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Epinephrine and the Ca^{2+} ionophore A23187 synergistically induce platelet aggregation without protein kinase C activation

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Aspirin-pretreated, ^{32}P-prelabeled, washed human platelets resuspended in a buffer containing apyrase and 2% plasma were exposed to epinephrine and the Ca^{2+} ionophore A23187. Epinephrine potentiated platelet aggregation (not secretion), the production of [^{32}P]phosphatidic acid and myosin light chain phosphorylation induced by A23187. No phosphorylation of the 40 kDa protein, the substrate of protein kinase C, was observed. We conclude that G_i-protein activation evoked by epinephrine and Ca^{2+} mobilization caused by A23187 represents a novel synergism for platelet aggregation and that protein kinase C activation, under these conditions is not needed for platelet aggregation.

Ca^{2+} ionophore; Epinephrine; Synergism; Platelet aggregation; Protein kinase C; Phosphatidic acid

1. INTRODUCTION

Ca^{2+} mobilization and protein kinase C activation are two known pathways mediating platelet activation by physiological agonists that activate phospholipase C [1]. Ca^{2+} ionophores and protein kinase C activators synergize in inducing platelet aggregation and secretion [2], and we could demonstrate recently that Ca^{2+} mobilization primes protein kinase C in human platelets leading to increased aggregation and secretion [3].

Epinephrine as a physiological platelet stimulus is unique. It does not induce phospholipase C activation in human platelets, if the formation or action of positive feedback stimulators such as ADP and endoperoxides/thromboxane A_2 is inhibited [4]. Also, epinephrine does not – in aspirinized platelets – elevate significantly cytosolic Ca^{2+} and activate Ca^{2+}-dependent kinases and protein kinase C [5]. Epinephrine binds to α_2-adrenoceptors on human platelets [6,7]. These are coupled to the guanine nucleotide-binding protein G_i which mediates inhibition of adenylate cyclase [8,9]. However, inhibition of adenylate cyclase is not the mechanism by which epinephrine induces and potentiates aggregation [10–12]. Thus, the effector mechanism by which α_2-adrenoceptor activation causes and potentiates platelet aggregation is not known.

We have observed recently that α_2-adrenoceptor agonists, known to interact with G_i, synergized with the protein kinase C pathway in inducing platelet aggregation. The synergism of epinephrine and protein kinase C activators in inducing platelet aggregation involved a novel mechanism [5]. The present study was undertaken to determine whether and how α_2-adrenoceptor activation by epinephrine would synergize with the Ca^{2+} pathway stimulated by Ca^{2+} ionophores in human platelets.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{32}P]Orthophosphate was obtained from NEN (Boston, MA). Acetylsalicylic acid, A23187, prostaglandin E_1 (PGE_1), prostacyclin (PGI_2), 1-epinephrine bitartrate and apyrase (A 6132) were from Sigma (St. Louis, MO). Indo-1/AM was from

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Calbiochem (La Jolla, CA). All other materials were obtained as described [3,4].

2.2. *Methods*

Platelet-rich plasma from 200 ml of freshly drawn blood anticoagulated with 1/10 vol. of 3.8% (w/v) trisodium citrate was incubated with aspirin (1 mM) for 15 min at 37°C. PGI_2 (50 ng/ml) was then added, and platelets were pelleted by centrifugation at 800 × *g* for 10 min. Platelets were resuspended in 3 ml of a buffer prewarmed to 37°C (pH 7.4) containing Hepes (20 mM), NaCl (138 mM), KCl (2.9 mM), $MgCl_2$ (1 mM), glucose (5 mM), autologous platelet-poor plasma (PPP, 2%), apyrase (3 U/ml ADPase) and PGE_1 (2 μM), and were incubated with 3–4 mCi ^{32}P for 1.5 h at 37°C. Then 20 ml of buffer containing 1 mM EGTA were added, and platelets were pelleted by centrifugation (800 × *g* for 10 min). Platelets were resuspended in 10–15 ml of buffer without EGTA and PGE_1, but containing apyrase (0.6 U ADPase/ml), and 2% PPP. The platelet suspension was kept at room temperature. Samples (0.8 ml) were transferred into aggregometer cuvettes, and were incubated for 2 min at 37°C whilst stirring (1100 rpm) and then exposed to A23187 dissolved in DMSO. The final concentration of DMSO in the platelet sample was <0.2%. Epinephrine or buffer was added 10–15 s before A23187. Aggregation was measured in a laboratory aggregometer. Aliquots (0.05 ml) were transferred either into 0.05 ml of sample buffer containing 1% SDS and dithiothreitol (15 mg/ml) for measurement of protein phosphorylation or into 0.375 ml chloroform/methanol (1:2) for determination of phosphatidic acid [3,4,13]. Phosphorylation or [^{32}P]phosphorylation in control samples ranged in various experiments for the 20 kDa protein from 150 to 650 cpm, for the 40 kDa protein from 180 to 750 cpm and for [^{32}P]phosphatidic acid from 200 to 1000 cpm. Secretion of [^3H]serotonin was measured as described [13]. Ca^{2+} mobilization was measured using Indo-1/AM as in [3].

