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Alpha-Adrenergic Stimulation of Phosphatidylinositol Synthesis in Human Platelets as an Alpha-2 Effect Secondary to Platelet Aggregation

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Epinephrine and adenosine diphosphate (ADP) stimulated ^{3}H-glycerol uptake into phosphatidylinositol of human platelets. Yohimbine, an alpha-2 adrenoceptor antagonist, markedly reduced epinephrine-stimulated ^{3}H-glycerol uptake into phosphatidylinositol; while prazosin, an alpha-1 antagonist, was without effect. Likewise, yohimbine, but not prazosin, blocked epinephrine-induced platelet aggregation. Furthermore, clonidine, a specific agonist for alpha-2 adrenoceptors, stimulated incorporation of ^{3}H-glycerol into phosphatidylinositol and promoted platelet aggregation in the presence of low concentrations of ADP. These studies indicate that the effects of epinephrine on platelet aggregation and phosphatidylinositol synthesis are mediated through alpha-2 adrenoceptors. Further, since the stimulation of phosphatidylinositol synthesis seen with epinephrine was also observed with ADP, this suggests that the increased ^{3}H-glycerol labeling is an indirect result of platelet aggregation.

Key words: phosphatidylinositol, human platelets, alpha catecholamines, clonidine, yohimbine, prazosin

Alpha catecholamine effects have been divided into alpha-1 and alpha-2 actions [1-3]. There are separate receptors for each effect. Activation of alpha-1 catecholamine receptors is associated with an elevation of cytosol Ca^{2+} and phosphatidylinositol turnover [3]. In contrast, alpha-2 catecholamine receptor activation is associated with an inhibition of adenylate cyclase [3]. Clonidine is a relatively specific agonist and yohimbine an antagonist for alpha-2 catecholamine receptors. Prazosin is an antagonist of alpha-1 catecholamine effects [1-3].

In platelet membranes [4-6] or intact platelets [7], there appear to be only alpha-2 catecholamine receptors. Alpha-2 catecholamine agonists inhibit cyclic AMP accumulation in intact platelets [8, 9] as well as adenylate cyclase activity of platelet membranes [9, 10] and stimulate platelet aggregation [11-13].

The present studies were designed to investigate the mechanisms by which epinephrine stimulates phosphatidylinositol synthesis in platelets. Deykin and

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Snyder [14] found that epinephrine markedly increased glycerol incorporation into platelet phosphatidylinositol. The present studies were designed to determine whether alpha-2 catecholamine receptor activation might indirectly increase phosphatidylinositol synthesis in platelets since there are no alpha-1 catecholamine receptors in platelets.

METHODS

Fresh human blood was collected in 0.1 ml volume of 3.8% sodium citrate and platelet-rich plasma (PRP) was obtained by differential centrifugation [15]. Platelet aggregation was measured by the turbidometric method of Born and Cross [16]. Subjects were free from any medication for two weeks prior to blood collection.

The incorporation of radioactive [2-^{3}H]glycerol (9.5 Ci/mmole) into phospholipids was determined by incubating 0.5 ml of PRP in the presence or absence of hormone plus 5 or 10 μCi/ml [^{3}H]glycerol for 30 min at 37°C in a water bath shaker. To extract phospholipids, the reactions were terminated by adding 2.8 ml of MeOH: $CHCl_3$ (2:1). This mixture was shaken for 30 min at room temperature after which 1 ml of $CHCl_3$ then 1 ml of 2 M KCl was added. The samples were thoroughly mixed and the phases separated by centrifugation at 4°C. One milliliter aliquots of the lower $CHCl_3$ phase were then evaporated to dryness in vacuo. Samples were redissolved in $CHCl_3$ and the phospholipids separated by one-dimensional thin-layer chromatography on silica gel H plates with 1.5% magnesium acetate using a solvent system of $CHCl_3$:MeOH:28%NH_4OH:H_2O (130:70:6:4). The phospholipids were visualized with I_2 vapor staining and then scraped into scintillation vials for radioactivity measurements [17]. This method clearly separated phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol from other phospholipids but did not separate phosphatidylserine from phosphatidic acid.

The following compounds were obtained from the indicated sources: prazosin, Pfizer; clonidine, Boehringer-Mannheim; yohimbine, Nutritional Biochemical Corp; ADP and (−)epinephrine, Sigma; [2-^{3}H]glycerol from New England Nuclear Corp.

RESULTS

The stimulation of platelet aggregation by epinephrine was much more sensitive to inhibition by yohimbine than by prazosin as shown in Figure 1. The data indicate that both primary and secondary aggregation were affected by yohimbine, while prazosin affected neither phase of aggregation except at high concentrations.

