Recycling of Platelet Phosphorylation and Cytoskeletal Assembly

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ABSTRACT   The shape change and aggregation of washed platelets induced by 10 μM arachidonic acid (AA) can be reversed by 20 ng/ml prostacyclin (PGI$_2$), but these platelets can be reactivated by treatment with 30 μM epinephrine and subsequent addition of 10 μM AA mixture. These events may be modulated by cAMP since 2 mM dibutyryl cAMP also reversed activation without reactivation by epinephrine and AA. We examined protein phosphorylation and formation of cytoskeletal cores resistant to 1% Triton X-100 extraction of these platelets and correlated these processes with aggregation, fibrinogen binding, and changes in ultrastructure. Unactivated platelet cores contained <15% of the total actin and no detectable myosin or actin-binding protein. AA-induced cytoskeletal cores, which contained 60–80% of the total actin, myosin, and actin-binding protein as the major components, were disassembled back to unactivated levels by PGI$_2$ and then fully reassembled by epinephrine and AA. Phosphorylation of myosin light chain and a 40,000-dalton protein triggered by AA (two- to fivefold) was reversed to basal levels by PGI$_2$ but was completely restored to peak levels upon addition of the epinephrine and AA mixture. The reversibility of actin-binding protein phosphorylation could not be established clearly because both PGI$_2$ and dibutyryl cAMP caused its phosphorylation independent of activation. With this possible exception, cytoskeletal assembly with associated protein phosphorylation, aggregation, fibrinogen binding, and changes in ultrastructure triggered by activation are readily and concertedly recyclable.

That platelet activation with all of its structural rearrangements basically reflects the activity of cytoskeletal processes has long been recognized (1). Our previous studies have demonstrated a de novo cytoskeletal assembly process upon platelet activation. One of the initial steps in this process is extensive actin polymerization from a large pool of monomeric actin present in the resting platelet. These newly formed actin filaments interact with actin-binding protein (ABP)[1] to form pseudopodal projections and with myosin to form a contractile microfilament network responsible for centralization of secretory granules (2). The contractile activity of this actomyosin has been shown to be regulated through phosphorylation of the 20,000-dalton light chains of myosin (MLC)

by myosin kinase (3, 4). Calcium flux either through the plasma membrane or from the dense tubular system (5) regulates this myosin kinase through its interaction with calmodulin (6).

In addition to MLC, ABP (7) and a 40,000-dalton protein (8) (40P)[2] are phosphorylated during platelet activation. MLC has <0.1 mol phosphate/mol protein before activation and >0.9 mol phosphate after activation (4). The phosphorylation of 40P, estimated after isolation from activated platelets, is in excess of 0.5 mol of phosphate/mol protein (9). Based on the stoichiometry of MLC phosphorylation and the pool sizes of these proteins, calculations suggest that phosphorylation of actin-binding protein approaches 1 mol phosphate/mol monomer after activation (7). Unlike the case with MLC activa-

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[1] *Abbreviations used in this paper:*   AA, arachidonic acid; ABP, actin-binding protein; MLC, light chains of myosin; 40P, 24P, and 22P: 40,000-, 24,000-, and 22,000-dalton polypeptide, respectively; PGI$_2$, prostacyclin.

[2] This protein actually has a molecular mass of 47,000 daltons (9) but electrophoresis anomalously as a 40,000-dalton protein in the gel system used in this study.

tion, the reasons for the phosphorylations of ABP and 40P during platelet stimulation have not been established.

In addition to the internal processes discussed above, platelet cytoskeletal assembly has been shown to have specific interactions with plasma membrane proteins. Amongst these interacting membrane proteins is the glycoprotein IIb-III complex shown by Phillips et al. (10) to mediate platelet aggregation. This complex was subsequently identified as the binding site for fibrinogen (11) known to be involved in platelet aggregation (12).

Platelets in plasma, once activated and aggregated, can be redispersed by addition of prostacyclin (PGI$_2$) and then subseqeuently reactivated by a combination of arachidonic acid (AA) and epinephrine (13). Epinephrine, which reduces the cAMP levels induced by PGI$_2$, resensitizes the platelets to AA. Platelets redispersed by PGI$_2$ regain their unactivated ultrastructure, and the reaggregation occurs normally. In this study, we examined the reversibility of cytoskeletal assembly and actin polymerization during the cyclic activation of washed platelets and correlated these processes with changes in ultrastructure, platelet aggregation, fibrinogen binding, and protein phosphorylation.

MATERIALS AND METHODS

Platelet Preparation and Labeling with [³²P]Orthophosphate:
Platelets were isolated from citrate anticoagulated whole blood, as has been described previously (2), and suspended at 1 × 10⁹ platelets/ml in Tangen-HEPES-BSA buffer (145 mM sodium chloride, 5 mM potassium chloride, 0.1 mM magnesium chloride, 0.05 mM calcium chloride, 5.5 mM glucose, 1 mg/ml BSA, and 15 mM HEPES, pH 7.4). The platelets were allowed to incorporate [³²P]orthophosphate (1 mCi/ml) for 45 min at 37°C. Residual plasma protein and unincorporated ³²P-label were then removed by gel filtering at 4°C on a Sepharose 2B column equilibrated and eluted with Tangen-HEPES-BSA buffer. The bed volume of the column was 10 times the applied sample volume. The platelets were collected, counted, and adjusted to 5 × 10⁸ platelets/ml with buffer. After 30-min incubation at 37°C the platelets recovered their discoid shape as detected by light microscopy.

