating the normal process of aggregation induced by physiological stimuli has been questioned (4, 6, 7). Arachidonate is released from phospholipids when platelets are stimulated by thrombin, ionophore A23187, and collagen. This arachidonate is utilized immediately by the cycloxygenase and lipoxygenase enzyme systems (8–15).

Thrombin- and ionophore A23187-induced platelet aggregation can be blocked by dibutyryl adenosine 3′:5′-cyclic monophosphate (Bt_2cAMP), cyclic AMP (cAMP) phosphodiesterase inhibitors, and stimulators of adenylate cyclase (10, 12, 14–17). Very recently, it has been shown that elevation of cAMP inhibits release of arachidonate from phospholipids, and it was suggested that this action of cAMP might, at least in part, explain its inhibitory effect on platelet aggregation (10, 12, 14, 15). It seems, however, that the cycloxygenase products that appear during platelet stimulation may not be involved in mediating the aggregation or release reaction since aspirin and indomethacin do not block thrombin- and ionophore A23187-induced aggregation (16–19). If this interpretation is correct, then it is apparent that if the phospholipid/arachidonate system is to be involved at all in a causative role, some step proximal to arachidonate metabolism must be considered.

We have shown that phospholipase activation and aggregation induced by thrombin or ionophore A23187 are blocked by increased levels of cAMP. Indomethacin inhibits cycloxygenase activity and eicosatetraynoic acid inhibits both cycloxygenase activity. Since neither of these inhibitors prevents aggregation by thrombin or ionophore A23187, the possible role of arachidonate metabolites, but not of other products of membrane phospholipases, must be further questioned as causative agents.

MATERIALS AND METHODS

Most of these have already been essentially described (10). Platelet-rich plasma was obtained from 1 unit of horse blood anticoagulated with anticoagulant citrate dextrose. Samples (50 ml) of the plasma were incubated with 2 μCi of [*1*-^{14}C]arachidonate at 37° for 2 hr. Platelets were then isolated by centrifugation, resuspended in Tris/saline/EDTA buffer (NaCl, 134 mM/15 mM Tris·HCl, pH 7.4/1 mM EDTA/5 mM D-glucose), centrifuged again, and finally resuspended in 10–20 ml of the same buffer. Samples of 0.5 ml (3–6 mg of protein) were incubated with ionophore A23187 (2 μM) (Eli Lilly and Co.), or thrombin (1 unit/ml) for 5 min. Preincubations with or without Bt_2cAMP, aminophylline plus methylisobutylxanthine, or eicosatetraynoic acid (Hoffman-La Roche, Inc.) were for 10–15 min followed by a 5-min incubation with or without thrombin or ionophore A23187. Platelets were treated with indomethacin during the 2 hr of [^{14}C]arachidonate-labeling or they were not treated with indomethacin. Duplicate incubations were stopped with chloroform/methanol and partitioned with chloroform and water. Lipids from the lower phases were dried under N_2 and redissolved in chloroform (10). One of the duplicate incubation mixtures was placed on thin-layer chromatography plates for phospholipid separation (chloroform/methanol/acetic acid/water, 50:30:8:4, vol/vol) (10, 20) and the other one for separation of phosphatidic acid, TXB_2, 12-hydroxy-5,8,10,-heptadecatrienoic acid (HHT), 12-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), and arachidonate (top phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water, 90:50:20:100, vol/vol) (10).

In all experiments of thrombin-induced release of [^{14}C]arachidonate metabolites we noticed a marked increase of radioactivity in a spot that runs very close to the origin. This spot does not chromatograph with known standard prostaglandins and it is degraded by phospholipase A_2 with release of [^{14}C]arachidonate. The compound incorporates $^{32}P_i$ and it is quantitatively recovered with the solvent front in our usual phospholipid separations where phosphatidic acid is present. Authentic phosphatidic acid cochromatographs with this active spot and not with other phospholipds, which remain together

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Abbreviations: cAMP, cyclic AMP; Bt_2cAMP, dibutyryl cAMP; TX, thromboxane; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid.

