Biochem. J. (1984) **222**, 351–361
Printed in Great Britain

Potentiation by thrombin of the secretion of serotonin from permeabilized platelets equilibrated with Ca^{2+} buffers

Relationship to protein phosphorylation and diacylglycerol formation

Richard J. HASLAM and Monica M. L. DAVIDSON
Department of Pathology, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

(*Received 21 February 1984/Accepted 8 May 1984*)

After human platelets have been rendered permeable to small molecules by high voltage electric discharges, addition of buffered micromolar concentrations of Ca^{2+} causes an ATP-dependent secretion of dense granule serotonin [Knight & Scrutton (1980) *Thromb. Res.* **20**, 437–446]. In the present study, platelets permeabilized by this technique were found to show an up to 10-fold increase in their sensitivity to Ca^{2+} after exposure to thrombin. In permeabilized platelets, as in the intact cells, release of serotonin was associated with the Ca^{2+}-dependent phosphorylation of 47000 and 20000 Da polypeptides (P47 and P20). Thrombin markedly increased the phosphorylation of P47 in the presence of 0.1–1.0 μM-Ca^{2+}_{free} but had a much smaller effect on phosphorylation of P20. Thrombin also stimulated the formation of 1,2-diacylglycerol in the presence of 0.1 μM-Ca^{2+}_{free} and was even more effective with 1.0 μM-Ca^{2+}_{free}, suggesting that receptor-activated hydrolysis of phosphoinositides to 1,2-diacylglycerol was preserved in permeabilized platelets and was potentiated by low intracellular concentrations of Ca^{2+}. The increase in phosphorylation of P47 on addition of thrombin may therefore be accounted for by the stimulatory action of 1,2-diacylglycerol on Ca^{2+}-activated, phospholipid-dependent protein kinase. However, in both the presence and absence of thrombin, higher Ca^{2+} concentrations were required for optimal secretion than for maximal phosphorylation of both P47 and P20, indicating that additional actions of Ca^{2+} and thrombin, perhaps also mediated by 1,2-diacylglycerol formation, may be involved in the release of serotonin.

Until recently, the Ca^{2+} ion was widely perceived to be the intracellular messenger responsible for all the biochemical and functional responses that follow stimulation of blood platelets by thrombin and other aggregating agents (Feinstein, 1978; Lüscher *et al.*, 1980). The best evidence for this view was the ability of the Ca^{2+} ionophore, A23187, to reproduce most of the effects of physiological stimuli on platelets (Feinman & Detwiler, 1974; White *et al.*, 1974) and the observation that exogenous Ca^{2+} ions could release dense granule serotonin (5-hydroxytryptamine) from platelets permeabilized by high voltage electric discharges (Knight & Scrutton, 1980). However, as secretion of the lysosomal enzyme, β-*N*-acetylglucosaminidase, from permeabilized platelets occurred over the same range of Ca^{2+} concentrations as secretion of dense granule constituents, agonist selectivity with respect to the release of the contents of different platelet granules implies the existence of an additional mechanism of stimulus–response coupling (Knight *et al.*, 1982). Measurements of the concentration of cytosol Ca^{2+}, using the fluorescent indicator quin2, have also indicated that the effects of thrombin on platelets may not be mediated by Ca^{2+} ions alone in that a pathway of secretion exists that can function in the absence of any increase in intracellular Ca^{2+} (Rink *et al.*, 1982, 1983). In the present study, we have investigated this question using platelets permeabilized to Ca^{2+} buffers by the high-voltage discharge method (Knight &

Abbreviations used: P47 and P20, the 47000 and 20000 Da polypeptides that are phosphorylated in stimulated platelets; Pipes, 1,4-piperazinediethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; SDS, sodium dodecyl sulphate; pCa, $-\log[Ca^{2+}_{free}]$.

Scrutton, 1980; Knight, 1981) and have shown that thrombin greatly enhances the sensitivity of these preparations to Ca^{2+} ions.

