Subcellular Distribution of the Different Platelet Proteins Phosphorylated on Exposure of Intact Platelets to Ionophore A23187 or to Prostaglandin E₁

POSSIBLE ROLE OF A MEMBRANE PHOSPHOPOLYPEPTIDE IN THE REGULATION OF CALCIUM-ION TRANSPORT

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Exposure of ³²P-labelled human platelets to ionophore A23187 results in an increased incorporation of ³²P into polypeptides with apparent mol.wts. of 47000 (P47) and 20000 (P20), whereas exposure to prostaglandin E₁ results in increased labelling of polypeptides with apparent mol.wts. of 24000 (P24) and 22000 (P22) [Haslam, Lynham & Fox (1979) *Biochem. J.* 178, 397–406]. Labelled platelets that had been incubated with ionophore A23187 or prostaglandin E₁ were sonicated and rapidly separated into three fractions by differential centrifugation. Electron microscopy and measurement of marker enzymes indicated that the 1300–19000g_{av.} particulate fraction was enriched in granules, mitochondria and plasma membranes, that the 19000–90000g_{av.} particulate fraction was enriched in both intracellular and plasma membranes and that the 90000g_{av.} supernatant contained only soluble proteins. ³²P-labelled phosphopolypeptide P47 was present almost exclusively in the 90000g_{av.} supernatant, whereas phosphopolypeptide P20 was largely dephosphorylated under fractionation conditions that protected other phosphopolypeptides. ³²P-labelled phosphopolypeptide P24 was enriched in both particulate fractions, but particularly in the 19000–90000g_{av.} fraction, and may therefore be present in both the intracellular and plasma membranes. Phosphopolypeptide P22 appeared to be similarly distributed. Both particulate fractions were capable of the ATP-dependent oxalate-stimulated uptake of Ca²⁺. When the 19000–90000g_{av.} membrane fraction was prepared from platelets that had been incubated with ionophore A23187, active uptake of Ca²⁺ did not occur, but when this fraction was isolated from platelets that had been exposed to prostaglandin E₁, uptake of Ca²⁺ was significantly greater than observed with the corresponding membranes from control platelets. It is suggested that phosphorylation of polypeptide P24 (or P22) by a cyclic AMP-dependent protein kinase may promote the active transport of Ca²⁺ out of the platelet cytosol.

Exposure of human platelets that have been pre-incubated with [³²P]P_i to collagen or the bivalent-ionophore A23187 results in increased incorporation of ³²P into polypeptides with apparent mol.wts. of 47000 (P47) and 20000 (P20), as determined by SDS/polyacrylamide-gel electrophoresis (Haslam *et al.*, 1979). Similar observations have been reported with thrombin as the stimulus (Lyons *et al.*, 1975; Haslam & Lynham, 1977). These phosphorylation reactions were closely associated with the secretion of platelet granule constituents, such as 5-hydroxytryptamine (the platelet-release reaction) (Haslam & Lynham, 1977) and were apparently mediated, directly or indirectly, by an increase in the

concentration of Ca²⁺ in the platelet cytosol (Haslam & Lynham, 1978). Treatment of platelets with PGE₁, an agent that increases the cyclic AMP concentration in platelets, inhibited both the collagen-induced phosphorylation of polypeptides P47 and P20 and the release reaction (Haslam *et al.*, 1979). Moreover, the addition of PGE₁ alone resulted in the phosphorylation of two other polypeptides with apparent mol.wts. of 24000 (P24) and 22000 (P22). We have suggested that the cyclic AMP-dependent phosphorylation of polypeptide P24 or polypeptide P22 may initiate processes leading to a decrease in cytosol Ca²⁺ concentration (Haslam *et al.*, 1979), for example by stimulating the active transport of Ca²⁺ across platelet membranes (Käser-Glanzmann *et al.*, 1977), and by this mechanism may secondarily inhibit phosphorylation of polypeptides P47 and P20 and the release reaction. To investigate the possible roles of polypeptides P47 and P20 in the platelet release

Abbreviations used: P47, P24, P22 and P20 are phosphopolypeptides with apparent mol.wts. of 47000, 24000, 22000 and 20000 respectively; SDS, sodium dodecyl sulphate; PGE₁, prostaglandin E₁; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

reaction and of polypeptides P24 and P22 in inhibiting this reaction, we have now studied the subcellular distribution of these polypeptides after they have been labelled with ^{32}P in intact platelets. We have also investigated the relationship between phosphorylation of membrane-bound polypeptides in intact platelets and the active transport of Ca^{2+} . Some of the results have been reported briefly elsewhere (Fox *et al.*, 1978).

Experimental

Materials

Carrier-free $^{32}\text{P}\text{P}_i$, carrier-free Na^{125}I and $^{45}\text{CaCl}_2$ (15–30 Ci/g) were obtained from NEN (Canada), Lachine, Que., Canada. 5-Hydroxy-[G- ^3H]tryptamine (sp. radioactivity approx. 20 Ci/mmol) and liquid scintillation 'cocktail' (ACS) were from Amersham Corp., Oakville, Ont., Canada. Reagents for preparation of suspensions of washed platelets and for SDS/polyacrylamide-gel electrophoresis of platelet proteins were obtained from sources given previously (Haslam *et al.*, 1979). Chloromethyl *N*- α -*p*-tosyl-L-lysyl ketone hydrochloride, EGTA, Hepes buffer, cytochrome *c*, *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide, lactoperoxidase, Tris/ATP, NADH, protein standard solution, aprotinin and leupeptin were from Sigma Chemical Co., St. Louis, MO, U.S.A. Other reagents were of analytical grade. Ionophore A23187 was a gift from Dr. R. L. Hamill, Eli Lilly and Co., Indianapolis, IN, U.S.A., and PGE_1 was a gift from Dr. J. Pike, Upjohn Company, Kalamazoo, MI, U.S.A. A stock neutral solution of 5 mM- PGE_1 in 50% (v/v) ethanol was prepared.

Preparation of washed platelet suspensions

Suspensions of human platelets were labelled by preincubation with $^{32}\text{P}\text{P}_i$ and 5-hydroxy[^3H]tryptamine as described by Haslam & Lynham (1977), except that 10 mM-Hepes adjusted to pH 7.35 with NaOH was included in the modified Tyrode's solution used during the preincubation step. The platelets were finally resuspended at a concentration of $(6-7) \times 10^8/\text{ml}$ in Tyrode's solution containing 5 mM-EDTA; the resuspension medium was adjusted to a pH of 7.35 with NaOH.

