Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 9197–9201, December 1986
Medical Sciences

Activation of phospholipases A and C in human platelets exposed to epinephrine: Role of glycoproteins IIb/IIIa and dual role of epinephrine

(fibrinogen/Na^+/H^+ exchange/thromboxane A_2/phosphatidylinositol phosphates/arachidonic acid)

HARJIT SINGH BANGA*, ELIZABETH R. SIMONS†, LAWRENCE F. BRASS‡, AND SUSAN E. RITTENHOUSE*§

*Department of Biochemistry, University of Vermont College of Medicine, Burlington, VT 05405; †Department of Biochemistry, Boston University School of Medicine, Boston MA 02118; ‡Division of Hematology/Oncology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

Communicated by Eugene Braunwald, August 12, 1986

ABSTRACT **Human platelets stimulated by epinephrine undergo enhanced turnover of phosphatidylinositol 4,5-bisphosphate, accumulate inositol trisphosphate, diacylglycerol, and phosphatidic acid, and phosphorylate a 47-kDa protein. All of these phenomena indicate stimulation of phospholipase C. These responses are blocked completely by inhibitors of α_2-adrenergic receptors (yohimbine), cyclooxygenase (aspirin or indomethacin), phospholipase A [2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RS-082)], Na^+/H^+ exchange [ethylisopropylamiloride (EIPA)], fibrinogen binding to glycoprotein IIb/IIIa (antibody A2A9), Ca^{2+}/Mg^+ binding (EDTA), or removal of fibrinogen. Epinephrine evokes (*i*) an increased turnover of ester-linked arachidonic acid in aspirin-treated platelets that is inhibited by ONO-RS-082, EDTA, yohimbine, or the absence of fibrinogen and (*ii*) a rapid cytoplasmic alkalinization that is inhibited partially by blockage of cyclooxygenase activity and completely by A2A9 or EIPA. In contrast, when incubated with subaggregatory concentrations of the prostaglandin H_2/thromboxane A_2 analogue [(15*S*)-hydroxy-11α,9α-(epoxymethano)prosta-5,13-dienoic acid (U46619) and epinephrine, aspirin-treated platelets show a potentiation of phospholipase C activation that is unaffected by the above inhibitors. We propose that epinephrine, in promoting exposure of glycoprotein IIb/IIIa sites for fibrinogen binding, leads to a cytoplasmic alkalinization, which, in conjunction with local shifts in Ca^{2+}, promotes low-level activation of phospholipase A. The resulting free arachidonic acid is converted to cyclooxygenase products, which, potentiated by epinephrine, activate phospholipase C. This further amplifies the initial stimulatory response.**

Human platelets undergo aggregation and secretion of granule contents following exposure to epinephrine in the presence of fibrinogen and Ca^{2+}. Accompanying such activation is an enhanced turnover of phosphatidylinositol catalyzed by phospholipase C (PLC), indicated in earlier studies by stimulated incorporation of [^{3}H]glycerol (1, 2), diacylglycerol accumulation, and activation of protein kinase C (3). However, human platelet adrenergic receptors have been characterized as being primarily of the α_2 subclass (4, 5), which, in other tissues, is not linked with phosphatidylinositol phosphate turnover (6).

A resolution of this paradox may lie in a report that epinephrine-stimulated generation of [^{32}P]phosphatidic acid (PtdOH), an indirect indicator of PLC activation, can be inhibited by aspirin (7). This implies a possible role for the cyclooxygenase-catalyzed oxygenation of arachidonic acid in mediating the process of PLC activation in response to epinephrine. Recently, investigators have found that epinephrine promotes arachidonic acid mobilization (a precondition for cyclooxygenase action) in a manner dependent upon Na^+/H^+ exchange (8). This mobilization was quantitated by measuring thromboxane B_2 (TXB_2), the stable metabolite of thromboxane A_2, which is, in turn, formed by way of cyclooxygenase.

Clearly, to sort out epinephrine-initiated events from those resulting from the action of stimulatory cyclooxygenase metabolites, it is necessary to characterize those events that occur in the presence of cyclooxygenase inhibitors, such as aspirin. One such change elicited by epinephrine even in the presence of aspirin is a shift in the conformation of glycoprotein IIb/IIIa (GPIIb/IIIa) complex on the platelet surface (9, 10) that facilitates the binding of fibrinogen (11, 12). Another observation is that epinephrine potentiates the aggregatory response of aspirin-treated platelets exposed to subthreshold levels of other agonists, such as TXA_2/prostaglandin H_2 (PGH_2) analogues (13). Therefore, we hypothesized that the binding of epinephrine to the platelet surface might evoke a mobilization of arachidonic acid independently (at least initially) of any involvement of PLC. The oxygenation of arachidonic acid to PGH_2 and TXA_2 would then provide receptor-directed agonists capable of activating PLC (14). Such activation could be potentiated by epinephrine, leading to an amplified response, including further release of arachidonate. In testing this hypothesis, we have investigated the roles of Na^+/H^+ exchange and occupancy of GPIIb/IIIa by fibrinogen in the initiation and potentiation processes.

