

# (-)-Adrenaline-induced, Calcium-dependent Phosphorylation of Proteins in Human Platelets

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## Abstract

In human platelets, adrenaline stimulated, approximately four-fold, as compared with controls, the phosphorylation of primarily two proteins of apparent molecular weights of 20,000 and 40,000, respectively. Maximum phosphorylation occurred after incubation for 1 min and was inhibited by the addition of either yohimbine, prostaglandin  $E_1$ , or EGTA. Phosphorylation of the two proteins was accompanied by diacylglycerol formation. The (-)-adrenaline-induced phosphorylation of proteins corresponds to the activation of a calcium-dependent protein kinase partially purified by DEAE-cellulose and Sephadex G150 column chromatography. The enzymatic activity was modulated by addition of (-)-adrenaline and  $CaCl_2$ , by diolein, and in the presence of membranes or phosphatidylinositol but not phosphatidylethanolamine and phosphatidylcholine. A phospholipid-dependent reaction appears to be involved in the molecular mechanism of action of adrenaline.

## Introduction

The alpha adrenergic effects of adrenaline occur subsequent to binding of the neurotransmitter to alpha adrenergic receptors (1). 3',5'-cyclic adenosine monophosphate (cAMP) appears to act antagonistically and it has been proposed that calcium ions play a crucial role in the activation of adrenaline-stimulated cellular functions; however, the precise mechanism of this transmembrane signal has not yet been clarified. The stimulatory effect of alpha adrenergic agonists on calcium fluxes (2-4), the calcium-dependent mimicry of the effect of alpha adrenergic activation by such agents as vasopressin, angiotensin II, and the ionophore A23187 (5, 6), which alter  $Ca^{2+}$  fluxes, as well as the inhibitory effects of calcium antagonists on many alpha responses (7, 8), suggest a  $Ca^{2+}$ -dependent regulatory step in the molecular mechanism of action of adrenaline.

Recent reports by Michell and co-workers (9-11) have indicated that alpha adrenergic stimulation increases the incorporation of  $^{32}P_i$  into cellular phospholipid; thus, an altered metabolism of phospholipid appears to be importantly involved in the activation of alpha receptors. Similarly, Kawahara et al. (12) demonstrated a thrombin-induced phospholipid turnover

that may serve as a transmembrane signal for the phosphorylation of proteins during platelet activation in the presence of calcium ions. Since stimulation of platelets by thrombin might be analogous to hormonal control of cellular processes (13), we attempted to clarify the regulatory mechanism involved during alpha adrenergic activation by using human platelets that contain the pharmacologically relevant components: alpha-2 receptors, the cytoplasmic organelles for the regulation of intracellular  $Ca^{2+}$  concentration, i.e., dense tubular system and mitochondria) and the contractile proteins, actin and myosin.

In the following, we report that adrenaline acts by stimulation of calcium-dependent phosphorylation of contractile proteins, one of which has been shown to be the light chain of myosin.

## Methods

**Pharmacological agents.** (-)-Adrenaline was purchased from Fluka Chemical Co. (Buchs, Switzerland); prostaglandin  $E_1$  ( $PGE_1$ )<sup>1</sup> and thrombin from Serva Feinbiochemika (Zürich, Switzerland); pronase, ribonuclease, monoolein, diolein, triolein, tripalmitin arachidonic acid, cholesterol, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine from Sigma Chemical Co. (St. Louis, MO). Sephadex G-150 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and DEAE cellulose from E. Merck (Darmstadt, Germany). [ $\gamma$ - $^{32}P$ ]ATP ( $6 \times 10^4$  cpm/nmol), [ $^3H$ ]arachidonic acid (78.1 Ci/mmol), [ $^3H$ ]5-hydroxytryptamine (11 Ci/mmol), and  $^{32}P_i$  carrier free (1,000  $\mu$ Ci/ml) were purchased from New England Nuclear, Boston, MA, and  $H_1$ -histone from Boehringer, Mannheim, Federal Republic of Germany.

**Isolation and preparation of human platelets.** Platelets were isolated by differential centrifugation from individual units of fresh human blood of healthy volunteers, who had not ingested any drug known to affect platelet function for at least 3 wk before donation, using the method of Baenziger and Majerus (14). Briefly, 400 ml of blood were added to 50 ml plastic centrifuge tubes containing 0.85 ml of 0.25 M  $Na_2EDTA$  while anticoagulant platelet-rich plasma was collected and centrifuged (2,250 g; 15 min; 23°C), and the resulting pellets were pooled and washed by resuspending in a buffer containing 0.113 M NaCl, 4.3 mM  $K_2HPO_4$ , 4.3 mM  $Na_2HPO_4$ , 24.4 mM  $NaH_2PO_4$ , and 5.5 mM glucose (pH 6.5). To remove contaminating leukocytes and erythrocytes, the samples were centrifuged at 120 g for 7 min. The supernatant fluids were collected and again centrifuged (2,000 g; 15 min; 23°C); the precipitate was washed once and finally resuspended in a buffer containing 0.14 M NaCl, 15 mM Tris-HCl, and 5.5 mM glucose (pH 7.5). The isolated platelets ( $5 \times 10^{10}$ ) were virtually pure, as evidenced by staining procedures, and were maintained at room temperature.