2.3. *Statistical analysis*

Statistical analysis was performed using Student's *t*-test for paired and unpaired data. Results are expressed as means ± SE where *n* = number of separate experiments.

3. RESULTS

Epinephrine (10 μM), which by itself did not induce aggregation, added 10 s before A23187 increased several fold platelet aggregation induced by A23187. The potentiation of platelet aggregation by epinephrine was particularly pronounced at 250 nM A23187 (figs 1,2). Aggregation was partially reversible and no secretion of serotonin was observed at that concentration of A23187. Epinephrine also potentiated the action of a higher concentration of A23187 (500 nM) on platelet aggregation without, however, increasing the amount

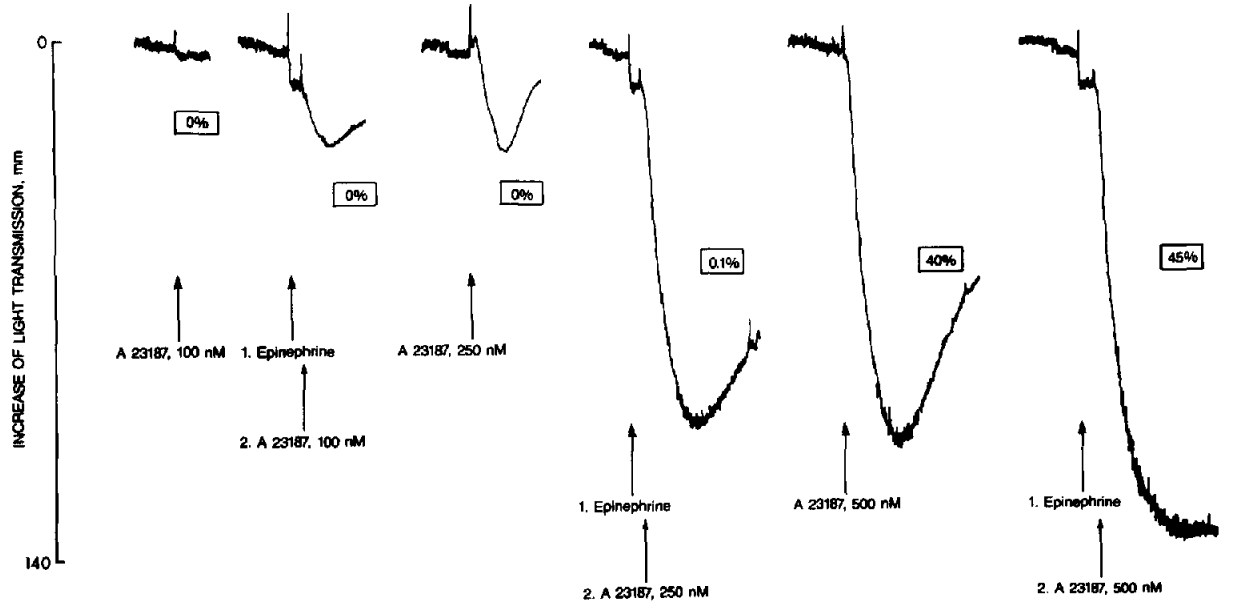

Fig.1. Potentiation of Ca^{2+} ionophore A23187-induced platelet aggregation by epinephrine (10 μM). Suspensions of aspirinized, washed platelets labeled with [^3H]serotonin were incubated at 37°C and stirred for 2 min in aggregometer cuvettes, and then stimulated with various concentrations of A23187. Epinephrine which by itself did not induce platelet aggregation was added 10 s before A23187. Secretion of [^3H]serotonin (% of total, indicated in rectangles) was measured 2 min after addition of A23187. The experiment is representative for at least ten other experiments.