MATERIALS AND METHODS

Preparation of Platelets. Human platelet-rich plasma, free of erythrocytes, was prepared as described (15) in the presence of 0.5 μM prostaglandin E_1 (Upjohn) and spun at 3000 × *g* × 5 min at room temperature. Platelets were suspended in 4 ml of 20% plasma/80% gel-filtration buffer (GFB) (pH 6.5) (14) containing 0.1% gelatin and creatine phosphate (5 mM)/creatine phosphokinase (40 units/ml). For labeling with ^{32}P and/or [^{3}H]arachidonic acid, the platelets were incubated as described (14) for 60 min at 37°C with 0.3 mC_i of [^{32}P]P_i per ml, carrier free, and/or 6.5 μC_i of [^{3}H]arachidonic acid per ml (New England Nuclear). Platelets were then filtered through a 25-ml column (5-cm inner

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PLC, phospholipase C; PLA_2, phospholipase A_2; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; InsP_3, *myo*-inositol trisphosphate; U46619, (15*S*)-hydroxy-11α,9α-(epoxymethano)prosta-5,13-dienoic acid; ONO-RS-082, 2-(*p*-amylcinnamoyl)-amino-4-chlorobenzoic acid; EIPA, ethylisopropylamiloride; A2A9, monoclonal antibody to glycoprotein IIb/IIIa complex (GPIIb/IIIa); TXB_2, thromboxane B_2; PGH_2, prostaglandin H_2; PtdOH, phosphatidic acid; 12-HETE, 12-hydroxyicosatetraenoic acid.

§To whom reprint requests should be addressed.

diameter) of Sepharose 2B equilibrated with GFB (pH 7.3) without creatine phosphate/creatine phosphokinase.

Stimulated [^{32}P]PtdOH and TXB_2 Formation. Filtered human platelets (10^9 per ml; labeled or unlabeled) were incubated with Ca^{2+} (1 mM) in the presence of human fibrinogen (100–500 μg/ml; Kabi Diagnostica, Stockholm, or the generous gift of Jack Hawiger, New England Deaconess Hospital, Boston) for up to 4 min at 37°C in a Payton aggregometer before exposure to (−)-epinephrine (0–100 μM) or buffer. Platelets were incubated for up to 120 s. The incubations were terminated by the addition of aliquots to 3.75 vol of $CHCl_3$/MeOH, 1:2 (vol/vol). Lipid extracts were chromatographed and quantified as described (15). The aqueous phases of the extracts were assayed for TXB_2 by radioimmunoassay (Upjohn). In some experiments, fibrinogen was replaced by or added with A2A9 (40 μg/ml), a monoclonal antibody against GPIIb/IIIa complex that binds equally well to resting and stimulated platelets, prepared as described (9, 10). A2A9 completely blocked aggregation induced by epinephrine in the presence of 50 μg of fibrinogen per ml. 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RS-082) (3.5 μM; Ono Pharmaceutical, Osaka, Japan) was used to block phospholipase A (PLA), and ethylisopropylamiloride [40 μM (EIPA), the gift of E. J. Cragoe, Merck Sharp & Dohme] was used as an inhibitor of Na^+/H^+ exchange (8). Inhibitors were added 2 min prior to the addition of stimulus. The effect of a passive vehicle for Na^+/H^+ exchange (the ionophore monensin, 10 μM) in promoting TXB_2 formation was also examined. In other studies, 10 μM sodium arachidonate (NuChek) was added to unlabeled platelets 4 min after buffer or inhibitors and incubated for 2 min in the absence of fibrinogen and epinephrine before extraction as above. Any effects of inhibitors on TXB_2 formation (cyclooxygenase) were assessed.

Phosphatidylinositol Phosphate Turnover, Protein Phosphorylation, and Secretion. Platelets labeled with ^{3}H or ^{32}P were incubated as above in an aggregometer with and without aspirin (1 mM) for 15 min, followed by epinephrine. Aliquots were removed at various times for electrophoresis on 11% $NaDodSO_4$/polyacrylamide gels, as described before (16), or extraction and thin-layer chromatography (14). When *myo*-inositol trisphosphate ($InsP_3$) was to be quantitated, unlabeled platelets were incubated in 10-ml cuvettes such that aliquots containing 3×10^9 platelets could be removed and extracted (17). $InsP_3$ was quantitated by capillary gas chromatography (17, 18). Secretion of dense granule substituents was monitored by the release of [^{14}C]serotonin from platelets (14).