**Activation of endogenous phosphorylation.** To activate endogenous phosphorylation, the platelet suspension was again centrifuged (2,000 g; 15 min; 23°C) and resuspended in the incubation buffer (0.14 M NaCl, 15 mM Tris-HCl; 5.5 mM glucose, and 0.5 mM  $CaCl_2$ , pH 7.5, adjusted to a concentration of  $2 \times 10^9$  cells/ml, and incubated at

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1. Abbreviations used in this paper: PAGE, polyacrylamide gel electrophoresis;  $PGE_1$ , prostaglandin  $E_1$ .

room temperature for 60 min with carrier-free  $^{32}\text{P}_i$  (New England Nuclear; 1,000  $\mu\text{Ci}/\text{ml}$ ). The cells were then centrifuged and washed twice at 2,000  $g$  for 15 min with the resuspension buffer and then suspended in 5 ml of the incubation buffer.

Platelet suspensions (0.2 ml or 90  $\mu\text{g}$  cell protein each;  $2 \times 10^8$  platelets) were introduced into plastic tubes containing (–)-adrenaline (0.5 or 1  $\mu\text{M}$ ) and (–)-adrenaline (1  $\mu\text{M}$ ) plus prostaglandin  $\text{E}_1$  (10  $\mu\text{M}$ ) in 0.1 ml incubation buffer. To assess the effect of calcium ions on (–)-adrenaline-induced protein phosphorylation, EGTA (1 mM) or  $\text{CaCl}_2$  (0.5 mM) were added. After incubation for 1 min (unless otherwise indicated in legends to figures), the reaction was stopped by addition of 250  $\mu\text{l}$  of stop solution (10%, wt/vol, SDS; Tris-HCl, 100 mM (pH 7.4); beta mercaptoethanol, 0.5 mM; sucrose, 0.1 g/ml; and bromphenol blue tracking dye, 0.02 mg/ml) with immediate placement in a bath of boiling water for 2 min.

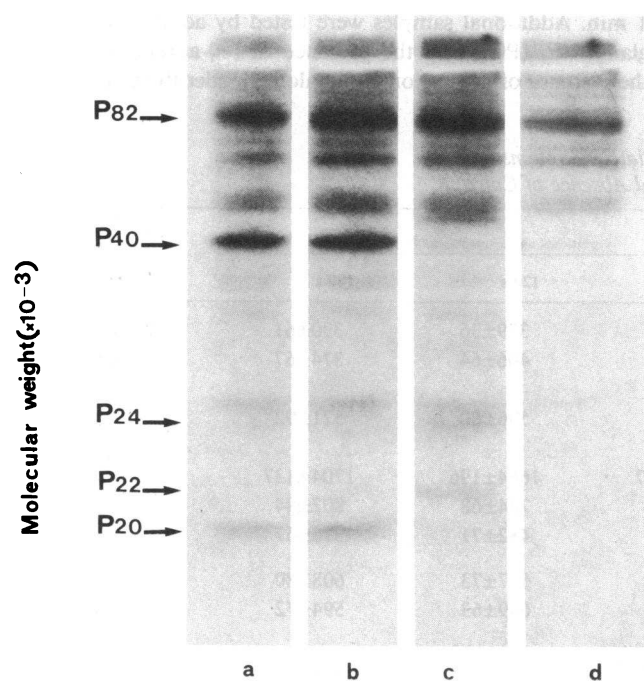
The effectiveness of adrenaline in stimulating the platelet release was determined in some experiments. In these, 1.5 mmol  $[\text{H}]5$ -hydroxytryptamine was added to suspension of freshly prepared platelets at the same time as  $^{32}\text{P}_i$  was to label contents of platelet dense bodies. Release of this 5-hydroxy $[\text{H}]$ tryptamine by adrenaline was measured as described by Haslam et al. (15).

**SDS-polyacrylamide gel electrophoretic analysis of platelet lysates and autoradiography.** The platelet proteins were separated by 12.5% polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% SDS, according to Laemmli (16). The gels were stained with Coomassie Blue and destained overnight in a solution containing 50% methanol and 7% acetic acid. All radioactive bands disappeared after treatment

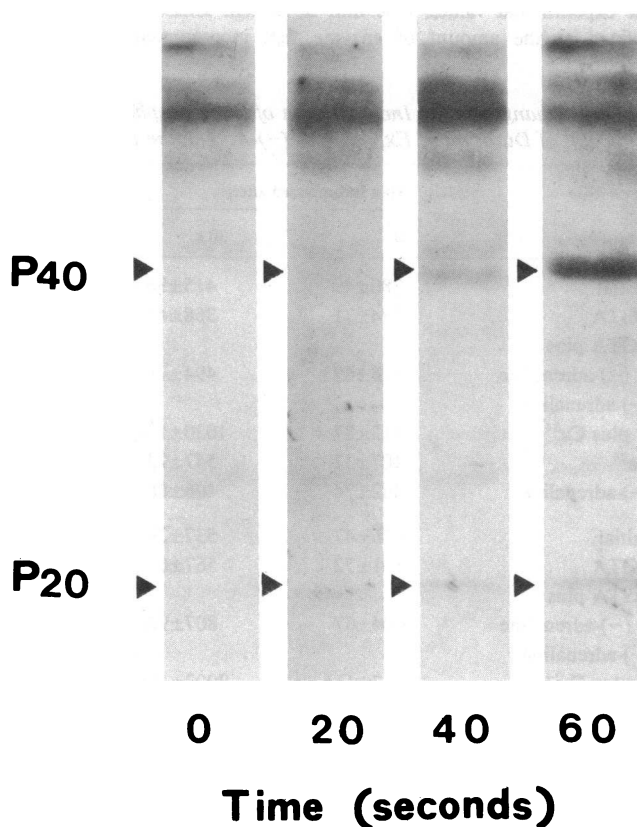
of the samples with pronase, but not with ribonuclease; and thus, they represent proteins. The apparent molecular weights of the protein bands previously shown to contain phosphate were determined by calibrating the gel with standard proteins of known molecular weight. After the staining procedure, each slab gel was dried on filter papers and exposed to Kodak Royal X-Omat film (Eastman Kodak Co., Rochester, NY) to prepare autoradiograms. The relative intensity of each band was quantitated by densitometric tracings of the autoradiograms at 430 nm using a chromatogram scanner. Protein was determined according to Lowry et al. (17). In a parallel set of experiments, gels were sliced into 2-mm sections. Each section was dissolved by shaking in 0.5 ml of 30% hydrogen peroxide at 60°C for 4 h and the  $^{32}\text{P}_i$  content of the resulting solution was determined by liquid scintillation spectroscopy.