Incubation of suspensions of ^{32}P -labelled platelets

Samples of platelet suspension (2 ml) were stirred at 37°C in silicone-treated glass tubes; 4 μl of 0.154 M-NaCl (control) or 4 μl of 1 mM- PGE_1 in 0.154 M-NaCl (final PGE_1 concentration, 2 μM) were added at zero time and stirring was continued for 2 min. When ionophore A23187 was studied, samples were first stirred for 1.5 min and 4 μl of dimethyl sulphoxide

containing ionophore was then added to give a final ionophore concentration of 0.4 μM ; stirring was then continued for a further 0.5 min. Incubations with PGE_1 contained ethanol at a final concentration of 0.02% (v/v). Neither dimethyl sulphoxide nor ethanol had any effects on phosphorylation of platelet proteins or on the platelet release reaction at the concentrations used (Haslam *et al.*, 1979). At the end of the incubations with these various additions, which were carried out in duplicate, a sample (50 μl) was immediately removed from one tube out of each pair for measurement of the release of platelet 5-hydroxy[^3H]tryptamine as described previously (Haslam *et al.*, 1979), whereas 200 μl was removed from the second tube out of each pair for analysis of protein phosphorylation. The remaining platelet suspension from each pair of duplicate incubations was pooled and rapidly cooled to 0°C by placing in a mixture of ice and salt at about -15°C. Sodium pyrophosphate solution (100 μl ; final concentration 5 mM) was then added to inhibit phosphoprotein phosphatases (Kato & Bishop, 1972) and the suspension was used immediately for the preparation of platelet subcellular fractions.

Preparation of subcellular fractions

Platelet suspension at 0°C was usually sonicated continuously for 60 s in a tube surrounded by ice by using a Branson Sonifier, model W-140D (Heat Systems-Ultrasonics Inc., Plainview, NY, U.S.A.), which was operated with a microtip and the output control set at 4.5. In some experiments, indicated in the Results section, the proteinase inhibitors aprotinin (0.3 trypsin units/ml) and leupeptin (1 $\mu\text{g}/\text{ml}$) were added to the platelet suspension before sonication, which was then carried out in four 15 s bursts with cooling to 0°C between bursts. Sonicated platelet suspensions were centrifuged at 1300 g_{av} for 5 min at 0°C to remove any intact platelets that remained. The supernatants were first centrifuged at 19000 g_{av} for 20 min at 4°C to obtain a 1300–19000 g_{av} particulate fraction (International B20 centrifuge; A-321 rotor) and then centrifuged at 90000 g_{av} for 60 min at 4°C to obtain a 19000–90000 g_{av} particulate fraction and a 90000 g_{av} supernatant (International B50 centrifuge, A-321 rotor).

Characterization of subcellular fractions

Washed platelets were prepared as described previously except that they were not labelled with ^{32}P . Subcellular fractions were isolated as above, but in the absence of sodium pyrophosphate. The particulate fractions were resuspended at 1–3 mg of protein/ml in Tyrode's solution containing 5 mM-EDTA, with a Dounce homogenizer fitted with a loose pestle. Marker enzymes assayed were cyto-

chrome *c* oxidase (Cooperstein & Lazarow, 1951), β -*N*-acetylglucosaminidase (Haslam *et al.*, 1975), glucose 6-phosphatase (Hübscher & West, 1965) and lactate dehydrogenase (Bergmeyer *et al.*, 1965). 5-Hydroxy[3 H]tryptamine, incorporated into the platelets during the washing procedure, was used to identify dense bodies.

Plasma membranes were identified by the presence of covalently bound 125 I, incorporated by lactoperoxidase-catalysed iodination of intact platelets (Phillips, 1972). Iodination was carried out at the end of the preincubation step using platelets that were otherwise unlabelled. To ensure complete removal of non-covalently bound 125 I, platelets were washed two additional times before sonication, using Tyrode's solution containing bovine serum albumin (0.35%, w/v) and 0.1 mM- $\text{Na}_2\text{S}_2\text{O}_3$. Moreover, the subcellular fractions obtained were extracted three times with 0.3 M- HClO_4 before they were dissolved in electrophoresis buffer (see below) and counted for 125 I in a Searle gamma counter (model 128S). Electrophoresis on SDS/polyacrylamide gels confirmed that all the radioactivity remaining was protein-bound.

Preparation of samples for electron microscopy

Samples were fixed overnight in 2.5% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4, containing 7.5% (w/v) sucrose, were postfixed for 1 h in 1% (w/v) OsO_4 in 0.1 M-sodium cacodylate buffer, pH 7.4, and were dehydrated in graded concentrations of ethanol. The specimens were embedded in Spurr's resin, sectioned and stained with uranyl acetate and lead citrate for examination under the electron microscope (Phillips EM 300). Details of the methods used are described by Glauert (1975).

Analysis of phosphorylated polypeptides by SDS/polyacrylamide-gel electrophoresis

Platelet particulate fractions were dissolved in 300 μ l of a solution containing 3% (w/v) SDS, 62 mM-Tris/HCl, pH 6.8, and 1 mM-chloromethyl *N*- α -*p*-tosyl-L-lysyl ketone. Platelet suspension or 90000 g_{av} supernatant (200 μ l) was prepared for electrophoresis by the addition of 35 μ l of 20% (w/v) SDS and 7.5 μ l of 2.0 M-Tris/HCl, pH 6.8, containing 32 mM-chloromethyl *N*- α -*p*-tosyl-L-lysyl ketone. Samples were immediately heated at 100°C for 2 min. Particulate fractions did not dissolve easily, so after heating they were sonicated and then left overnight at room temperature. Protein concentrations were determined and 2-mercaptoethanol was then added to a final concentration of 5% (v/v), glycerol to 6% (w/v) and Bromophenol Blue to 0.0025% (w/v). Samples were again heated at 100°C for 2 min and a precise amount of dissolved protein (usually 100 μ g) was analysed by

SDS/polyacrylamide-gel electrophoresis on cylindrical gels containing 13% (w/v) acrylamide (Haslam *et al.*, 1979). Gels were stained for protein, scanned at 550 nm, sliced, counted and 32 P profiles were plotted, all as described previously (Haslam & Lynham, 1977; Haslam *et al.*, 1979). The 32 P in individual phosphopolypeptides was calculated as the sum of the radioactivity in the slices containing a peak of radioactivity. When peaks of radioactivity were not apparent, as in some subcellular fractions, the radioactivity was determined in the same number of slices from the region of the gel in which a peak was seen in other subcellular fractions. The apparent molecular weights of platelet phosphopolypeptides were determined by comparison of their electrophoretic mobilities with those of standard proteins, as reported previously (Haslam *et al.*, 1979).