Potentiation Studies. ^{32}P-labeled platelets, exposed to aspirin, were incubated at 37°C for 4 min in the presence of Ca^{2+}, fibrinogen, and/or A2A9. ONO-RS-082 or EIPA was also included where appropriate. Subaggregatory concentrations of (15*S*)-hydroxy-11α,9α-(epoxymethano)prosta-5,13-dienoic acid [(U46619) a stable thromboxane receptor agonist; Upjohn] were determined for each preparation of platelets following dose–response assays. The platelets were then mixed with such concentrations of U46619, epinephrine (100 μM), both, or neither, for 75 s. The reaction was determined and products were resolved as above.

Arachidonic Acid Turnover. ^{3}H-labeled platelets were exposed to aspirin or buffer and incubated as above in the presence of 0.05% delipidated albumin, with and without epinephrine (100 μM) for up to 10 min. Incubations were terminated and supernatants containing [^{3}H]arachidonic acid were resolved by HPLC as described (19). Fractions containing arachidonic acid and 12-hydroxyicosatetraenoic acid (12-HETE) were either assayed by scintillation spectrophotometry or dried, derivatized (20), and quantitated by capillary gas chromatography on a CP Sil88 column (Chrompack) in a Hewlett–Packard 5880A gas chromatograph at 175°C (injection, 200°C; detector, 200°C; flow, 2.43 ml/min; split, 50:1). In aspirin-free platelets, maximum 12-HETE formation occurred 5 min after the addition of epinephrine (40 ng per 10^9 platelets). However, since no rise in arachidonic acid (or 12-HETE) was observed in response to epinephrine/aspirin, studies were undertaken to detect accelerated turnover of arachidonate. For "cold chase" experiments, [^{3}H]arachidonic acid/^{32}P-labeled platelets, treated with aspirin, were incubated for 10 s with epinephrine (50 μM) in the presence of fibrinogen (200 μg/ml) and Ca^{2+} (0.5 mM) and then exposed to ethanol vehicle (0.1%) or unlabeled arachidonic acid (1 or 10 μM) for 120 s. Aspirin completely inhibited all cyclooxygenase activity, as gauged by failure of treated platelets to aggregate in response to 3 μM arachidonate or form TXB_2. In some studies, buffer was substituted for epinephrine or fibrinogen, or 1 mM EDTA replaced Ca^{2+}, or the α_2-antagonist yohimbine (10 μM; Sigma) was present. Incubations were stopped with $CHCl_3$/MeOH/HCl (1:2:0.02), and lipids were resolved and quantitated (15). The effects of epinephrine on incorporation of [^{3}H]arachidonic acid into phospholipid were also measured. Aspirin-treated platelets were incubated as above with and without epinephrine. After 10 s, 9 nM [^{3}H]arachidonic acid was added. In one study, ONO-RS-082 (3.5 μM) was included. Aliquots were removed after different times and lipids were resolved by two-dimensional silicic acid paper chromatography (21).

Cytoplasmic Alkalinization. The changes in intracellular pH of human platelets in response to epinephrine were measured essentially as described (22). Filtered platelets (10^9 per ml) were allowed to equilibrate with 9-aminoacridine (4 μM; Sigma) in the presence of Ca^{2+} and fibrinogen or A2A9 for 4 min in a Perkin–Elmer 650-10S spectrofluorimeter equipped with thermostating and stirring devices. The fluorescence was monitored continuously (λ_{ex} = 400 nm; λ_{em} = 456 nm). When the fluorescence reached a constant level, epinephrine or monensin was added and the change in fluorescence was monitored. The change in transmembrane pH gradient, $\delta(\Delta pH)$, was calculated as described (22). Aspirin was found to interfere with uptake of 9-aminoacridine and fluorescence measurements. Therefore, indomethacin was substituted and the cells were allowed to equilibrate with 9-aminoacridine before adding 2 μg of indomethacin per ml. This was sufficient to block conversion of 3 μM arachidonic acid to TXB_2. After 3 min, epinephrine and/or U46619 were added and the change in fluorescence was monitored as above. In some experiments, A2A9, EIPA, or creatine phosphate/creatine phosphokinase was added 3 min prior to epinephrine. In all experiments, aggregation in response to epinephrine was closely monitored and used as an index of platelet "responsiveness," which was unimpaired by 9-aminoacridine.

