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Effects of Collagen, Ionophore A23187 and Prostaglandin E_1 on the Phosphorylation of Specific Proteins in Blood Platelets

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Human platelets that had been preincubated with 5-hydroxy[^{3}H]tryptamine and [^{32}P]P_i were stirred with various agents; the secretion of 5-hydroxy[^{3}H]tryptamine from platelet granules and the radioactivity of platelet [^{32}P]phosphopolypeptides separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were then measured. Exposure of the platelets to collagen fibres or ionophore A23187 selectively increased the phosphorylation of polypeptides with apparent mol.wts. of 47000 (P47) and 20000 (P20) by approx. 3-fold, in association with the release of 5-hydroxy[^{3}H]tryptamine. The 47000-mol.wt. phosphopolypeptide (P47) was clearly separated from platelet actin by the electrophoresis system used. Prostaglandin E_1, which inhibits platelet function by increasing platelet cyclic AMP, decreased the phosphorylation of polypeptides caused by collagen as well as the release of 5-hydroxy[^{3}H]tryptamine. Prostaglandin E_1 also selectively increased the phosphorylation of distinct polypeptides with apparent mol.wts. of 24000 (P24) and 22000 (P22) by approx. 2-fold. As the phosphorylation reactions caused by collagen are probably mediated by an increase in Ca^{2+} concentration in the platelet cytosol and may have a role in the release reaction [Haslam & Lynham (1977) *Biochem. Biophys. Res. Commun.* **77**, 714–722; (1978) *Thromb. Res.* **12**, 619–628], we suggest that a cyclic AMP-dependent phosphorylation of the 24000- and/or 22000-mol.wt. polypeptides caused by prostaglandin E_1 may initiate processes that decrease the Ca^{2+} concentration in the cytosol, so inhibiting both the Ca^{2+}-dependent phosphorylation reactions and the release reaction. Treatment of platelets with prostaglandin E_1 did not inhibit the increased phosphorylation of polypeptides with apparent mol.wts. of 47000 and 20000 (P47 and P20) caused by ionophore A23187, which may therefore short-circuit cyclic AMP-dependent mechanisms that decrease the Ca^{2+} concentration in the platelet cytosol. As prostaglandin E_1 did inhibit the release of 5-hydroxy[^{3}H]tryptamine by ionophore A23187, cyclic AMP may also inhibit the release reaction by additional mechanisms.

When platelets are exposed to materials such as collagen fibres, thrombin or the bivalent-cation ionophore A23187, they aggregate and discharge their granule contents in a secretory process known as the platelet release reaction. These effects probably result from increases in the concentration of Ca^{2+} in the platelet cytosol (Feinman & Detwiler, 1974; Massini & Lüscher, 1976; Charo *et al.*, 1976; Feinstein *et al.*, 1976; Le Breton *et al.*, 1976) and are inhibited by agents, such as PGE_1, that increase platelet cyclic AMP concentrations (Salzman, 1972; Haslam, 1975; Haslam *et al.*, 1978*a*). Since Ca^{2+} and cyclic AMP can each activate specific protein kinases (Brostrom *et al.*, 1971; Rubin & Rosen, 1975; Hosey & Tao, 1977) and since platelets are known to contain phosphorylase kinase (Gear & Schneider, 1975; Chaiken *et al.*, 1975), cyclic AMP-dependent protein kinases (Kaulen & Gross, 1974; Steiner, 1975; Lyons *et al.*, 1975; Booyse *et al.*, 1976) and a myosin-light-chain kinase (Daniel & Adelstein, 1976) that is activated by Ca^{2+} ions (Hathaway *et al.*, 1978), protein phosphorylation could have several major roles in the regulation of platelet function.

The physiological relationships of changes in the phosphorylation of specific platelet proteins can be studied in intact platelets labelled by preincubation with [^{32}P]P_i if, after addition of various activators or inhibitors, individual [^{32}P]phosphopolypeptides are isolated by SDS/polyacrylamide-gel electrophoresis or other techniques (Lyons *et al.*, 1975; Apitz-Castro *et al.*, 1976; Haslam & Lynham, 1976, 1977, 1978; Daniel *et al.*, 1977). Using this approach, Lyons *et al.* (1975) found that treatment of ^{32}P-labelled platelets with thrombin resulted in increased phosphorylation of polypeptides with apparent mol.wts. of 40000 and 20000. In this laboratory, it was found that poly-

Abbreviations used: PGE_1, prostaglandin E_1; SDS, sodium dodecyl sulphate; P47, P24, P22 and P20 are phosphopolypeptides with apparent mol.wts. of 47000, 24000, 22000 and 20000 respectively.

peptides with similar mol.wts. (48000–40000 and 25000–19000) were phosphorylated, apparently by Ca^{2+}-dependent mechanisms, when platelets were treated with collagen or ionophore A23187 (Haslam & Lynham, 1977, 1978). Moreover, the latter studies showed that, in general, the increased phosphorylation of both of these classes of polypeptides correlated quantitatively with the extent of the platelet release reaction and was not observed when platelet aggregation occurred without release. PGE_1 was found to inhibit the increase in phosphorylation of both the higher- and lower-molecular-weight polypeptides caused by thrombin (Lyons *et al.*, 1975) and that of the higher-molecular-weight polypeptides caused by collagen (Haslam & Lynham, 1976, 1978). However, we found that PGE_1 did not appear to decrease the net incorporation of ^{32}P into the phosphopolypeptides of mol.wt. 25000–19000 observed in the presence of collagen and was itself able to increase the labelling of these polypeptides (Haslam & Lynham, 1978). This suggested that this segment of the gels might contain at least two different phosphopolypeptides, one phosphorylated by a Ca^{2+}-dependent mechanism and the other by a cyclic AMP-dependent mechanism. In the present study, we have used an improved method of SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) to resolve these platelet phosphopolypeptides and to investigate further the relationships between protein phosphorylation and platelet function. Some of these results have been reported briefly elsewhere (Haslam *et al.*, 1977, 1978*a*).