Measurement of $^{45}\text{Ca}^{2+}$ uptake

Particulate fractions, prepared from suspensions of unlabelled platelets, were resuspended at 1–4 mg of protein/ml in a solution containing 40 mM-Tris buffered to pH 6.8 with histidine hydrochloride, 5 mM- MgCl_2 , 100 mM-KCl and 5 mM-potassium oxalate, by using a Dounce homogenizer fitted with a tight pestle. Triplicate samples (20 μ l) were incubated at 25°C in a shaking water bath with 1.58 ml of medium containing 40 mM-Tris/histidine buffer, pH 6.8, 5 mM- MgCl_2 , 100 mM-KCl, 5 mM-potassium oxalate, 5 mM-Tris/ATP, 25.8 μ M- $^{45}\text{CaCl}_2$ (sp. radioactivity approx. 50 mCi/mmol) and 88.5 μ M-EGTA, in which the free Ca^{2+} concentration was 0.75 μ M (Katz *et al.*, 1970). At intervals, 0.35 ml portions were removed from each of the three incubation mixtures, filtered (Millipore type HA, 0.45 μ m pore size) and washed with 3 $\times$ 15 ml of ice-cold incubation medium lacking Tris/ATP and containing unlabelled CaCl_2 . Filters were dried in scintillation vials and then covered with 7.5 ml of liquid-scintillation 'cocktail', 1 ml of water was added and the vials were counted for ^{45}Ca with an efficiency of approx. 90%. The radioactivity of the $^{45}\text{CaCl}_2$ used was determined by counting a sample of the total incubation mixture dried on a Millipore filter.

Protein determinations

Protein was assayed by the method of Lowry *et al.* (1951), with a protein standard solution containing 5% (w/v) human albumin and 3% (w/v) human globulin. Buffer blanks were subtracted.

Results

Characterization of platelet subcellular fractions

Sonication of platelet suspensions resulted in disruption of about 95% of the platelets. After

removal of the remaining intact platelets, two particulate fractions and one supernatant fraction were isolated as described in the Experimental section; $20 \pm 2\%$ (mean $\pm$ S.E.M. from seven experiments) of the protein in these three fractions was in the 1300–19000 g_{av} . particulate fraction, whereas $10 \pm 2\%$ (mean $\pm$ S.E.M.) was in the 19000–90000 g_{av} . particulate fraction. Electron microscopy showed that the 1300–19000 g_{av} . particulate fraction was heterogeneous, containing mitochondria, α -granules, dense bodies and membrane fragments and vesicles (Plate 1a). The 19000–90000 g_{av} . particulate fraction was more homogeneous, containing only membrane vesicles of various sizes (Plate 1b). As shown in Table 1, cytochrome *c* oxidase, β -*N*-acetylglucosaminidase and 5-hydroxy[3H]tryptamine were significantly enriched relative to whole-platelet lysate only in the 1300–19000 g_{av} . particulate fraction, which is consistent with the presence of mitochondria and various granule types in this fraction. However, approx. 50% of the total 5-hydroxy[3H]tryptamine and β -*N*-acetylglucosaminidase were found in the 90000 g_{av} . supernatant, indicating that many dense bodies and lysosomes had been disrupted during the sonication and fractionation procedures. After lactoperoxidase-catalysed iodination of intact platelets, ^{125}I -labelled plasma membrane was recovered in both particulate fractions. Significant enrichment

ratios of 2.04 and 2.64 were obtained in the 1300–19000 g_{av} . and 19000–90000 g_{av} . fractions respectively (Table 1), but the difference between the two particulate fractions was not statistically significant (two-sided paired *t* test). Although $53 \pm 7\%$ (mean $\pm$ S.E.M. from four determinations) of the platelet glucose 6-phosphatase was found in the soluble fraction, the enzyme was significantly enriched in the 19000–90000 g_{av} . fraction (3.2-fold), suggesting that this fraction at least contains intracellular membranes (see the Discussion section). The enrichment ratios for glucose 6-phosphatase in both particulate fractions may be decreased by a factor of two as a result of the existence of the soluble glucose 6-phosphatase. The 1300–19000 g_{av} . particulate fraction as a whole contained approximately one-half as much glucose 6-phosphatase as the smaller 19000–90000 g_{av} . fraction. Lactate dehydrogenase was recovered almost entirely in the 90000 g_{av} . supernatant.

Distribution of phosphopolypeptides in subcellular fractions

SDS/polyacrylamide-gel electrophoresis was used to resolve the [^{32}P]phosphopolypeptides of intact platelets and of subcellular fractions prepared from them. When intact ^{32}P -labelled platelets suspended in Tyrode's solution containing EDTA were exposed

Table 1. *Characterization of subcellular fractions of sonicated platelets*

Subcellular fractions were prepared and measurements of enzymes and other components carried out as described in the Experimental section. The mean specific activities of enzymes in unfractionated platelet sonicated material were: cytochrome *c* oxidase, 0.079 min $^{-1}$ /mg of protein (20°C); β -*N*-acetylglucosaminidase, 13.5 nmol/min per mg of protein (30°C); glucose 6-phosphatase, 3.71 nmol/min per mg of protein (37°C); lactate dehydrogenase, 1.20 μ mol/min per mg of protein (20°C). The enrichment of an enzyme or another component in a subcellular fraction was calculated as the ratio of its specific activity in that subcellular fraction to its specific activity in the whole platelet sonicate from which it was prepared. Values given are mean ratios $\pm$ S.E.M. from three or four separate experiments. Significant enrichment or depletion of a component is indicated: **P* < 0.05 in a two-sided paired *t* test.

Subcellular fraction	Enrichment of component assayed (ratio relative to whole platelet sonicated material)					
	Cytochrome <i>c</i> oxidase	β - <i>N</i> -Acetyl- glucosaminidase	5-Hydroxy[3H]- tryptamine	Lactoperoxi- dase-catalysed ^{125}I incorporation	Glucose 6- phosphatase	Lactate dehydrogenase
1300–19000 g_{av} . particulate fraction	4.03 $\pm$ 0.64 (3)*	2.22 $\pm$ 0.18 (3)*	1.94 $\pm$ 0.11 (3)*	2.04 $\pm$ 0.22 (4)*	0.84 $\pm$ 0.21 (4)	0.15 $\pm$ 0.06 (3)*
19000–90000 g_{av} . particulate fraction	0.05 $\pm$ 0.05 (3)*	0.54 $\pm$ 0.08 (3)*	0.09 $\pm$ 0.02 (3)*	2.64 $\pm$ 0.34 (4)*	3.18 $\pm$ 0.62 (4)*	0.26 $\pm$ 0.06 (3)*
90000 g_{av} . supernatant	0.0 $\pm$ 0.0 (3)*	0.46 $\pm$ 0.09 (3)*	0.83 $\pm$ 0.15 (3)	0.23 $\pm$ 0.04 (4)*	0.55 $\pm$ 0.11 (4)*	1.49 $\pm$ 0.15 (3)

EXPLANATION OF PLATE I

Electron micrographs of particulate fractions from platelets

Subcellular fractions of human platelets were prepared and processed for electron microscopy as described in the Experimental section. (a) 1300–19000 g_{av} . particulate fraction (the bar represents 1 μ m); (b) 19000–90000 g_{av} . particulate fraction (the bar represents 0.3 μ m).