**Isolation of calcium-dependent protein kinase from human platelets.** Calcium-dependent protein kinase (protein kinase C), which can be obtained from a variety of tissues, was isolated from human platelets ( $5 \times 10^{10}$ ) by column chromatography using the method of Kawahara et al. (12). Collected fractions, 1.8 ml each, were assayed for protein kinase C activity in the presence of  $\text{Ca}^{2+}$ , and because of the known dependency of the enzyme on phospholipids, also in the presence of diolein and 5'nucleotidase-positive fractions of platelet membranes (prepared with slight modifications according to Ray [18], whereby platelet membranes were substituted for liver cell membranes) or acidic phospholipids.

The major fractions containing protein kinase activity (fractions 11–23) were pooled and concentrated to 5 ml, using an Amicon ultrafiltration cell (Amicon Corp., Danvers, MA) equipped with PM-



**Figure 1.** Effect of (–)-adrenaline on endogenous phosphorylation in human platelets. After preincubation for 1 h with  $[\text{P}]$ phosphate (carrier free) at 23°C, intact human platelets (90  $\mu\text{g}$  protein/sample) were exposed to: (a) (–)-adrenaline, 0.5  $\mu\text{M}$ ; (b) (–)-adrenaline, 1  $\mu\text{M}$ ; (c) (–)-adrenaline, 1  $\mu\text{M}$ , plus  $\text{PGE}_1$ , 10  $\mu\text{M}$ ; and (d) incubation buffer alone for 1 min. After the incubation period, electrophoresis on 12.5% polyacrylamide gels, in the presence of SDS, and autoradiography were performed, as described in Methods. The apparent molecular weight of the phosphorylated bands indicated by the arrows (82,000, P82; 40,000, P40; 24,000, P24; 22,000, P22; 20,000, P20) were determined by calibrating the gel with standard proteins of known molecular weight. The data are representative of eight independently performed experiments.



**Figure 2.** (–)-Adrenaline-induced phosphorylation of platelet proteins as a function of time of incubation. After preincubation with  $[\text{P}]$ phosphate for 1 h at 23°C, intact human platelets (90  $\mu\text{g}$  cell protein/sample) were exposed to (–)-adrenaline (1  $\mu\text{M}$ ) for the indicated time intervals. Arrows indicate the 20,000- and 40,000-mol wt region. Similar results were obtained in four additional experiments.

10 filter membrane. To exclude the contamination of the enzyme preparations with other protein kinases, calmodulin, interfering enzymes, and endogenous phosphate-acceptor proteins, the enzyme preparation was further purified by essentially using the method of Kikkawa et al. (19). Protein, 2.5 mg in 5 ml, was subjected to gel filtration on a column of Sephadex G-150 (30 × 1.2 cm) equilibrated with 20 mM Tris-HCl, adjusted to pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and containing 50 mM 2-mercaptoethanol. The elution was performed with the same solution at a flow rate of 2 ml/h; fractions of 0.5 ml were collected. When each fraction was assayed, protein kinase C eluted as an apparently single peak (fractions 45–54). These fractions were pooled and concentrated to 3 ml by ultrafiltration as described above.

**Enzyme assays.** Protein kinase C activity was routinely determined by measuring the incorporation of  $^{32}\text{P}$  from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  into calf thymus  $\text{H}_1$ -histone. The reaction mixture (250  $\mu\text{l}$  volume) contained 5  $\mu\text{M}$  Tris-HCl at pH 7.5, 1.25  $\mu\text{M}$  magnesium acetate, 50  $\mu\text{g}$   $\text{H}_1$ -histone, 2.5 nmol of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  ( $6 \times 10^4$  cpm/nmol), 0.2  $\mu\text{g}$  of diolein, 5  $\mu\text{g}$  of platelet membrane protein with 5'-nucleotidase activity or varying concentrations of acidic phospholipids, enzyme preparations to be assayed, varying amounts of (–)-adrenaline, and varying concentrations of  $\text{CaCl}_2$ . After incubation at 37°C, the reaction was stopped by addition of trichloroacetic acid (25%) and the acid-precipitable material was collected on Whatman cellulose nitrate membrane filters (pore size, 0.45  $\mu\text{m}$ ; Whatman Inc., Clifton, NJ).

Protein kinase A activity was similarly assayed, except that 250 pmol of cyclic AMP was added, instead of diolein and  $\text{CaCl}_2$ . The basal activity, which was obtained in the presence of 0.5 mM EGTA, instead of diolein and  $\text{CaCl}_2$  (for protein kinase C activity), or in the absence of cAMP (for protein kinase A activity) was subtracted from the experimental values. One unit of protein kinase C and A was defined as the amount of enzyme that incorporated 1 nmol of

phosphate from ATP into  $\text{H}_1$ -histone per minute, under each of the standard assay conditions described above. During this assay, the reaction proceeded linearly with time, and the activity was proportional to the amount of enzyme employed.

Measurements of the accumulation of cAMP were performed according to Block et al. (20).