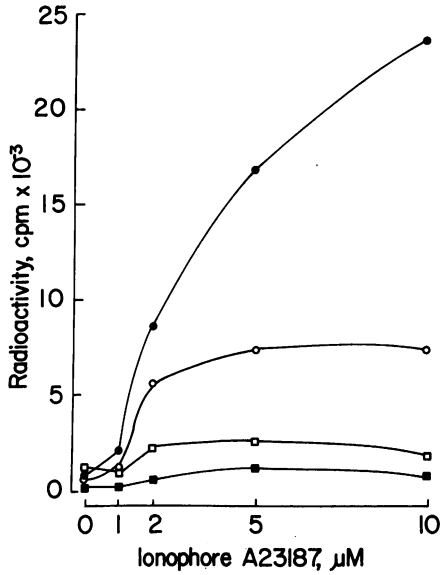

FIG. 1. Effect of different concentrations of ionophore A23187 on [^{14}C]arachidonate-labeled platelets. ●, HETE; ○, HHT; ■, TXB_2; □, phosphatidic acid.

at the origin (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin).

Radioactive spots were localized by radioautography and quantitated by liquid scintillation counting. Platelet aggregation of 0.5-ml samples of platelet-rich plasma or washed platelets (0.5–2 mg of protein) was measured by continuous recording of light transmission at 37° (Chronolog Corp., Broomall, PA)

RESULTS

Effect of Ionophore A23187 on Redistribution of Radioactivity from [^{14}C]Arachidonate-Labeled Platelets. As shown previously (10, 14), incubation of platelet-rich plasma with [^{14}C]arachidonate for 2 hr leads to its incorporation into the phospholipids of horse platelets. When the labeled platelets are washed and treated with ionophore A23187, radioactivity is detected in arachidonate and products of lipoxygenase (HETE) and cycloxygenase (HHT and TXB_2), and there is increased incorporation of label in phosphatidic acid (Fig. 1). Except for

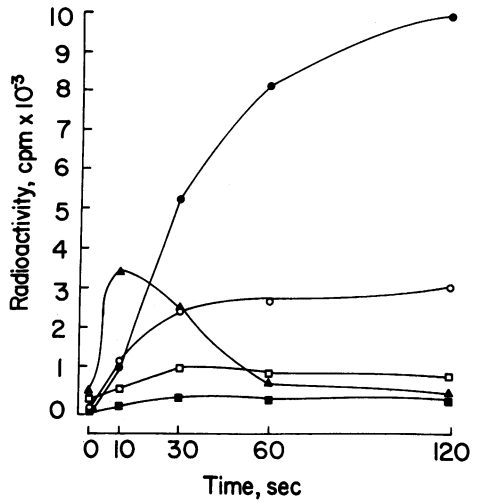

FIG. 2. Time-course of ionophore A23187 (2 μM) effect on [^{14}C]arachidonate-labeled platelets. ▲, Arachidonate; other symbols as in legend of Fig. 1.