Experimental

Materials

Carrier-free $[^{32}P]P_i$ was obtained from NEN Canada Ltd., Lachine, Que., Canada, and 5-hydroxy[G-^{3}H]tryptamine (approx. 20Ci/mmol) from Amersham Corp., Oakville, Ont., Canada. Bovine serum albumin was from ICN Canada Ltd., Montreal, Que., Canada, and SDS from BDH Chemicals, Toronto, Ont., Canada. Acrylamide, *NN'*-methylenebisacrylamide, ammonium persulphate, *NNN'N'*-tetramethylethylenediamine, Coomassie Brilliant Blue R, Bromophenol Blue, heparin, Trizma (Tris) base, 4-methylumbelliferone and bovine tendon collagen were from Sigma Chemical Co., St. Louis, MO, U.S.A. Collagen fibres were prepared for use as described previously (Haslam *et al.*, 1975). Purified proteins used for calibration of discontinuous SDS/polyacrylamide gels with respect to molecular weight were also obtained from Sigma Chemical Co., except for brain tubulin, which was kindly supplied by Dr. R. Keates, Department of Biochemistry, McMaster University. Potato apyrase (activity 3μmol/min per mg of protein with 100μM-ADP at 37°C) was generously provided by Dr. J. F. Mustard, Department of Pathology, McMaster University. Ionophore A23187 was supplied by Dr. R. L. Hamill of Eli Lilly and Co., Indianapolis, IN, U.S.A., and PGE_1 by Dr. J. Pike of the Upjohn Co., Kalamazoo, MI, U.S.A. A stock neutral solution of 10mM-PGE_1 in 95% (v/v) ethanol was prepared. Other reagents were of analytical grade.

Preparation of suspensions of labelled platelets

Washed human platelets were prepared by the method of Mustard *et al.* (1972) as modified by Haslam & Lynham (1977). The platelets were first washed with phosphate-free Tyrode's solution containing heparin (50 units/ml), bovine serum albumin (0.35%, w/v) and apyrase (60μg/ml). They were then resuspended at about 3×10^9 platelets/ml in the same medium without heparin but containing 0.8mCi of carrier-free $[^{32}P]P_i$/ml and 1μM-5-hydroxy[^{3}H]tryptamine (approx. 20Ci/mmol). This suspension was incubated for 60min at 37°C, during which time 4–8% of the $[^{32}P]P_i$ and almost all of the 5-hydroxyl[^{3}H]tryptamine were taken up. The platelets were then washed in Tyrode's solution (with phosphate) containing added bovine serum albumin and apyrase as before and were finally resuspended at 4×10^8 platelets/ml in albumin-free Tyrode's solution containing 6μg of apyrase/ml. When only platelet aggregation and the release reaction were studied (e.g. Table 3), labelling with $[^{32}P]P_i$ was omitted.

Incubations

Samples of platelet suspension (0.9ml) with additions to a final volume of 1.0ml were stirred at 37°C in an apparatus for the turbidimetric measurement of platelet aggregation (Payton Associates Ltd., Scarborough, Ont., Canada). Aggregating agents or corresponding solvent were added after stirring the platelets for 1min in the presence or absence of PGE_1, and the incubations were then continued for up to 2min. In general, platelets were incubated with 50μg of collagen/ml for 2min and with 0.4μM-ionophore A23187 for 0.5min, as these conditions were previously found to give optimal increases in protein phosphorylation (Haslam & Lynham, 1977). PGE_1 and collagen were usually each added in 50μl of 0.154M-NaCl; ionophore A23187 was added in 2μl of dimethyl sulphoxide. The final incubation volume was always adjusted to 1.0ml with 0.154M-NaCl. In samples containing PGE_1, ethanol was present at a final concentration of 0.02% (v/v), and dimethyl sulphoxide was present at a final concentration of 0.2% (v/v) in experiments with ionophore A23187. Neither of these solvents had any effects on the parameters studied.