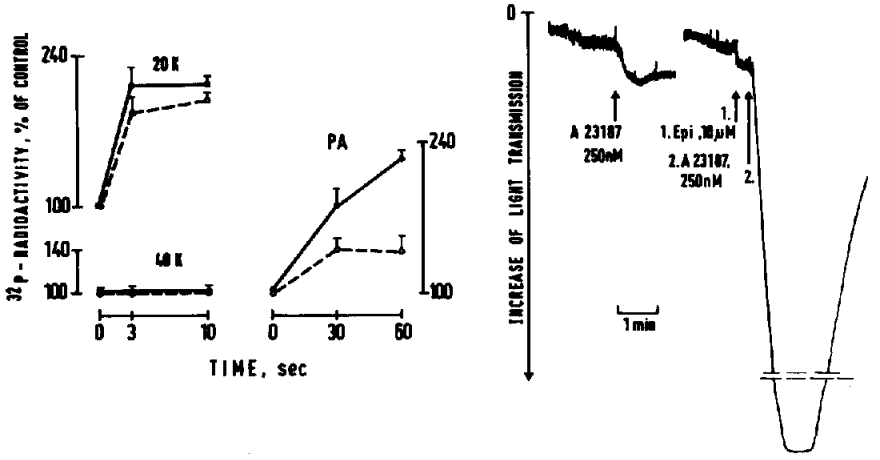

Fig.2. Effect of epinephrine (10 μM) on [^{32}P]phosphorylation of 20 kDa and 40 kDa proteins, formation of [^{32}P]phosphatidic acid (PA) (left panel) and platelet aggregation (right panel) in platelets stimulated by A23187 (250 nM). Suspensions of aspirinized, washed platelets were prelabeled with ^{32}P. Results are means ± SE from four experiments. A23187 alone (o---o); epinephrine for 10 s, then A23187 (•——•). Phosphorylation of 40 kDa protein was also not observed 20, 30 and 60 s after stimulation (not shown).

of serotonin released by that concentration of A23187 (fig.1).

A23187 induced the formation of [^{32}P]phosphatidic acid. The increase did not occur before 5 s (not shown) and was found to be concentration-dependent (fig.3). Prior addition of epinephrine potentiated phosphatidate formation induced by A23187 (figs 2,3). After platelet activation with 500 nM A23187, aggregation and formation of phosphatidic acid were comparable to the values

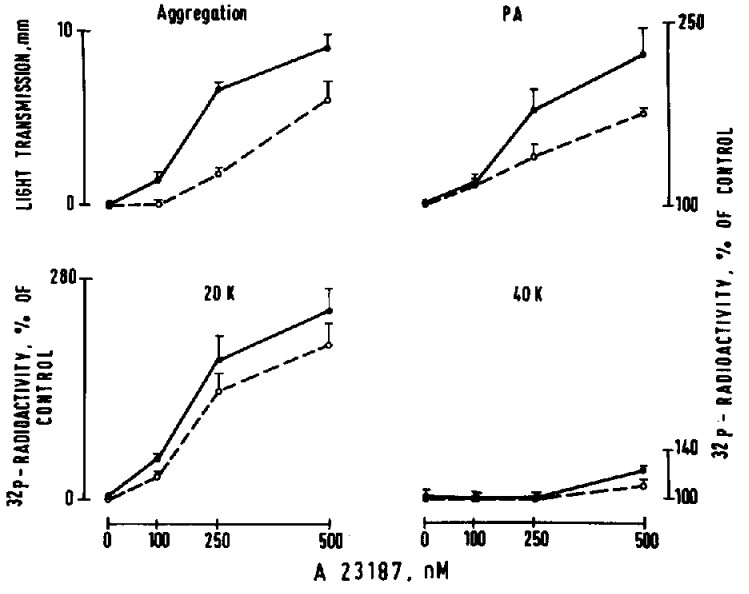

Fig.3. Effect of epinephrine (10 μM) on aggregation, [^{32}P]phosphatidic acid formation, and [^{32}P]phosphorylation of 20 and 40 kDa proteins in platelets stimulated by various concentrations of A23187. Phosphorylation of the 20 kDa protein was measured 3 s after addition of A23187, and phosphorylation of the 40 kDa protein 10 s after addition of A23187. Aggregation and phosphatidic acid were measured 30 s after addition of A23187. Symbols as in legend to fig.2. Results are means ± SE from four experiments.

obtained after platelet activation with 250 nM A23187 plus epinephrine, indicating a close association of the amount of phosphatidate production and platelet aggregation.

A23187 (100 and 250 nM) stimulated the rapid (faster than 3 s) phosphorylation of the 20 kDa myosin light chain. A23187 at these concentrations did not stimulate the phosphorylation of the 40 kDa substrate of protein kinase C (figs 2,3). Phosphorylation of the 40 kDa protein was found to be increased only after platelet stimulation with 500 nM A23187 (fig.3). Prior incubation of platelets with epinephrine enhanced the 20 kDa myosin light chain phosphorylation induced by A23187 without affecting the 40 kDa protein phosphorylation.