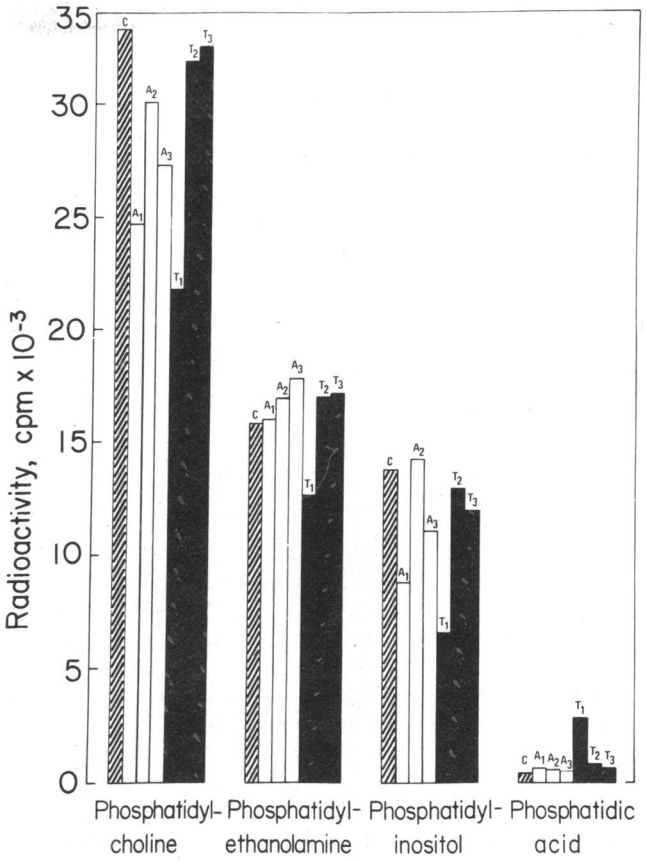

FIG. 3. Effect of cAMP phosphodiesterase inhibitors and Bt_2cAMP on ionophore A23187 and thrombin action on [^{14}C]arachidonate-labeled phospholipids. Preincubations with or without aminophylline (2.5 mM) plus methylisobutylxanthine (0.22 mM) or Bt_2cAMP (1 mM) were for 10 min followed by a 5-min incubation with or without ionophore A23187 (2 μM) or thrombin (1 unit/ml). C, control without additions; A_1, action of ionophore A23187; A_2, preincubation with aminophylline plus methylisobutylxanthine followed by ionophore A23187; A_3, preincubation of Bt_2cAMP followed by ionophore A23187. T_1, T_2, and T_3 are as in A_1, A_2, and A_3, respectively, but incubations were with thrombin.

HETE, between 2 and 5 μM of ionophore A23187 gives the maximal incorporation into the [^{14}C]arachidonate products. At very short time intervals (e.g., 10 sec) there is release of [^{14}C]arachidonate which is immediately metabolized to HETE, HHT, and TXB_2 (Fig. 2). Increase labeling of phosphatidic acid is also observed at short time intervals. After 30 sec there is virtually no further increase in HHT, TXB_2, and phosphatidic acid, but there is continued incorporation of radioactivity into HETE.

cAMP Inhibits Thrombin- and Ionophore A23187-Induced Phospholipid Breakdown. Ionophore A23187 decreases the [^{14}C]arachidonate radioactivity present as phosphatidylcholine and phosphatidylinositol, but not phosphatidylethanolamine (Fig. 3). This ionophore-induced degradation is substantially inhibited by aminophylline and methylisobutylxanthine and by Bt_2cAMP (Fig. 3). Thrombin degrades all these phospholipid classes, and this is prevented by Bt_2cAMP, aminophylline plus methylbutylxanthine, and by prostacyclin, a potent stimulant of adenylate cyclase (refs. 10 and 14; Fig. 3). Thrombin also increases incorporation of [^{14}C]arachidonate into phosphatidic acid, perhaps even more effectively than the ionophore, and this is also blocked by cAMP (Figs. 3 and 5). The ionophore A23187- and thrombin-induced phospholipid degradation occurs in parallel to formation of HETE, HHT, TXB_2, and

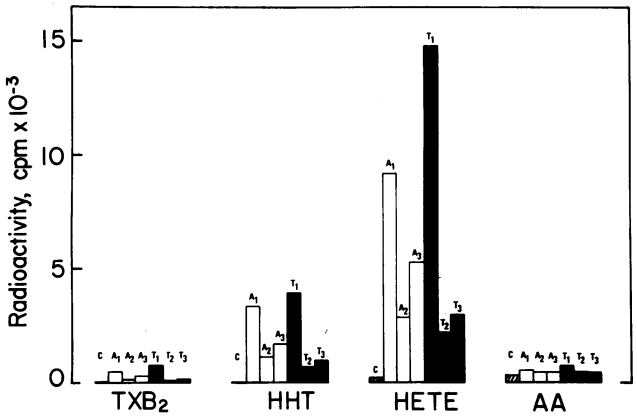

FIG. 4. Effect of cAMP phosphodiesterase inhibitors and Bt_2cAMP on ionophore A23187- and thrombin-induced production of oxygenated metabolites from [^{14}C]arachidonate-labeled platelets. All details as in legend of Fig. 3. AA, arachidonate.

arachidonate, the formation of which is also concomitantly blocked by Bt_2cAMP and by aminophylline plus methylisobutylxanthine (Fig. 4) in a concentration-dependent manner (Fig. 5).