SDS/polyacrylamide-gel electrophoresis

In experiments in which protein phosphorylation was studied, incubations were carried out in duplicate; one was stopped by addition of 0.1 ml of 3M-$HClO_4$ for subsequent electrophoresis of platelet protein and one was used to determine release of 5-hydroxy[^{3}H]-tryptamine (see below). Protein precipitated with $HClO_4$ was isolated by centrifugation (1500*g* for 10min) and dissolved in 200μl of solution containing 3% (w/v) SDS, 0.0025% (w/v) Bromophenol Blue, 62mM-Tris/HCl, pH6.8, 6% (w/v) glycerol and 5% (v/v) mercaptoethanol. These samples were heated at 100°C for 2min. Precise volumes (usually 50μl, containing approx. 160μg of protein) were electrophoresed on cylindrical gels by using a modification of the method of Laemmli (1970). Separating gels (approx. 9cm long) contained 13% (w/v) acrylamide, 0.35% (w/v) *NN'*-methylenebisacrylamide, 0.1% (w/v) SDS, 0.025% (v/v) *NNN'N'*-tetramethylethylenediamine, 0.075% (w/v) ammonium persulphate, 0.1% (w/v) glycerol and 0.375M-Tris/HCl, pH8.8. Stacking gels (approx. 1cm long) contained 5% (w/v) acrylamide, 0.13% (w/v) *NN'*-methylenebisacrylamide, 0.1% (w/v) SDS, 0.038% (v/v) *NNN'N'*-tetramethylethylenediamine, 0.3% (w/v) ammonium persulphate, 0.05% (w/v) glycerol and 0.125M-Tris/HCl, pH6.8. The electrode buffer was as described by Laemmli (1970). Protein samples were electrophoresed at room temperature for 1h at 130mV followed by about 4h at 150mV. Gels were stained for 5–6h with 0.1% (w/v) Coomassie Brilliant Blue R in methanol/acetic acid/water (5:1:5, by vol.), destained for about 12h in methanol/acetic acid/water (5:1:5, by vol.), and then further destained in methanol/acetic acid/water (3:1:7, by vol.) for about 1 day.

Gels were scanned at 550nm with a Gilford spectrophotometer with a linear transport and were then frozen and divided into 1mm slices with a Bio-Rad gel slicer (model 190). Slices were placed in polythene vials containing 2ml of 0.01% (w/v) 4-methylumbelliferone in water and counted for Čerenkov radiation in a Beckman LS 230 scintillation counter (efficiency approx. 60%). The results were stored on Teletype tape and were superimposed on the densitometric scans of the corresponding gels by using a Hewlett–Packard tape reader (HP 9863A), calculator (HP 9810A) and plotter (HP 9862A). Corrections for background radioactivity, for decay of ^{32}P and for any discrepancies between the recorded lengths of gels and number of slices obtained were applied. The ^{32}P incorporated into a particular phosphopolypeptide was assumed to equal the sum of that found in all the slices corresponding to a particular peak of radioactivity. When a radioactive peak was not clearly defined, as in some control gels, the radioactivity was determined in the same number of slices from the polypeptide region in which stimulation was seen in other gels.

SDS/polyacrylamide gels were calibrated with respect to molecular weight by parallel electrophoresis of approx. 10μg of each of the following purified proteins: phosphorylase *a*, bovine serum albumin, brain tubulin, ovalbumin, lactate dehydrogenase, carboxypeptidase A, carbonic anhydrase, α-chymotrypsinogen A, trypsin and β-lactoglobulin. A plot of the negative logarithms of the mobilities of these proteins relative to the tracking dye ($-\log R_F$) against their molecular weights was linear (Neville, 1971). The average relative mobilities of platelet phosphopolypeptides that showed increased labelling on incubation of platelets with collagen, ionophore A23187 or PGE_1 were determined from a total of 16 different polyacrylamide gels. The apparent molecular weights of these phosphopolypeptides were then calculated from the linear regression of $-\log R_F$ against molecular weight for the above protein standards.

Measurement of the release reaction and of the extent of platelet aggregation

The release of ^{3}H from platelets in which granule 5-hydroxytryptamine had been labelled by preincubation of the platelets with 5-hydroxy[^{3}H]tryptamine was determined after mixing platelet suspension with 0.2vol. of 9% (w/v) paraformaldehyde to prevent subsequent release or re-uptake of 5-hydroxy-[^{3}H]tryptamine (Costa & Murphy, 1975). In the experiments in which phosphorylation of platelet proteins was studied, release of 5-hydroxy[^{3}H]tryptamine was determined in one of each pair of identical incubation mixtures at the end of the incubation. In other experiments, in which release of 5-hydroxy-[^{3}H]tryptamine was measured at more than one time interval (Table 3), 50μl samples of incubation mixture were withdrawn and mixed with paraformaldehyde solution. The formaldehyde-treated samples were centrifuged at 12000*g* for 30s in an Eppendorf Microcentrifuge and the supernatant was counted for ^{3}H radioactivity by liquid-scintillation spectrometry with an efficiency under dual-label counting conditions of about 16% (Haslam *et al.*, 1978*b*). ^{3}H counts were corrected for background radioactivity, for ^{32}P cross-over when ^{32}P-labelled platelets were used and for any ^{3}H present in the supernatant from control incubations. Released 5-hydroxy[^{3}H]tryptamine was expressed as a percentage of the total platelet-bound 5-hydroxy-[^{3}H]tryptamine. Lysis of platelets was monitored by measurement of the percentage of platelet lactate dehydrogenase released, by using the method of Bergmeyer *et al.* (1965). Aggregation was recorded as the decrease in absorbance (ΔA) of the stirred platelet suspension by using a potentiometric recorder

operated in a logarithmic mode (Photovolt Corp., New York, NY, U.S.A.).