A possible explanation for this effect is provided by the hypothesis of Nishizuka and colleagues (Kishimoto *et al.*, 1980; Kawahara *et al.*, 1980; Takai *et al.*, 1982), who have proposed that 1,2-diacylglycerol, formed as a result of the receptor-activated hydrolysis of phosphoinositides, may play a role in the activation of platelets and other cells. Thus, 1,2-diacylglycerol greatly increases the activity of Ca^{2+}-activated, phospholipid-dependent protein kinase at micromolar Ca^{2+} concentrations (Kishimoto *et al.*, 1980). This enzyme is present in high concentrations in platelets, in which its principal substrate is a 40000–47000 Da polypeptide (Kawahara *et al.*, 1980; Takai *et al.*, 1982; Sano *et al.*, 1983; Imaoka *et al.*, 1983). Earlier studies with intact platelets (Lyons *et al.*, 1975; Haslam & Lynham, 1977; Haslam *et al.*, 1979, 1980) showed that phosphorylation of this polypeptide (P47), as well as that of a 20000 Da polypeptide (P20), is closely associated with platelet degranulation. P20 has been identified as the P-light chain of platelet myosin (Daniel *et al.*, 1981) but, although native P47 has now been purified to homogeneity and characterized (Imaoka *et al.*, 1983), its precise function in platelets is still unknown. However, recent studies have suggested that phosphorylation of P47 may be necessary, though not sufficient, for secretion to occur. Thus, low concentrations of exogenous compounds that can substitute for endogenous 1,2-diacylglycerol, such as phorbol ester or 1-oleoyl-2-acetylglycerol, selectively stimulate the phosphorylation of P47, but only induce secretion of serotonin in the presence of concentrations of ionophore A23187 that cause selective phosphorylation of P20 (Yamanishi *et al.*, 1983; Kaibuchi *et al.*, 1983). For these reasons, we have investigated the effects of thrombin on the phosphorylation of P47 and P20 and on 1,2-diacylglycerol formation in permeabilized platelets.

Experimental

Materials

[*side chain*-2-^{14}C]Serotonin (55mCi/mmol), [γ-^{32}P]ATP (3000Ci/mmol) and ACS aqueous counting scintillant were from Amersham (Oakville, Ont., Canada). [2,8-^{3}H]Adenine (38Ci/mmol) was from ICN (Irvine, CA, U.S.A.) and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (87Ci/mmol) from New England Nuclear (Lachine, Que., Canada). EDTA, EGTA, ATP, Pipes, Hepes, heparin, glutamic acid and bovine serum albumin (fraction V) were purchased from Sigma (St. Louis, MO, U.S.A.). Human thrombin (3200 NIH units/mg of protein) was from Calbiochem–Behring (La Jolla, CA, U.S.A.). Highly purified $CaCl_2,4H_2O$ (Suprapur) was obtained from E. Merck (Darmstadt, Germany) and $MgCl_2,6H_2O$ (AnalaR) from BDH Chemicals (Toronto, Ont., Canada). Sepharose CL-4B was from Pharmacia (Canada) (Dorval, Que., Canada) and silica gel G (SIL G-25) t.l.c. plates from Brinkmann (Rexdale, Ont., Canada). Potato apyrase was prepared as described elsewhere (Molnar & Lorand, 1961).

Methods

Preparation of human platelets for exposure to high-voltage electric discharges. Blood was collected into ACD anticoagulant (Aster & Jandl, 1964) and centrifuged at 200*g* for 15min at room temperature to give platelet-rich plasma. After centrifugation of the latter at 2400*g* for 15min, the platelets were resuspended in one-third of the supernatant plasma. This platelet-enriched plasma [$(0.6–1.3)\times10^9$ platelets/ml] was incubated for up to 1h at 37°C with appropriate isotopically labelled compounds, as described below. The labelled platelets were isolated by centrifugation and resuspended at room temperature in Ca^{2+}-free Tyrode's solution containing 5mM-Pipes buffer, pH6.5, 0.35% (w/v) bovine serum albumin, 50units of heparin/ml, and apyrase (30μg/ml). This suspension was re-centrifuged and the platelets finally suspended at $(1–2)\times10^9$ platelets/ml in the same medium without heparin or apyrase.