Effects of Indomethacin and Eicosatetraynoic Acid. Pretreatment of platelet-rich plasma with indomethacin is effective in blocking the cycloxygenase products, as reflected by HHT and TXB_2 when platelets are stimulated with ionophore A23187 and thrombin (Fig. 6). The decrease in HHT and TXB_2 is accompanied by increased labeling in HETE. Further addition of indomethacin to washed platelets before or during

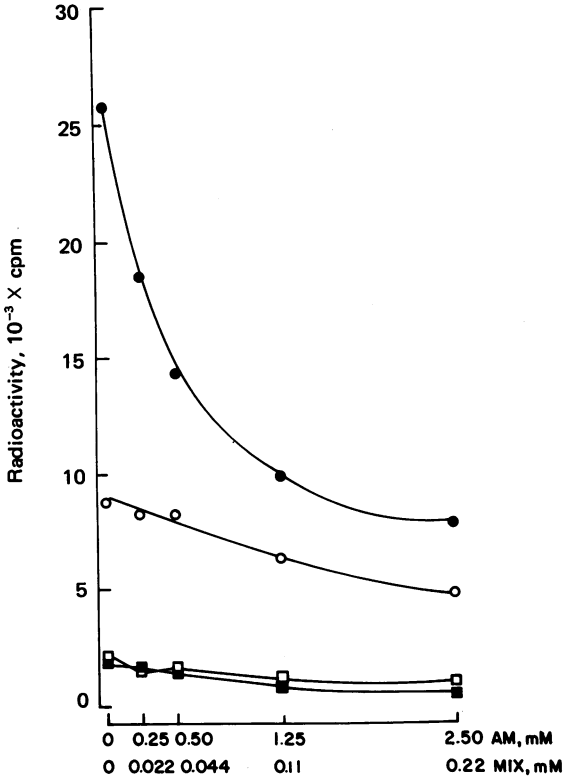

FIG. 5. Action of different concentrations of cAMP phosphodiesterase inhibitors on the ionophore A23187 effect on [^{14}C]arachidonate-labeled platelets. Preincubations with aminophylline (AM) plus methylisobutylxanthine (MIX) were for 15 min followed by ionophore A23187 for 5 min. Symbols: see legend of Fig. 1.

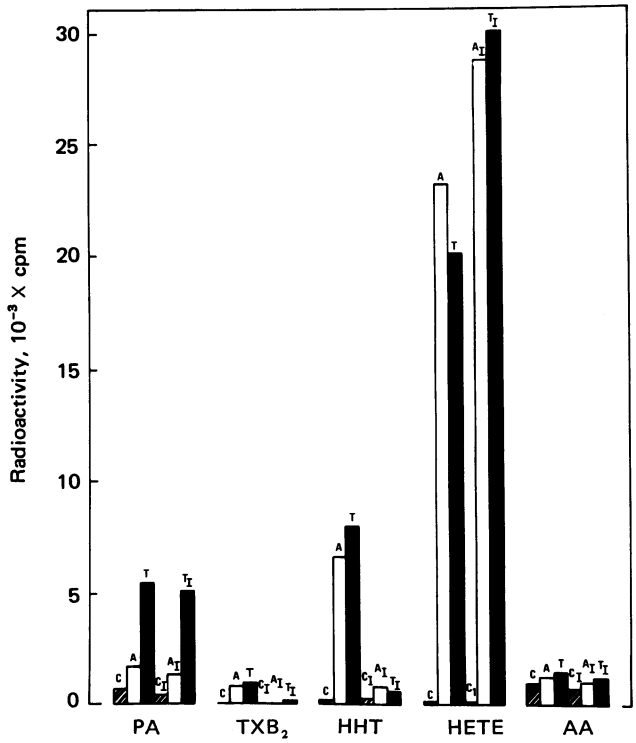

FIG. 6. Effect of ionophore A23187 and thrombin on [^{14}C]arachidonate-labeled platelets pretreated with indomethacin. Platelet-rich plasma was labeled with [^{14}C]arachidonate for 2 hr with and without indomethacin (10 μM). Platelets were then isolated and incubated without further additions (C) or with 2 μM ionophore (A) or 1 unit of thrombin per ml (T) for 5 min. Subscript I refers to platelets that have been pretreated with indomethacin. PA, phosphatidic acid; AA, arachidonate.

treatment with ionophore A23187 and thrombin has no further effect. The increased ^{14}C labeling into phosphatidic acid induced by ionophore A23187 or by thrombin is not affected by indomethacin.

Pretreatment of washed platelets with eicosatetraynoic acid (7–28 μM) inhibits nearly completely the formation of products derived from lipoxygenase and cycloxygenase activities induced by ionophore A23187 and thrombin (Fig. 7). Radioactive HETE, HHT, and TXB_2 are profoundly decreased, whereas free [^{14}C]arachidonate is increased. Eicosatetraynoic acid also slightly inhibits phospholipase activity, as reflected by the total phospholipid radioactivity (Fig. 7).

