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Prostacyclin inhibits platelet aggregation induced by phorbol ester or Ca^{2+} ionophore at steps distal to activation of protein kinase C and Ca^{2+}-dependent protein kinases

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Suspensions of aspirin-treated, ^{32}P-prelabelled, washed platelets containing ADP scavengers in the buffer were activated with either phorbol 12,13-dibutyrate (PdBu) or the Ca^{2+} ionophore A23187. High concentrations of PdBu ($\geqslant$ 50 nM) induced platelet aggregation and the protein kinase C (PKC)-dependent phosphorylation of proteins with molecular masses of 20 (myosin light chain), 38 and 47 kDa. No increase in cytosolic Ca^{2+} was observed. Preincubation of platelets with prostacyclin (PGI_2) stimulated the phosphorylation of a 50 kDa protein [EC_{50} (concn. giving half-maximal effect) 0.6 ng of PGI_2/ml] and completely abolished platelet aggregation [ID_{50} (concn. giving 50% inhibition) 0.5 ng of PGI_2/ml] induced by PdBu, but had no effect on phosphorylation of the 20, 38 and 47 kDa proteins elicited by PdBu. The Ca^{2+} ionophore A23187 induced shape change, aggregation, mobilization of Ca^{2+}, rapid phosphorylation of the 20 and 47 kDa proteins and the formation of phosphatidic acid. Preincubation of platelets with PGI_2 (500 ng/ml) inhibited platelet aggregation, but not shape change, Ca^{2+} mobilization or the phosphorylation of the 20 and 47 kDa proteins induced by Ca^{2+} ionophore A23187. The results indicate that PGI_2, through activation of cyclic AMP-dependent kinases, inhibits platelet aggregation at steps distal to protein phosphorylation evoked by protein kinase C and Ca^{2+}-dependent protein kinases.

INTRODUCTION

Most physiological platelet agonists stimulate inositol phospholipid hydrolysis by activation of phospholipase C [1]. Two products generated by this reaction have specific functions: 1,2-diacylglycerol activates protein kinase C (PKC), and inositol 1,4,5-trisphosphate mobilizes Ca^{2+} from intracellular stores, leading to activation of Ca^{2+}/calmodulin-dependent kinases such as myosin light-chain kinase (MLCK) [2–4]. Phorbol esters and Ca^{2+} ionophores by-pass receptor activation and do not stimulate inositol phospholipid hydrolysis in platelets if the various positive-feedback mechanisms (ADP, endoperoxides/thromboxane A_2) have been properly blocked [5,6]. Phorbol ester and Ca^{2+} ionophores activate platelets through different mechanisms and can be used specifically to probe the PKC-dependent pathway and the Ca^{2+} pathway of platelet activation, respectively [7,8]. Phorbol esters, by intercalation into the platelet membrane without mobilization of Ca^{2+}, translocate PKC from the cytosol to the membrane and induce the PKC-dependent phosphorylation of various proteins, such as the 47 kDa polypeptide and the 20 kDa myosin light chain [2,9]. PKC phosphorylates the 20 kDa protein at sites different from those phosphorylated by MLCK [9]. Ca^{2+} ionophores such as ionophore A23187 activate platelets through Ca^{2+} influx and Ca^{2+} mobilization from intracellular stores [10,11]. The increase in cytosolic Ca^{2+} leads to the activation of Ca^{2+}/calmodulin-dependent kinases, such as MLCK, which phosphorylates myosin light chain (20 kDa), and to a Ca^{2+}-dependent activation of PKC, which phosphorylates a 47 kDa protein [12,13]. Many studies indicate that phosphorylation of the 20 and 47 kDa proteins are closely related to platelet functional responses [1]: MLCK-dependent phosphorylation of myosin light chain may trigger shape change, whereas PKC-dependent phosphorylation of the 47 kDa polypeptide may regulate aggregation and secretion [14–16].

Prostacyclin (PGI_2) is the most powerful inhibitor of platelet function [17]. It increases cyclic AMP in human platelets, leading to activation of protein kinase A. In human platelets, protein kinase A phosphorylates proteins with molecular masses of 22, 24, 42, 50 and 130 kDa [18–22]. Cyclic AMP-dependent phosphorylation of the microsomal 22 kDa protein, recently named thrombolamban, stimulates Ca^{2+} uptake into platelet membrane vesicles [23–26]. From those results it is suggested that cyclic AMP acts through the stimulation of Ca^{2+} transport from the cytosol into the dense-tubular system. Such a mechanism of action might explain the rapid fall in cytoplasmic Ca^{2+} and the reversal of protein phosphorylation that is observed when adenylate cyclase stimulators are added after the maximal platelet responses evoked by platelet stimuli [27–29], but it does not explain the prevention of stimulus-induced platelet activation. It is currently assumed that the prevention of stimulus-induced platelet activation by cyclic AMP is caused by an inhibition of receptor-mediated phosphoinositide hydrolysis and subsequent PKC activation and Ca^{2+} mobilization [29–32].

Different studies, however, have indicated that other target sites exist for cyclic AMP. For example, cyclic AMP inhibits Ca^{2+}-dependent secretion and aggregation

Abbreviations used: PKC, protein kinase C; MLCK, myosin light-chain kinase; PdBu, phorbol 12,13-dibutyrate; OAG, 1-oleoyl-2-acetylglycerol; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PGI_2, prostacyclin; PGE_1, prostaglandin E_1.

at steps distal to Ca^{2+} mobilization [33–36]. Also, it has been reported that the catalytic subunit of cyclic AMP-dependent kinase phosphorylates the purified Ca^{2+}/calmodulin-dependent MLCK, thereby decreasing its ability to phosphorylate myosin light chain [37]. Furthermore, modest increments in platelet cyclic AMP have been reported to abolish platelet-activating-factor-induced aggregation and secretion, but to have little effect on phosphoinositide hydrolysis and elevation of cytosolic Ca^{2+} induced by platelet-activating factor [38]. The present study was undertaken to find out whether increasing platelet cyclic AMP could suppress aggregation by inhibiting PKC or Ca^{2+}-dependent kinases involved in the activation of intact platelets.