Technique for permeabilizing platelets by high voltage electric discharges. The apparatus used was based on that described by Knight (1981). Platelet suspension (20–23°C), prepared as above, was mixed with 0.05 vol. of 100mM-EGTA (K^+ salt, pH6.5) and 2ml portions were placed in a chamber formed by 10cm^2 stainless steel electrodes 0.2cm apart. The suspension was then subjected to 10 electric discharges (1 discharge/s) from a 4.5μF capacitor charged at 3.0kV. The temperature of the suspension did not rise above 24°C. The permeabilized platelets were cooled to 4°C and isolated from the suspending medium by gel filtration at 4°C through a column of Sepharose CL-4B, which was eluted with a medium (pH7.4) that contained 12.5mM-$MgCl_2$ and the potassium salts of glutamic acid (160mM), Hepes (20mM), EGTA (2.5mM) and EDTA (2.5mM) (buffer A). Prior to use, columns were washed with 0.15 vol. of buffer A containing 10% (w/v) bovine serum albumin, followed by 4 vol. of buffer A. As soon as the platelets were eluted, they were diluted with buffer A containing sufficient ATP (Na^+ salt) to give a final ATP concentration of 5mM and the required platelet count (see later). This suspension was stored at 0°C and samples were used for up to 2h.

Measurement of the effects of $[Ca^{2+}_{free}]$ *and thrombin on release of* [^{14}C]*serotonin from permeabilized platelets.* In these experiments, 10ml of platelet-enriched plasma was incubated with one or two additions of 1μM-[^{14}C]serotonin to incorporate about 0.05μCi of ^{14}C/10^9 platelets. Permeabilized platelets were isolated as above and incubated in mixtures (100μl) containing 80μl of platelet suspension (usually 0.36×10^9 platelets/ml), 10μl of buffer A and 10μl of various concentrations of $CaCl_2$ in buffer A. The concentrations of $CaCl_2$ required to give appropriate pCa values were calculated as described by Fabiato & Fabiato (1979). The pH values of the $CaCl_2$ solutions were adjusted so that a final pH of 7.4 was obtained after mixing with the other additions. These mixtures were usually incubated for 15min at 0°C to permit equilibration of the Ca^{2+} buffers with the intracellular medium before transfer to 25°C and further incubation for up to 10min. Thrombin, if present, was added in 1 μl of buffer A at the time of transfer to 25°C. Incubations were terminated by addition of 5 vol. of 0.15M-NaCl containing 1.8% (w/v) paraformaldehyde and 6mM-EDTA, the mixtures were centrifuged at 12000*g* for 1.5min and the ^{14}C in the supernatants was counted in ACS scintillation fluid. Release of [^{14}C]serotonin was calculated as a percentage of the ^{14}C found in platelets in incubation mixtures lacking $CaCl_2$ and incubated at 0°C.

Measurement of the effects of $[Ca^{2+}_{free}]$ *and thrombin on protein phosphorylation in permeabilized platelets.* Platelets that had been labelled with [^{14}C]serotonin were permeabilized and isolated by gel filtration. [γ-^{32}P]ATP (200μCi/ml) was added to suspension containing unlabelled ATP 15min before 100μl incubation mixtures containing Ca^{2+} buffers were constituted as above. After incubation of these samples for 15min at 0°C and then at 25°C with or without thrombin, the reactions were terminated by addition of 0.5ml of 10% (w/v) trichloroacetic acid. The precipitated protein was analysed by discontinuous SDS/polyacrylamide-slab gel electrophoresis using 13% (w/v) acrylamide in the separating gel and labelled polypeptides were located by autoradiography (Imaoka *et al.*, 1983). Polypeptides P47 and P20 were cut out and counted for ^{32}P (Haslam & Lynham, 1977). Incorporation of ^{32}P was expressed as nmol/10^9 platelets. On many SDS/polyacrylamide slab gels, P20 was resolved into three components that we have previously designated P20a, P20b and P19 (Haslam *et al.*, 1980). As recent work in this laboratory has shown that these are different forms of the myosin P-light chain (R. J. Haslam & J. A. Lynham, unpublished work), the ^{32}P present in all three was counted and included under 'P20'.

Measurement of the effects of $[Ca^{2+}_{free}]$ *and thrombin on 1,2-diacylglycerol formation in permeabilized platelets.* The methods of Rittenhouse-Simmons (1979) were used with minor modifications. Platelet-enriched plasma (up to 44ml) was incubated for 45min at 37°C with 6nM-[^{3}H]arachidonic acid (0.5μCi/ml) and 1μM-[^{14}C]serotonin; 70–80% of the [^{3}H]arachidonic acid was incorporated. These platelets were washed and finally resuspended at about 2×10^9/ml before being permeabilized. After isolation of the platelets by gel filtration and addition of ATP, incubation mixtures with a final volume of 1ml containing $(0.4–0.8) \times 10^9$ platelets in appropriate Ca^{2+} buffers were constituted and equilibrated at 0°C for 15min, before final incubation at 25°C with or without thrombin. At the end of each incubation, 0.05ml of suspension was used for measurement of the release of [^{14}C]serotonin as above, and the lipids were extracted from the remainder (Bligh & Dyer, 1959). 1,2-Diacylglycerol was isolated by t.l.c. on silica gel G (system II; Rittenhouse-Simmons, 1979) and counted in ACS scintillation fluid.