Effect of Indomethacin and Eicosatetraynoic Acid on Platelet Aggregation. Ionophore A23187 (0.5–4 μM) induces aggregation of horse blood platelet-rich plasma, and this aggregation is not blocked by preincubating the plasma with 10 μM indomethacin for 2 hr (data not shown). Ionophore A23187 (0.5–1 μM) also induces aggregation of washed platelets that have been isolated from platelet-rich plasma treated or not treated with 10 μM indomethacin for 2 hr. In both cases aggregation is inhibited by aminophylline plus methylisobutylxanthine or Bt_2cAMP (Fig. 8). Although thrombin-induced aggregation (washed platelets) is also similarly inhibited by cAMP (10, 14), aggregation is not prevented by preincubation of the platelet-rich plasma with indomethacin (Fig. 8). Further addition of indomethacin (10–50 μM) to the washed platelets from the pretreated platelet-rich plasma also does not prevent aggregation by 0.2–0.4 unit of thrombin per ml (data not shown). Furthermore, ionophore A23187- and thrombin-induced aggregation is also not blocked by preincubation (1–4

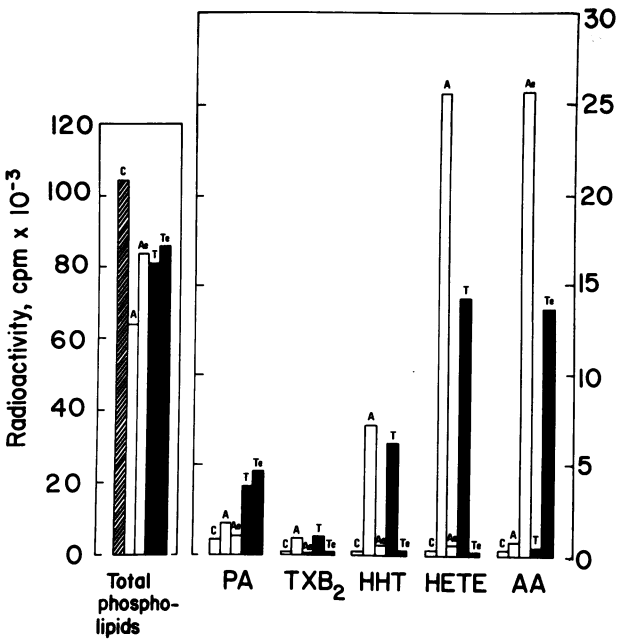

FIG. 7. Effect of eicosatetraynoic acid on ionophore A23187 and thrombin action on ^{14}C-labeled platelets. Preincubation with or without eicosatetraynoic acid (28 μM) was for 10 min followed by a 5-min incubation with or without ionophore A23187 (2 μM) or thrombin (1 unit/ml). C, Control without additions; A, action of ionophore A23187; T, action of thrombin. Subscript e refers to platelets preincubated with eicosatetraynoic acid. PA, phosphatidic acid; AA, arachidonate.