Aggregation and Reagents:
Aggregation was monitored in a Payton dual channel aggregometer (Payton Associates, Buffalo, NY) thermostated at 37°C. Platelet samples of 1-5 ml were treated as indicated in the figure legends with 10 μM AA (NuChek Prep, Elysian, MN) added from a 1-mM solution, 20 ng/ml PGI$_2$ from a 10 μg/ml-stock solution, 30 μM epinephrine (Sigma Chemical Co., St. Louis, MO) from a 1-mM stock solution, 2 mM dibutyryl cAMP (Sigma Chemical Co.) from a 100-mM solution, or 10 μM phorbol 13-myristate 12-acetate (PMA from P-L Biochemicals, Inc., Milwaukee, WS) from a 1-mM stock solution. Dibutyryl cAMP solutions were freshly made for each experiment. PMA in dimethylsulfoxide was stored at −20°C while PGI$_2$ solutions, adjusted to pH 9, were stored at −70°C for not more than a 4-wk period.

Fibrinogen Binding:
Human fibrinogen (Type L, Kabi Group, Greenwich, CT) was treated with diisopropylfluorophosphate (12). This solution was either dialyzed into 150 mM NaCl, 20 mM HEPES, pH 7.4 for a stock solution or dialyzed into 100 mM sodium borate, pH 8.5 for radiolabeling by the method of Bolton and Hunter (14). After labeling, this fibrinogen was also dialyzed into the same buffer as the stock solution. Fig. 1 shows a densitometric tracing of the autoradiogram of SDS PAGE gel of reduced fibrinogen. The labeled fibrinogen was denatured in 2% SDS, containing 2% 2-mercaptoethanol, and electrophoresed according to the procedure of Studier (15). The three closely spaced peaks arise from the A-alpha, B-beta, and gamma polypeptides of fibrinogen, and the high molecular weight contaminant is probably plasma fibronectin which contained 10% of the total radioactivity found in the fibrinogen polypeptides. 92% of the label was coagulable. Concentrations of fibrinogen were calculated using a value of 15.1 (16) for $A_{cm}^{1\%}$, 280 nm.

Fibrinogen binding was measured using the technique described for coagulation Factor Xa (17). Aliquots (0.2 ml) of platelet suspension containing the [¹²⁵I]fibrinogen were layered onto a mixture of one part Apiezon oil (Biddle Instruments, Blue Bell, PA) and nine parts n-butylphthalate. The sample was immediately centrifuged at 10,000 g for 3 min. An aliquot of the supernatant was counted to determine the concentration of unbound fibrinogen, and the pellet was counted to determine the amount bound.

Preparation of Cytoskeletal Cores:
Cytoskeletal cores were isolated from unlabeled platelets by centrifugation at 11,000 g for 5 min after extraction with 1% Triton X-100, 50 mM Tris-HCl, 5 mM EGTA, pH 7.4 as described by Phillips et al. (10). To facilitate comparisons, all samples applied to the SDS PAGE gels were obtained from the same number of platelets. Therefore, the sum of protein at any molecular weight region of the gel from the cytoskeletal core and supernatant lanes would equal the protein in that same region from a whole platelet lane. To accomplish this, the following procedure was adopted. A denaturation buffer was prepared that contained 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, 1 mM EDTA, 4 mM EGTA, 0.01% bromophenol blue, 150 mM Tris-HCl, pH 6.8 and another denaturation buffer at five times the concentrations of each ingredient. A 0.2-ml aliquot of a platelet sample was mixed with 0.2 ml of extraction buffer and the cores collected. A 0.2-ml aliquot of the supernatant was denatured with 0.04 ml of the fivefold concentrated denaturation buffer. After thoroughly removing the remaining supernatant, the cytoskeletal-core pellets were denatured in a volume of denaturation buffer equal to the original platelet volume. A 0.1-ml aliquot of the denatured supernatant and a 0.04-ml aliquot of the cytoskeletal core fraction, both equivalent to 0.04 ml of the original platelet suspension, were electrophoresed on 6-17.5% polyacrylamide gradient gels prepared as described by Studier (15), then fix-stained with Coomassie Blue, destained, and scanned on a LKB Ultrascan Laser Densitometer to quantify protein bands.

DNAase I Assay For Monomeric Actin:
Total actin content in cytoskeletal core and supernatant fractions and in whole platelet lysates after depolymerization for 30 min at 0°C in 0.75 M guanidine-HCl, 0.5 M sodium acetate, 0.5 mM sodium-ATP, 10 mM Tris-HCl, 0.5 mM calcium chloride, pH 7.5 was measured by a DNAase-I-inhibition assay originally described by Blikstad et al. (18) and applied to platelets (19) but slightly modified by Carroll et al. (2).

The monomeric actin content in unfractionated platelet lysates was measured within 3 min after adding the extraction buffer containing Triton X-100 plus EGTA. Monomeric actin contents are expressed as a percentage of the total platelet actin content determined as described above. The validity of these assays for total and monomeric actin in platelets was established previously (19).

Phosphorylation of Platelet Proteins:
Phosphorylation of platelet ABP, 20,000-dalton MLC, 40P, 24,000-dalton peptide (24P), and 22,000-dalton peptide (22P) was determined during the course of activation, reversal, and reactivation. Aliquots (0.05 ml) were taken at the indicated times and immediately denatured by heating for 3 min at 100°C after adding 0.025 ml of a threefold concentrated denaturation buffer (see above). These samples were stored at −20°C and reheated for 2 min at 100°C before electrophoresis as described above. The gels were soaked for 30 min at 60°C in 10% trichloroacetic acid before fix-staining, destaining, drying, autoradiographing, and quantifying ³²P-CPM/gel band as previously described (7). Molecular weights of phosphorylated bands were determined by co-electrophoresis of platelet actin-binding
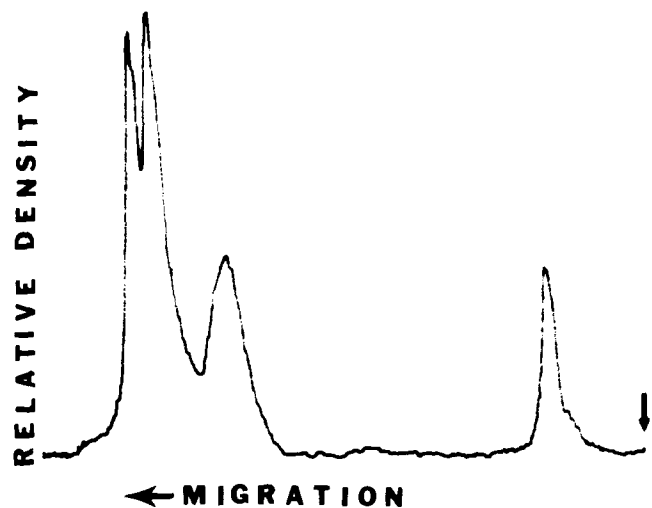