Results

Effects of collagen and PGE_1 on the phosphorylation of platelet polypeptides

Discontinuous SDS/polyacrylamide-gel electrophoresis of platelet protein resolved at least 14 stainable polypeptides with molecular weights less than that of actin, the most abundant platelet protein, and even more with higher molecular weights (Fig. 1). Moreover, electrophoresis of protein from ^{32}P-labelled platelets demonstrated at least 16 different phosphopolypeptides present in widely different amounts and with mol.wts. ranging from 250000 to 18000 (Fig. 1*a*). When labelled platelets were exposed to collagen (Fig. 1*b*), there were, in addition to small non-specific increases in the phosphorylation of most platelet phosphopolypeptides, major increases in the phosphorylation of two polypeptides with apparent mol.wts. of 47000 (P47) and 20000 (P20), as determined by the electrophoresis of standard proteins (see the Experimental section). The radioactivity found in polypeptide P47 superimposed on a stained protein band that was close to but clearly separate from platelet actin. This protein had an electrophoretic mobility lower than that of actin, but higher than that of brain tubulin. In four experiments in which platelet suspension was stirred with 50μg of collagen/ml for

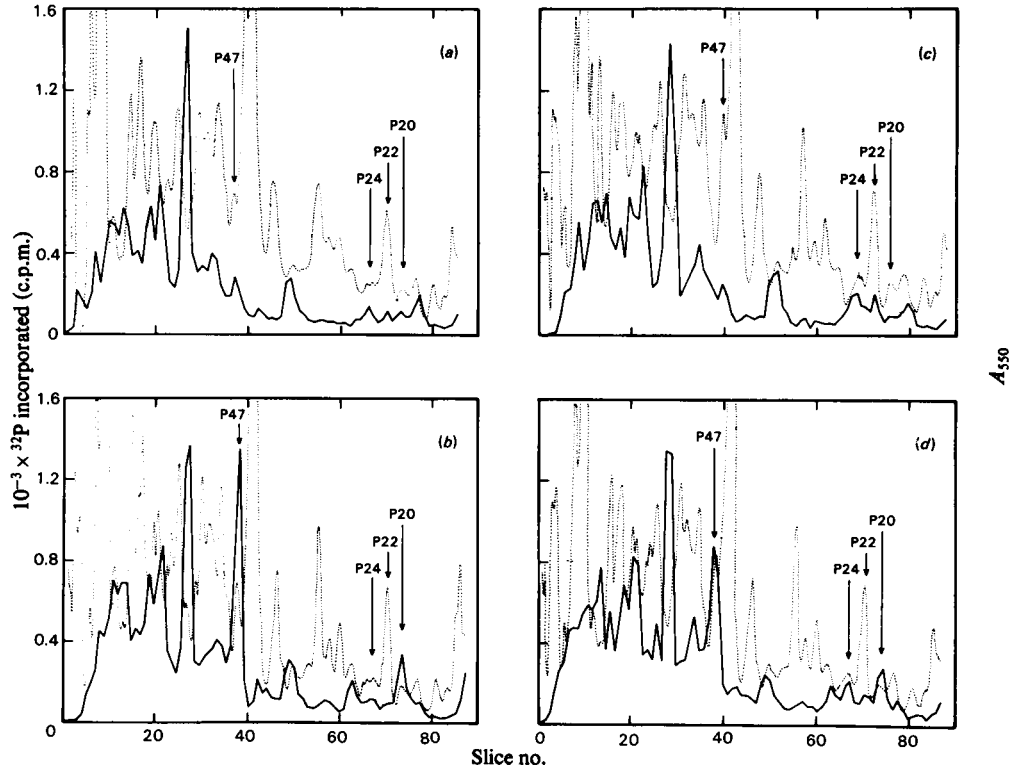

Fig. 1. *Densitometric scans of stained SDS/polyacrylamide gels with superimposed ^{32}P profiles showing the effects of collagen and PGE_1 separately or together on the phosphorylation of polypeptides in intact platelets*

Suspensions of human platelets that had been labelled with 5-hydroxy[^{3}H]tryptamine and [^{32}P]P_i were stirred with no additions other than 0.154M-NaCl (*a*, control), with 50μg of collagen/ml (*b*), with 2μM-PGE_1 (*c*) or with 2μM-PGE_1 and 50μg of collagen/ml (*d*). The total incubation time was 3min; PGE_1 was added at zero time and collagen after 1min. Platelet polypeptides were then separated by discontinuous SDS/polyacrylamide-gel electrophoresis and the gels were stained, sliced and counted for radioactivity. ^{32}P profiles were superimposed on densitometric scans as described in the Experimental section. Slices are numbered from the cathode buffer end of the gel. Those polypeptides that were selectively phosphorylated in the presence of collagen are designated P47 and P20, and those phosphorylated in the presence of PGE_1 are designated P24 and P22. In this experiment, collagen caused the release of 44 and 15% of the platelet 5-hydroxy[^{3}H]tryptamine in the absence and presence of PGE_1 respectively. ——, ^{32}P incorporated; ····, A_{550}.

2min, the increased phosphorylation of both polypeptide P47 and P20 was highly significant ($2P < 0.005$, paired t test), averaging 3.51-fold and 2.75-fold respectively, and was associated with a mean release of 41% of platelet 5-hydroxy[^{3}H]tryptamine (Table 1). In control gels from platelets that were not exposed to collagen, the segments including polypeptides P47 and P20 contained $4.6 \pm 0.5\%$ and $1.8 \pm 0.2\%$ respectively of the radioactivity found above the tracking dye (means ± S.E.M. from four experiments). In many experiments (e.g. Fig. 1*b*), selective increases in the radioactivity incorporated into minor phosphopolypeptides with apparent mol.wts. of 40000 and 27000 were also observed, whereas a decrease was noted in the incorporation of ^{32}P into a phosphopolypeptide with an apparent mol.wt. of 18000. Although detectable in the majority of experiments, these changes varied in extent and therefore did not reach statistical significance in the experiments on which Table 1 is based.