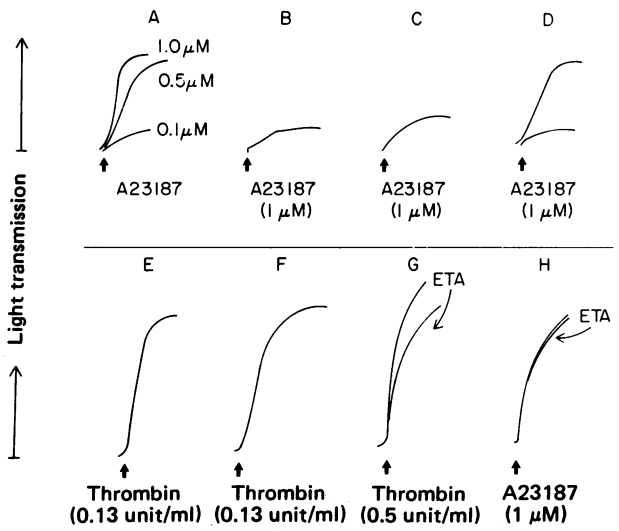

FIG. 8. Action of cAMP phosphodiesterase inhibitors, Bt_2cAMP, indomethacin, and eicosatetraynoic acid, on ionophore A23187- and thrombin-induced aggregation of washed platelets. (*A*) Aggregometer tracings of different concentrations of ionophore A23187; (*B*) effect of a 1-min preincubation with aminophylline (2.5 mM) plus methylisobutylxanthine (0.22 mM); (*C*) effect of a 1-min preincubation with Bt_2cAMP (1 mM); (*D*) as in *B* but platelets were pretreated with 10 μM indomethacin as indicated in Fig. 6; (*E*) thrombin; (*F*) thrombin-induced aggregation of indomethacin-pretreated platelets; (*G*) action of a 1-min preincubation with 10 μM eicosatetraynoic acid (ETA) on thrombin-induced aggregation; (*H*) action of a 4-min preincubation with 10 μM eicosatetraynoic acid on ionophore A23187-induced aggregation.

min) with 10 μM eicosatetraynoic acid under conditions under which all arachidonate metabolism is virtually blocked (Fig. 8).

DISCUSSION

Indomethacin, which is clearly shown to block cycloxygenase product formation (ref. 8 and Fig. 6), does not inhibit thrombin- or ionophore A23187-induced aggregation. Eicosatetraynoic acid inhibits both cycloxygenase and lipoxygenase activities profoundly (ref. 8 and Fig. 7) but does not affect aggregation. Several reports from other laboratories describe the inability of aspirin and indomethacin to block thrombin- and ionophore-induced aggregation (16–19). All these observations suggest that products derived from known metabolic routes of arachidonate metabolism are not essential mediators of platelet aggregation.* However, these data do not completely exclude the possibility that the production of only minute or undetectable amounts of endoperoxides or thromboxanes are sufficient to initiate aggregation.

Despite the lack of evidence for an obligatory role of an arachidonate metabolic product for aggregation, much evidence still suggests that some process related to phospholipid degradation may be an early and specific event. The early expression of phospholipase activity, which also leads to the formation of products other than arachidonate, is greatly favored by thrombin and ionophore A23187 as well as virtually all other stimuli of primary platelet aggregation. Further, inhibition of phospholipase activity by cAMP correlates well with the blockade of platelet aggregation. Since phospholipase activity is calcium dependent (22), the action of cAMP could in theory be explained by a reduction of this divalent action. It has recently been shown that platelet vesicles can concentrate calcium when incubated in the presence of cAMP, ATP, and protein kinase (23). In order to examine the possible role of phospholipase activity independent of calcium mobilization, we tried a more specific phospholipase A_2 inhibitor, bromophenacylbromide (24), which produces inhibition of aggregation in rabbit platelets (7). However, under our usual experimental design, bromophenacylbromide (1–50 μM) fails to inhibit thrombin- and ionophore A23187-induced phospholipid degradation. Mepacrine is reputedly another potential phospholipase A_2 inhibitor that also inhibits rabbit platelet aggregation, but it is questionable whether its action on phospholipase is specific (7, 9).

At present, the precise physiological role of this phospholipase activity cannot be ascertained. It is quite possible, as has been supposed before (11, 13), that the phospholipase activation and phospholipid breakdown during platelet stimulation is simply secondary to this stimulation or an independent consequence of Ca^{2+} mobilization. However, virtually no information is currently available about the calcium requirements, if indeed any such exist, for activation of a critical phospholipase during platelet stimulation. The activity of a phospholipase that specifically releases arachidonate in platelet homogenates and membranes is greatly stimulated in the presence of 10 mM Ca^{2+} (22).

Thrombin and ionophore A23187 increase [^{14}C]arachidonate labeling of phosphatidic acid (Figs. 1–3 and 5–7) almost certainly on the basis of phospholipid degradation since washed, labeled platelets are virtually devoid of free [^{14}C]arachidonate. Increased labeling of phosphatidic acid is detected 2 sec after addition of thrombin, and probably reflects the production of diacylglycerol and its phosphorylation by diacylglycerol kinase.

* It is well known that indomethacin inhibits platelet "aggregation," but, as summarized recently (21), this indomethacin-sensitive aggregation is of a "secondary" (i.e., "second wave") type and thus is different from the "primary" aggregation events studied here.