Epinephrine did not significantly affect Ca^{2+} mobilization evoked by Ca^{2+} ionophore. A23187 (250 nM) increased the cytosolic Ca^{2+} from 80 nM in resting platelets to 394 ± 17 nM (maximum after 10 s). Addition of epinephrine prior to 250 nM A23187 did not significantly increase this value further (424 ± 36 nM; mean ± SD, $n = 3$).

4. DISCUSSION

Epinephrine potentiated platelet aggregation and [^{32}P]phosphatidate production induced by the Ca^{2+} ionophore A23187. [^{32}P]Phosphatidate formation correlated closely with aggregation. The origin of phosphatidate formation after platelet stimulation with A23187 is obscure. Phospholipase C-induced breakdown of phosphoinositides is not involved, since the formation of endoperoxides/thromboxane A_2 and ADP had been blocked [14]. Also, if traces of diglyceride were formed after A23187 stimulation of platelets, a large protein kinase C activation would have been expected due to the priming of protein kinase C by the cytosolic Ca^{2+} increase [3]. But no protein kinase C activation as measured by 40 kDa protein phosphorylation was found. Possible sources of [^{32}P]phosphatidate formation in platelets stimulated by A23187 are a stimulated conversion of diglyceride to phosphatidic acid or, more likely, an inhibition of the reconversion of phosphatidic acid to phosphatidylinositol. Interruption of the phosphatidylinositol cycle by A23187 in platelets has been previously demonstrated [15]. Alternatively, [^{32}P]phosphatidate could derive from phospholipid degradation by phospholipase D. Phospholipase D hydrolyzing phosphatidylinositol has recently been shown in neutrophils activated by A23187 [16]. Because epinephrine increased the production of phosphatidate after A23187 stimulation of platelets, one can speculate that a G_i-protein might be involved in the activation of phospholipase D. Evidence for a G-protein coupled to hormone-induced phospholipase D activation has been presented in hepatocytes [17].

The close association of phosphatidic acid formation and aggregation is interesting. Phosphatidic acid can translocate Ca^{2+} across organic phases [18] and, if added at micromolar concentrations to multilamellar liposomes, increases their permeability for Ca^{2+} [19]. In addition, it has been shown that 1-oleoyl-2-acetylglycerol added to saponized platelets causes the mobilization of Ca^{2+} from the endoplasmic reticulum. This effect was found to be dependent on its conversion to phosphatidic acid [20]. However, bioactive diacylglycerols added to intact platelets are also transformed to phosphatidic acid [21] but they do not increase cytosolic Ca^{2+} [22]. Furthermore, inhibition of diglyceride kinase by specific drugs in intact platelets enhances rather than inhibits platelet aggregation and secretion induced by exogenous diacylglycerols [23,24]. Thus, the function of phosphatidate for platelet aggregation is uncertain.

Most surprising was the observation that the combination of epinephrine with a low concentration of A23187 induced about 70% of maximal aggregation without any activation of protein kinase C. Previous studies [2,3,13,25–28] indicated the significance of protein kinase C in regulating platelet aggregation and secretion. The present results indicate that it is possible to circumvent that pathway by the synergistic interaction of Ca^{2+} mobilization and α_2-adrenergic receptor activation. α_2-Adrenergic receptors in platelets are coupled selectively to G_i [4,6–9]. Therefore, the novel synergism described in the present study seems to depend on G_i-protein activation and Ca^{2+} mobilization. That synergism could be responsible for shape change and primary aggregation induced by low concentrations of physiological stimuli. For example, ADP and the thromboxane A_2 analogs STA_2 mobilize intracellular Ca^{2+}, their receptors

are coupled to G_i proteins [29,30], but at low concentrations they do not induce the phosphorylation of the 40 kDa protein [31,32].

In summary, there may be at least three pathways in platelets which synergize with each other [1]: the Ca^{2+} pathway that causes myosin light chain phosphorylation, the protein kinase C pathway and an unknown pathway triggered by α_2-adrenergic receptor/G_i-protein activation. Synergism has been demonstrated for each pair of these pathways: (a) Ca^{2+} mobilization and protein kinase C activation [2,3], (b) epinephrine and protein kinase C activation [5], and (c) epinephrine and Ca^{2+} mobilization (this study). Interestingly, each pathway can synergize with the other independently of the third pathway. The synergism of the Ca^{2+} pathway and the α_2-adrenergic receptor/G_i-protein pathway concerns platelet aggregation but not secretion, and it is, in that regard, comparable to the synergism between the protein kinase C and G_i-protein pathways [5]. In contrast, the Ca^{2+} and protein kinase C pathways synergize in inducing both platelet aggregation and secretion [2,3].

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