EXPERIMENTAL

Materials

Phorbol 12,13-dibutyrate (PdBu) and Ca^{2+} ionophore A23187 were from Sigma. Stock solutions of PdBu were prepared in dimethyl sulphoxide, and ionophore A23187 was dissolved in ethanol. PGI_2 was solubilized in glycine (1.88 mg/ml) and NaCl (1.47 mg/ml), and its pH adjusted with NaOH to 10.5. Indo-1/AM and quin-2/AM, both penta-acetoxylmethyl esters, were from Calbiochem (La Jolla, CA, U.S.A.) and were dissolved in dimethyl sulphoxide. All other materials were obtained as previously described [15,16].

Isolation and ^{32}P labelling of human platelets

Platelet-rich plasma from 160 ml of freshly drawn blood anticoagulated with 0.1 vol. of 3.8% (w/v) trisodium citrate was incubated with aspirin (1 mM) for 15 min at 37 °C. PGI_2 (20 ng/ml) was then added, and platelets were pelleted by centrifugation at 800 *g* for 10 min. Platelets were resuspended in 3 ml of 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), apyrase (3 units of ADPase/ml), PGE_1 (2 μM) and 10% (v/v) autologous platelet-poor plasma. Platelets were incubated with 8–10 mCi of ^{32}P for 90 min at 37 °C. Then 20 ml of prewarmed buffer containing 1 mM-EGTA, 2 μM-PGE_1 or PGI_2 (50 ng/ml) and 0.36 mM-NaH_2PO_4 was added, and platelets were pelleted by centrifugation (800 *g* for 10 min). Platelets were resuspended in 15 ml of buffer (700000–900000 platelets/μl) without platelet-poor plasma, EGTA and PGE_1, but containing $CaCl_2$ (10 μM) and apyrase (0.6 unit of ADPase /ml) or phosphocreatine (2 mM)/creatine kinase (20 units/ml). The platelet suspension was kept at room temperature for 1 h before the start of the experiment.

Samples (0.8 ml) were transferred into aggregometer cuvettes and incubated for 2 min at 37 °C with stirring before addition of PdBu or ionophore A23187. Aggregation was measured with a Chronolog-Aggregometer adjusted for maximal sensitivity, or a Lumi-Aggregometer (Chronolog, Havertown, PA, U.S.A.). The platelet suspension in the Lumi-Aggregometer cuvette was calibrated against 0.3 ml of platelet suspension diluted with 0.1–0.2 ml of buffer. Samples (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, v/v) for determination of phosphatidic acid [32]. Phosphorylation of ^{32}P-labelled proteins was measured after separation by SDS/polyacrylamide (12.5%)-gel electrophoresis [39]. In control samples, ^{32}P radioactivity ranged from 600 to 1500 c.p.m. for the 20 kDa protein, from 1000 to 4000 c.p.m. for the 47 kDa protein, and from 400 to 1000 c.p.m. for phosphatidic acid. All experiments shown are representative of at least three other experiments which gave similar results.

Measurement of Ca^{2+} mobilization

Platelet-rich plasma from 160 ml of freshly drawn blood anticoagulated with trisodium citrate (0.38%, w/v) was incubated with 5 μM-Indo-1/AM dissolved in dimethyl sulphoxide and 1 μM-PGE_1 for 45 min at 37 °C. Aspirin (1 mM) was then added, and incubation was continued for an additional 15 min. Platelets were pelleted by centrifugation and resuspended in 20 ml of buffer containing $CaCl_2$ (10 μM) and apyrase (0.6 unit of ADPase/ml). Samples (1 ml) of platelet suspension were prewarmed at 37 °C for 1 min, and Ca^{2+} measurements were performed in a Perkin–Elmer 512 double-beam fluorescence spectrophotometer. The emission wavelength was 390 nm and the excitation wavelength was 340 nm. F, $F_{max.}$, F_{Mn} and F_{leak} were recorded, and the intracellular Ca^{2+} concentrations were calculated as in refs. [40] and [41].

RESULTS

Effect of PGI_2 on the stimulation of platelets by phorbol ester

Suspensions of aspirin-treated, ^{32}P-prelabelled, washed platelets containing Ca^{2+} (10 μM) and apyrase (Figs. 2–5, 7) or phosphocreatine/creatine kinase (Figs. 1, 6, 8) in the buffer were exposed to PdBu. As with previous observations in platelet-rich plasma [15], PdBu induced slow aggregation without prior shape change. Aggregation was decreased in the presence of EGTA (2 mM) and almost completely suppressed after preincubation of platelets with PGI_2 (250 ng/ml) (Fig. 1). Even at high concentrations (100 nM), PdBu did not increase platelet cytosolic Ca^{2+} (Table 1). Also, loading platelets with the Ca^{2+} chelator quin-2 enhanced platelet aggregation induced by PdBu (results not shown), whereas aggregation elicited by ionophore A23187 (see below), vasopressin and ADP was delayed.

PdBu-induced aggregation was associated with the PKC-dependent phosphorylation of several proteins (Fig. 2). Phosphorylation of the 47 kDa protein preceded both aggregation and the phosphorylation of myosin light chain (20 kDa). Phosphorylation of the 20 kDa protein and platelet aggregation induced by PdBu showed similar kinetics. Addition of EGTA decreased aggregation, and decreased specifically 20 kDa protein phosphorylation elicited by PdBu (Fig. 3). Omission of stirring of the platelet suspension eliminated platelet aggregation, but it did not affect 20 kDa protein phosphorylation elicited by PdBu (Fig. 3). These results suggest a role for PKC-dependent myosin light-chain phosphorylation in platelet aggregation evoked by phorbol ester. However, studies using PGI_2 showed that the PKC-dependent myosin light-chain (20 kDa) phosphorylation was not directly involved in platelet aggregation induced by PdBu. Preincubation of platelets with PGI_2 inhibited aggregation in a dose-dependent manner, but it did not affect protein phosphorylation induced by PdBu, including the phosphorylation of the 20 kDa protein (Figs. 4–6). PGI_2, through the activa-