FIGURE 1   SDS PAGE of [¹²⁵I]fibrinogen. The radiolabeling of the human fibrinogen and the electrophoresis of the reduced, denatured product was performed as described in Materials and Methods. The autoradiogram was obtained from the dried gel that was exposed for 1 d to Kodak BB-5 x-ray film. The densitometer tracing was obtained using an LKB laser densitometer. The arrow indicates the beginning of the gradient gel.

protein (250,000 daltons), myosin (200,000 daltons heavy chains, 20,000- and 16,000-dalton light chains), and actin (43,000 daltons) isolated as previously described (7) as well as standard proteins obtained from Bio-Rad Laboratories (Richmond, CA) consisting of myosin (200,000-dalton heavy chain), beta-galactosidase (116,000 daltons), phosphorylase B (94,000 daltons), BSA (68,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), soybean trypsin inhibitor (21,000 daltons), and lysozyme (14,300 daltons). In addition, bovine pancreas alpha-chymotrypsinogen (23,500 daltons, from Worthington Biochemical Corp., Freehold, NJ) was utilized as a molecular weight marker for the 24P band.

Ultrastructure: Samples of platelets during the course of the recycling activation were fixed for examination in the electron microscope according to methods reported in detail in previous publications (20–22). Briefly, the samples were combined with an equal volume of 0.1% glutaraldehyde in White's saline, pH 7.3. After 15 min at 37°C, the fixed platelets were centrifuged into pellets and the supernatant was replaced with 3% glutaraldehyde in the same buffer. Fixation was continued at 4°C for 60 min. The cells were then washed in buffer and further fixed with 1% osmium tetroxide in veronal acetate buffer. After exposure to the second fixation for 60 min, the samples were dehydrated in a graded series of alcohol and embedded in Epon 812. Contrast of thin sections cut from plastic blocks on an ultramicrotome was enhanced with uranyl acetate and lead citrate. Observations were made in a Philips 301 electron microscope.

RESULTS

Electron Microscopy-Aggregometry

The platelets used in these studies were concentrated by centrifugation and further washed by gel filtration. They maintained most of their discoid form, but some platelets had short pseudopod projections. Thin sections as shown in Fig. 2A revealed the lentiform shape supported by circumferential microtubule bundles. Mitochondria as well as secretory alpha-granules and dense bodies were randomly dispersed in the cytoplasm, which also contained discrete particles and large masses of glycogen.

Addition of 10 μM AA to platelets with constant stirring led to extensive aggregation as shown in Fig. 3. Examination by electron microscopy of a sample withdrawn just before the indicated addition of PGI$_2$ revealed changes typical of platelets exposed to potent activators (Fig. 2B). The cells lost their discoid form and became irregular with many intertwining pseudopods in large platelet aggregates. The platelet profiles in which alpha-granules and dense bodies were visible showed a concentration of those granules in the centers of most cells. Many of these centralized granules were seen to be encircled by close-fitting rings of microtubules and microfilaments (as indicated by arrowheads).

The platelet aggregates induced by AA could be dissociated by the addition of PGI$_2$ (Fig. 3). Samples prepared for electron microscopy after the return of the aggregometry tracings to a plateau close to the control platelet baseline showed complete disaggregation of the cells (Fig. 2C). Most of these cells had recovered a discoid form, and only occasional short pseudopodal projections were observed. Indeed, the general appearance of the PGI$_2$-treated cells was somewhat more regular and discoid than the untreated control platelet. Bundles of microtubules could again be observed at the polar ends of cross-sectioned cells, supporting the lentiform shape. The granules, although fewer in number, had also returned to a randomly dispersed pattern.

The platelets, after disaggregation induced by PGI$_2$, were reactivated by a combination of epinephrine plus arachidonic acid. The platelets again underwent a full aggregation response (Fig. 3) and when fixed for electron microscopy revealed a return to the activated state with pseudopodal development and granule centralization (Fig. 2D).

Fibrinogen Binding

Since aggregation by AA as well as ADP has been shown to be mediated by fibrinogen binding to the platelets, we examined the expression of fibrinogen-binding site as a function of activation-reversal-reactivation. The experimental conditions were identical to those used in Fig. 2, except that 0.69 μM human fibrinogen was added to the gel-filtered platelets 10 min before activation with AA. Sample aliquots were taken before activation, at a point about halfway up the aggregation curve during the initial aggregation, during the plateau in the disaggregation response after PGI$_2$ addition, and at the plateau of reaggregation by epinephrine and AA. Binding was measured at two separate radiospecific activities obtained by mixing the labeled fibrinogen with unlabeled fibrinogen. No difference in the amount of fibrinogen bound was detected over a threefold difference in radiospecific activity, indicating that the labeled and unlabeled fibrinogen were binding similarly and the nonspecific binding was within the experimental error of the binding assay. The amount of radioactivity in the platelet pellet was corrected for a trapped volume of 0.23 μl per 10^8 platelets (23). The results of these binding studies are listed in Table I. Although a sample was taken at the peak of the first aggregation, binding could not be determined, since large, loose aggregates were present that would not penetrate the butylphthalate layer. However, the results clearly demonstrate the cyclic binding of fibrinogen that occurred during the cycles of aggregation and deaggregation.