**Assay for lipid metabolism.** The platelet-rich plasma (60 ml) was labeled with 25  $\mu\text{Ci}$  of  $[\text{H}^3]\text{arachidonic acid}$  according to the methods described by Rittenhouse-Simmons (21). Platelets were isolated and washed as previously described (12). The radioactively labeled platelets were stimulated by addition of (–)-adrenaline (1  $\mu\text{M}$ ) for various time intervals at 37°C. The incubations were terminated by addition of 20 vol chloroform/methanol (2:1) and radioactive lipids were extracted using the method of Folch et al. (22). Diacylglycerol, phospholipids, and arachidonic acid metabolites were separated by Silicia G plates (0.5 mm, Merck AG Darmstadt, Federal Republic of Germany). The solvent systems employed were dichloroethane/methanol (196:4, vol/vol), or benzene/diethyl ether/EtOH/ $\text{NH}_3$  (100:80:4:0.2, vol/vol). Resolved lipids were made visible with  $\text{I}_2$ . The area corresponding to each lipid was scrapped off the plates, transferred into vials, and the radioactivity was determined.

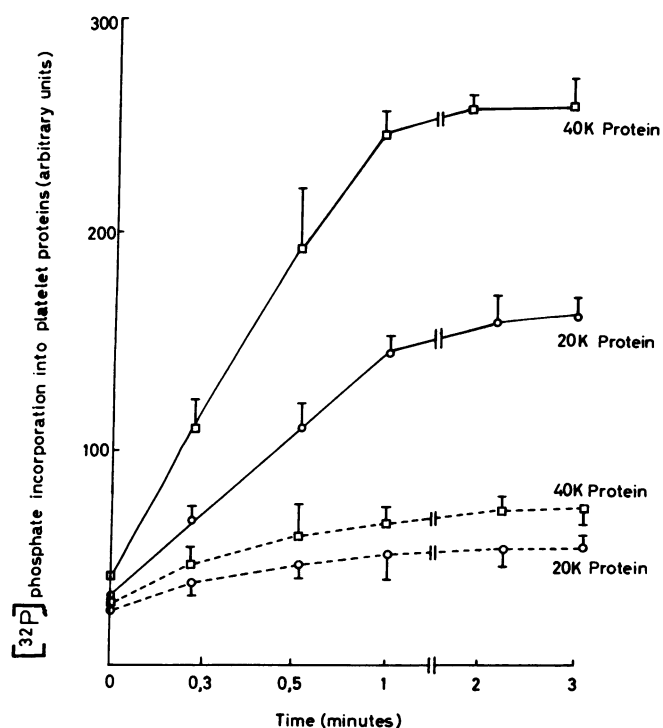
## Results

**Effect of (–)-adrenaline on  $^{32}\text{P}$ -incorporation into specific platelet proteins.** To demonstrate the effect of (–)-adrenaline on protein phosphorylation in human platelets, intact cells were prelabeled with  $^{32}\text{P}_i$  and subsequently exposed to the catecholamine for 1 min. Additional samples were tested by addition of prostaglandin  $\text{E}_1$  ( $\text{PGE}_1$ ) in the presence of (–)-adrenaline and in the presence of incubation buffer alone. Under these conditions,

Table I. Changes in the Incorporation of  $[\text{H}^3]\text{Phosphate}$  into Human Platelet Proteins as a Function of Duration of Exposure to (–)-Adrenaline in the Presence and Absence of Calcium

	cpm incorporated after:					Specific proteins
	0	30 s	60 s	120 s	180 s	
Initial	380±49	415±36	367±36	379±36	390±61	20,000 mol wt protein
EGTA	374±71	368±41	390±49	406±64	374±67	
EGTA plus						
(–)-adrenaline	416±69	494±78	526±57	596±80	571±92	
(–)-adrenaline plus $\text{Ca}^{2+}$	512±87	1020±154	1662±147	1664±196	1704±137	
$\text{Ca}^{2+}$	407±37	547±72	697±62	704±68	802±94	40,000 mol wt protein
(–)-adrenaline	462±74	406±86	576±67	482±71	376±57	
Initial	476±47	537±29	550±67	597±73	608±80	
EGTA	501±72	567±63	616±92	609±63	594±72	
EGTA plus						
(–)-adrenaline	604±67	807±92	1562±216	1497±174	1592±204	40,000 mol wt protein
(–)-adrenaline plus $\text{Ca}^{2+}$	617±114	2002±198	2496±306	2496±402	2506±3156	
$\text{Ca}^{2+}$	509±49	874±63	997±103	1013±204	1097±124	
(–)-adrenaline	526±62	627±82	817±92	786±112	586±103	

Phosphorylation of human platelets as a function of duration of exposure to (–)-adrenaline in the presence and absence of calcium. After preincubation with carrier-free  $[\text{H}^3]\text{phosphate}$  for 1 h at 23°C, the platelets (90  $\mu\text{g}$  protein/sample) were preincubated with (a) EGTA, 1 mM; (b) (–)-adrenaline, 1 mM, plus EGTA; (c) (–)-adrenaline plus  $\text{CaCl}_2$ , 0.5  $\mu\text{M}$ ; (d)  $\text{CaCl}_2$  alone; or (e) (–)-adrenaline alone. At the times indicated, stop solution was added and the phosphorylation of platelets was analyzed by SDS-PAGE and autoradiography. After the protein-staining pattern was recorded by spectrophotometry, the gels were sliced into 2-mm sections. Each section was dissolved by shaking in 0.5 ml of 30% hydrogen peroxide at 60°C for 4 h. The  $^{32}\text{P}_i$  content of the resulting solution was determined by liquid scintillation spectroscopy. The data represent the  $^{32}\text{P}_i$  content of gel sections of the 40,000- and 20,000-mol wt region. The results represent the mean of four independently performed experiments ( $\pm\text{SD}$ ).