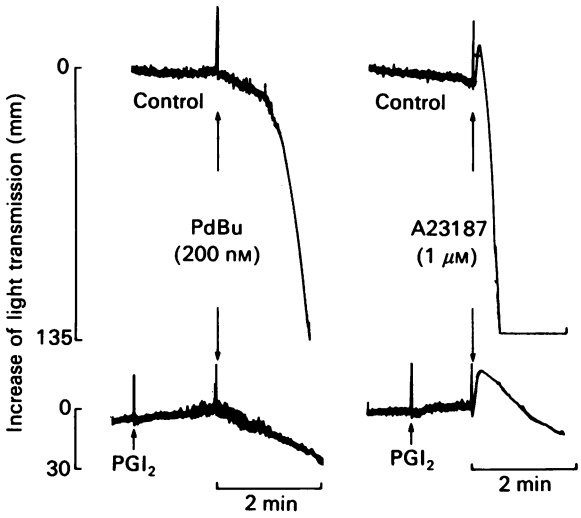

Fig. 1 Effects of PGI_2 on platelet aggregation induced by PdBu or A23187

Suspensions of aspirin-treated ^{32}P-labelled, washed platelets were prepared as detailed in the Experimental section and resuspended in buffer containing phosphocreatine/creatine kinase and Ca^{2+} (10 μM). PGI_2 (500 ng/ml) was added 1 min before the agonists. Representative aggregation tracings of one experiment are shown.

Table 1. Effect of PGI_2 on increase in cytosolic Ca^{2+} induced by various concentrations of ionophore A23187

Aspirin-treated platelets had been loaded with Indo-1/AM as detailed in the Experimental section. PGI_2 (500 ng/ml) was added 1 min before ionophore A23187. The platelet suspension was incubated at 37 °C for 1 min before addition of ionophore A23187. Aggregation was measured in parallel samples. The experiment is representative of two others.

Treatment	Intracellular Ca^{2+} (nM)	Increase in light transmission (mm)
Control	80	0
PdBu (100 nM)	80	0
A23187 (20 nM)	102	0
PGI_2 plus A23187 (20 nM)	112	0
A23187 (50 nM)	231	15
PGI_2 plus A23187 (50 nM)	243	Shape change
A23187 (100 nM)	298	28
PGI_2 plus A23187 (100 nM)	367	Shape change
A23187 (200 nM)	984	80
PGI_2 plus A23187 (200 nM)	970	3
A23187 (500 nM)	2571	145
PGI_2 plus A23187 (500 nM)	2571	7

tion of cyclic AMP-dependent kinases, induced the phosphorylation of a 50 kDa protein with an EC_{50} (concn. giving half-maximal effect) of 0.6 ng/ml, which is similar to the ID_{50} (concn. giving 50% inhibition) (0.5 ng/ml) for inhibition of platelet aggregation (Figs. 2

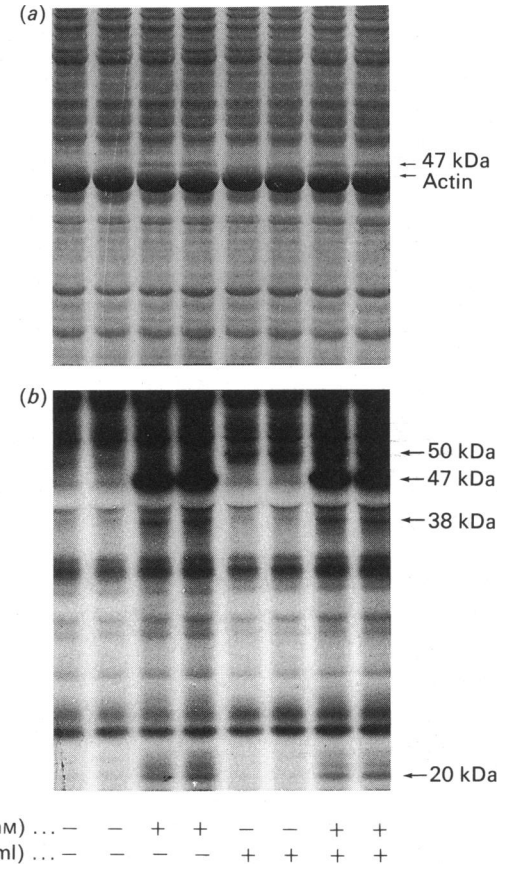

Fig. 2. Effects of PdBu and PGI_2 on protein phosphorylation

A Coomassie Blue stain (*a*) and an autoradiograph (*b*) of [^{32}P]phosphorylated platelet proteins are shown. PGI_2 was added 1 min before PdBu. Samples were taken before the addition of PdBu (control) and 1 min after addition of PdBu. Note the mobility shift (above actin) of the phosphorylated 47 kDa protein after stimulation with PdBu in the Coomassie-Blue-stained gel. Also PGI_2 induces phosphorylation of a 50 kDa protein, which is not modified by PdBu.

and 4). Increases in phosphorylation of the 22 and 24 kDa proteins induced by PGI_2 was less evident, since both proteins were already phosphorylated in control platelets (Fig. 2).

Effect of PGI_2 on the stimulation of platelets with ionophore A23187

In contrast with PdBu, platelets activated by Ca^{2+} ionophore A23187 showed rapid aggregation and protein phosphorylation. Low concentrations of ionophore A23187 (0.1 μM) induced selective phosphorylation of the 20 kDa myosin light chain, shape change and a small, reversible, aggregation. At a slightly higher concentration (0.2 μM), ionophore A23187 evoked another small increase in 20 kDa-protein phosphorylation, but a large increase in aggregation and phosphorylation of the 47 kDa protein. Platelet aggregation induced by these concentrations of ionophore A23187 was almost completely blocked by preincubation of platelets with PGI_2; phosphorylation of the 20 and 47 kDa proteins and shape change were, however, not affected by PGI_2 (Figs. 7, 8). A concentration of 0.5 μM-ionophore A23187