Measurement of the effectiveness of high voltage electric discharges in permeabilizing platelets. In some experiments, platelet-enriched plasma was incubated for 1h with both 2μM-[^{3}H]adenine (10μCi/ml) and 1μM-[^{14}C]serotonin. Platelet and supernatant ^{3}H and ^{14}C were then measured both before and after exposure of the platelets to electric discharges and after their isolation by gel filtration. The release of ^{3}H-labelled adenine nucleotides and of [^{14}C]serotonin by these procedures were thus determined. Leakage of platelet lactate dehydrogenase, assayed according to Bergmeyer *et al.* (1965), was determined in some samples.

Results and discussion

Development of a method for preparing stable permeabilized platelets

The procedures followed by Knight & Scrutton (1980) for preparing permeabilized platelets were modified to remove compounds released from the platelet cytosol by the electric discharges and to obtain stable preparations that could be used in several sequential biochemical investigations (see the Experimental section). These objectives were achieved by gel filtration of the platelets through Sepharose CL-4B and by their subsequent storage at 0°C in the presence of ATP. In addition, leakage of [^{14}C]serotonin was much reduced by use of a glutamate medium buffered at pH7.4, rather than pH6.6, and by addition of ATP as soon as the platelets were eluted from the Sepharose column. As EGTA alone does not buffer Ca^{2+}_{free} over a pCa range of 6 to 4 at pH7.4, a system containing EGTA, EDTA and $MgCl_2$ that provided effective buffering from a pCa of 8 to 4 was used. The high

voltage discharge procedure selected (10 × 3 kV) released 80% of the ^{3}H-labelled adenine nucleotides from platelets labelled with [^{3}H]adenine, but <2% of the platelet [^{14}C]serotonin and <1% of platelet lactate dehydrogenase were liberated at this time. Although subsequent handling led to slight leakage of platelet ^{14}C and lactate dehydrogenase, the total present in supernatants from the final preparations of permeabilized platelets was always <5% of the platelet content. Part of the residual ^{3}H present in platelets that had been labelled with [^{3}H]adenine, probably protein-bound adenine nucleotide, appeared in the supernatant after addition of unlabelled ATP. No significant release of [^{14}C]serotonin (relative to controls at 0°C) was observed on incubation of these permeabilized platelets at 25°C in the absence of added Ca^{2+} (pCa >8 and probably about 9) or in the presence of a Ca^{2+} buffer giving a pCa of 7 (Fig. 1). However, as reported previously (Knight & Scrutton, 1980), 10–100-fold higher concentrations of Ca^{2+}_{free} caused release of [^{14}C]serotonin from the platelets. The optimal period of equilibration of the platelets with Ca^{2+} buffers was determined (Fig. 1). The release of [^{14}C]serotonin during 10min incubations at 25°C in the presence of Ca^{2+} buffers giving pCa values of 6 or 5 was increased by preincubation with the buffers at 0°C for up to 15min. Longer preincubation periods had no further effect at these pCa values, though at a pCa of 4 inhibition of secretion was observed, apparently as a result of Ca^{2+}-dependent proteolysis (R. J. Haslam & M. M. L. Davidson, unpublished work). These findings indicated that 15min at 0°C was sufficient for complete equilibration of the added Ca^{2+} buffers with the intracellular medium and this period was therefore used subsequently.