Cytoskeletal Cores and Actin Polymerization

Previous studies have supported a role for the polymerization of actin and its interaction with myosin and actin-binding protein in platelet activation. These processes have been followed by isolation of cytoskeletal cores resistant to 1% Triton X-100 extraction and by quantifying both monomeric and total actin content in platelet whole lysate as well as core and supernatant fractions (2).

Fig. 4 shows the protein composition of the typical cytoskeletal core developed during 10 μM AA activation (lane 3) and the loss of these proteins when the core was completely disassembled by PGI$_2$ treatment (lane 5) and the return to the original protein composition as the core was reassembled after addition of epinephrine plus AA (lane 7). A similar cycling of actin polymerization was detected in whole platelet lysates by the DNase I assay as shown in Table II. The sampling of the platelets during the time course corresponded exactly to those taken for the electron microscopy study, and both studies were performed on the same preparation of platelets.

Table II also shows the total content of actin following guanidine depolymerization of multimeric actin in supernatant and pelleted cytoskeletal core fractions prepared from aliquots taken at the same time as samples denatured for SDS PAGE (gel shown in Fig. 4). The small amount of cytoskeletal core found in control platelets and the 60–80% of actin, myosin, and ABP incorporated into cytoskeletal core of activated platelets were similar to the values previously reported for control platelets and those fully activated by thrombin (2). The drop in monomeric actin as a percentage of the total platelet actin from 68 ± 4% to 25 ± 6% was also similar to that observed for fully activated platelets (2). The increase of monomeric actin content to near control levels by PGI$_2$
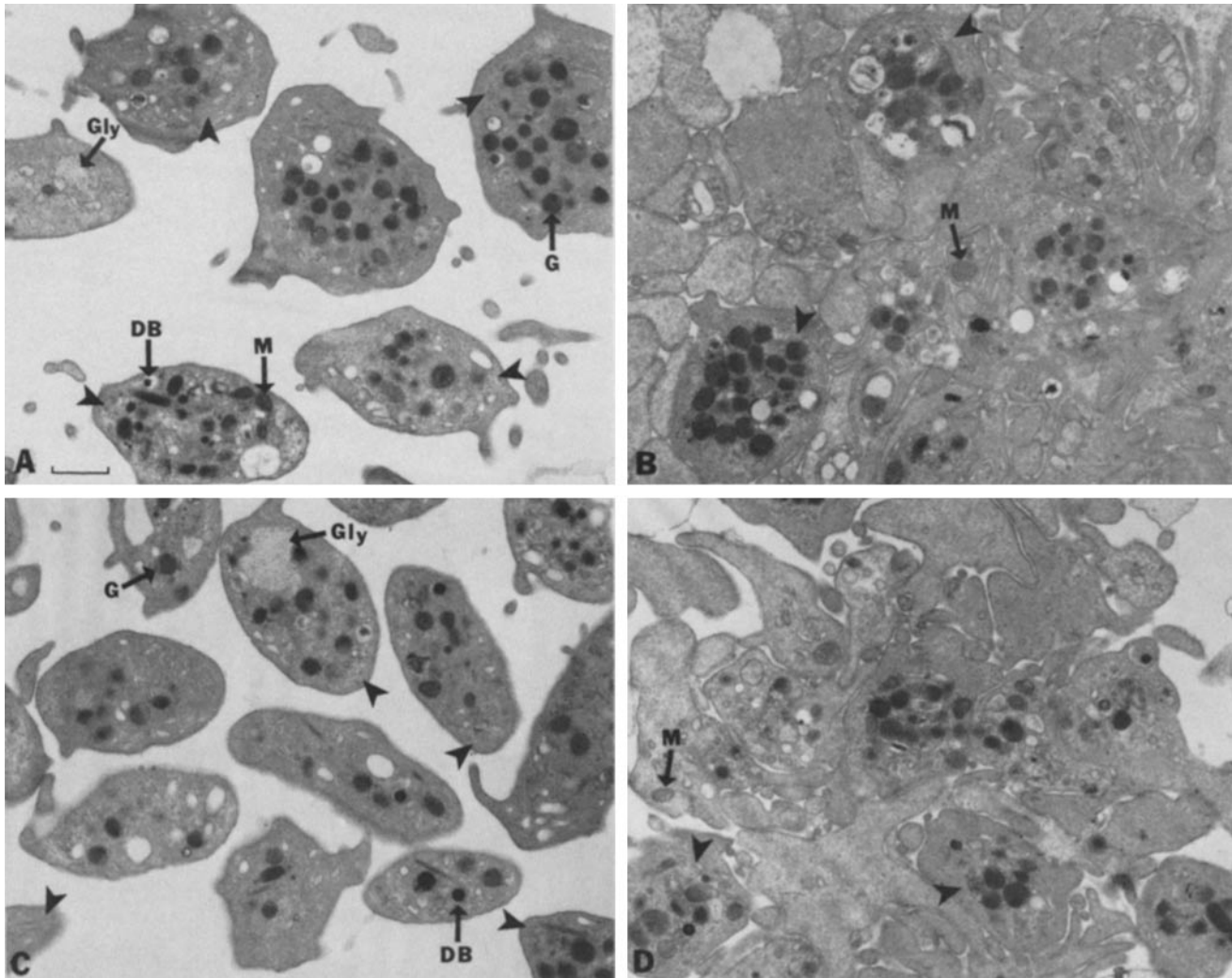
FIGURE 2   Platelet ultrastructure during cyclic activation. The preparation of the samples is described in Materials and Methods. The electron micrographs are of the following samples: (A) unactivated washed platelets, (B) platelets at maximum aggregation after addition of 10 μM arachidonic acid, (C) platelets disaggregated by addition of 20 ng/ml PGl$_2$, and (D) platelets reaggregated by addition of 30 μM epinephrine and 10 μM arachidonic acid. Cellular features identified in the figures are microtubules (arrowheads), dense bodies (DB), alpha-granules (G), mitochondria (M), and glycogen particles (Gly). Bar, 1 μm (A). × 9,600.
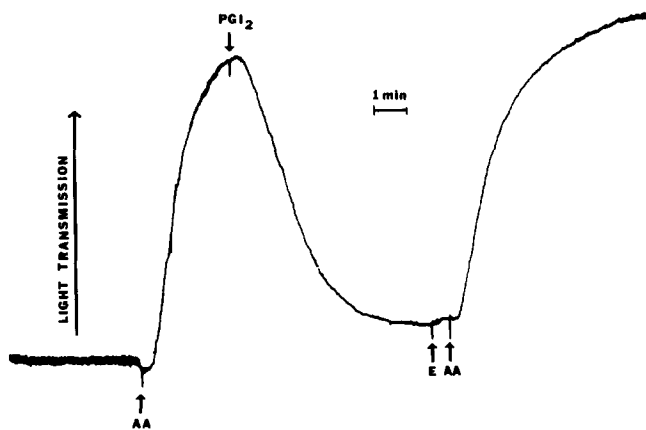