RESULTS AND DISCUSSION

The studies to be described establish four sequential phases of human platelet activation in response to α_2-adrenergic stimulation: (*i*) activation of Na^+/H^+ exchange (alkalinization) that is dependent upon the binding of fibrinogen to receptive GPIIb/IIIa complexes, (*ii*) mobilization of arachidonic acid by PLA_2 in a manner partially dependent upon Na^+/H^+ exchange, (*iii*) conversion of arachidonic acid to PGH_2/TXA_2, thereby initiating the activation of PLC, and (*iv*) potentiation of the activation of PLC. The fourth, potentiating, phase can be mimicked by epinephrine and low concentrations of the PGH_2/TXA_2 analogue U46619 and is independent of fibrinogen binding, Na^+/H^+ exchange, and PLA.

The factors affecting platelet cytoplasmic alkalinization are represented graphically in Fig. 1. Alkalinization is a function of fibrinogen interaction with GPIIb/IIIa complex, insofar as

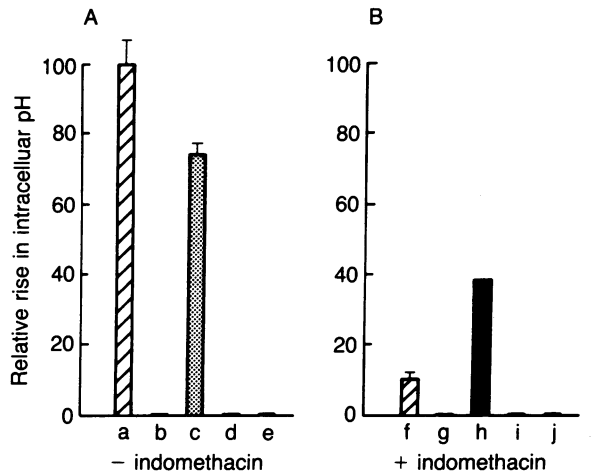

FIG. 1. Relative rise in transmembrane pH as measured by 9-aminoacridine in the presence or absence of indomethacin. Platelets (10^9 per ml), equilibrated with 9-aminoacridine, were incubated in the presence of Ca^{2+} (1 mM) and epinephrine (100 μM) and the following additions: fibrinogen (200 μg/ml, a and f), A2A9 (40 μg/ml, b and g), fibrinogen + A2A9 (c), fibrinogen + EIPA (40 μM, d), fibrinogen + creatine phosphate/creatine phosphokinase (e), fibrinogen + U46619 (7.5 nM, h), A2A9 + U46619 (i), or fibrinogen + U46619 *without* epinephrine (j). These results were compared with those in the absence of agonist, and the effects of indomethacin (2 μg/ml) were determined. Fluorescence of 9-aminoacridine was measured before and after the addition of stimulus (λ_{ex} = 400 nM; λ_{em} = 456 nM). ΔpH was calculated as described (22). A maximum response (+0.3 pH unit) was achieved with 10 μM monensin. The full epinephrine response was equivalent to +0.16 pH unit over the resting intracellular pH of 7.0 (22). Results are presented as the % of the rise in pH achieved with epinephrine (a) and represent the mean of two experiments performed in duplicate ± SEM, except for single determinations (e and h).

it is blocked competitively by A2A9 and by the omission of fibrinogen. Cytoplasmic alkalinization most likely occurs by way of Na^+/H^+ exchange, since it can be blocked by an amiloride analogue, EIPA, which itself does not affect fibrinogen binding (8). Epinephrine-induced alkalinization is observed in the absence of cyclooxygenase activity and secretion (both of which are eliminated by indomethacin), although it is significantly greater if cyclooxygenase is active. This observation may be related to the known role for PGH_2/TXA_2 in promoting the secretion of ADP and GPIIb/IIIA/fibrinogen complex formation. Interestingly, alkalinization of indomethacin-treated platelets can be potentiated by epinephrine plus U46619 as long as fibrinogen is present. Thus, in the normal platelet exposed to epinephrine, additional factors can be recruited to promote alkalinization, provided that fibrinogen can bind to the platelet.

Maximum alkalinization can be achieved by the addition of monensin to platelets, which facilitates passive equilibration of external and internal pH. Monensin (10 μM) causes some TXB_2 formation (21 ± 2 pg per 10^7 platelets per 30–60 s), which is more rapid than that induced by epinephrine and unaffected by the omission of fibrinogen. However, the amount of TXB_2 formed is much less than that achieved with epinephrine (70 ± 5 pg per 10^7 platelets per 60 s), and aggregation does not occur. TXB_2 is the stable metabolite of TXA_2, which results from the action of cyclooxygenase on free arachidonic acid. Alkalinization (or Na^+ influx, or both) thus appears to be necessary for mobilization of arachidonic acid (measured as TXB_2) in response to epinephrine or ADP (8). However, alkalinization by itself does not appear to be sufficient cause for full mobilization. Other possible factors could be G_i regulatory protein and/or an epinephrine-induced Ca^{2+} rise. Ware *et al.* (23) have reported that aspirin-treated platelets undergo an aequorin-detectable increase in Ca^{2+}, most likely localized, in response to epinephrine. Notably, these are conditions in which $InsP_3$ is not formed (see Fig. 2).