The addition of 1 μM epinephrine to human platelets resulted in a marked increase in incorporation of [^{3}H]glycerol into phosphatidylinositol (Table I). There was only a small and rather variable increase by epinephrine of glycerol incorporation into phosphatidylcholine and phosphatidic acid in the experiments shown in Table I. Actual values from another series of experiments are shown in Table II. Both epinephrine and ADP specifically stimulated the incorporation of glycerol into phosphatidylinositol while having little effect on the much larger basal formation of phosphatidylcholine. The smaller and more variable effect of epinephrine on phosphatidic acid formation is comparable to what has been noted in adipocytes [17].

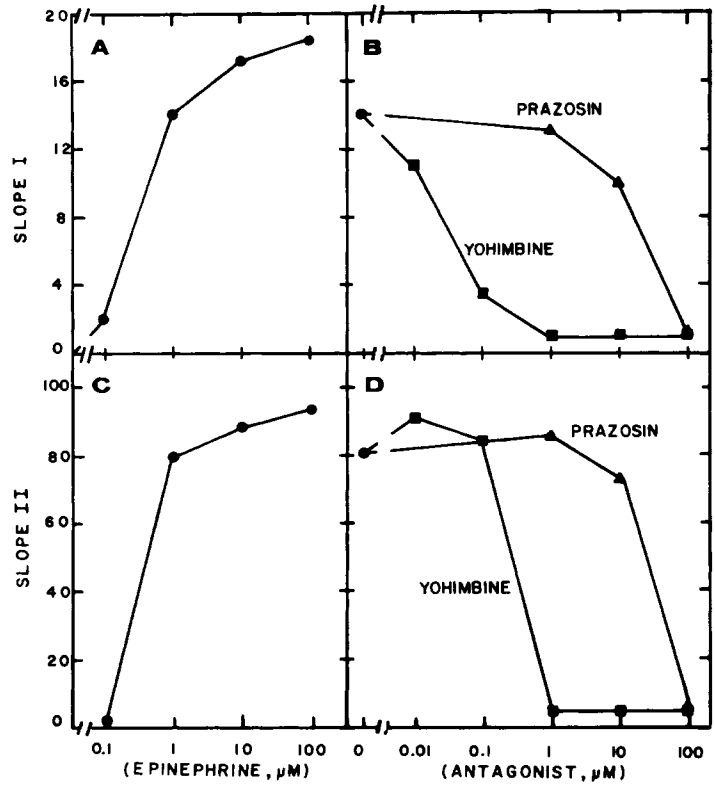

Fig. 1. Dose-response to epinephrine and effect of alpha-adrenergic antagonists on platelet aggregation. Panels A and C show dose responses to epinephrine for primary (A) and secondary (C) platelet aggregation. Panels B and D depict the effects of alpha-adrenergic antagonists on primary (panel B) and secondary (panel D) platelet aggregation in the presence of 1 μM epinephrine. The platelets were preincubated for 5 min with different concentrations of prazosin (triangles), or yohimbine (squares) followed by the addition of epinephrine. Platelet aggregation was recorded as the change in light transmission per minute during the primary phase of aggregation (slope I) and during the secondary phase of aggregation (slope II). The results are the means of at least three experiments performed on different days.

TABLE I. Effect of Epinephrine, Alpha-Adrenergic Antagonists, and ADP on the Incorporation of [^{3}H]-Glycerol Into Platelet Phosphatidylinositol*

Addition	Phosphatidylinositol cpm	Phosphatidylinositol % increase due to added agents
None	88 ± 4	0
Prazosin, 1 μM	89 ± 16	+ 1%
Yohimbine, 1 μM	115 ± 5	+ 31%
Epinephrine, 1 μM	173 ± 21	+ 96%
Epinephrine, 1 μM + prazosin, 1 μM	280 ± 21	+218%
Epinephrine, 1 μM + yohimbine, 1 μM	114 ± 15	+ 30%
ADP, 10 μM	269 ± 51	+205%

*Platelet-rich plasma (humans) was incubated for 30 min with 5 μCi/ml of [^{3}H]glycerol. The values in cpm are the mean ± SEM of three to six experiments and are the increments over background.