When ^{32}P-labelled platelets were incubated with 2μM-PGE_1 for 3min, selective increases were observed in the phosphorylation of polypeptides with apparent mol.wts. of 24000 (P24) and 22000 (P22) (Fig. 1*c*). These increases averaged 1.79-fold and 1.98-fold for polypeptides P24 and P22 respectively, and were statistically significant (Table 1). The specific effects of PGE_1 on the labelling of these phosphopolypeptides were substantially greater than the apparently non-specific effects observed with collagen (Table 1). In control gels from platelets that were not exposed to PGE_1 or collagen, the segments of the gels containing polypeptides P24 and P22 contained $2.6 \pm 0.2\%$ and $1.4 \pm 0.1\%$ respectively of the radioactivity found above the tracking dye (means ± S.E.M. from four experiments). In some experiments, PGE_1 also appeared to cause increased phosphorylation of a polypeptide with an apparent mol.wt. of 50000, but this change did not reach statistical significance in the experiments on which Table 1 is based.

Exposure of a suspension of platelets to both PGE_1 (2μM) and collagen (50μg/ml) (Fig. 1*d*) resulted in increased phosphorylation of the polypeptides that were phosphorylated in the presence of PGE_1 alone (P24 and P22) and also of those that were phosphorylated in the presence of collagen alone (P47 and P20). The increases in the phosphorylation of the latter polypeptides, however, were significantly lower in platelets exposed to both PGE_1 and collagen than in platelets exposed to collagen alone (Fig. 1; Table 1; $2P < 0.02$, paired t test). This inhibition of the collagen-induced phosphorylation reactions by PGE_1 (by about 50%) was associated with an inhibition of the release of 5-hydroxy[^{3}H]tryptamine of $71 \pm 4\%$ (mean of values from four individual experiments ± S.E.M.).

Effects of ionophore A23187 and PGE_1 on the phosphorylation of platelet polypeptides

Incubation of ^{32}P-labelled platelets with 0.4μM-ionophore A23187 for 0.5min (Fig. 2*b*; Table 2) had the same effects on the phosphorylation of platelet polypeptides as exposure to collagen at 50μg/ml for 2min (Table 1). In a total of five experiments, phosphorylation of polypeptides P47 and P20 increased by an average of 3.43-fold and 2.97-fold respectively, and the mean release of platelet 5-hydroxy[^{3}H]-tryptamine was 46%. Although exposure of ^{32}P-labelled platelets to 2μM-PGE_1 for 1.5min (Fig. 2*c*; Table 2) increased the phosphorylation of polypeptide P24 to the same extent as after 3min (Table 1), a significantly smaller increase (1.37-fold) in the phosphorylation of polypeptide P22 was observed ($2P < 0.01$; Student's t test). In the presence of both PGE_1 and ionophore A23187, significant increases in the phosphorylation of polypeptides P47, P24, P22 and P20 occurred (Fig. 2*d*; Table 2). However, in contrast with the effect of PGE_1 on the collagen-induced phosphorylation reactions, PGE_1 did not decrease the phosphorylation of polypeptides P47 and P20 induced by ionophore A23187, despite inhibiting

Table 1. *Effects of collagen and PGE_1 on the phosphorylation of platelet polypeptides*

Samples of platelet suspension that had been labelled with 5-hydroxy[^{3}H]tryptamine and [^{32}P]P_i were stirred with no addition other than 0.154M-NaCl (control), with 50μg of collagen/ml, with 2μM-PGE_1 or with 2μM-PGE_1 and 50μg of collagen/ml. The total incubation time was 3min; PGE_1 was added at zero time and collagen after 1min. Release of 5-hydroxy[^{3}H]tryptamine was measured and the phosphopolypeptides were separated by discontinuous SDS/polyacrylamide-gel electrophoresis. The incorporation of ^{32}P into each phosphopolypeptide peak was expressed as its ratio relative to the ^{32}P in the corresponding region of the gel from the control incubation in the same experiment. Values are means ± S.E.M. from four different experiments. The statistical significance of changes in phosphorylation relative to the controls was calculated by a paired t test; * $2P < 0.05$; ** $2P < 0.005$.

Additions	^{32}P incorporated (ratio relative to control)				Release of 5-hydroxy[^{3}H]tryptamine (%)
	P47	P24	P22	P20	
Collagen	3.51 ± 0.03**	1.22 ± 0.09	1.33 ± 0.06*	2.75 ± 0.23**	41 ± 8
PGE_1	1.17 ± 0.11	1.79 ± 0.13*	1.98 ± 0.10**	0.96 ± 0.20	—
PGE_1+collagen	2.44 ± 0.17**	1.59 ± 0.17*	1.74 ± 0.07**	1.88 ± 0.13*	12 ± 3