Notably, cAMP also inhibits thrombin- and ionophore A23187-induced labeling of phosphatidic acid in parallel with the inhibition of aggregation. It is reasonable to speculate that the appearance of phosphatidic acid may be reflecting the activity of a very specific phospholipase (probably of the C-type) possibly related in an important way to the early events of platelet aggregation. It has also been reported that ADP, thrombin, and collagen increase ^{32}P labeling into phosphatidic acid in rabbit platelets (25, 26). Very recently, the same group has also reported that [^{14}C]arachidonate labeling of phosphatidic is increased by thrombin (27).

1. Hamberg, M. & Samuelsson, B. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 899–903.
2. Hamberg, M., Svensson, J. & Samuelsson, B. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 2994–2998.
3. Needleman, P., Moncada, S., Bunting, S., Vane, J. R., Hamberg, M. & Samuelsson, B. (1976) *Nature* **261,** 558–560.
4. Needleman, P., Minkes, M. & Raz, A. (1976) *Science* **193,** 163–165.
5. Needleman, P., Raz, A., Ferrendelli, J. A. & Minkes, M. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1716–1720.
6. Bult, H. & Bonta, I. L. (1976) *Nature* **264,** 449–451.
7. Vargaftig, B. B. (1977) *J. Pharm. Pharmacol.* **29,** 222–228.
8. Bills, T. K., Smith, J. B. & Silver, M. J. (1976) *Biochim. Biophys. Acta* **424,** 303–314.
9. Blackwell, G. L., Duncombe, W. G., Flower, R. J., Parsons, M. F. & Vane, J. R. (1977) *Br. J. Pharmacol.* **59,** 353–366.
10. Lapetina, E. G., Schmitges, C. J., Chandrabose, K. & Cuatrecasas, P. (1977) *Biochem. Biophys. Res. Commun.* **76,** 828–835.
11. Pickett, W. C., Jesse, R. L. & Cohen, P. (1977) *Biochim. Biophys. Acta* **486,** 209–213.
12. Minkes, M., Stanford, N., Shi, M. M. Y., Roth, G. J., Raz, A., Needleman, P. & Majerus, P. N. (1977) *J. Clin. Invest.* **59,** 449–454.
13. Rittenhouse-Simmons, S. & Deykin, D. (1977) *J. Clin. Invest.* **60,** 495–498.
14. Lapetina, E. G., Schmitges, C. J., Chandrabose, K. & Cuatrecasas, P. (1978) in *Advances in Prostaglandin and Thromboxane Research,* eds. Galli, C., Galli, G. & Porcellati G. (Raven Press, New York), Vol. 3, pp. 127–135.
15. Gerrard, J. M., Peller, J. D., Krick, T. P. & White, J. G. (1977) *Prostaglandins* **14,** 39–50.
16. White, J. G., Rao, G. H. R. & Gerrard, J. M. (1974) *Am. J. Pathol.* **77,** 135–149.
17. Feinstein, M. B. & Fraser, C. (1975) *J. Gen. Physiol.* **66,** 561–581.
18. O'Brien, J. R. (1968) *Lancet* **i,** 779–783.
19. Smith, J. B. & Willis, A. L. (1971) *Nature* **231,** 235–237.
20. Skipski, V. P., Peterson, R. F. & Barclay, M. (1964) *Biochem. J.* **90,** 374–378.
21. Charo, I. F., Feinman, R. D. & Detwiler, T. C. (1977) *J. Clin. Invest.* **60,** 866–873.
22. Derksen, A. & Cohen, P. (1975) *J. Biol. Chem.* **250,** 9342–9347.
23. Käser-Glanzmann, R., Jakábová, M., George, J. N. & Lüscher, E. F. (1977) *Biochim. Biophys. Acta* **466,** 429–440.
24. Volwerk, J. J., Pieterson, W. A. & De Haas, G. H. (1974) *Biochemistry* **13,** 1446–1454.
25. Lloyd, J. V., Nishizawa, E. E., Joist, J. H. & Mustard, J. F. (1973) *Br. J. Haematol.* **24,** 589–604.
26. Lloyd, J. V. & Mustard, J. F. (1974) *Br. J. Haematol.* **26,** 243–253.
27. Leung, N. L., Kinlough-Rathbone, R. L. & Mustard, J. F. (1977) *Thromb. Haematol.* **38,** 7 (Abstr.)