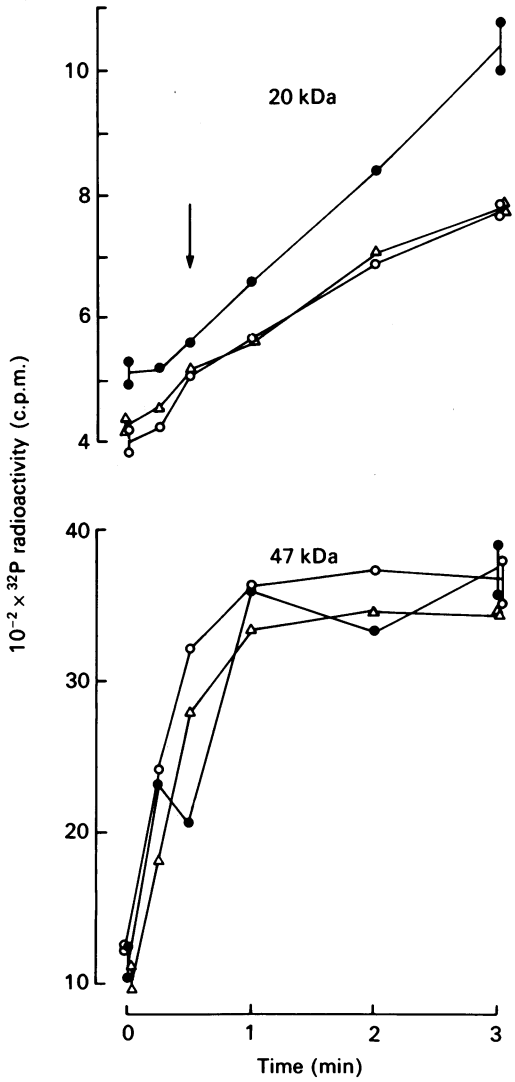

Fig. 3. Effect of EGTA and stirring on phosphorylation of the 20 kDa and 47 kDa proteins induced by PdBu

EGTA was added 1 min before PdBu (75 nM). Arrow indicates when aggregation starts as measured in the Chronolog-Aggregometer. Aggregation at 1 min after addition of PdBu was 41 mm in the absence of EGTA and 16 mm in the presence of EGTA. Symbols: ●, PdBu; ○, 2 mM-EGTA + PdBu; △, 2 mM-EGTA + PdBu, no stirring.

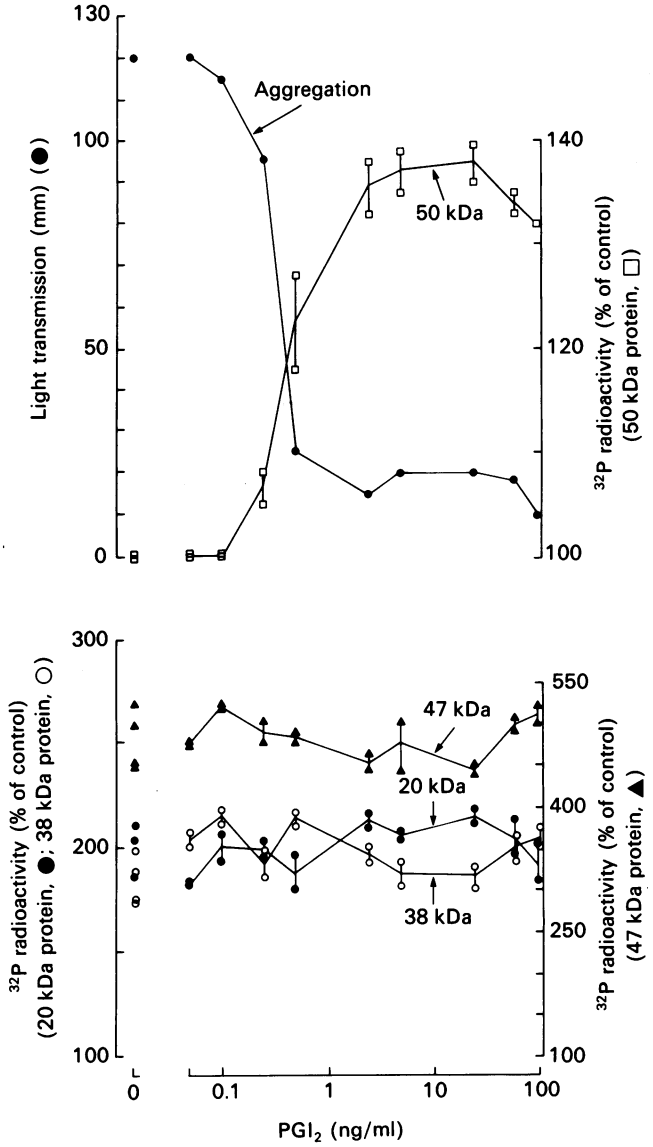

Fig. 4. Effects of various concentrations of PGI_2 on aggregation and protein phosphorylation induced by PdBu

PGI_2 was added 1 min before PdBu. Phosphorylation of 20, 38 and 47 kDa proteins and aggregation were measured 1 min after addition of PdBu (66 nM). The 50 kDa-protein phosphorylation induced by PGI_2 was measured 45 s after addition of PGI_2. The results are duplicates of one experiment, which is representative of three other experiments.

induced irreversible aggregation and a further increase in the 47 kDa-protein phosphorylation. In one experiment, the extent of protein phosphorylation was decreased in parallel with aggregation by preincubation of platelets with PGI_2. However, the early increase in protein phosphorylation at 10 s was not affected by PGI_2 (Fig. 7). Protein phosphorylation induced by a high concentration (1 μM) of ionophore A23187 was not inhibited by PGI_2, although aggregation was much decreased (Figs. 1, 7 and 8). Ionophore A23187 induced a dose-dependent increase in [^{32}P]phosphatidic acid that was affected by PGI_2 (Fig. 7). The maximal increase observed after 1 μM-A23187 was small (2-fold) as compared with other platelet stimuli.