FIGURE 3   Platelet aggregation during cyclic activation. The aggregometer tracing of a suspension of washed platelets indicates the course of aggregation during activation with 10 μM arachidonic acid (AA), reversal of activation by addition of 20 ng/ml PGl$_2$ and resensitization by addition of 30 μM epinephrine (E), and reactivation by a second addition of arachidonic acid. The arrows indicate time of additions.

TABLE I

Fibrinogen Binding during Cyclic Activation of Platelets

Platelet sample	Molecules/Platelet*
Before activation	4,500
During initial activation‡	12,600
At minimum of deaggregation	6,900
At maximum of reaggregation	18,000

* Average of the number of fibrinogen molecules bound per platelet calculated from two separate experiments.
‡ The average number calculated at about halfway through the maximum aggregation response.

reversal of AA activation indicated that the actin polymerization process was also reversible.

Phosphorylation of Platelet Protein

Platelet activation has been shown to be accompanied by two- to fivefold increases in ^{32}P-labeling of ABP, 20,000-dalton MLC, as well as the 40P (7–9) of unknown function. As we have previously reported (24), ABP, MLC, and 40P

FIGURE 4 Protein content of cytoskeletons during cyclic activation. Platelet cytoskeletal core and supernatant fractions were prepared, electrophoresed on 6 to 17.5% polyacrylamide gradient SDS slab gels, and stained with Coomassie Blue as described in Materials and Methods. Shown on the gel are the cytoskeletal core and supernatant fractions, respectively, for unactivated washed platelets (lanes 1 and 2); arachidonic acid-activated platelets (lanes 3 and 4); activated platelets after PGI₂-reversal (lanes 5 and 6), and subsequent reactivation with epinephrine and arachidonic acid (lanes 7 and 8). Molecular weights ($\times 10^{-3}$) of standards are shown on the left. Indicated on the right margin are bands corresponding to actin-binding protein (*ABP*), 200,000-dalton heavy chain of myosin (*MHC*), a platelet alpha-actinin-like protein (*α-A*), and *actin*.
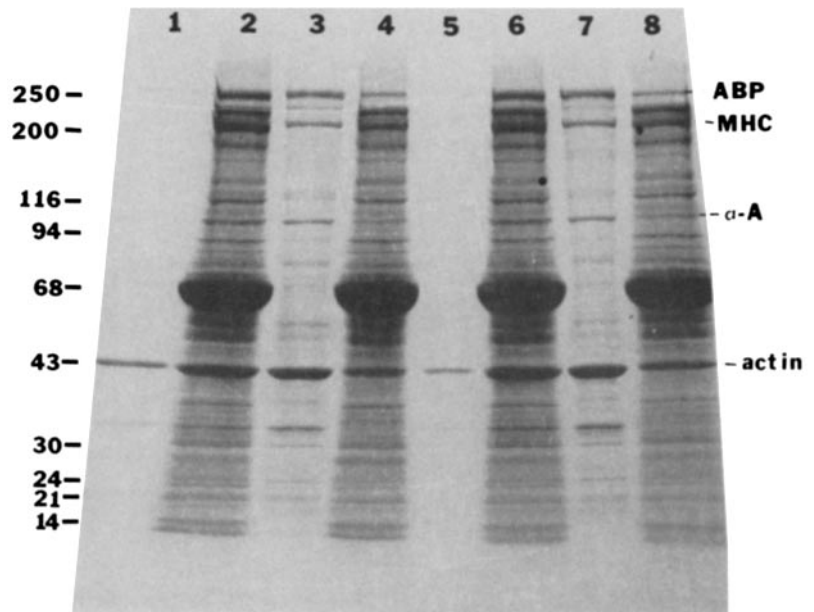
TABLE II

Monomeric Actin Content in Whole Platelet Lysates and Actin Content in Isolated Cytoskeletal Cores during Cyclic Activation

	Monomeric actin content in platelet lysates	Actin content in isolated cytoskeletal cores
	%*	%†
Control	68	11
AA-activated	25	78
PGI₂-reversed	62	5
Epinephrine-AA reactivated	32	71

* Percentages (of total platelet actin) were calculated by dividing the DNAaseI-inhibitory units in lysates by the inhibitory units in these same lysates, but after depolymerization with 0.75 M guanidine-HCl as described in Materials and Methods. These values are averages from three assays and the standard error of the mean was 6%.