to ionophore A23187 for 0.5 min, the amount of ^{32}P incorporated into two specific polypeptides, P47 and P20, was markedly increased relative to that in the same polypeptides in unstimulated platelets (5.3- and 2.4-fold respectively; Table 2). Analysis of subcellular fractions from stimulated platelets showed that $92 \pm 1\%$ (mean $\pm$ S.E.M.) of the ^{32}P -labelled polypeptide P47 recovered was found in the $90000g_{\text{av}}$ supernatant and that this phosphopolypeptide was significantly enriched in this fraction (Figs. 1a-1d; Table 2). Although some ^{32}P -labelled polypeptide

P47 was present in the $90000g_{\text{av}}$ supernatant from untreated platelets, there was too little ^{32}P incorporated for significant enrichment to be detected under these conditions. Most (approx. 86%) of the radioactivity present in polypeptide P47 in intact platelets was recovered after subcellular fractionation. In contrast, only about 23% of the ^{32}P present in polypeptide P20 in intact ionophore-treated platelets was recovered after fractionation; of this, an average of 73% was in the $90000g_{\text{av}}$ supernatant and the remainder was distributed between the two particulate

Table 2. *Effects of exposure of intact platelets to ionophore A23187 or PGE_1 on the phosphorylation of platelet polypeptides in subcellular fractions prepared from them*

Samples of platelet suspension that had been labelled with [^{32}P]P_i were stirred either with no additions other than 0.154 M-NaCl, with $0.4 \mu\text{M}$ -ionophore A23187 or with $2 \mu\text{M}$ - PGE_1 . The total incubation time was 2 min; PGE_1 was added at zero time and ionophore after 1.5 min. Release of platelet 5-hydroxy[^3H]tryptamine by ionophore A23187 ranged from 22 to 66% in these experiments. Subcellular fractions were prepared and polypeptides were separated by SDS/polyacrylamide-gel electrophoresis as described in the Experimental section. The incorporation of ^{32}P into each phosphopolypeptide peak affected by ionophore A23187 or PGE_1 was expressed as a ratio relative to the ^{32}P in the corresponding region of the gel obtained by electrophoresis of the same amount of protein from the same fraction of control platelets. Values given are the mean ratios $\pm$ S.E.M. from five different experiments. The statistical significance of changes in phosphorylation relative to the controls was calculated by a two-sided paired *t* test ($*P < 0.05$). The enrichment of a phosphopolypeptide in a subcellular fraction was calculated as the ratio of the radioactivity in that phosphopolypeptide peak to the radioactivity in the corresponding peak obtained on electrophoresis of the same amount of protein from some of the intact platelets used to prepare the subcellular fraction. Values given are the mean ratios $\pm$ S.E.M. from the same five experiments; significant enrichment or depletion of phosphopolypeptides is shown: $*P < 0.05$ in a two-sided paired *t* test. The recovery of a [^{32}P]phosphopolypeptide in the total protein of all subcellular fractions was expressed as a percentage of that found in the intact platelets from which the fractions were prepared. These results (means $\pm$ S.E.M.) are based on four of the above five experiments, in which the distribution of total protein in the three fractions was measured.

		^{32}P incorporated into specific phosphopolypeptides (ratio relative to control platelets)			
Addition to incubation	Fraction analysed	P47	P24	P22	P20
Ionophore A23187	Intact platelets	$5.33 \pm 0.36^*$	$1.20 \pm 0.04^*$	1.14 ± 0.11	$2.38 \pm 0.26^*$
	1300–19000 g_{av} particulate fraction	$1.98 \pm 0.14^*$	—	—	$2.53 \pm 0.33^*$
	19000–90000 g_{av} particulate fraction	$1.62 \pm 0.09^*$	—	—	1.44 ± 0.16
	90000 g_{av} supernatant	$4.40 \pm 0.59^*$	—	—	0.90 ± 0.16
PGE_1	Intact platelets	0.98 ± 0.08	$1.81 \pm 0.13^*$	$1.27 \pm 0.06^*$	$0.76 \pm 0.03^*$
	1300–19000 g_{av} particulate fraction	—	$1.69 \pm 0.22^*$	$1.39 \pm 0.10^*$	—
	19000–90000 g_{av} particulate fraction	—	$1.82 \pm 0.22^*$	$1.48 \pm 0.15^*$	—
	90000 g_{av} supernatant	—	$1.38 \pm 0.13^*$	1.16 ± 0.12	—
		Enrichment of specific phosphopolypeptides (ratio relative to intact platelets)			
		P47	P24	P22	P20
Ionophore A23187	1300–19000 g_{av} particulate fraction	$0.27 \pm 0.02^*$	—	—	$0.25 \pm 0.02^*$
	19000–90000 g_{av} particulate fraction	$0.29 \pm 0.02^*$	—	—	$0.27 \pm 0.03^*$
	90000 g_{av} supernatant	$1.39 \pm 0.11^*$	—	—	$0.31 \pm 0.05^*$
PGE_1	1300–19000 g_{av} particulate fraction	—	$1.59 \pm 0.12^*$	0.96 ± 0.11	—
	19000–90000 g_{av} particulate fraction	—	$1.99 \pm 0.08^*$	1.21 ± 0.09	—
	90000 g_{av} supernatant	—	$0.48 \pm 0.05^*$	0.76 ± 0.09	—
		Recovery of specific phosphopolypeptides (%)			
		P47	P24	P22	P20
Ionophore A23187	All subcellular fractions combined	85.9 ± 5.2	—	—	23.4 ± 3.8
PGE_1	All subcellular fractions combined	—	76.7 ± 7.7	73.3 ± 8.9	—

Fig. 1. Subcellular distribution of [^{32}P]phosphopolypeptides present in ^{32}P -labelled platelets that have been exposed to ionophore A23187 or PGE_1 .

Samples of a platelet suspension that had been labelled with [^{32}P]P_i and 5-hydroxy[^3H]tryptamine were stirred either for 1.5 min with no addition followed by 0.5 min with $0.4\ \mu\text{M}$ -ionophore A23187 or for 2 min with $2\ \mu\text{M}$ - PGE_1 . In this experiment, ionophore A23187 caused the release of 56% of the platelet 5-hydroxy[^3H]tryptamine. Polypeptides of intact platelets and of subcellular fractions prepared from them were separated by SDS/polyacrylamide-gel electrophoresis; the same amount of protein was applied to each gel. The ^{32}P profiles of the gels are as follows: (a) and (e), intact platelets; (b) and (f), 1300–19000 g_{av} . particulate fractions; (c) and (g), 19000–90000 g_{av} . particulate fractions; (d) and (h), 90000 g_{av} . supernatants. (a), (b), (c) and (d) are derived from the ionophore-treated platelets, whereas (e), (f), (g) and (h) are from the PGE_1 -treated platelets. Those polypeptides that were selectively phosphorylated in the presence of ionophore A23187 are designated P47 and P20, whereas those phosphorylated in the presence of PGE_1 are designated P24 and P22. The segments of the gels containing these phosphopolypeptides are indicated by distinctive shading.

fractions (Table 2). However, because of the extensive dephosphorylation of polypeptide P20, no enrichment could be demonstrated in any fraction. Ionophore A23187 also caused small increases in the labelling of a minor phosphopolypeptide with an apparent

mol.wt. of 40000 (P40) in some experiments; this ^{32}P -labelled polypeptide was found in the particulate fractions from sonicated platelets.