Sweatt *et al.* (8) have reported that omission of Na^+ (which also prevents Na^+/H^+ exchange) blocks TXB_2 formation by inhibiting the release of arachidonic acid rather than by inhibiting cyclooxygenase, since it does not interfere with the conversion of arachidonic acid to TXB_2. As seen in Table 1, the stimulated increases in TXB_2 are also inhibited by A2A9, which, as noted above, decreases fibrinogen/GPIIb/IIIa complex formation and cytoplasmic alkalinization, and by ONO-RS-082, an agent that impairs PLA activity. We have found that neither of these agents blocks conversion of arachidonic acid to TXB_2; therefore they must be acting at the level of arachidonic acid release. In parallel, formation of [^{32}P]PtdOH, a sensitive monitor of PLC activation, is also inhibited by EIPA, A2A9, and ONO-RS-082. These findings are consistent with a role for TXA_2 in mediating the activation of PLC (see also Fig. 2). We have found that none of these inhibitors interferes with the formation of PtdOH induced by U46619, an agonist that, as an analogue of TXA_2, bypasses the need for arachidonic acid mobilization and TXA_2 formation. Further, from these observations we know that the inhibitors block neither diacylglycerol kinase (required for PtdOH synthesis from diacylglycerol) nor PLC (which forms diacylglycerol).

We have attempted to quantitate arachidonic acid release (or lysophospholipid formation) in aspirin-treated platelets exposed to epinephrine, using HPLC, thin-layer chromatography, and gas chromatography, but without success. No statistically significant release is observed, despite increases in TXB_2 and 12-HETE in aspirin-free platelets exposed to epinephrine. It seemed possible that, in the absence of cyclooxygenase activity to convert arachidonic acid to its metabolites, the small amounts of arachidonic acid released might be reincorporated rapidly into phospholipids. It is unlikely that this arachidonic acid would be used by lipoxygenase, which requires substantially more availability of arachidonic acid than does cyclooxygenase (24), and, in fact, we detect no rise in 12-HETE under these conditions. We therefore have employed two approaches to detect arachidonic acid turnover in phospholipid, examining the effects of epinephrine on (*i*) incorporation of [^{3}H]arachidonic acid (trace levels) into phospholipid and (*ii*) mobilization of [^{3}H]arachidonic acid from phospholipid in prelabeled platelets, using unlabeled arachidonic acid. Epinephrine enhances uptake of [^{3}H]arachidonic acid into several classes of phospholipid 1.5- to 2-fold in 60 s (not shown). The PLA inhibitor ONO-RS-082 inhibits this uptake. Such platelets undergo a low level ("primary wave") of aggregation but do not secrete storage pool components such as ADP.

Epinephrine also enhances the replacement of labeled arachidonic acid by unlabeled arachidonic acid in a manner dependent upon fibrinogen, divalent cation, and α_2 receptor occupancy (Table 2). Levels of ^{32}P in phospholipid are

Table 1. Effects of inhibitors on epinephrine-stimulated PtdOH and TXB_2 production in human platelets

Addition	[^{32}P]PtdOH	TXB_2
Epinephrine	405 ± 28	1426 ± 48
+ ONO-RS-082	125 ± 4	101 ± 4
+ EIPA	112 ± 3	ND
+ A2A9	118 ± 13	222 ± 99

Platelets were stirred in an aggregometer with Ca^{2+} and fibrinogen (200 μg/ml) in the presence or absence of A2A9 (100 μg/ml), ONO-RS-082 (3.5 μM), or EIPA (40 μM) for 4 min, followed by epinephrine or buffer for 75 s. Results are expressed as the % of epinephrine-free controls and represent the mean ± SEM. ND, not determined.

Table 2. Effect of epinephrine on exchange of labeled arachidonic acid in lipid pools

Lipid	Condition	Unlabeled arachidonate, μM 1	10
PtdIns	Epinephrine	0.940 ± 0.013	0.921 ± 0.016
	+ yohimbine	ND	0.979 ± 0.010
	− fibrinogen	0.972 ± 0.011	0.960 ± 0.020
	+ EDTA	ND	0.985 ± 0.015
	Buffer	0.991 ± 0.021	0.982 ± 0.018
Free arachidonic acid	Epinephrine	1.41 ± 0.09	1.90 ± 0.12
	+ yohimbine	ND	1.12 ± 0.10
	− fibrinogen	1.19 ± 0.13	1.32 ± 0.12
	+ EDTA	ND	1.10 ± 0.07
	Buffer	1.05 ± 0.05	1.16 ± 0.08