† Percentages (of total platelet actin) were calculated by dividing the DNAase-I-inhibitory units in isolated cytoskeletal cores after depolymerization with guanidine-HCl by the inhibitory units present in a total platelet lysate after guanidine depolymerization. The standard error of the mean for these samples was 9%. These cytoskeletal core samples were taken as duplicates to the cytoskeletal cores shown in Fig. 2.

were rapidly phosphorylated after AA activation, as shown in Fig. 4, with the peak of ³²P-labeling between 30 and 60 s. Following this peak labeling, an aggregation-dependent dephosphorylation was observed that reduced the ³²P-labeling of ABP and MLC to basal levels by 5 min after AA addition. The ³²P-labeling of 40P typically falls off at a slower rate with ~40–50% levels remaining after 10 min (full dephosphorylation time courses were shown previously (7, 25)).

Also shown in Fig. 5 is the effect on the phosphorylation of ABP, MLC, and 40P of PGI₂-induced reversal of platelet activation. Accelerated loss of ³²P from MLC was observed, so that it was reduced below basal levels within 30 s of PGI₂ addition. The 40P labeling also showed a dramatic decrease in ³²P-labeling falling to basal levels by 3 min after PGI₂ addition. In contrast, ³²P-labeling of ABP increased after PGI₂ addition to levels above the initial activation peak response. Upon reactivation of the platelets by epinephrine and AA, MLC and 40P bands again showed increased ³²P-labeling, returning to peak levels obtained during the initial phosphorylation response. ABP that was already heavily labeled after

PGI₂ introduction showed an additional modest increase upon reactivation.

A somewhat inverse response was observed for a 24,000-dalton band (24P) when compared to MLC and 40P phosphorylation over these time courses (Fig. 5). The ³²P-labeling of 24P increased 1.5-fold upon AA activation and showed a further twofold increase after addition of PGI₂ opposite to the rapid decline in MLC and 40P phosphorylation. The reactivation by epinephrine and AA led to a decline of the ³²P-labeling of 24P back to levels observed before PGI₂ addition but not to preactivation, basal levels. Greater than twofold increases in ³²P-labeling of a 22,000-dalton protein (22P) was also observed after PGI₂ addition, but, in contrast to 24P, no reduction in ³²P-labeling of this band was observed after reactivation (data not shown).

The phosphorylation patterns in Fig. 5 suggested that PGI₂ caused the dephosphorylation of 40P and MLC but stimulated phosphorylation of ABP. These conclusions were obscured by the aggregation-induced dephosphorylation. For this reason, the experiments were repeated in the presence of EGTA which prevents aggregation and thereby aggregation-initiated dephosphorylation. Fig. 6 shows the phosphorylation of 40P and MLC induced by AA and reversed by PGI₂ in the absence of aggregation. Clearly, PGI₂ leads to the dephosphorylation of these two proteins. During the course of this study, Feinstein et al. (26) reported that prostaglandin D₂ (PGD₂) plus theophylline and forskolin, both of which cause elevated levels of cAMP in platelets, reversed the phosphorylation of these two proteins as well as core assembly induced by thrombin in the presence of EGTA.

Those proteins phosphorylated on addition of agents that raise cAMP levels have been previously reported but the molecular-weight range did not include ABP. Fig. 7 shows the radioautogram of the SDS PAGE gel as well as quantification of the time course of phosphorylation induced by PGI₂. Not only was phosphorylation of 22P and 24P stimulated but so was ABP phosphorylation to an extent similar to that seen during activation (Fig. 5). Included in this figure is one sample where the phosphorylation induced by dibutyryl cAMP can be compared with that induced by PGI₂. Like PGI₂, 2 mm dibutyryl cAMP reversed aggregation; however, the disaggre-

gated platelets were not reaggregated upon addition of epinephrine plus AA (data not shown). ABP was phosphorylated when dibutyryl cAMP was included, an observation that suggests that PGI$_2$ stimulation of the phosphorylation of this protein is also mediated through cAMP. Such phosphorylation of ABP by cAMP-dependent protein kinases in vitro has been reported by Davies et al. (25).

DISCUSSION

The results presented here reveal that the interaction of myosin and ABP with filamentous actin and the polymeri-

zation of actin caused by platelet activation are completely reversed by agents elevating platelet cAMP levels. In the case of PGI$_2$ reversal of AA activation, the platelets remained completely functional and could be completely reactivated by treatment with epinephrine followed by exposure to AA. The relief of PGI$_2$ inhibition correlates with reduction in cAMP levels by epinephrine as established in previous studies (13). Cytoskeletal disassembly, as well as the rapid MLC and 40P dephosphorylation induced by PGI$_2$, correlated with increased phosphorylation of 24P and 22P. During the reactivation by epinephrine and AA, only the phosphorylation of 24P was reduced to levels observed before PGI$_2$ addition.