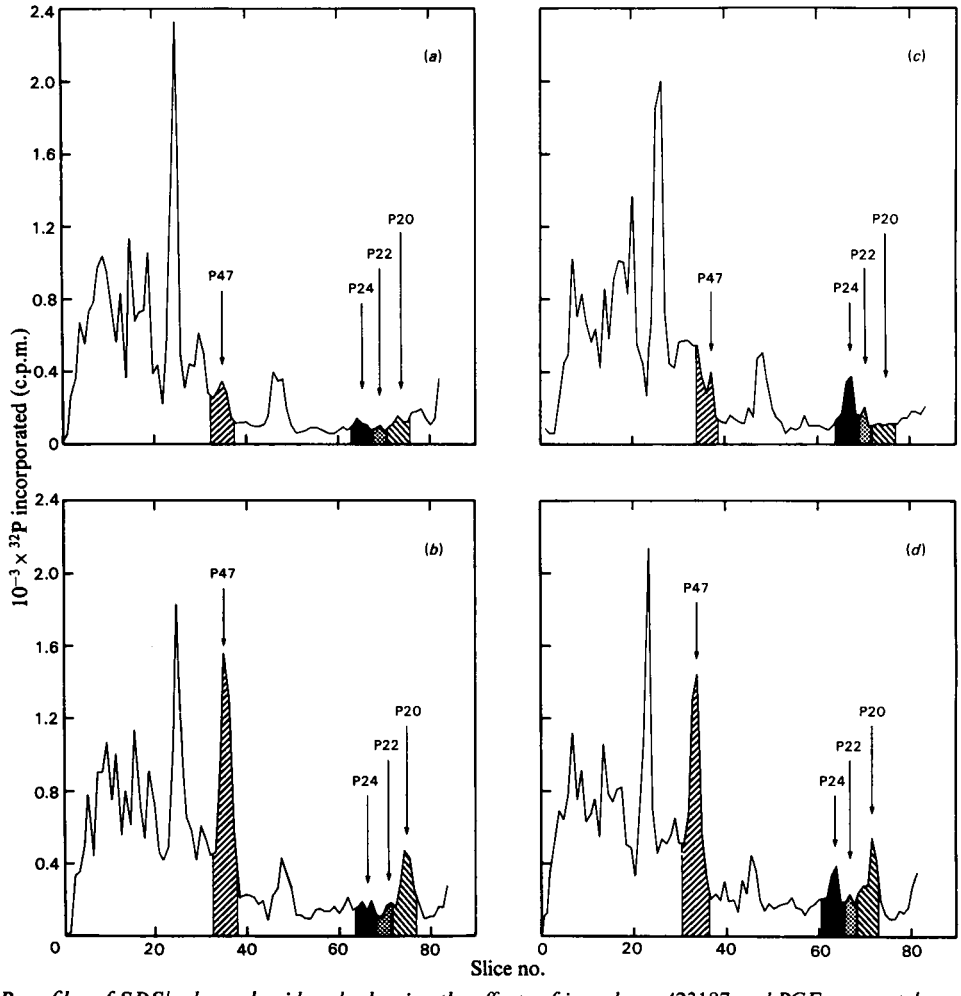

Fig. 2. *^{32}P profiles of SDS/polyacrylamide gels showing the effects of ionophore A23187 and PGE_1 separately or together on the phosphorylation of polypeptides in intact platelets*

Suspensions of human platelets that had been labelled with 5-hydroxy[^{3}H]tryptamine and [^{32}P]P_i were stirred with no additions other than 0.154M-NaCl and dimethyl sulphoxide (*a*, control), with 0.4μM-ionophore A23187 (*b*), with 2μM-PGE_1 (*c*) or with 2μM-PGE_1 and 0.4μM-ionophore A23187 (*d*). The total incubation time was 1.5min; PGE_1 was added at zero time and ionophore A23187 after 1 min. Platelet polypeptides were then separated by discontinuous SDS/polyacrylamide-gel electrophoresis and the gels were stained, sliced and counted for radioactivity. ^{32}P profiles were superimposed on densitometric scans as described in the Experimental section, but for clarity only the former are shown. Slices are numbered from the cathode buffer end of the gel. Those polypeptides that were selectively phosphorylated in the presence of ionophore A23187 are indicated and are designated P47 and P20, whereas those that were phosphorylated in the presence of PGE_1 are designated P24 and P22. The shaded areas indicate the slices included in calculations of the ^{32}P incorporated into these polypeptides (e.g. in Table 2). In this experiment, ionophore A23187 caused the release of 17 and 12% of the platelet 5-hydroxy[^{3}H]tryptamine in the absence and presence of PGE_1 respectively.

the release reaction to a significant though variable extent (i.e. by 50±18%, mean of values from five individual experiments±S.E.M.; $2P<0.05$, paired *t* test). Further experiments showed that this variability was due to the fact that increases in the concentration of ionophore A23187 over a narrow range close to that used in the phosphorylation studies markedly decreased the effectiveness of PGE_1 as an inhibitor of the release reaction (Table 3). The highest concentrations of ionophore A23187 used in this study (0.6μM) caused release of only 2% of platelet lactate dehydrogenase, indicating that release of 5-hydroxy[^{3}H]tryptamine was not due to platelet lysis. Release of platelet 5-hydroxy[^{3}H]tryptamine

Table 2. *Effects of ionophore A23187 and PGE_1 on the phosphorylation of platelet polypeptides*

Samples of platelet suspension that had been labelled with 5-hydroxy[^{3}H]tryptamine and [^{32}P]P_i were stirred either with no additions other than 0.154M-NaCl and dimethyl sulphoxide (control), with 0.4μM-ionophore A23187, with 2μM-PGE_1, or with 2μM-PGE_1 and 0.4μM-ionophore A23187. The total incubation time was 1.5min; PGE_1 was added at zero time and ionophore A23187 after 1min. Release of 5-hydroxy[^{3}H]tryptamine was measured and the phosphopolypeptides were separated by discontinuous SDS/polyacrylamide-gel electrophoresis. The incorporation of ^{32}P into each phosphopolypeptide peak was expressed as its ratio relative to the ^{32}P in the corresponding region of the gel from the control incubation in the same experiment. Values are means ± S.E.M. from five different experiments. The statistical significance of changes in phosphorylation relative to the controls was calculated by a paired *t* test; * $2P<0.05$; ** $2P<0.005$.