PGI_2 did not inhibit Ca^{2+} mobilization induced by 0.02–0.5 μM-ionophore A23187, although it inhibited aggregation (Table 1). PGI_2 also did not accelerate the return of elevated Ca^{2+} concentration to basal values during the first 2 min after platelet stimulation with A23187 (results not shown). EGTA, which chelates extracellular Ca^{2+}, and quin-2, which chelates intracellular Ca^{2+}, or a combination of both delayed and diminished aggregation induced by ionophore A23187 (Fig. 9). Interestingly, A23187 induced secretion of ATP with EGTA present, but not when it was absent. Similar findings have been reported previously, and might be explained by the relative impermeability of the platelet plasma membrane to calcium complexes of A23187 [42].

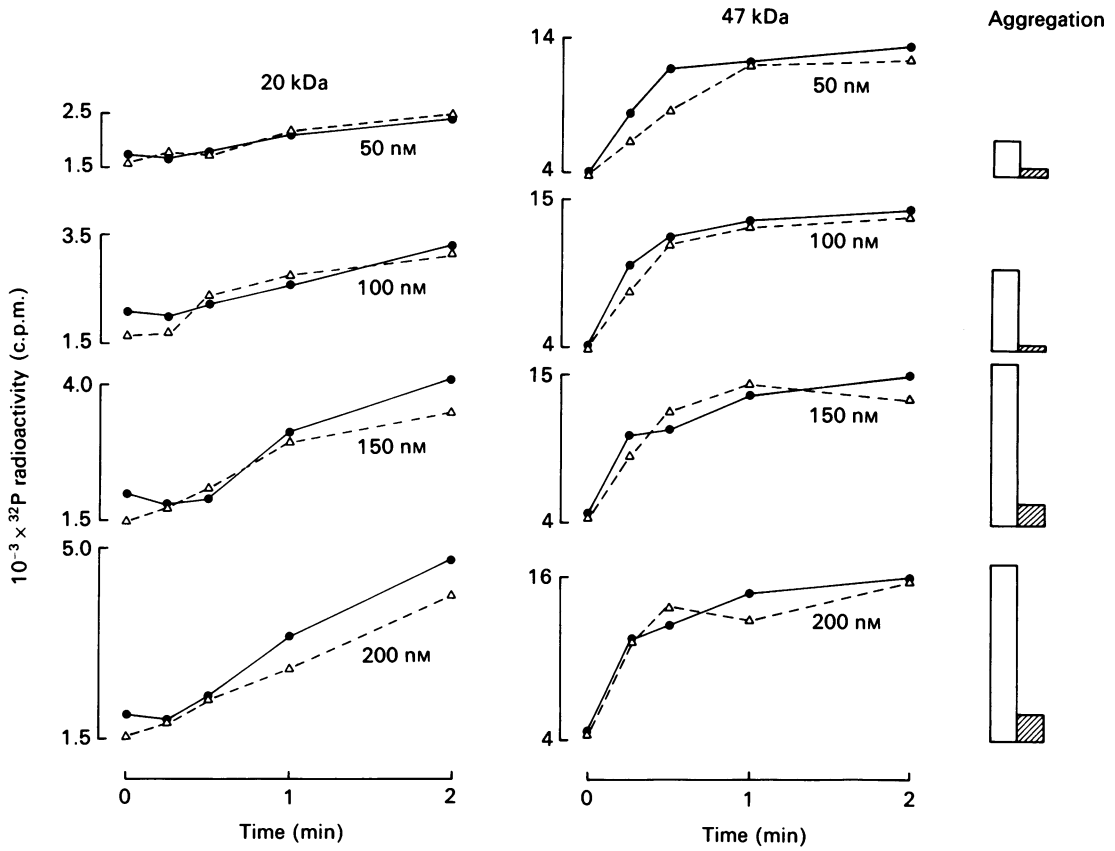

Fig. 5. Effects of PGI_2 on protein phosphorylation and aggregation elicited by various concentrations of PdBu

PGI_2 (500 ng/ml) (△; ▨) or buffer (control, ●; □) were added 1 min before PdBu.

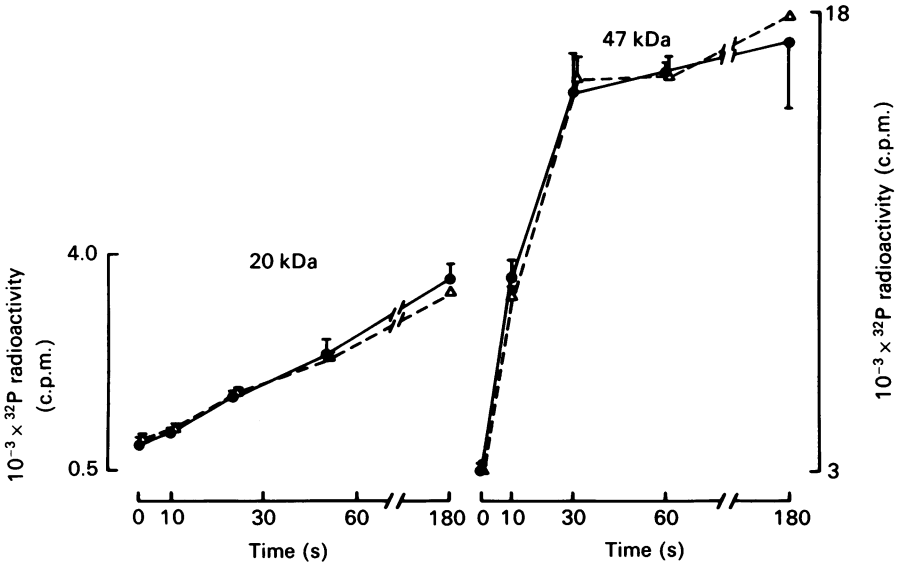

Fig. 6. Effects of PGI_2 on protein phosphorylation elicited by PdBu

The platelet buffer contained phosphocreatine/creatine kinase instead of apyrase. Values are means ± S.D. of triplicates of one experiment representative of another two. Platelet aggregation induced by 200 nM-PdBu after 2 min was 102 ± 33, versus 24 ± 8 mm in the presence of PGI_2. Symbols are as in the legend to Fig. 5.