FIGURE 5 Platelet protein phosphorylation during cyclic activation. During the time course, each of the following reagents was added: 10 μM arachidonic acid (AA) to initiate activation, 20 ng/ml PGI$_2$ to reverse activation, and a combination of 30 μM epinephrine (Epi) plus 10 μM AA for reactivation (additions indicated by arrows). At each indicated time point, aliquots of total platelets were removed and immediately denatured, and later these samples were resolved by SDS PAGE as described in Materials and Methods. The radioactivity was measured for the following platelet polypeptides: MLC (■), 24P (□), and ABP (◆). 40P (●) is plotted on a fourth scale, i.e., 1,000 cpm is plotted as 250 cpm.
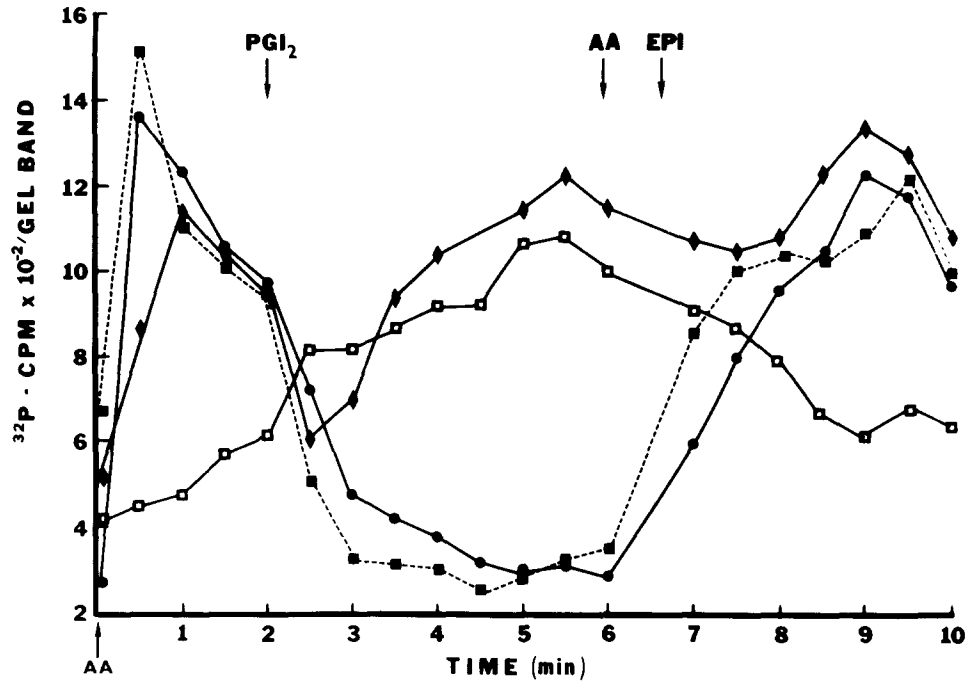

FIGURE 6 Reversal by PGI$_2$ of 40P and myosin light chain phosphorylation in the absence of aggregation. Duplicate samples of ^{32}P-labeled platelets were activated at zero time with 10 μm arachidonic acid in the presence of 2 mM EGTA to prevent aggregation. After 2 min, 20 ng/ml PGI$_2$ was added to the one sample (closed symbols) while the other sample served as an activation control. To the sample that received PGI$_2$, a combination of 30 μM epinephrine and 10 μM AA was added at 7 min. At each indicated time point, aliquots of total platelets were removed, immediately denatured, and later resolved by SDS PAGE as described in Materials and Methods. The radioactivity was measured for the following platelet peptides: control activated, MLC (◇) and 40P (○), and activated-PGI$_2$ reversed at 2 min MLC (■) and 40P (●). As in Fig. 5, the 40P radioactivity is plotted on a fourth scale.
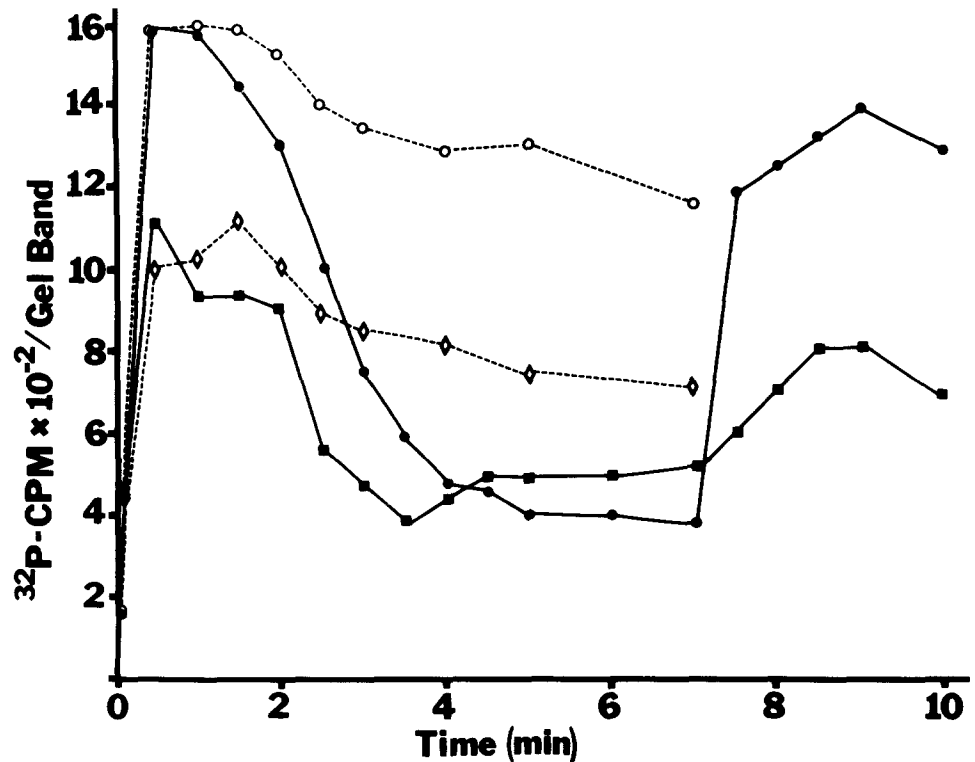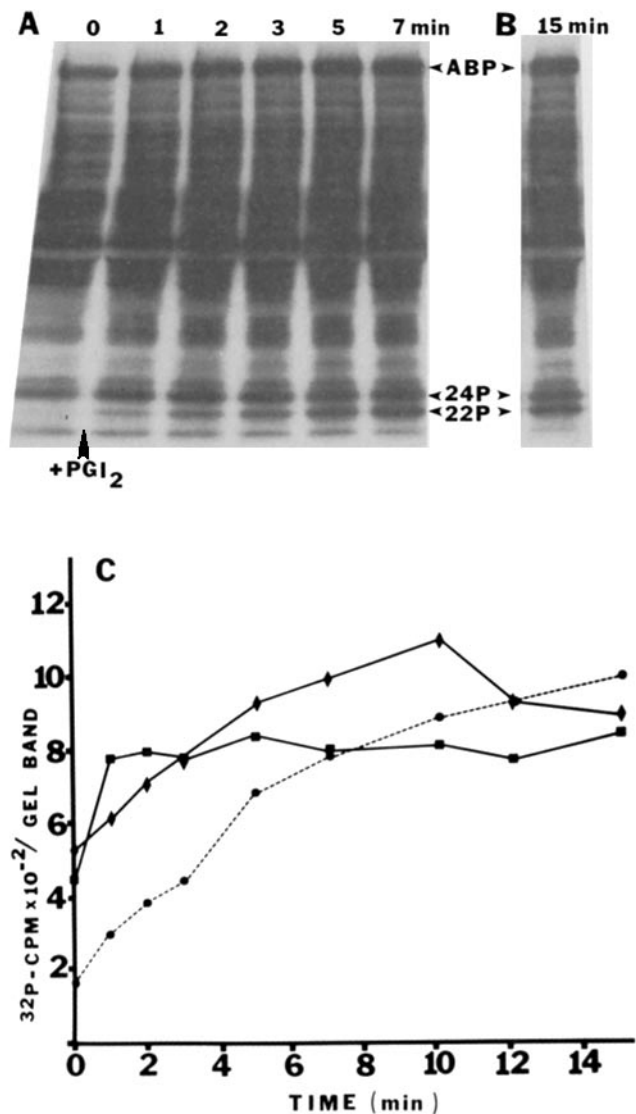
FIGURE 7 Platelet protein phosphorylation induced by PGI₂ in the absence of platelet activation. Aliquots were removed at the indicated times and processed as described in Fig. 5. The parts of the figure are designated as A, radioautogram of total platelet proteins during 20 ng/ml PGI₂ treatment taken at the indicated times above the lanes; B, radioautogram of total platelet proteins treated for 15 min with 2 mM dibutyryl cyclic AMP; and C, quantification of the radioactivity during PGI₂ treatment in the following peptides: ABP (◆), 24P (■), and 22P (●).