TABLE II. Stimulation of [^{3}H]-Glycerol Incorporation Into Phosphatidylinositol by Clonidine*

	% Increases in glycerol incorporation over control values into														
	Phosphatidylcholine					Phosphatidic acid + phosphatidylserine					Phosphatidylinositol				
Additions Experiments:	1	2	3	4	$\bar{X}$	1	2	3	4	$\bar{X}$	1	2	3	4	$\bar{X}$
Epinephrine, 1 μM	23	9	-6	19	11	8	–	-5	81	28	72	55	13	109	62
Epinephrine, 5 μM	39	1	–	29	23	16	–	-5	132	74	135	130	–	147	137
Clonidine, 20 μM	25	-6	11	40	18	1	–	-2	140	46	11	103	13	135	66
ADP, 0.8 μM	16	8	38	-10	13	35	–	-31	57	21	58	55	28	123	66
ADP, 0.8 μM + epinephrine 1 μM	8	10	43	24	21	5	–	45	199	83	13	72	104	199	97
ADP, 0.8 μM + epinephrine, 5 μM	13	4	–	50	22	134	–	–	171	152	145	89	–	155	130
ADP, 0.8 μM + clonidine, 20 μM	0	22	64	52	35	42	–	26	75	48	354	110	101	165	183
ADP, 4 μM	14	81	-5	57	37	-2	–	88	110	65	23	178	185	127	128

*Platelet-rich plasma (humans) was incubated for 30 min with 10 μCi/ml of [^{3}H]glycerol and the indicated additions. The values are shown as the percent increases or decreases (–) from platelets incubated without any additions. The average uptake of [^{3}H]glycerol into phosphatidylcholine was 17,040 cpm, into phosphatidic acid was 250 cpm, and into phosphatidylinositol was 465 cpm. The individual values for four experiments (each experiment was done on a separate day) are shown as well as the means. The dashes indicate that the samples were lost or not run in a particular experiment.

The increase in phosphatidylinositol synthesis due to epinephrine was actually potentiated by the alpha-1 antagonist prazosin and inhibited by yohimbine, which is an alpha-2 antagonist. The effect of yohimbine was specific since 10 μM yohimbine had no effect on ADP (4 μM)-stimulated labeling of phosphatidylinositol. In a separate series of three experiments, the average percent increase of [^{3}H]glycerol label in phosphatidylinositol due to 4 μM ADP was 389 ± 32 while that of 4 μM ADP + 10 μM yohimbine was 375 ± 25.

If the epinephrine effects are due to alpha-2 adrenoceptor activation, then clonidine should mimic the effects of epinephrine. Clonidine was an effective stimulator of [^{3}H]glycerol uptake into phosphatidylinositol, especially in the presence of 0.8 μM ADP (Table II). Previously, Hsu et al [12] noted that clonidine stimulated platelet aggregation very little except in the presence of ADP. We have observed that some individuals show a response to clonidine alone with respect to platelet aggregation, while others show no response except in the presence of low concentrations of ADP. In one individual whose platelets aggregated to the same extent with 20 μM clonidine as with 1 μM epinephrine, the percent increase in incorporation of glycerol into phosphatidylinositol was 89% with clonidine and 90% with epinephrine. These data support the conclusion that epinephrine-induced platelet aggregation and the increase in phosphatidylinositol synthesis are both alpha-2 responses.

DISCUSSION

Thrombin-induced platelet aggregation is associated with an increased synthesis of phosphatidylinositol and phosphatidic acid [18]. However, the earliest effect of thrombin on platelet phospholipid metabolism is a breakdown of about half of