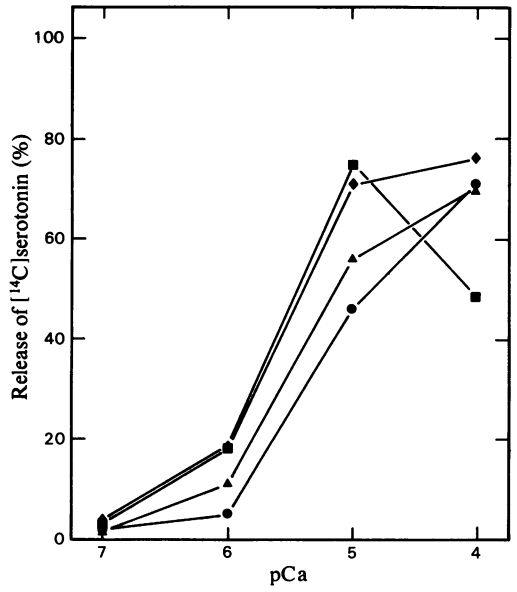

Fig. 1. *Effects of preincubation with Ca^{2+} buffers on the release of [^{14}C]serotonin from permeabilized platelets*
Samples of permeabilized platelets were incubated at 0°C with the Ca^{2+} buffers indicated for 0min (●), 8min (▲), 15min (♦) or 30min (■) before transfer to 25°C for 10min and measurement of the release of [^{14}C]serotonin.

Effect of thrombin on the release of [^{14}C]serotonin from permeabilized platelets

In the absence of thrombin, a maximum release of 80–90% of platelet [^{14}C]serotonin was observed at a pCa of 4.5, and 50% of platelet [^{14}C]serotonin was released at a pCa of 5.4–5.2 (about 5 μM-Ca^{2+}_{free}). Addition of 2 units of thrombin/ml caused a roughly one log unit (10-fold) shift to the left in this dose–response curve for Ca^{2+} (Fig. 2). Release of 50% of platelet [^{14}C]serotonin now required a pCa of only 6.6–6.2, though again no ^{14}C was liberated in the complete absence of added Ca^{2+}. The same effect of thrombin on the Ca^{2+} log dose–response curve was observed whether the platelets were equilibrated with Ca^{2+} buffers for 0, 8, 15 or 30min (results not shown). While this work was in progress, Knight & Scrutton (1983) briefly reported a similar leftward shift in the log dose–response curve for release of serotonin from permeabilized platelets on addition of thrombin. As the maximum release of [^{14}C]serotonin from permeabilized platelets was only slightly greater in the presence

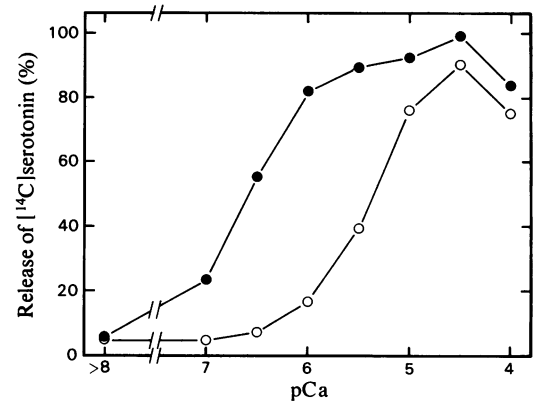

Fig. 2. *Potentiation by thrombin of the release of [^{14}C]serotonin from permeabilized platelets at different Ca^{2+}_{free} concentrations*
Samples of permeabilized platelets containing [^{14}C]serotonin were equilibrated with the Ca^{2+} buffers indicated for 15min at 0°C and then incubated for 10min at 25°C in the absence (○) or presence (●) of thrombin (2units/ml).

than in the absence of thrombin (Fig. 2), the action of this enzyme cannot be explained by the release of a separate pool of [^{14}C]serotonin that is not affected by Ca^{2+} alone. Thus, the results suggest that thrombin acts by enhancing the sensitivity of the permeabilized platelets to added Ca^{2+}. When intact platelets containing [^{14}C]serotonin were suspended in the same medium as the permeabilized platelets, thrombin released identical amounts of ^{14}C whether extracellular Ca^{2+} ions were present or not (Fig. 3*b*). This shows not only that the Ca^{2+} buffers used do not act at the external surface of the platelet but also that our preparations of permeabilized platelets contained no residual intact platelets in which mobilization of intracellular Ca^{2+} by thrombin could substitute for added Ca^{2+}. Although, in principle, thrombin might transiently increase the intracellular $[Ca^{2+}_{free}]$ above that in the added Ca^{2+} buffer, we found that use of a more concentrated Ca^{2+} buffer (7.5mM-EGTA, 7.5mM-EDTA and 17.5mM-$MgCl_2$) had no effect on the relationships between pCa and release of [^{14}C]serotonin observed with and without thrombin (results not shown). This suggests that thrombin potentiated the release of [^{14}C]serotonin by enhancing one or more of the actions of Ca^{2+} within the platelet.