When ^{32}P -labelled platelets were exposed to $2\ \mu\text{M}$ - PGE_1 for 2 min, the amount of ^{32}P in two

Table 3. Effect of exposure of intact platelets to PGE₁ on the uptake of ⁴⁵Ca²⁺ by platelet particulate fractions

Platelets were incubated with or without 2 μ M-PGE₁ for 2 min, after which they were sonicated for four periods of 15 s in the presence of aprotinin and leupeptin, as described in the Experimental section. The 1300–19000g_{av.} and 19000–90000g_{av.} particulate fractions were prepared and the uptake of ⁴⁵Ca²⁺ by these was measured after incubation for the times indicated. The values for ⁴⁵Ca²⁺ uptake given (means $\pm$ S.E.M.) were derived from either five separate experiments (1300–19000g_{av.} fraction) or six separate experiments (19000–90000g_{av.} fraction).

Particulate fraction used	Period of incubation with ⁴⁵ Ca ²⁺ (min)	⁴⁵ Ca ²⁺ uptake (nmol/mg of protein)		Increase in ⁴⁵ Ca ²⁺ uptake caused by PGE ₁ (%)
		Particulate fractions from control platelets	Particulate fractions from platelets treated with PGE ₁	
1300–19000g _{av.}	3	3.58 $\pm$ 0.40	3.74 $\pm$ 0.42	4
	6	6.56 $\pm$ 0.58	7.15 $\pm$ 0.86	9
	10	10.16 $\pm$ 1.15	10.84 $\pm$ 1.50	7
	15	14.35 $\pm$ 1.61	16.17 $\pm$ 2.50	13
19000–90000g _{av.}	3	2.44 $\pm$ 0.44	3.37 $\pm$ 0.39	38
	6	3.94 $\pm$ 0.63	5.35 $\pm$ 0.55	36
	10	6.37 $\pm$ 1.04	9.51 $\pm$ 1.10	49
	15	9.46 $\pm$ 1.59	13.82 $\pm$ 1.71	46

polypeptides, P24 and P22, increased significantly (Table 2). The larger increase (1.8-fold) was in polypeptide P24. Moreover, the total ³²P incorporated into polypeptide P24 after 2 min was 2–3-fold that incorporated into polypeptide P22, maximum labelling of which required longer incubations (Haslam *et al.*, 1979). Incubation of ³²P-labelled platelets with PGE₁ significantly increased the ³²P found in polypeptides P24 and P22 in both platelet particulate fractions (Table 2). The 1300–19000g_{av.} particulate fraction of ionophore-treated platelets also contained increased amounts of ³²P in polypeptides P24 and P22 (Fig. 1), but this was due to the loss of secreted granule proteins from this fraction of ionophore-treated platelets, which increased the specific radioactivities of many phosphopolypeptides. Analysis of subcellular fractions from PGE₁-treated platelets showed that the radioactivity in polypeptide P24 was not only enriched in both particulate fractions relative to intact platelets, but was depleted from the soluble fraction (Figs. 1e–1h; Table 2); its enrichment in the 19000–90000g_{av.} fraction (1.99-fold) was significantly greater than in the 1300–19000g_{av.} fraction (1.59-fold) ($P < 0.02$; two-sided paired *t* test). Polypeptide P22 also appeared to be present in the particulate fractions, though its enrichment in these was significant ($P < 0.05$; two-sided paired *t* test) only with respect to the soluble fraction and not with respect to intact platelets, probably because of some dephosphorylation (27%) during fractionation (Figs. 1e–1h; Table 2). In addition to its effect on the phosphorylation of polypeptides P24 and P22, PGE₁ also appeared to increase ³²P incorporation into a 50000-mol.wt. polypeptide in some experiments; this phosphopolypeptide was found in the 90000g_{av.} supernatant fraction of platelet sonicates.

Fractionation of platelets also revealed marked differences in the subcellular distribution of two major phosphopolypeptides that did not show changes in ³²P incorporation on exposure of platelets to either ionophore A23187 or PGE₁. A phosphopolypeptide with an apparent mol.wt. of 65000 was found predominantly in the 90000g_{av.} supernatant, whereas one with an apparent mol.wt. of 34000 was found exclusively in the particulate fractions (Fig. 1).

Active transport of ⁴⁵Ca²⁺ by platelet particulate fractions

Both particulate fractions took up ⁴⁵Ca²⁺ by mechanisms that were dependent on the presence of ATP, were stimulated by 5 mM-oxalate (100–300%) and were unaffected by 5 mM-NaN₃ (indicating that mitochondria were not involved). In eight experiments, the effect of treatment of the intact platelets with 0.4 μ M-ionophore A23187 for 0.5 min on the uptake of ⁴⁵Ca²⁺ by the 19000–90000g_{av.} particulate fraction was measured; in each the uptake of ⁴⁵Ca²⁺ was abolished (e.g. Fig. 2a). This may indicate either that ionophore A23187 was retained in the platelet membranes during fractionation or that it caused irreversible changes in the membranes that sequester Ca²⁺. The effect of exposure of platelets to 2 μ M-PGE₁ for 2 min on ⁴⁵Ca²⁺ uptake by the 19000–90000g_{av.} particulate fraction was measured over a number of different incubation times (2–15 min) in each of 15 different experiments (e.g. Fig. 2b). After transformation ($-1/\sqrt{x}$) of the results (*x*) to suppress the increase in variance with increasing incubation time, an unbalanced repeated measures analysis of variance (Winer, 1971; Searle, 1971) indicated that, despite considerable variability in the rates of ⁴⁵Ca²⁺ uptake from experiment to experiment, there was a

Fig. 2. Effect of exposure of intact platelets to ionophore A23187 or to PGE₁ on the uptake of $^{45}\text{Ca}^{2+}$ by membrane vesicles prepared from these platelets

Results from two separate experiments are shown. (a) $^{45}\text{Ca}^{2+}$ uptake by 19000-90000 g_{av} . particulate fractions from control platelets (●) and from the same platelet preparation after incubation with 0.4 μM -ionophore A23187 for 0.5 min (■); (b) $^{45}\text{Ca}^{2+}$ uptake by 19000-90000 g_{av} . particulate fractions from control platelets (●) and from the same platelet preparation after incubation with 2 μM -PGE₁ for 2 min (▲). Mean values $\pm$ S.E.M. from three determinations of $^{45}\text{Ca}^{2+}$ uptake after each of four periods of incubation with $^{45}\text{Ca}^{2+}$ are shown.

highly significant increase in the uptake of $^{45}\text{Ca}^{2+}$ by membranes from platelets that had been treated with PGE₁ ($P < 0.001$). The average increase was 48% in 6 min incubations of these membranes with $^{45}\text{Ca}^{2+}$. In contrast, comparable increases in $^{45}\text{Ca}^{2+}$ uptake were not observed with the 1300-19000 g_{av} . particulate fraction from PGE₁-treated platelets.