**Figure 3.** Inhibitory effect of yohimbine on (-)-adrenaline-induced stimulation of protein phosphorylation in human platelets. After preincubation with [ $^{32}$ P]phosphate for 1 h at 23°C, human platelets (90  $\mu$ g protein/sample) were incubated with (and without) yohimbine (0.1  $\mu$ M) for 15 min before (-)-adrenaline (1  $\mu$ M) was added and incubation was continued for the time intervals indicated. The samples were processed as described in the legend to Fig. 1. The data represent the mean of four independent experiments. (---) treated with yohimbine; (—) without yohimbine. The results were expressed in arbitrary units (33).

incubation with the hormone for 1 min resulted in the release of  $62 \pm 8\%$  of the [ $^3$ H]hydroxytryptamine present in platelets labeled with both [ $^3$ H]5-hydroxytryptamine and  $^{32}$ P $_i$  (mean  $\pm$  SE,  $n = 5$ ). PAGE and subsequently performed scanning of the gels revealed no changes in the gel pattern under the different incubation conditions. However, autoradiography showed an approximately fourfold increase in phosphorylation, which was primarily noted in two proteins (of apparent molecular weights of 20,000 and 40,000) (Fig. 1). This effect was totally inhibited in the presence of PGE $_1$ , which antagonizes adrenaline-induced platelet activation. The inhibitory effect of PGE $_1$  (10  $\mu$ M) on adrenaline activity was consistent even in the presence of varying concentrations of the catecholamine (0.1–100  $\mu$ M). The effect of (-)-adrenaline on phosphorylation of platelet proteins was time-dependent: maximum values were reached after incubation for 1 min (Fig. 2). Incubation periods up to 5 min did not result in further increments of phosphorylation.

The addition of (-)-adrenaline to intact platelets did not result in the stimulation of accumulation of intracellular cAMP in the presence of the phosphodiesterase inhibitor, isobutylmethylxanthine (0.1 mM).

**Influence of calcium-ions on adrenaline-stimulated phosphorylation of proteins.** The inability of (-)-adrenaline to stimulate the elevation of intracellular cAMP in human platelets suggests that the action of the catecholamine involves a calcium-dependent mechanism. To test the influence of Ca $^{2+}$

on the adrenaline-dependent phosphorylation of protein, intact platelets were incubated in the presence and absence of hormone and in the presence and absence of Ca $^{2+}$ . As shown in Table I, when exposed in the absence of both, the phosphorylation of proteins in platelets may be regarded as "base line." A slight increase in  $^{32}$ P-incorporation was seen in the presence of (-)-adrenaline alone. Under optimum conditions, in a Ca $^{2+}$ -enriched medium, (-)-adrenaline stimulated a further increment in the phosphorylation of platelet proteins. Cells exposed to Ca $^{2+}$  alone demonstrated only a minor incorporation of  $^{32}$ P $_i$  into the platelet proteins. After maximum  $^{32}$ P-incorporation into platelet proteins, different rates of dephosphorylation were observed; however, the time-courses of dephosphorylation of the 20,000-mol wt protein and that of the 40,000-mol wt protein were similar.

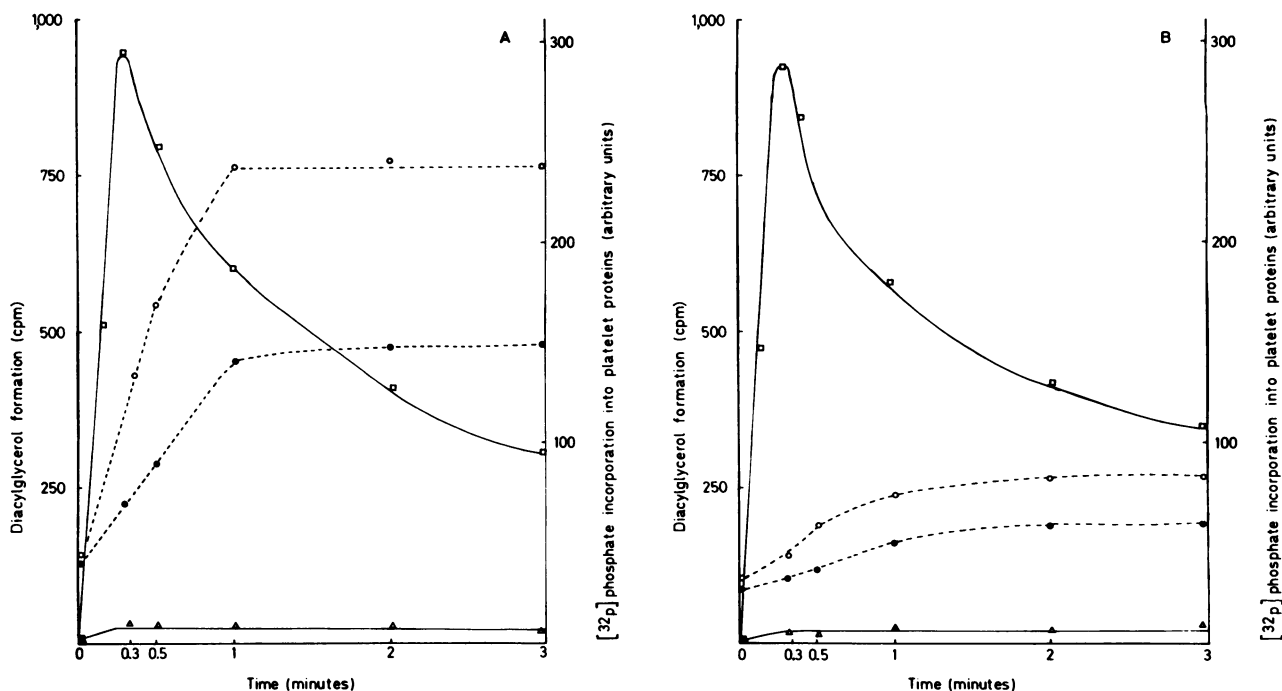
**Inhibitory effect of yohimbine on the adrenaline-induced phosphorylation of platelet-proteins.** Human platelets have been reported to possess alpha-2-receptors that mediate alpha adrenergic stimulation. To evaluate whether this effect can be prevented by a specific alpha-2-antagonist, the prelabeled cells were incubated for 15 min with yohimbine before the addition on (-)-adrenaline. As shown in Fig. 3, platelets incubated in the presence of yohimbine demonstrated a marked inhibition in phosphorylation of proteins, as compared with controls (cells without yohimbine). This effect could be overcome by removal of yohimbine by washing of the platelets and subsequent addition of increasing concentrations of (-)-adrenaline (data not shown).