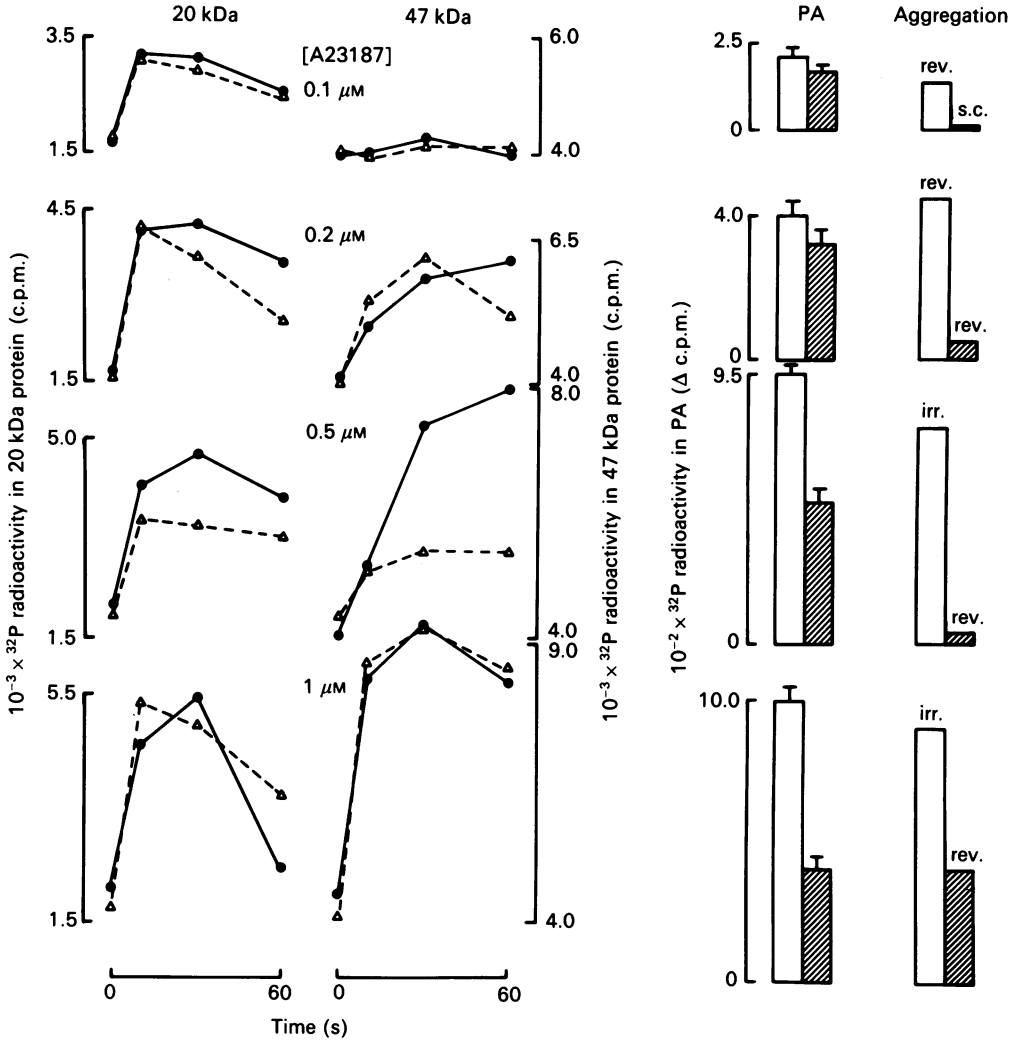

Fig. 7. Effects of PGI_2 on protein phosphorylation, phosphatidate formation and aggregation induced by various concentrations of the Ca^{2+} ionophore A23187

Experimental conditions are the same as described in the legends to Figs. 1 and 5. Formation of phosphatidate (PA) and aggregation were measured 1 min after addition of ionophore A23187. Abbreviations: rev., reversible aggregation; irr., irreversible aggregation; s.c., shape change. △, ▨, $+PGI_2$; ●, □, control.

DISCUSSION

In the present study, PdBu and ionophore A23187 were used to stimulate separately the two known pathways of platelet activation, the PKC- and the Ca^{2+}-dependent pathways respectively. Possible positive-feedback mechanisms were eliminated by irreversibly blocking platelet cyclo-oxygenase with aspirin and blocking the action of ADP by using ADP scavengers. Thus the effects of PdBu and ionophore A23187 on platelets can be considered direct actions not mediated or synergized by endoperoxide/thromboxane A_2 or trace amounts of ADP.

PdBu, in the absence of any increase of cytosolic Ca^{2+}, evoked the phosphorylation of proteins with molecular masses of 20, 38 and 47 kDa. Dose-dependency and time courses of the 20 kDa myosin light-chain phosphorylation were different from those for the 47 kDa-protein phosphorylation, which agrees with findings from other laboratories [9,43]. Interestingly, PdBu, phorbol 12-myristate 13-acetate and *sn*-1,2-dioctanoylglycerol stimulated the phosphorylation of proteins in the 38–41 kDa range, which may include the 41 kDa α_i-subunit of G_i (Fig. 2) [43,44]. High concentrations (> 50 nM) of PdBu (which are supramaximal for the phosphorylation of the 47 kDa protein) were needed to observe a significant aggregation response. A close temporal relationship between phorbol ester-induced 20 kDa protein phosphorylation and platelet aggregation (Fig. 3) was found, suggesting a role for PKC-dependent myosin light-chain phosphorylation in inducing platelet aggregation. However, we found that preincubation of platelets with PGI_2 completely suppressed aggregation without affecting the phosphorylation of the proteins stimulated by PdBu. PGI_2 stimulation of the phosphorylation of the 50 kDa substrate of cyclic AMP-dependent protein kinase closely correlated with the inhibition of PdBu-induced aggregation by PGI_2. From these data we