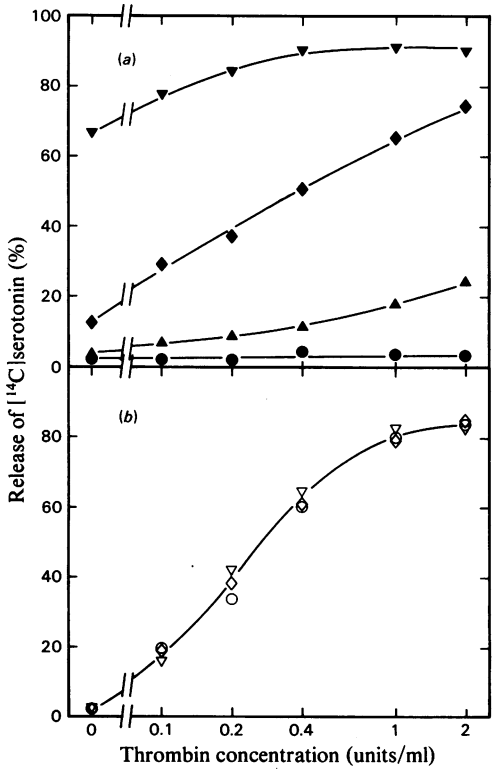

Fig. 3. *Effects of different thrombin concentrations on the release of* [^{14}*C*]*serotonin from permeabilized and intact platelets incubated with* Ca^{2+} *buffers*

(*a*) Results with permeabilized platelets prepared as described in the Experimental section. (*b*) Results with intact platelets transferred by gel filtration to the same medium as the permeabilized platelets (to which ATP was then added). Both permeabilized and intact platelets were incubated with Ca^{2+} buffers, first for 15min at 0°C without thrombin and then for a further 10min at 25°C with the indicated thrombin concentrations. Filled symbols, permeabilized platelets; open symbols, intact platelets; ● ○, pCa >8; ▲, pCa = 7; ♦ ◇, pCa = 6; ▼ ▽, pCa = 5. All values are means of duplicate determinations in the same experiment.

Investigation of the effects of different thrombin concentrations on the release of [^{14}C]serotonin from permeabilized platelets showed that the concentration causing half-maximal stimulation was inversely related to $[Ca^{2+}_{free}]$ (Fig. 3*a*). With a pCa of 6, at which the effect of thrombin was most pronounced, half-maximal stimulation of the release of [^{14}C]serotonin required about 0.3unit/ml, similar to the concentration required when intact platelets were suspended in the same medium (Fig. 3*b*). Neither Ca^{2+} alone (pCa5) nor Ca^{2+} with thrombin caused any loss of lactate dehydrogenase from permeabilized platelets, indicating that [^{14}C]-serotonin was not released as a result of platelet lysis.

Protein phosphorylation in relation to the release of [^{14}*C*]*serotonin*

Because added [γ-^{32}P]ATP gains direct access to the protein kinases of permeabilized platelets, the relationship between protein phosphorylation and secretion could be studied in this preparation without some of the complications inherent in previous work in which intact platelets were pre-incubated with $^{32}P_i$ (Lyons *et al.*, 1975; Haslam *et al.*, 1979, 1980; Wallace & Bensusan, 1980). In the present experiments, permeabilized platelets were first incubated with [γ-^{32}P]ATP for 15min at 0°C in the absence of added Ca^{2+} to permit equilibration of the label with intracellular ATP. Virtually no phosphorylation of P47 or P20 occurred during this period. However, incubation of these preparations with Ca^{2+} buffers for 15min at 0°C followed by 10min at 25°C then led to substantial labelling of both of these polypeptides with ^{32}P, but no other major phosphorylation reactions were detected (Fig. 4). In the absence of thrombin, maximum incorporation of ^{32}P into both P47 (0.75nmol/10^9 platelets) and P20 (0.43nmol/10^9 platelets) were observed at a pCa of 5.5, that is at a $[Ca^{2+}_{free}]$ an order of magnitude lower than required for maximum release of [^{14}C]serotonin (Fig. 5). In the presence of thrombin, the maximum labelling of P47 and P20 was only marginally greater than in the absence of this stimulus but the values of