**Effect of (-)-adrenaline on diacylglycerol formation and protein phosphorylation in human platelets.** Assuming that phospholipid turnover is the signal for activation of protein kinase C, an enzyme that may be directly involved in the transmembrane control of protein phosphorylation (when exposed to (-)-adrenaline), correspondingly rapid phospholipid turnover and phosphorylation of platelet proteins should occur. Therefore, we studied the effect of the hormone on calcium activation of phosphatidylinositol turnover and compared this effect with phosphorylation of the two platelet proteins. As indicated in Fig. 4 A, when platelets were stimulated with (-)-adrenaline, 40,000- and 20,000-mol wt proteins were phosphorylated and this reaction was preceded by transient formation of endogenous diacylglycerol. The latter effect did not depend upon the presence of Ca $^{2+}$  in the incubation medium (Fig. 4 B).

**Activation of calcium-dependent protein kinase C by (-)-adrenaline.** Since (-)-adrenaline-induced phosphorylation of proteins was not associated with a rise in intracellular cAMP level, the effect of the catecholamine on calcium- and phospholipid-dependent protein kinase was examined. After isolation of the enzyme from the platelets on a DE-52 column, the  $^{32}$ P $_i$ -incorporation from [ $\gamma$ - $^{32}$ P $_i$ ]ATP into H $_1$ -histone was tested in the presence of Ca $^{2+}$ , diolefin, and platelet membranes.

As shown in Fig. 5, the enzyme activity (fractions 11–23) was increased by approximately 18-fold as compared with fractions containing protein kinase A. The enzyme activity of fractions containing protein kinase G was insignificant. To assess the effect of (-)-adrenaline on protein kinase C activity and to exclude the possibility of contamination with calmodulin, interfering enzymes, and endogenous acceptor proteins, the enzyme preparation was further purified on Sephadex G-150.

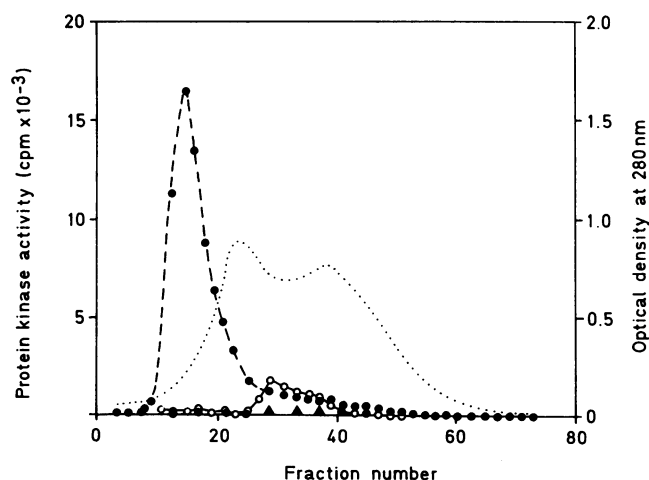
Partially purified protein kinase C was usually inactive, but was activated by the simultaneous addition of Ca $^{2+}$ ,



**Figure 4.** Time courses of diacylglycerol formation and phosphorylation of 40,000- and 20,000-mol wt proteins in human platelets. Washed human platelets ( $10^{10}$  per milliliter) which were labeled with [ $^3\text{H}$ ]arachidonic acid or  $^{32}\text{P}_i$  were stimulated at  $37^\circ\text{C}$  with (—)adrenaline ( $1\ \mu\text{M}$ ) in presence (A) and absence (B) of calcium ( $\text{CaCl}_2$ ,  $0.5\ \text{mM}$ ) for time intervals indicated. The incubations were terminated with 20 vol of chloroform/methanol (2:1). Formation of diacyl-

glycerol and phosphorylation of 40,000- and 20,000-mol wt proteins were assayed as described in the Methods. Diacylglycerol formation with (—)adrenaline (—□—) or without hormone (—△—); (---○---) 40,000- and (---●---) 20,000-mol wt protein phosphorylation. Results are presented as averages for an experiment performed in duplicate.

diolein, and platelet membranes. Further enhancement of enzymatic activity was achieved by addition of (—)adrenaline. Fig. 6 shows the effect of increasing concentrations of (—)