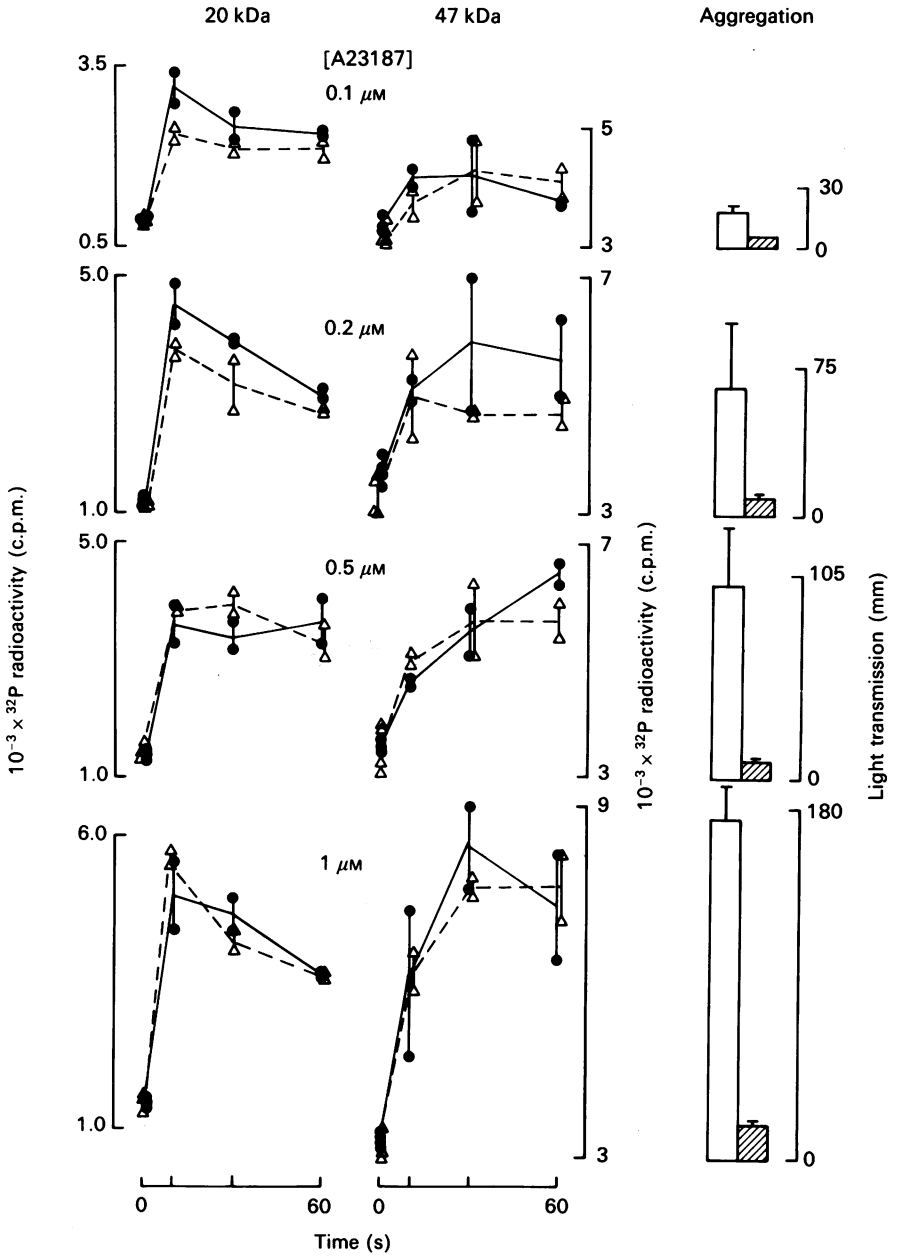

Fig. 8. Effects of PGI_2 on protein phosphorylation and aggregation induced by various concentrations of the Ca^{2+} ionophore A23187

The resuspension platelet buffer contained phosphocreatine/creatine kinase instead of apyrase. Other conditions are the same as described in the legend to Fig. 1. Symbols are as in Fig. 7. This experiment is representative of another three that gave essentially similar results.

conclude that PdBu induces platelet aggregation through the PKC-dependent phosphorylation of specific proteins, but that distal to that event there are steps crucial for the regulation of platelet aggregation that are critically affected by cyclic AMP-dependent protein kinases. It has recently been demonstrated that glycoprotein Ibβ is phosphorylated by protein kinase A in intact platelets [45]. A similar phosphorylation could involve the glycoprotein IIb/IIIa complex, which represents the fibrinogen receptor and undergoes a conformational change upon platelet activation [1]. This hypothetical phosphorylation by protein kinase A might prevent conformational changes of the fibrinogen receptor brought about by PKC-dependent phosphorylation of specific proteins. Alternatively, cyclic AMP might directly (independently of stimulation of protein kinases A) affect the conformation of proteins [46] involved in aggregation. Cyclic AMP could resemble (in that regard) other platelet-inhibiting drugs. For example, Holmsen's group recently reported that non-lytic concentrations of chlorpromazine inhibited dense-granule and lysosomal secretion in platelets, but not 47 kDa- and 82 kDa-protein phosphorylation induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA). These authors concluded that chlorpromazine acts at a step after protein kinase activation [47].