Aspirin-treated platelets, labeled with ^{32}P and [^{3}H]arachidonic acid, were incubated for 10 s with epinephrine (50 μM) in the presence of fibrinogen (200 μg/ml) and Ca^{2+} (0.5 mM) and then exposed to ethanol vehicle (0.1%) or unlabeled arachidonic acid (1 or 10 μM) for 120 s. Other incubations substituted buffer for epinephrine or fibrinogen, substituted 1 mM EDTA for Ca^{2+}, or included yohimbine (10 μM). Results are expressed as the mean ± SD of ^{3}H cpm in the cold chase lipid sample divided by those in the ethanol control lipid sample for two experiments performed in duplicate. ND, not determined. No change was observed in ^{32}P content of phospholipids vs. controls. Effects of trace contamination by fibrinogen in "− fibrinogen" samples cannot be excluded, since A2A9 was not present.

unchanged, indicating no loss of phospholipid (not shown). The most consistent changes are observed in phosphatidylinositol (PtdIns), although we cannot rule out contributions from other phospholipids, obscured by statistical variation. We conclude that epinephrine stimulates a low level of PLA activation in aspirin-treated platelets and that binding of fibrinogen is required for this response. The requirement for divalent cation may reflect its role in promoting the association of GPIIb/IIIa complex (9), the binding of fibrinogen to the complex, and a possible need for Ca^{2+}influx.

Fig. 2 clearly demonstrates that α_2-adrenergic stimulation is linked only indirectly to PLC by way of PGH_2/TXA_2. This conclusion differs from that of Block *et al.* (3), who did not monitor the effects of aspirin on the epinephrine response. Platelets exposed to epinephrine accumulate InsP_3, diacylglycerol (not shown, but peaking here at 60 s), and PtdOH, and phosphorylated 47-kDa and 20-kDa (myosin light chain) proteins (Fig. 2, solid lines). These changes are blocked completely by aspirin (Fig. 2, broken lines) or indomethacin (not shown). Inhibition cannot be overcome by up to 500 μM epinephrine. Although not clearly evident here, a small wave of aggregation does occur in aspirin-treated platelets, but secretion (ordinarily 68% ± 4% SEM of the total storage pool after a 120-s exposure to epinephrine) is prevented completely by aspirin. We have also observed that the presence of yohimbine or omission of fibrinogen will block these changes equally effectively. Finally, despite the lack of measurable kinase C activation in aspirin-treated platelets exposed to epinephrine, we know from the foregoing results that alkalinization can occur. Thus, in contrast to the case for other systems (25), kinase C is not necessary for initiation of this event.

The function of epinephrine in potentiating PLC activation in response to U46619 (and presumably PGH_2/TXA_2) is distinct from that as a promoter of PLA activation in the sequence described above. Aspirin-treated platelets show no rise in the mass of InsP_3 in response to epinephrine above basal levels of 40 pmol per 10^9 platelets (Fig. 2) and a slight rise in response to 7.5 nM U46619 (137% of control in 60 s; 159% in 90 s). In the presence of epinephrine plus 7.5 nM