Additions	^{32}P incorporated (ratio relative to control)				Release of 5-hydroxy[^{3}H]tryptamine (%)
	P47	P24	P22	P20	
Ionophore A23187	3.43±0.29**	1.25±0.14	1.18±0.18	2.97±0.17**	46±11
PGE_1	1.10±0.06	1.71±0.25*	1.37±0.12*	0.88±0.06	—
PGE_1+ionophore A23187	2.99±0.18**	1.99±0.26*	1.86±0.26*	3.03±0.17**	24±12

Table 3. *Effects of PGE_1 on the release of platelet 5-hydroxy[^{3}H]tryptamine and on platelet aggregation induced by different concentrations of ionophore A23187*

Suspensions of platelets labelled with 5-hydroxy[^{3}H]tryptamine were prepared and samples were stirred for 1min with or without PGE_1 before addition of ionophore A23187; final concentrations of these additions are given. Aggregation was recorded, and 50μl samples were removed after 0.5min and 2min for measurement of the release of 5-hydroxy-[^{3}H]tryptamine.

Expt.	Additions: Ionophore A23187 (μM)	Additions: PGE_1 (μM)	Release of 5-hydroxy[^{3}H]tryptamine (%): 0.5min	Release of 5-hydroxy[^{3}H]tryptamine (%): 2min	Extent of aggregation (ΔA): 0.5min	Extent of aggregation (ΔA): 2min
1	0.4	—	53	49	0.19	0.58
		2.0	3	3	0.01	0.05
	0.6	—	79	68	0.24	0.56
		2.0	68	73	0.24	0.37
2	0.3	—	16	18	0.26	0.73
		2.0	0	1	0.01	0.05
	0.4	—	63	61	0.38	0.71
		2.0	42	38	0.12	0.59

was no greater after 2min than after 0.5min exposure to ionophore A23187, whether PGE_1 was present or not, though aggregation continued to increase during this period (Table 3). Thus, regardless of the time at which release was measured, there was a lack of correlation between the effects of PGE_1 on ionophore A23187-induced release of 5-hydroxy[^{3}H]tryptamine and on ionophore A23187-induced phosphorylation of polypeptides P47 and P20.

Chemical linkage of the ^{32}P associated with platelet proteins

We have previously shown that, after extraction of phospholipid from acid-precipitated platelet material with chloroform/methanol (2:1, v/v), most of the remaining ^{32}P is recoverable as [^{32}P]P_i after prolonged alkaline hydrolysis (0.5M-NaOH for 8h at 37°C). This indicates that the latter ^{32}P was initially present as protein phosphate in the form of alkali-labile phosphoester (Haslam & Lynham, 1977). In the present study, extraction of acid-precipitated platelet material (from approx. 4×10^8 platelets) with 5ml of chloroform/methanol (2:1, v/v) for 90min at 0°C was shown not to affect the pattern of ^{32}P incorporation into polypeptides P47, P24, P22 and P20 or other phosphopolypeptides observed after SDS/polyacrylamide-gel electrophoresis of platelet protein, whether control platelets or platelets exposed to collagen or PGE_1 were used. This procedure did remove a large radioactive peak of ^{32}P-labelled phospholipid that migrated with the tracking dye. Incubation of acid-precipitated platelet material with 0.8M-hydroxylamine in 0.05M-sodium acetate buffer, pH5.4, for 10min at 30°C was also without effect on the labelling of the phosphopolypeptides studied, indicating that the ^{32}P they contain is not present in

acyl phosphate residues. However, after incubation of chloroform/methanol-extracted platelet material with 0.5M-NaOH for only 30min at 37°C, 56% of the ^{32}P present was solubilized. No peaks of ^{32}P incorporation into phosphopolypeptides identifiable as polypeptides P47, P24, P22 and P20 were observed after electrophoresis of the remaining acid-precipitable material, though loss of some protein by alkaline hydrolysis prevented unequivocal interpretation of these results.

Discussion

We have previously reported that exposure of intact platelets to collagen or ionophore A23187 results in increased phosphorylation of polypeptides with apparent mol.wts. of 48000–40000 and 25000–19000 (Haslam & Lynham, 1977). These effects correlated with secretion of 5-hydroxy[^{3}H]tryptamine from platelet granules rather than with platelet aggregation. The phosphorylation of 48000–40000-mol.wt. polypeptides was detected as increases in ^{32}P in the region of SDS/polyacrylamide gels that included platelet actin. Similarly, Lyons *et al.* (1975) found that the approx. 40000-mol.wt. polypeptide phosphorylated in platelets treated with thrombin co-electrophoresed with platelet actin. Use of discontinuous SDS/polyacrylamide-gel electrophoresis has now enabled us to separate a major phosphopolypeptide with an apparent mol.wt. of 47000 (P47) from actin. This phosphopolypeptide accounts for most of the ^{32}P radioactivity in the 48000–40000-mol.wt. range in stimulated platelets. Thus this study supports our earlier conclusion, based on partial purification of actin from stimulated ^{32}P-labelled platelets, that actin is not phosphorylated (Haslam & Lynham, 1977).