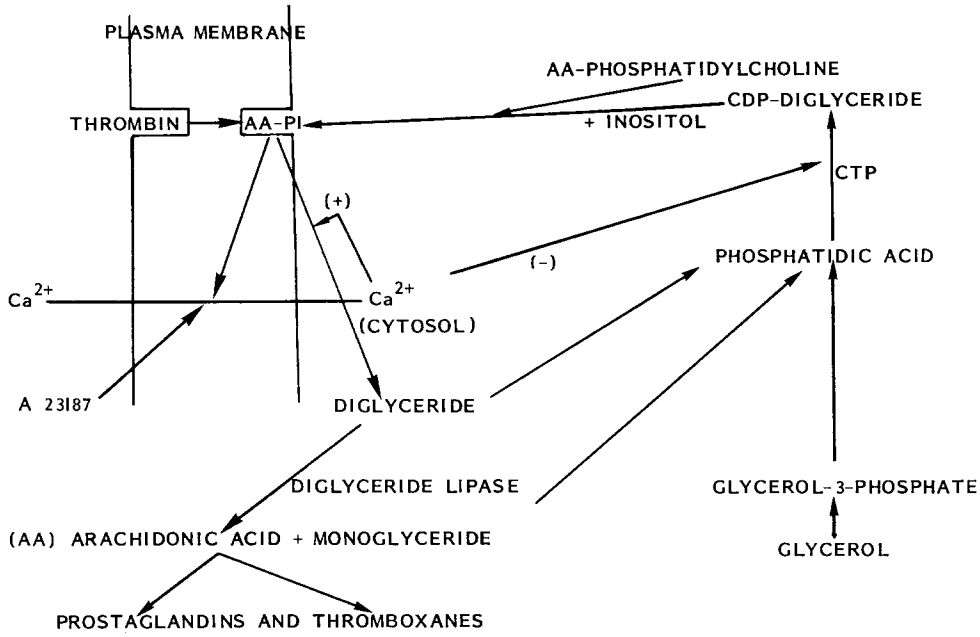

Fig. 2. Model for phosphatidylinositol (PI) turnover in platelets. The breakdown of phosphatidylinositol is postulated to be directly activated by thrombin and also indirectly by elevation of Ca^{2+} with the divalent cation ionophore A 23187. The initial breakdown of phosphatidylinositol may be linked to Ca^{2+} gating, while the subsequent breakdown primarily results in the release of arachidonic acid (AA), which is converted to the various prostaglandins and other metabolites. The increase in resynthesis of phosphatidic acid and phosphatidylinositol may be due to release of an inhibitory constraint on phosphatidylinositol synthesis due to release of Ca^{2+} from the site of synthesis to the cytosol.

the total phosphatidylinositol in human platelets within 10 sec after thrombin addition [19]. The pathways for phosphatidylinositol turnover in platelets are summarized in Figure 2. The breakdown of phosphatidylinositol after thrombin addition is associated with a rise in cytosol Ca^{2+}. It is known that the breakdown of phosphatidylinositol can be increased by addition of the Ca^{2+} ionophore A 23187 [19]. Possibly, the primary event in thrombin action is activation of a phosphatidylinositol-specific phospholipase [20], which is associated with release of bound intracellular Ca^{2+} and entry of extracellular Ca^{2+}. The elevation of Ca^{2+} further activates the phospholipase C, which can account for the ionophore response.

The diglyceride formed from phosphatidylinositol breakdown [21] may activate protein phosphorylation in platelets [22]. The diglyceride can be cleaved by diglyceride lipase resulting in the release of arachidonic acid, which serves as a precursor for prostaglandins and thromboxanes (Fig. 2). Resynthesis of phosphatidylinositol occurs via either de novo synthesis or conversion of monoglycerides and diglycerides to phosphatidic acid. The newly synthesized phosphatidylinositol undergoes a deacylation-reacylation cycle, which results in the unique 1-stearoyl-2-arachidonyl pattern of platelet phosphatidylinositol [23, 24].

Binding studies with platelet membranes have shown that the alpha-adrenergic binding sites are of the alpha-2 subtype [4–6]. Our findings indicate that the aggregation of platelets due to epinephrine is secondary to activation of alpha-2

adrenoceptors, confirming the conclusion of Hsu et al [12]. The inhibition of platelet adenylate cyclase due to alpha catecholamines may play a significant role in platelet aggregation. However, other agents such as 2′-5′-dideoxy-adenosine [25] and 9-(tetrahydro-2-furyl)adenine (SQ 22536) [26] inhibit platelet adenylate cyclase but do not produce aggregation [27]. Conversely, several groups have found no reduction in total cyclic AMP of platelets during aggregation [(review) 28]. However, there was a reduction in cyclic AMP accumulation during aggregation of platelets previously labeled by incubation with adenosine but not with adenine [28]. Possibly, the inhibition by epinephrine of adenylate cyclase activity in platelets is limited to a small compartment linked to aggregation or some unknown signal is linked to catecholamine induced aggregation. All present evidence supports the hypothesis that catecholamine effects mediated through alpha-2 adrenoceptors are secondary to inhibition of adenylate cyclase [3].