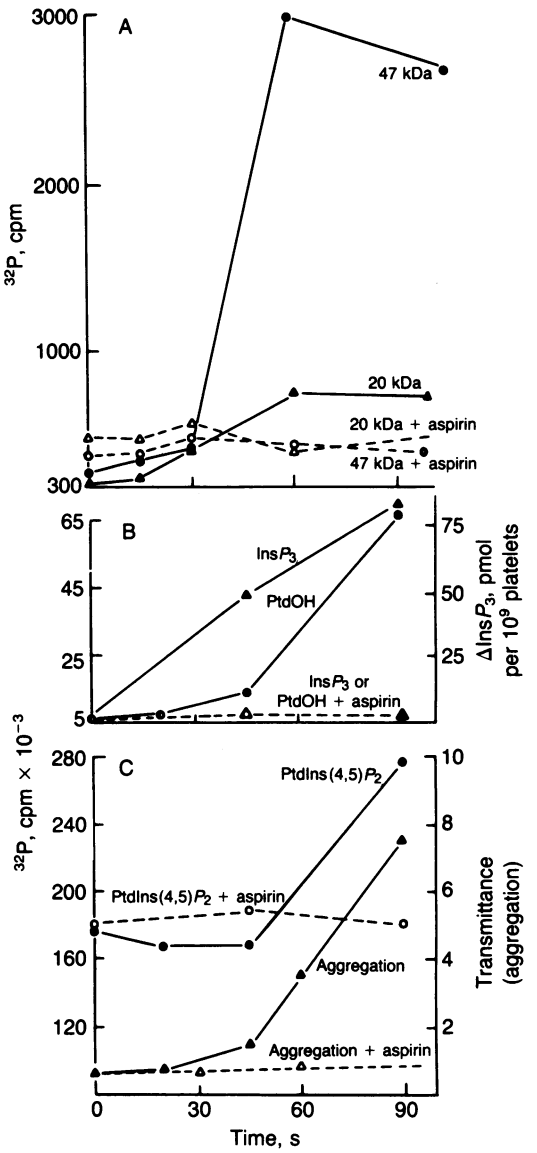

FIG. 2. Effect of aspirin on epinephrine-induced phosphatidylinositol phosphate turnover, protein phosphorylation, and aggregation in human platelets. Platelets, labeled with ^{32}P, were incubated for 15 min with or without aspirin (1 mM) in an aggregometer, after which fibrinogen (200 μg/ml), Ca^{2+} (1 mM), and epinephrine (50 μM) or buffer were added. Aliquots were removed after various times for resolution and quantitation of phosphorylated protein (*A*) or phosphatidylinositol phosphate and metabolites (*B* and *C*). Aggregation was followed simultaneously (*C*). PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate.

U46619, a significantly potentiated rise in InsP_3 is observed (251% of control in 60 s; 304% in 90 s). Potentiation by epinephrine of PtdOH accumulation also occurs as well as potentiated phosphorylation of 47-kDa protein and aggregation. Thus, in two experiments performed in duplicate, epinephrine induces no change in PtdOH accumulation by aspirin-treated platelets, 7.5 nM U46619 induces a rise of 105% ± 3%, but 7.5 nM U46619 plus epinephrine cause a potentiated rise of 154% ± 4% (±SEM). Significantly, this potentiation is not impaired by any of the inhibitors of arachidonic acid mobilization: A2A9, ONO-RS-082, or EIPA. Most probably, it reflects some other parallel action of epinephrine, perhaps a G_i protein-promoted enhancement of U46619 binding to its receptor or an enhanced coupling of the receptor to PLC. Thus, the functioning of the α_2 receptor in

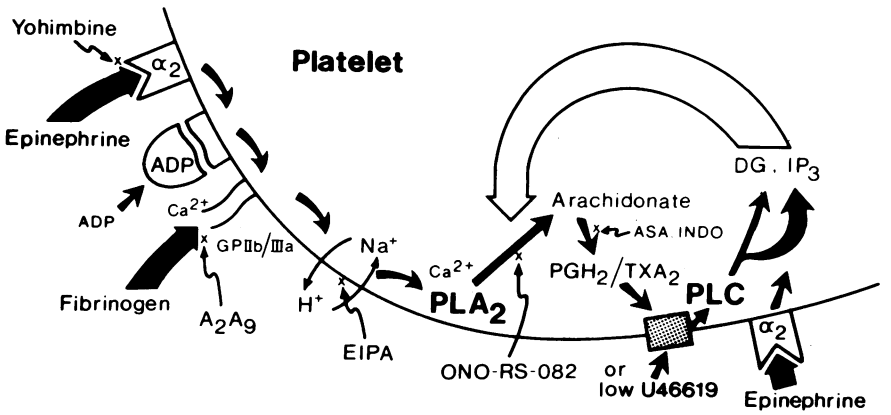

FIG. 3. Dual function of epinephrine in platelet activation. The scheme shown indicates our knowledge of the mechanism of action of epinephrine based upon data reported here and in the literature. Jagged arrows indicate receptor antagonists and inhibitors. It is assumed, but not proven, that the TXA_2 and U46619 receptor(s) is on the plasma membrane. DG, diacylglycerol; IP_3, InsP_3; ASA, aspirin; INDO, indomethacin.

platelets is a dual one, as an initiator of events leading to TXA_2 formation and as a potentiator of TXA_2-caused events. The second function is at least as important as the first, since only small amounts of thromboxane are formed in the first 30 s of exposure to epinephrine (2–4 pg per 10^7 platelets). This small local concentration (≈5–10 nM, based upon an intracellular volume of 10 μl per 10^9 platelets), although barely capable of activating PLC alone, can have its effects enhanced by epinephrine, leading to increased PLC activation. The consequent formation of InsP_3 and diacylglycerol should promote more Ca^{2+} mobilization, arachidonic acid release, and secretion, further amplifying the initial stimulus.