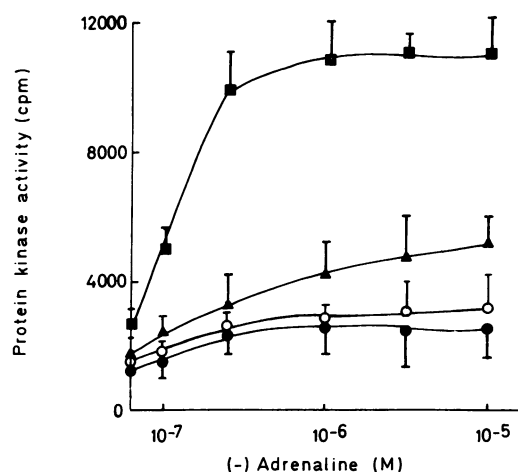


**Figure 5.** Activity and elution profile of protein kinase C on DEAE-cellulose. The enzyme was eluted by application of 96 ml of a linear concentration gradient of NaCl (0–0.4 M) at a flow rate of 7.5 ml/h. The fractions (1.8 ml each) were collected and assayed for enzymatic activity (see Methods). (—▲—) Enzymatic activity in the presence of cAMP, platelet membranes, and diolein, (—●—) in the presence of  $\text{CaCl}_2$  plus diolein and platelet membranes, (—○—) and in the presence of platelet membranes and diolein. (---) optical density at 280 nm.

adrenaline in the presence of fixed amounts of  $\text{Ca}^{2+}$ , diolein, and platelet membranes. Inversely, the enzymatic activity was markedly and progressively inhibited by removal of either  $\text{Ca}^{2+}$  or platelet membranes, or both. Yohimbine inhibited (—)adrenaline-induced enzyme activation to base-line values.

To explore the role of  $\text{Ca}^{2+}$  on (—)adrenaline-induced activation of protein kinase C more explicitly, the platelet membranes were exposed to increasing amounts of  $\text{Ca}^{2+}$  in the presence of constant concentrations of diolein and (—)adrenaline. As illustrated in Fig. 7, the enzymatic activity was increased to maximum values by increasing  $\text{Ca}^{2+}$  in the presence of the four additions, but was decreased in the absence of (—)adrenaline and even more in the absence of (—)adrenaline and platelet membranes.

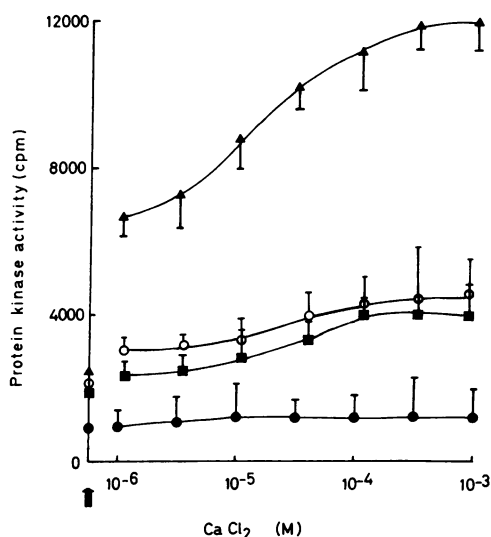
It was previously shown that unsaturated diacylglycerol increased the affinity of the protein kinase C for  $\text{Ca}^{2+}$ , thereby rendering the enzyme fully active (23). Therefore, we repeated the experiments using phospholipids in place of platelet membranes. In addition, the effects of yohimbine, (+)adrenaline, and (—)adrenaline on the activation of the enzyme in the presence and absence of  $\text{Ca}^{2+}$  were studied. As indicated in Table II, among various phospholipids tested only phosphatidylinositol was active in support of enzyme activation, whereas (+)- or (—)adrenaline and yohimbine (alone or in combination with the hormones) were ineffective. Activation of the enzyme was dependent upon the presence of  $\text{Ca}^{2+}$ . The inability of phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine to stimulate the formation of diglyceride suggests phosphatidylinositol to be the most likely source of diacylglycerol.



**Figure 6.** The effect of various concentrations of (–)-adrenaline on reaction velocity of protein kinase C at constant concentrations of  $\text{CaCl}_2$  ( $0.5 \mu\text{M}$ ),  $0.8 \mu\text{g/ml}$  diolein, and  $20 \mu\text{g}$  of platelet membranes/ml and in the presence of yohimbine ( $0.1 \mu\text{M}$ ). (—■—), in the presence of diolein,  $\text{CaCl}_2$ , and platelet membranes; (—○—) at the same conditions plus yohimbine; (—▲—), in the presence of platelet membranes and diolein; and (—●—) in the presence of (–)-adrenaline and diolein. The data represent the mean of four independent experiments. Each value is the mean of triplicate determinations  $\pm$  SD. The effect of yohimbine was studied following preincubation of membranes for 15 min before the addition of (–)-adrenaline.

## Discussion

Despite extensive studies on the mechanism of the action of adrenaline, the cellular events leading to its end-function, i.e.,



**Figure 7.** The effect of (–)-adrenaline on reaction velocity of protein kinase C at various concentrations of  $\text{CaCl}_2$ . Protein kinase C activity was assayed in the presence of fixed amounts of (–)-adrenaline ( $1 \mu\text{M}$ ), platelet membranes ( $20 \mu\text{g/ml}$ ), and diolein ( $2.4 \mu\text{g/ml}$ ). Where indicated with an arrow, EGTA ( $0.5 \mu\text{M}$ ) was added, instead of  $\text{CaCl}_2$ , at final concentrations indicated, (—▲—) in the presence of (–)-adrenaline, platelet membranes and diolein, (—○—) at the same conditions plus yohimbine, (—■—) in the presence of platelet membranes and diolein, (—●—) in the presence of (–)-adrenaline plus diolein. The data represent the mean of five independent experiments. Each value is the mean of triplicate determinations  $\pm$  SD.