In later experiments (Table 3), an attempt was made to decrease the variability in the rates of $^{45}\text{Ca}^{2+}$ uptake by platelet particulate fractions by sonication of the platelets for four 15 s periods with cooling to 0°C in between, and by addition of proteinase inhibitors at the time of sonication (see the Experimental section). When balanced results (Table 3) obtained under these conditions were analysed by the same statistical methods as before, it was found

that although mean rates of $^{45}\text{Ca}^{2+}$ uptake by 19000-90000 g_{av} . particulate fractions were not significantly altered, the overall variation in the rates of $^{45}\text{Ca}^{2+}$ uptake was significantly decreased ($P < 0.05$). For example, the uptake of $^{45}\text{Ca}^{2+}$ by 19000-90000 g_{av} . particulate fractions in 6 min incubations was 4.97 ± 1.14 nmol/mg of protein (mean $\pm$ S.E.M. from 12 experiments) in the initial study and 3.94 ± 0.63 nmol/mg of protein (mean $\pm$ S.E.M. from six experiments) after using the modified sonication method (Table 3). Rates of $^{45}\text{Ca}^{2+}$ uptake by the 1300-19000 g_{av} . particulate fraction from untreated platelets were on average about 50% higher than observed with the corresponding 19000-90000 g_{av} . fraction, when the improved method was used (Table 3). Analysis of variance showed that, although there was no significant difference between the rates of $^{45}\text{Ca}^{2+}$ uptake by the 1300-19000 g_{av} . fractions from control and PGE₁-treated platelets, the rates of $^{45}\text{Ca}^{2+}$ uptake by the 19000-90000 g_{av} . fraction from PGE₁-treated platelets were, as in the previous study, significantly higher than observed with the same fraction from control platelets ($P < 0.05$). On average, PGE₁ caused a 36-49% increase in rates of $^{45}\text{Ca}^{2+}$ uptake, depending on the incubation period (see Table 3).

Discussion

Method of subcellular fractionation

To study the subcellular distribution of the platelet phosphopolypeptides showing changes in ^{32}P incorporation that can be related to platelet function, we have carried out a rapid subcellular fractionation of ^{32}P -labelled platelets by sonication followed by differential centrifugation. Initial attempts to use the glycerol lysis method of rupturing platelets (Barber & Jamieson, 1970) were abandoned after it was found that centrifugation of platelets through a glycerol gradient increased the phosphorylation of platelet polypeptides, particularly P47 and P20. Sonication also has the advantage that membrane fractions isolated from sonicated platelets have been shown to take up $^{45}\text{Ca}^{2+}$ much more rapidly than platelet membranes prepared by other methods (Käser-Glanzmann *et al.*, 1978). In the present experiments, prior incubation of the platelets with ionophore A23187 or PGE₁ and sonication were carried out in the presence of EDTA for three reasons: first, to prevent aggregation of the platelets by the ionophore (while permitting the release reaction to occur) so that disruption of ionophore-treated and control platelets would be comparable; secondly, to decrease phosphoprotein phosphatase and protein kinase activities after sonication; thirdly, to inhibit Ca^{2+} -activated proteolysis of platelet proteins (Phillips & Jakábová, 1977). Pyrophosphate was also added to inhibit phosphatase activity (Kato & Bishop,

1972). These procedures enabled us to recover several [^{32}P]phosphopolypeptides in high yields.

Composition of subcellular fractions

Both the 1300–19000 $g_{av.}$ and 19000–90000 $g_{av.}$ particulate fractions contained plasma membranes, as indicated by the distribution of membranes that underwent lactoperoxidase-catalysed iodination. However, only the 19000–90000 $g_{av.}$ fraction appeared to be highly enriched in intracellular membranes, assuming that glucose 6-phosphatase is a marker for these membranes in the platelet. Other studies have indicated that although much of the platelet glucose 6-phosphatase is soluble (Harris & Crawford, 1973), the highest specific activity of this enzyme is found in a membrane fraction containing other presumed intracellular membrane markers, such as NADH-cytochrome *c* reductase and peroxidase (Broekman *et al.*, 1974; Cutler *et al.*, 1978). Thus, the 19000–90000 $g_{av.}$ particulate fraction probably consists of vesicles derived from the dense tubular membrane system of the platelet, together with the smaller of the plasma-membrane vesicles formed during sonication. Attempts by others to resolve these two membrane components by centrifugation on sucrose density gradients have not been successful (Käser-Glanzmann *et al.*, 1978). Although intact dense bodies, α -granules and lysosomes were recovered in the 1300–19000 $g_{av.}$ particulate fraction, we do not know which particulate fraction contained membranes of broken granules. The granule proteins of ^{32}P -labelled platelets probably do not contain ^{32}P , because of their sequestration in a different compartment from the metabolic pool of platelet ATP. It follows that release of granule proteins during sonication is unlikely to affect the subcellular distribution of ^{32}P -labelled phosphopolypeptides. However, we cannot rule out the possibility that sonication itself or an unphysiological component of the sonication medium (e.g. EDTA) may release membrane phosphopolypeptides into the supernatant fraction or cause some binding of soluble phosphopolypeptides by particulate fractions.

Subcellular distribution of polypeptides phosphorylated in the presence of ionophore A23187

In accord with a previous report from this laboratory (Haslam *et al.*, 1979), we have again found that stimulation of human platelets with ionophore A23187 at a concentration that causes release of platelet 5-hydroxytryptamine results in the selective phosphorylation of polypeptides with apparent mol.wts. of 47000 and 20000 (P47 and P20). However, in the experiments reported in the present paper, these phosphorylation reactions and the release of 5-hydroxytryptamine occurred in the presence of extracellular EDTA, indicating that these processes

are not dependent on aggregation or on extracellular Ca^{2+} . This is consistent with previous evidence that the phosphorylation of polypeptides P47 and P20 is mediated by an increase in cytosol Ca^{2+} derived from internal Ca^{2+} stores and is closely associated with the release of granule constituents (Haslam & Lynham, 1977, 1978).