Recently, investigators have demonstrated that the epinephrine-induced opening of GPIIb/IIIa binding sites for fibrinogen is dependent upon environmental ADP (26). Epinephrine enhances the binding of ADP to its receptor 10-fold (26), and it has been reported elsewhere (8) that ADP-induced arachidonic acid mobilization is inhibited by EIPA. Preliminary studies examining the effects of creatine phosphate/creatine phosphokinase (to remove ADP) on platelet activation by epinephrine have yielded variable results. Inhibition of alkalinization is observed (for example, see Fig. 1) but is not always complete. Further experiments, most probably with specific receptor-directed inhibitors, are needed to resolve this point definitively. However, given that Na^+/H^+ exchange appears to be dependent upon the binding of fibrinogen to GPIIb/IIIa, if exposure of cryptic GPIIb/IIIa complex is a function of ADP binding, it follows that alkalinization should also be a function of ADP binding. In such a case, activation of PLA would be dependent upon epinephrine-potentiated ADP binding, and PLC activation would be dependent upon epinephrine-potentiated TXA_2 effects. A scheme summarizing our findings and the possible role of ADP is presented in Fig. 3. The mechanisms by which epinephrine acts as a potentiator independently of fibrinogen, Na^+/H^+ exchange, and PLA and as a mobilizer of Ca^{2+} independently of phosphatidylinositol phosphate metabolism are important subjects for future study.

We thank Evan Benjamin and Steven McIntosh for technical assistance. This work was supported in part by Grants HL35797, HL35007, HL15335, and HL33852 from the National Institutes of Health and Established Investigator Awards (S.E.R. and L.F.B.) from the American Heart Association.

1. Wallace, M. A., Agarwal, K. C., Garcia-Sainz, J. A. & Fain, J. N. (1982) *J. Cell. Biochem.* **18,** 213–220.
2. Deykin, D. & Snyder, D. (1973) *J. Lab. Clin. Med.* **82,** 554–558.
3. Block, L. H., Jaksche, H., Erne, P., Bolli, P. & Buhler, F. R. (1985) *J. Clin. Invest.* **75,** 1600–1607.
4. Grant, J. A. & Scrutton, M. C. (1979) *Nature (London)* **277,** 659–661.
5. Hoffmann, B. B., Delean, A., Wood, C. L., Schocken, D. D. & Lefkowitz, R. J. (1979) *Life Sci.* **24,** 1739–1746.
6. Fain, J. & Garcia-Sainz, A. (1980) *Life. Sci.* **26,** 1183–1194.
7. Siess, W., Weber, P. C. & Lapetina, E. G. (1984) *J. Biol. Chem.* **259,** 8286–8292.
8. Sweatt, J. D., Johnson, S. L., Cragoe, E. J. & Limbird, L. E. (1985) *J. Biol. Chem.* **260,** 12910–12919.
9. Brass, L. F., Shattil, S. J., Kunicki, T. J. & Bennett, J. S. (1985) *J. Biol. Chem.* **260,** 7875–7881.
10. Bennett, J. S., Hoxie, J., Leitman, S., Vilaire, G. & Cines, D. B. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2417–2420.
11. Bennett, J. S. & Vilaire, G. (1979) *J. Clin. Invest.* **64,** 1393–1401.
12. Bennett, J. S., Vilaire, G. & Cines, D. B. (1982) *J. Biol. Chem.* **257,** 8049–8054.
13. Grant, J. A. & Scrutton, M. C. (1980) *Brit. J. Haematol.* **44,** 109–115.
14. Rittenhouse, S. E. (1984) *Biochem. J.* **222,** 103–110.
15. Rittenhouse, S. E. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5417–5420.
16. Rittenhouse, S. E. & Horne, W. C. (1984) *Biochem. Biophys. Res. Commun.* **123,** 393–397.
17. Rittenhouse, S. E. & Sasson, J. P. (1985) *J. Biol. Chem.* **260,** 8657–8660.
18. Rittenhouse, S. E., *Methods Enzymol.*, in press.
19. Russell, F. A. & Deykin, D. (1979) *Prostaglandins* **18,** 11–18.
20. Ackman, R. G. (1969) *Methods Enzymol.* **14,** 329.
21. Rittenhouse-Simmons, S., Russell, F. A. & Deykin, D. (1977) *Biochim. Biophys. Acta* **488,** 370–380.
22. Horne, W. C., Norman, N. E., Schwartz, D. B. & Simons, E. R. (1981) *Eur. J. Biochem.* **120,** 295–302.
23. Ware, J. A., Johnson, P. C., Smith, M. & Salzman, E. W. (1986) *J. Clin. Invest.* **77,** 878–886.
24. Sun, F. F., McGuire, J. C. & Metzler, C. M. (1981) *Prog. Lipid Res.* **20,** 275–278.
25. Moolenaar, W. H., Tertoolen, L. G. & deLaat, S. W. (1984) *Nature (London)* **312,** 371–374.
26. Figures, W. R., Scearce, L. M., Wachtfogel, Y., Chen, J., Colman, R. F. & Colman, R. W. (1986) *J. Biol. Chem.* **261,** 5981–5986.