**Table II.** Effects of Various Phospholipids (+)- and (–)-Adrenaline and Yohimbine on the Activation of Protein Kinase in Presence and Absence of Calcium

	Reaction velocity (cpm)	
	In presence of $0.5 \times 10^{-6} \text{ M CaCl}_2$	Without calcium
Phosphatidylinositol	$6700 \pm 1240$	$106 \pm 87$
Phosphatidylserine	$220 \pm 98$	$87 \pm 72$
Phosphatidylethanolamine	$160 \pm 76$	$105 \pm 62$
Phosphatidylcholine	$140 \pm 85$	$92 \pm 76$
Yohimbine	$105 \pm 28$	$96 \pm 37$
(+)-Adrenaline	$96 \pm 39$	$78 \pm 47$
Yohimbine plus (+)-adrenaline	$116 \pm 78$	$101 \pm 57$
(–)-Adrenaline	$104 \pm 67$	$107 \pm 62$
Yohimbine plus (–)-adrenaline	$87 \pm 42$	$92 \pm 36$
None	$110 \pm 74$	$87 \pm 49$

Activity of protein kinase C prepared from human platelets was assayed in the presence of  $0.8 \mu\text{g/ml}$  diolein,  $10 \mu\text{g/ml}$  each of the phospholipids or yohimbine ( $10 \mu\text{M}$ ), or (+)-adrenaline ( $1 \mu\text{M}$ ) or (–)-adrenaline ( $1 \mu\text{M}$ ) in the presence and absence of  $\text{CaCl}_2$  ( $0.5 \times 10^{-6} \text{ M}$ ). For conditions, see Methods. The results represent the mean of four independent experiments. Each value is the mean of triplicate determinations  $\pm$  SD.

activation of contractile proteins, are mainly unknown. Using human platelets, a biologically relevant target cell for this catecholamine, our data suggest a phosphorylation step of contractile proteins, one of 40,000 mol wt with unknown function, and one of 20,000 mol wt that has been identified to be the light chain of myosin (24), as the final event during alpha adrenergic activation. Similar observations with platelets have been made by Kawahara et al. (12), who demonstrated the stimulation of phosphorylation of proteins in the same molecular weight range by using thrombin. That the stimulatory effect of (–)-adrenaline on the phosphorylation of the two proteins was specific is evidenced from its inhibition by the alpha-2-antagonist, yohimbine.

The possible effect of adrenaline on phospholipid metabolism was indicated by the observation of Michell (9, 10) and others (11), who reported the breakdown of phosphatidylinositol as the primary event during alpha adrenergic activation. Subsequent studies have shown that phospholipid turnover can be stimulated in virtually any type of tissue stimulated by a variety of extracellular messengers (25). However, thus far, phosphatidylinositol turnover mediated by adrenergic agonists was believed to occur via alpha-1-receptors. The fact that the receptors in platelets are alpha-2 in type and the observation that the action of adrenaline can be inhibited by the specific alpha-2-antagonist, yohimbine, indicate that the phosphatidylinositol turnover cannot only be ascribed for alpha-1 but also for alpha-2-receptors.

The physiological responses of platelets to adrenaline (e.g., aggregation and secretion) are mediated by adrenergic receptors and were shown to depend upon the presence of calcium (21). Evidence that adrenaline influences the metabolism of calcium within the cells, with a resultant rise in cytosolic  $\text{Ca}^{2+}$  concentration, is obtained from the following observations: (a) the dependence upon calcium for many alpha adrenergic responses

(2, 3); (b) the stimulatory effect of alpha adrenergic agonists on calcium fluxes in several tissues (26–28); (c) the inhibition by calcium antagonists of many alpha responses (7, 8); and (d) the known effects of  $\text{Ca}^{2+}$  ions on processes influenced by alpha adrenergic stimulation (1).

Similarly, in our studies, EGTA inhibited adrenaline-induced phosphorylation of protein. A recent report, which describes the ability of adrenaline to increase the concentration of intracellular free  $\text{Ca}^{2+}$  in human platelets, suggests a gating function of the catecholamine on the transport of  $\text{Ca}^{2+}$  (29). The persistence of phosphatidylinositol turnover in platelets incubated in the absence of  $\text{Ca}^{2+}$  agrees with previous reports (30, 31), indicating that  $\text{Ca}^{2+}$  entry from the exterior cannot be required for the response to the hormone.

Human platelets contain a variety of protein kinases, as well as a variety of endogenous substrates for these enzymes (13). The experimental results presented above indicate that (–)-adrenaline stimulates protein kinase C and that membrane constituents (phospholipids), dioloin, and calcium play a co-operative role in the stimulation of the activity of the enzyme. This is strengthened by experiments using various acidic phospholipids in place of platelet membranes showing that phosphatidylinositol was by far the most active membrane phospholipid in support of enzyme activation. A similar observation was made by Rittenhouse-Simmons (21) that may be explained by existence of a specific phosphatidylinositol-phosphodiesterase in platelets producing diglyceride and inositol phosphate. The inability of (+)- and (–)-adrenaline (in presence and absence of yohimbine) to activate protein kinase C implies the necessity of receptor occupancy at intact platelets or membranes to elicit adrenergic response.

It may be considered that the human platelet represents a biologically relevant model, which features pharmacological characteristics that are important during adrenergic activation. Thus, the stimulation by (–)-adrenaline of the phosphorylation of contractile proteins, one of which is the light chain of myosin (24), provides a basis for the understanding of the catecholamine-stimulated mechanisms of contractile system (e.g., alpha-2-receptor-mediated vasoconstriction) (32). Accordingly, the demonstration of a calcium-dependent mechanism of protein phosphorylation, as the end function of (–)-adrenaline, may allow new insights into the cellular mechanisms of the circulating neurotransmitter.

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