Phosphatidylinositol turnover seems to play a key role in the stimulus-action coupling of agents that increase cytosol concentration of calcium [29]. The available data suggest that there exist at least two closely related phospholipid effects. One of these phenomena, that we will call primary in this discussion, has three characteristics: 1) the polar head of the phosphatidylinositol molecule (inositol and phosphate) turns over at a much greater rate than the rest of the molecule, 2) it involves turnover of only a small portion of the phosphatidylinositol present in the cell, and 3) it does not depend on the presence of extracellular calcium nor is it mimicked by calcium ionophores. This last characteristic is basic in supporting the hypothesis that phosphatidylinositol turnover is involved in the gating and mobilization of calcium [29]. There is another phospholipid effect that we consider to be a secondary effect and has the following characteristics: 1) the whole molecule seems to be broken down and resynthesized, 2) it involves turnover of a large fraction of the total phosphatidylinositol in the cell, and 3) it is dependent on extracellular calcium or mimicked by calcium ionophore. A similar division of phospholipid effects has been proposed by Griffin et al [30]. Obviously, both types of effects may occur in the same cell. Bell and Majerus [19] have shown that activation of platelets with thrombin produces a 50% cleavage of phosphatidylinositol. This effect is reproduced by the calcium ionophore A 23187, which suggests that an increase in cytosol Ca^{2+} can increase phosphatidylinositol breakdown. Our data suggest that the phospholipid effect observed in platelets activated with epinephrine probably involves a secondary or calcium-dependent phospholipid effect linked to aggregation since it can be mimicked by ADP. Furthermore, it is possible that the increased de novo synthesis of phosphatidylinositol from [2-^{3}H]glycerol by human platelets is secondary to a drop in Ca^{2+} in the endoplasmic reticulum. The only known factor regulating phosphatidylinositol synthesis is Ca^{2+}, which has inhibitory effects. The important point is that the effect of epinephrine noted in platelets is probably a secondary event linked in some way to changes in intracellular Ca^{2+} or cyclic AMP, or both.

Cyclic AMP and calcium are interrelated in the metabolic regulation of many cells [3]. In platelets, they show an inverse relationship, ie, agents that activate platelets such as epinephrine or ADP, decrease cyclic AMP, and increase the cytosolic concentration of calcium, whereas agents that increase cyclic AMP, such as adenosine, prostaglandin E_1 or beta-adrenergic amines, block platelet aggregation [31]. The mechanism by which cyclic AMP blocks aggregation is not clear. Two possibilities have been suggested: 1) a stimulation of calcium uptake by an intracel-

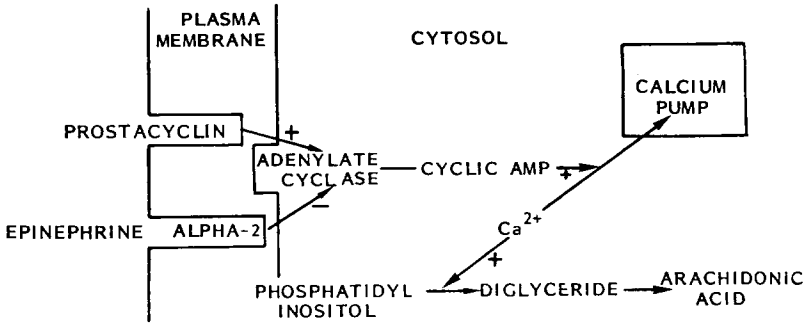

Fig. 3. A hypothesis for alpha-2 catecholamine regulation of phosphatidylinositol formation in platelets secondary to changes in cyclic AMP. The most potent known activator of platelet adenylate cyclase is prostacyclin, but whether under physiological conditions it inhibits platelets aggregation by increasing cyclic AMP remains to be demonstrated. Epinephrine through interaction with alpha-2 adrenoceptors is postulated to inhibit adenylate cyclase. The resulting decrease in intracellular cyclic AMP is postulated to elevate cytosol Ca^{2+}, possibly by reducing the activity of the cyclic AMP-dependent Ca^{2+} pumps. The increase in phosphatidylinositol synthesis may be secondary to the drop in calcium content of the endoplasmic reticulum. The elevation of cytosol Ca^{2+} also increases phosphatidylinositol degradation, but the primary effect seems to be a stimulation of platelet aggregation.

lular membrane system, thereby lowering the concentration of calcium in the cytosol [31], or 2) inhibition of the cytoplasmic phosphatidylinositol-specific phospholipase [32].

We conclude that the effects of epinephrine on platelet function are mediated through alpha-2 receptors and are possibly secondary to inhibition of adenylate cyclase as summarized in Figure 3. There is an increase in the turnover of phosphatidylinositol with epinephrine, but it seems to be a secondary response due to an increase in Ca^{2+}. In platelets, there is a reciprocal relationship between cyclic AMP and Ca^{2+} such that lowering intracellular cyclic AMP apparently results in an elevation of cytosol Ca^{2+}. This could be due to a cyclic AMP-dependent Ca^{2+} pump that stimulates Ca^{2+} uptake by the endoplasmic reticulum or extrusion into the medium.

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