The data of the present study contrast somewhat with

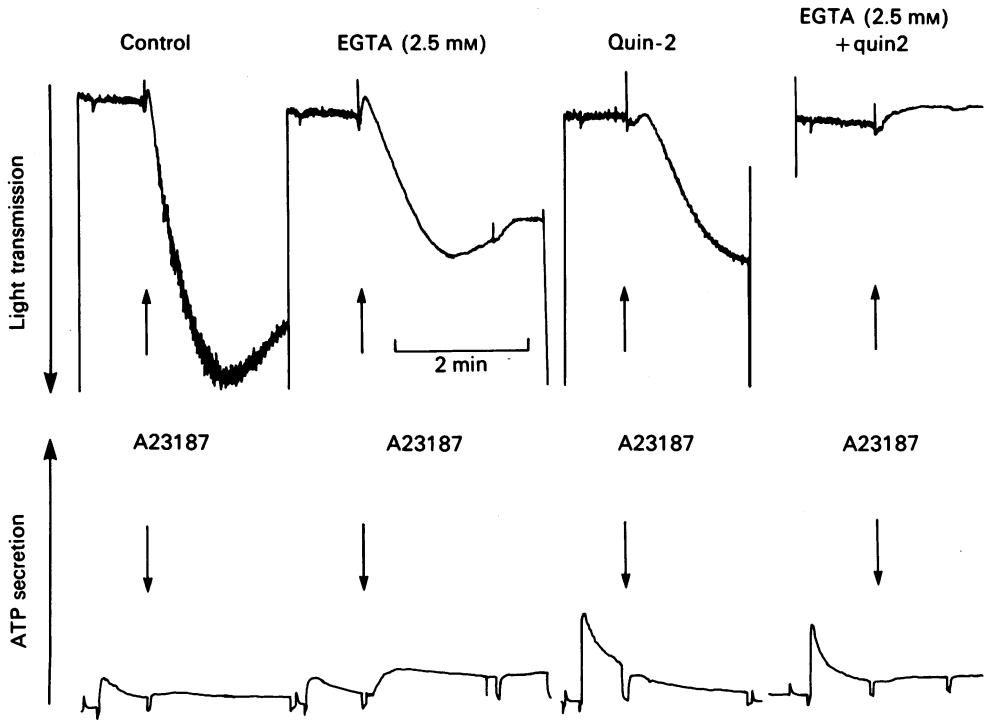

Fig. 9. Effects of intracellular Ca^{2+} chelation by quin-2 and of extracellular Ca^{2+} chelation by EGTA on platelet aggregation and ATP secretion induced by ionophore A23187

Platelet-rich plasma was incubated with 50 μM-quin-2/AM for 20 min at 37 °C. Platelets were then pelleted by centrifugation and resuspended in platelet-poor plasma. EGTA (2.5 mM) was added 1 min before ionophore A23187 (15 μM; arrow).

recent results published by de Chaffoy de Courcelles *et al.* [48]. They found that PGE_1, which increases cyclic AMP in platelets, induced a drastic inhibition of the phosphorylation of the 40 kDa (equivalent to our 47 kDa protein) protein induced by 1-oleoyl-2-acetylglycerol (OAG) and a slight inhibition of the 40 kDa protein phosphorylation induced by TPA. In their study, however, no precautions were taken to eliminate possible synergism or feedback activation by ADP or endoperoxides/thromboxane A_2. This is important, since the effect of OAG in platelets has been found to depend largely on the release of ADP [49]. In addition, TPA, in contrast with PdBu, may stimulate Ca^{2+} mobilization in platelets, since it has been reported to induce an aequorin signal [50] and a rapid Ca^{2+}/calmodulin-dependent phosphorylation of myosin light chain in human platelets [9]. It is possible that PGE_1 suppressed the Ca^{2+}-dependent activation of protein phosphorylation induced by TPA.

Ionophore A23187 at 0.1 μM induced shape change and a pronounced phosphorylation of the 20 kDa myosin light chain, but no or only a small phosphorylation of the 47 kDa protein. We observed that, by increasing the concentration of A23187 from 0.2 to 1 μM, aggregation and phosphorylation of the 47 kDa protein increased progressively, whereas the stimulation of 20 kDa myosin light-chain phosphorylation remained almost unchanged. These results support our recent hypothesis that myosin light-chain phosphorylation is related to shape change and is a prerequisite for aggregation, whereas PKC phosphorylation of the 47 kDa protein is more involved in regulation of aggregation [15,16]. The Ca^{2+}-dependent phosphorylation of the 20 and 47 kDa proteins induced by ionophore A23187 was not inhibited by PGI_2. In agreement with previous studies [10,33,34], aggregation but not shape change was inhibited by PGI_2. Since Ca^{2+} mobilization induced by ionophore A23187 was not inhibited by PGI_2 (see also refs. [33,34]), these results indicate that aggregation is inhibited by PGI_2 at steps distal to Ca^{2+}-dependent phosphorylation of 20 and 47 kDa proteins. This result is somewhat unexpected: biochemical studies using purified proteins have demonstrated that protein kinase A phosphorylates MLCK, thereby diminishing its enzymic activity and decreasing its affinity for calmodulin [4,37]. However, protein kinase A-dependent phosphorylation of MLCK has never been demonstrated in the intact cell.

The origin of phosphatidic acid formation after platelet activation with ionophore A23187 is not clear, because positive feedback activation of phospholipase C by cyclo-oxygenase products and ADP [6] had been eliminated in the present study. The increase in [^{32}P]-phosphatidic acid ($\sim$ 2-fold) observed was small compared with that with other stimuli, and might have escaped detection in earlier studies [6] because of lower ^{32}P labelling of platelets. Since Ca^{2+} mobilization is observed before [^{32}P]phosphatidate formation in platelets stimulated by A23187 (results not shown), it could have derived from Ca^{2+}-activated hydrolysis of phosphatidylinositol [51]. Other possible sources of [^{32}P]-phosphatidate formation in platelets stimulated by A23187 are a stimulated conversion of 1,2-diacylglycerol into phosphatidic acid or, more likely, an inhibition of

the reconversion of phosphatidic acid into phosphatidylinositol. Interruption of the phosphatidylinositol cycle by A23187 in platelets has been previously demonstrated [30,52]. Alternatively, [^{32}P]phosphatidate could derive from phospholipid degradation by phospholipase D. Hydrolysis of phosphatidylinositol and phosphatidylcholine by phospholipase D has recently been shown in neutrophils activated by Ca^{2+}-mobilizing agonists, including A23187 [53,54].

Our study shows that PGI_2, through activation of protein kinases A, inhibits platelet aggregation at steps distal to PKC and Ca^{2+}-dependent protein phosphorylation. At present, it seems that cyclic AMP inhibits platelet activation at multiple steps: it inhibits phosphoinositide hydrolysis by phospholipase C [1], fibrinogen receptor exposure [1], and secretion and aggregation independently of Ca^{2+} mobilization [33–36] and independently of the activation of PKC- and Ca^{2+}/calmodulin-dependent kinases (the present study). The function of proteins phosphorylated by protein kinases A is still largely unknown.

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