Over 90% of the phosphopolypeptide P47 recovered was found in the 90000 $g_{av.}$ supernatant fraction of platelets in the present study. It has been reported that a polypeptide with a mol.wt. of approx. 40000 is phosphorylated during exposure of platelets to thrombin and that 80% of this phosphopolypeptide was recovered in a 200000 g supernatant prepared from the platelets (Lyons *et al.*, 1975; Lyons & Atherton, 1979). This phosphopolypeptide is presumably identical with polypeptide P47. Both Lyons & Atherton (1979) and ourselves (Haslam *et al.*, 1979) have found conditions of SDS/polyacrylamide-gel electrophoresis under which this phosphopolypeptide migrates more slowly than actin. Moreover, this polypeptide has extraction properties (Haslam & Lynham, 1977) and an amino acid composition (Lyons & Atherton, 1979) distinct from those of actin. The latter workers have also shown that their phosphorylated polypeptide is neither tubulin nor the regulatory subunit of type II cyclic AMP-dependent protein kinase. Our studies have suggested that the phosphorylation of polypeptide P47 may play a role in the platelet release reaction (Haslam & Lynham, 1977) and therefore it is noteworthy that secretion of histamine from mast cells has also been reported to be associated with an increased phosphorylation of various polypeptides, including one with an apparent mol.wt. of 42000 (Sieghart *et al.*, 1978).

Although most of the [^{32}P]phosphopolypeptide P47 was recovered after subcellular fractionation, only about 23% of the original radioactivity remained in polypeptide P20 at the end of the fractionation procedures, despite the presence of phosphatase inhibitors. Lyons & Atherton (1979) similarly found that a platelet polypeptide with an apparent mol.wt. of 20000 (presumably P20) that underwent phosphorylation on exposure of the platelets to thrombin was partially dephosphorylated during subcellular fractionation, despite attempts to inhibit phosphatase activity. These workers found 55% of the residual radioactivity in the soluble fraction, whereas we found 73% in this fraction. These results suggest that dephosphorylation of polypeptide P20 is carried out by a different phosphatase from that acting on other platelet phosphopolypeptides. Polypeptide P20 may be identical with the P-light chain of platelet myosin, which has a mol.wt. of 20000 and has been reported to be phosphorylated during the exposure of intact platelets to thrombin (Daniel *et al.*, 1977). Platelet myosin is known to be phosphorylated by a protein

kinase and dephosphorylated by a phosphatase present in platelets (Daniel & Adelstein, 1976; Barylko *et al.*, 1977). As phosphorylation of the P-light chain of platelet myosin increases its actin-activated ATPase activity (Adelstein & Conti, 1975), it is possible that this reaction may trigger a contractile response involved in the platelet release reaction.

Subcellular distribution of polypeptides phosphorylated in the presence of PGE₁

The inhibitory effects of PGE₁ on platelet function result from an increase in the intracellular concentration of cyclic AMP and are probably mediated by activation of cyclic AMP-dependent protein kinases present in platelets (for review see Haslam *et al.*, 1978). As reported previously (Haslam *et al.*, 1979), brief exposure of intact platelets to PGE₁ increased the incorporation of ³²P into a polypeptide with an apparent mol.wt. of 24000 (P24) by up to 2-fold and also caused a smaller increase in the labelling of a minor phosphopolypeptide with an apparent mol.wt. of 22000 (P22). Because the increase in ³²P incorporation into polypeptide P22 was slower than into polypeptide P24, the phosphorylation of the latter is more likely to have a major role in the inhibition of platelet function, which is optimal after 0.5 min preincubation with PGE₁ (Haslam *et al.*, 1978). In the present study, we have shown that polypeptide P24 is membrane-bound. However, the evidence obtained does not permit us to assign this phosphopolypeptide specifically to either the plasma or intracellular membranes of the platelet, though it is unlikely to be present exclusively in the intracellular membranes (i.e. the dense tubular membrane system), as it was much more equally distributed between the two particulate fractions than glucose 6-phosphatase. The results suggested that the subcellular distribution of polypeptide P22 may be similar to that of polypeptide P24. Preliminary experiments with slab gel electrophoresis (Fox *et al.*, 1978) have confirmed this and also the presence in the 90000_{g_{av}} supernatant of a phosphopolypeptide with an apparent mol.wt. of 50000, increased labelling of which was sometimes detected after addition of PGE₁ in the present experiments.

Although studies by other workers (Steiner, 1975; Lyons *et al.*, 1975; Booyse *et al.*, 1976; Coquil *et al.*, 1977) have demonstrated the presence of cyclic AMP-dependent protein kinases and of substrates for these enzymes in platelet homogenates and membrane preparations, polypeptide P24 was not recognized as a major physiological substrate. Moreover, by using techniques similar to those described in the present paper, Lyons *et al.* (1975) were unable to detect cyclic AMP-dependent phosphorylation of platelet proteins in intact platelets, whereas Apitz-Castro *et al.* (1976) and Apitz-Castro & De Murciano (1978) reported only a cyclic

AMP-dependent phosphorylation of a high-molecular-weight membrane glycoprotein, which was not observed in the present or previous studies in this laboratory (Haslam & Lynham, 1978; Haslam *et al.*, 1979).

Possible relationship between the phosphorylation of polypeptide P24 (or P22) and the uptake of ⁴⁵Ca²⁺ by platelet particulate fractions

There is good evidence that cyclic AMP exerts its inhibitory effects on platelet function by suppressing a wide variety of Ca²⁺-dependent reactions that are necessary for normal platelet responses (Haslam *et al.*, 1978). For example, in intact platelets cyclic AMP inhibits the activation of both phosphorylase kinase (Chaiken *et al.*, 1975) and phospholipase A2 (Minkes *et al.*, 1977; Rittenhouse-Simmons & Deykin, 1978) by thrombin, as well as the phosphorylation of polypeptides P47 and P20 induced by thrombin or collagen (Lyons *et al.*, 1975; Haslam & Lynham, 1978; Haslam *et al.*, 1979). It is plausible to suggest that these effects may result from a decrease in the cytosol Ca²⁺ concentration or an inhibition of the effects of the Ca²⁺ ions. The existence of the former mechanism is supported by the ability of ionophore A23187 to overcome many of these inhibitory effects of increased platelet cyclic AMP (Chaiken *et al.*, 1975; Feinstein *et al.*, 1977; Rittenhouse-Simmons & Deykin, 1978; Haslam *et al.*, 1979).

Direct evidence of a mechanism by which cyclic AMP may decrease the cytosol Ca²⁺ concentration was obtained by Käser-Glanzmann *et al.* (1977), who observed that under certain conditions addition of cyclic AMP with cyclic AMP-dependent protein kinase to platelet membrane vesicles increased the initial rates of ATP-dependent uptake of ⁴⁵Ca²⁺, although the actual rates observed were, as in our experiments, very variable. The variation in rates of ⁴⁵Ca²⁺ uptake by supposedly identical platelet membrane preparations that we and others have encountered could be due to different degrees of damage during sonication of platelets, to variable extents of proteolysis and to differences in protein phosphorylation occurring during the ATP-dependent uptake of ⁴⁵Ca²⁺. Thus, in our later experiments less variation was observed when an attempt was made to control the first two of these factors. Phosphorylation of membrane protein was not demonstrated in the experiments of Käser-Glanzmann *et al.* (1977), but it seems possible that polypeptide P24 (or conceivably P22) could be the substrate of the added protein kinase mediating the increased uptake of ⁴⁵Ca²⁺. This hypothesis receives indirect support from studies on cardiac sarcoplasmic reticulum in which the cyclic AMP-dependent phosphorylation of an approx. 22000-mol.wt. polypeptide appears to stimulate the ATP-dependent uptake of ⁴⁵Ca²⁺ (Tada *et al.*, 1978). The results reported in the present paper