We have previously reported that, although most inhibitors of the release reaction blocked the increased phosphorylation of both 48000–40000- and 25000–19000-mol.wt. polypeptides caused by collagen, PGE_1 did not affect the net increase in labelling of those of lower molecular weight (Haslam & Lynham, 1978). As addition of PGE_1 by itself increased phosphorylation of the 25000–19000-mol.wt polypeptides, the results suggested that, whereas one or more of these polypeptides were phosphorylated in the presence of collagen, others were phosphorylated in the presence of PGE_1. By using discontinuous SDS/polyacrylamide-gel electrophoresis, we have now resolved three phosphopolypeptides in the 25000–19000-mol.wt. range, two of which (P24 and P22) were selectively phosphorylated during exposure of platelets to PGE_1 and one of which (P20) was selectively phosphorylated in the presence of collagen or ionophore A23187. It is possible that P20 is the 20000-mol.wt. light chain of platelet myosin, phosphorylation of which is known to increase the actin-activated adenosine triphosphatase activity of this protein (Adelstein & Conti, 1975). This view is supported by a report (Daniel *et al.*, 1977) that exposure of intact platelets to thrombin increases the phosphorylation of this polypeptide in myosin extracted from the platelets.

By using the methods described in this paper, smaller and less reproducible changes were seen in the phosphorylation of one or more other polypeptides with apparent mol.wts. of 50000, 40000, 27000 and 18000, depending on the stimulus used (see the Results section). However, preliminary studies using radioautography of slab gels have indicated that these changes do occur consistently (Fox *et al.*, 1978). In addition, Apitz-Castro *et al.* (1976, 1977) have reported that N^6,2′-*O*-dibutyryl cyclic AMP and PGE_1 increase phosphorylation of a high-molecular-weight platelet glycoprotein that does not penetrate the stacking gels and apparently contains acid-labile phosphate residues. This phosphoglycoprotein was not detected in the present experiments, in which only the separating gels were sliced, or in previous studies, in which stacking gels were not used (Haslam & Lynham, 1978), perhaps because of its lability under acid conditions. Investigation of the properties of the ^{32}P associated with the polypeptides described in the present paper indicated that the relevant segments of the gels did not contain ^{32}P in phospholipid or acyl phosphate residues. Since most of the protein-bound ^{32}P was present in alkali-labile phosphate residues, the phosphopolypeptides studied are probably serine or threonine phosphoesters.

Collagen and ionophore A23187 are each believed to induce the release of platelet granule contents via an increase in the concentration of Ca^{2+} in the platelet cytosol (Feinman & Detwiler, 1974; Massini & Lüscher, 1976; Charo *et al.*, 1976; Feinstein *et al.*, 1976; Le Breton *et al.*, 1976). Moreover, our previous evidence has suggested that the protein-phosphorylation reactions caused by inducers of the release reaction are also mediated by Ca^{2+} and could play a role in the release mechanism (Haslam & Lynham, 1977, 1978). Therefore, as PGE_1 inhibited both the release of platelet 5-hydroxy[^{3}H]tryptamine and the phosphorylation of platelet proteins caused by collagen, these actions of PGE_1 could both result ultimately from a diminished increase in the Ca^{2+} concentration in the cytosol. Since the immediate effect of PGE_1 responsible for inhibition of platelet function is to increase the formation of cyclic AMP in platelets (Haslam, 1975; Haslam *et al.*, 1978*a*,*b*) and since addition of cyclic AMP and platelet cyclic AMP-dependent protein kinase has been shown to increase the ATP-dependent uptake of Ca^{2+} by platelet membrane vesicles (Käser-Glanzmann *et al.*, 1977), it is probable that PGE_1 inhibits the release reaction at least in part by causing a cyclic AMP-dependent phosphorylation of platelet proteins that

stimulate the active transport of Ca^{2+} out of the platelet cytosol. If this hypothesis is correct, a close parallel may exist between the mechanisms of action of cyclic AMP in the platelet and in cardiac muscle, in which cyclic AMP is believed to enhance Ca^{2+} uptake into the sarcoplasmic reticulum by activating the phosphorylation of an approx. 22000-mol.wt. regulatory protein (LaRaia & Morkin, 1974; Tada *et al.*, 1974, 1975). It is likely that the increased phosphorylation of polypeptide P24 or P22 caused by PGE_1 in platelets relates to this proposed mechanism, as preliminary results indicate that these phosphopolypeptides are membrane-bound (Fox *et al.*, 1978). An additional effect of cyclic AMP on the entry of Ca^{2+} into the platelet cytosol is also conceivable.

PGE_1 did not inhibit the phosphorylation of polypeptides P47 and P20 induced by 0.4μM-ionophore A23187, although increased phosphorylation of polypeptides P24 and P22 was observed. This may be due to an ability of the ionophore to short-circuit cyclic AMP-dependent mechanisms for decreasing the concentration of Ca^{2+} in platelet cytosol. A similar explanation has been offered for the observation that PGE_1 was relatively ineffective in inhibiting the activation of platelet phospholipase A_2 by ionophore A23187, although it readily inhibited the activation of this enzyme by thrombin or collagen (Feinstein *et al.*, 1977). However, it is still necessary to explain how PGE_1 was able to inhibit the release of 5-hydroxy[^{3}H]tryptamine caused by 0.4μM-ionophore A23187 without affecting the phosphorylation of polypeptides P47 and P20. This is the first experimental situation that we have encountered in which the release of platelet 5-hydroxy[^{3}H]tryptamine and increased phosphorylation of polypeptides P47 and P20 were not closely correlated and implies that, even if the effect of PGE_1 on the phosphorylation of these polypeptides limits the extent of the release reaction when collagen is the stimulus, other factors must be limiting when ionophore A23187 is used. It is possible that PGE_1 may exert additional inhibitory effects via cyclic AMP on mechanisms facilitating the release process that operate distally to or in parallel with the phosphorylation of polypeptides P47 and P20.

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