The apparent opposite response of ABP phosphorylation which increased after PGI₂ addition and cytoskeletal disassembly was explained by examining the effects of PGI₂ and dibutyryl cAMP on unactivated platelets. Increased phosphorylation levels in ABP by these reagents, equal to those seen upon platelet activation, certainly obscure whether dephosphorylation occurs during PGI₂ reversal of platelet activation.

Inhibition of platelet activation by cAMP has been suggested to be the result of increased Ca++ sequestration (27) or direct inactivation of the myosin kinase (28). Since both of these inhibitory processes are suggested to be mediated by cAMP-dependent phosphorylation, we looked for the increased phosphorylation of any peptides correlating with PGI₂-induced reversal of platelet activation. As previously reported by Fox et al. (27), we observed significant increases

in 24P and 22P phosphorylation in response to addition of PGI₂ or dibutyryl cAMP. However, only 24P-phosphorylation levels showed any decline during epinephrine and AA reactivation, whereas 22P levels actually increased slightly.

Conti and Adelstein (29) have suggested that cAMP-dependent protein kinase phosphorylates myosin kinase at a site that prevents the binding of calmodulin that is necessary for myosin kinase activation. We were unable to identify significant ^{32}P-label increases by PGI₂ or dibutyryl cAMP treatment in the 130,000-dalton region of the gels where the phosphorylated myosin kinase would be expected to migrate. However, in view of the relatively small amount of this enzyme in the platelet and the presence of many phosphoproteins in this region of the gels employed in our study, the possibility remains that a cAMP-dependent phosphorylation of myosin kinase is involved in PGI₂ reversal of activation.

A direct association of the glycoprotein IIb-III complex of the platelet plasma membrane with the cytoskeletal cores of activated platelets that have aggregated has been shown by Phillips et al. (10). This complex has been shown to be the fibrinogen-binding site (11). In this study we demonstrate that increased fibrinogen binding correlates with cytoskeletal assembly. Previously, Hawiger et al. (30) had demonstrated fibrinogen binding that accompanies platelet activation and aggregation induced by ADP or thrombin was blocked by PGI₂ when added before ADP or thrombin. In this study, expression of this binding site during platelet activation was reversed by PGI₂ towards control levels, but was expressed again upon reactivation with epinephrine and AA. These results suggest reversible regulation of this binding site for fibrinogen that mediates platelet aggregation. Furthermore, the complex of glycoproteins IIb-III is not modified in an irreversible manner by PGI₂-induced disaggregation but remains readily activatable to support reaggregation.

The platelets contain a most dramatic display of the oppositional control of cellular reorganization whereby the calcium-mediate processes of cellular activation are pitted against the cAMP-mediate processes design to restore the resting state. The ultrastructural changes are extensive as are the assembly of the microtubular ring and the cytoskeleton. These organizations and disorganizations, with the exception of secretion, are reversible in both directions. While this reversibility would be expected in smooth muscle cell contraction, where this oppositional control also exists, it is remarkable that it is true in platelets where the changes are so extensive, including external as well as internal processes.

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