provide the first direct evidence linking phosphorylation of polypeptide P24 (or P22) with the uptake of $^{45}\text{Ca}^{2+}$ by platelet membranes. Thus, both particulate fractions studied were capable of the ATP-dependent oxalate-stimulated uptake of $^{45}\text{Ca}^{2+}$ and both contained these polypeptides. Moreover, treatment of intact platelets with PGE_1 increased both the phosphorylation of these polypeptides and the rate of $^{45}\text{Ca}^{2+}$ uptake by the isolated 19000–90000 g_{av} . particulate fraction. Although the latter observation strongly supports the view that phosphorylation of polypeptide P24 (or possibly P22) may promote the active transport of Ca^{2+} , a similar correlation was not obtained in experiments with the 1300–19000 g_{av} . particulate fraction. This apparent anomaly could result from a variety of causes; phosphorylation or dephosphorylation of membrane polypeptides may occur during the ATP-dependent uptake of $^{45}\text{Ca}^{2+}$, some membrane vesicles containing these phosphopolypeptides may be incompletely sealed (see Plate 1a) or have an incorrect orientation and, finally, other organelles (e.g. granules) or mechanisms could be responsible for much of the $^{45}\text{Ca}^{2+}$ uptake by the 1300–19000 g_{av} . particulate fraction. Thus, in general, our findings are consistent with the hypothesis that increases in platelet cyclic AMP inhibit platelet function at least in part through an increased phosphorylation of polypeptide P24 (or P22) and that this stimulates the sequestration of Ca^{2+} from the platelet cytosol.

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References

- Adelstein, R. S. & Conti, M. A. (1975) *Nature (London)* **256**, 597–598
- Apitz-Castro, R. & De Murciano, A. (1978) *Biochim. Biophys. Acta* **544**, 529–539
- Apitz-Castro, R., Ramírez, E., Maingon, R., De Murciano, A. & Ribbi, A. (1976) *Biochim. Biophys. Acta* **455**, 371–382
- Barber, A. J. & Jamieson, G. A. (1970) *J. Biol. Chem.* **245**, 6357–6365
- Barylko, B., Conti, M. A. & Adelstein, R. S. (1977) *Biophys. J.* **17**, 270a
- Bergmeyer, H.-U., Bernt, E. & Hess, B. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 736–741, Academic Press, New York
- Booyse, F. M., Marr, J., Yang, D. C., Guiliani, D. & Rafelson, M. E. (1976) *Biochim. Biophys. Acta* **422**, 60–72
- Broekman, M. J., Westmoreland, N. P. & Cohen, P. (1974) *J. Cell. Biol.* **60**, 507–519
- Chaiken, R., Pagano, D. & Detwiler, T. C. (1975) *Biochim. Biophys. Acta* **403**, 315–325
- Cooperstein, S. J. & Lazarow, A. (1951) *J. Biol. Chem.* **189**, 665–670
- Coquil, J.-F., Dupuis, M. & Hamet, P. (1977) *Union Méd. Can.* **106**, 457–464
- Cutler, L., Rodan, G. & Feinstein, M. B. (1978) *Biochim. Biophys. Acta* **542**, 357–371
- Daniel, J. L. & Adelstein, R. S. (1976) *Biochemistry* **15**, 2370–2377
- Daniel, J. L., Holmsen, H. & Adelstein, R. S. (1977) *Thromb. Haemostasis* **38**, 984–989
- Feinstein, M. B., Becker, E. L. & Fraser, C. (1977) *Prostaglandins* **14**, 1075–1093
- Fox, J. E. B., Lynham, J. A. & Haslam, R. J. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 799
- Glauert, A. M. (1975) *Fixation, Dehydration and Embedding of Biological Specimens*, American Elsevier Publishing Co., Inc., New York
- Harris, G. L. A. & Crawford, N. (1973) *Biochim. Biophys. Acta* **291**, 701–719
- Haslam, R. J. & Lynham, J. A. (1977) *Biochem. Biophys. Res. Commun.* **77**, 714–722
- Haslam, R. J. & Lynham, J. A. (1978) *Thromb. Res.* **12**, 619–628
- Haslam, R. J., Davidson, M. M. L. & McClenaghan, M. D. (1975) *Nature (London)* **253**, 455–457
- Haslam, R. J., Davidson, M. M. L., Davies, T., Lynham, J. A. & McClenaghan, M. D. (1978) *Adv. Cyclic Nucleotide Res.* **9**, 533–552
- Haslam, R. J., Lynham, J. A. & Fox, J. E. B. (1979) *Biochem. J.* **178**, 397–406
- Hübscher, G. & West, G. R. (1965) *Nature (London)* **205**, 799–800
- Käser-Glanzmann, R., Jakábová, M., George, J. N. & Lüscher, E. F. (1977) *Biochim. Biophys. Acta* **466**, 429–440
- Käser-Glanzmann, R., Jakábová, M., George, J. N. & Lüscher, E. F. (1978) *Biochim. Biophys. Acta* **512**, 1–12
- Kato, K. & Bishop, J. S. (1972) *J. Biol. Chem.* **247**, 7420–7429
- Katz, A. M., Repke, D. I., Upshaw, J. E. & Polascik, M. A. (1970) *Biochim. Biophys. Acta* **205**, 473–490
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lyons, R. M. & Atherton, R. M. (1979) *Biochemistry* **18**, 544–552
- Lyons, R. M., Stanford, N. & Majerus, P. W. (1975) *J. Clin. Invest.* **56**, 924–936
- Minkes, M., Stanford, N., Chi, M. M.-Y., Roth, G. J., Raz, A., Needleman, P. & Majerus, P. W. (1977) *J. Clin. Invest.* **59**, 449–454
- Phillips, D. R. (1972) *Biochemistry* **11**, 4582–4588
- Phillips, D. R. & Jakábová, M. (1977) *J. Biol. Chem.* **252**, 5602–5605
- Rittenhouse-Simmons, S. & Deykin, D. (1978) *Biochim. Biophys. Acta* **543**, 409–422
- Searle, S. R. (1971) *Linear Models*, J. Wiley and Sons, New York
- Sieghart, W., Theoharides, T. C., Alper, S. L., Douglas, W. W. & Greengard, P. (1978) *Nature (London)* **275**, 329–331
- Steiner, M. (1975) *Arch. Biochem. Biophys.* **171**, 245–254
- Tada, M., Ohmori, F., Kinoshita, N. & Abe, H. (1978) *Adv. Cyclic Nucleotide Res.* **9**, 355–369
- Winer, B. J. (1971) *Statistical Principles in Experimental Design*, 2nd ed., McGraw-Hill Book Co., New York