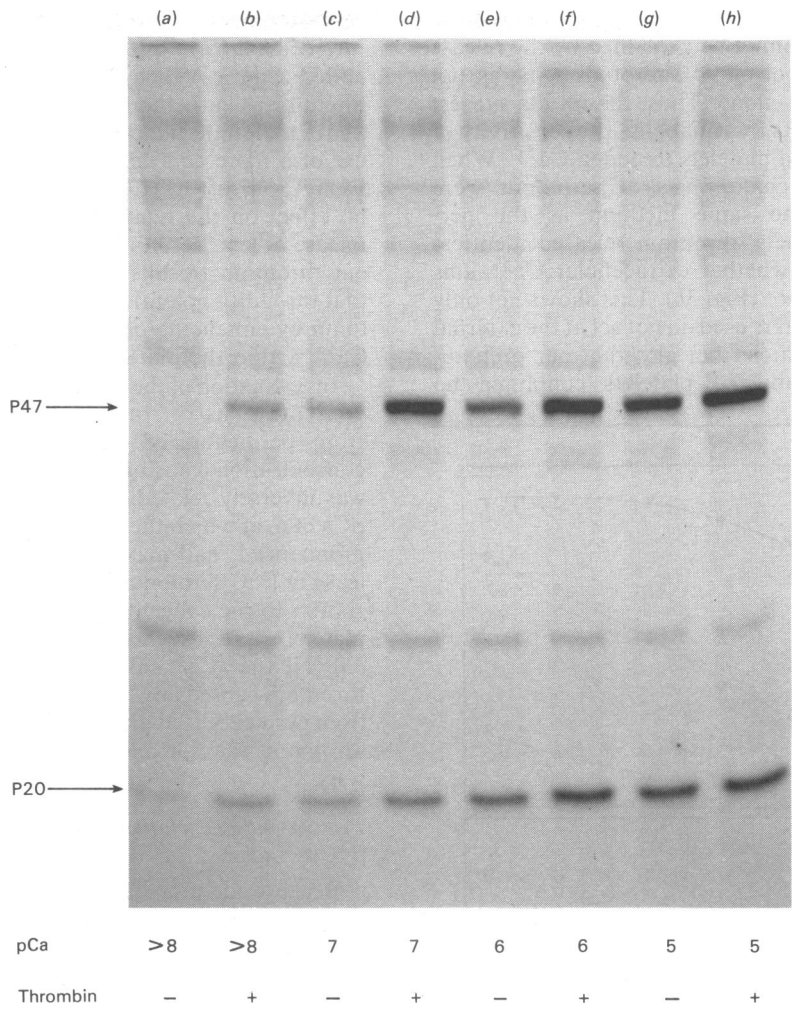

Fig. 4. *Effects of different concentrations of Ca^{2+}_{free} without and with thrombin on the phosphorylation of polypeptides in permeabilized platelets*

A suspension of permeabilized platelets was preincubated at 0°C for 15min with [γ-^{32}P]ATP and samples were then incubated with Ca^{2+} buffers giving pCa values of >8 (*a*, *b*), 7 (*c*, *d*), 6 (*e*, *f*) and 5 (*g*, *h*), first for 15min at 0°C without thrombin and then for 10min at 25°C in the absence (*a*, *c*, *e*, *g*) or presence (*b*, *d*, *f*, *h*) of 2 units of thrombin/ml. Protein was then precipitated with trichloroacetic acid and analysed by SDS/polyacrylamide-gel electrophoresis; an autoradiograph of the dried gel is shown. Other details are given in the Experimental section.

$[Ca^{2+}_{free}]$ required to achieve this were much lower, a pCa of 7 in the case of P47 and of 6.5 for P20 (Fig. 5). As a result, thrombin markedly stimulated the phosphorylation of P47 in the pCa range 7–6, but its effects on the phosphorylation of P20, though consistent, were proportionately much smaller (Figs. 4 and 5). Again, the extents of protein phosphorylation and of release of [^{14}C]serotonin were poorly correlated, though the discrepancy was more marked in the case of P47 than in that of P20.

Analysis of the time course of these events showed that phosphorylation of P47 and P20 preceded release of [^{14}C]serotonin, though from a quantitative point of view the correlation between the two was again poor (Fig. 6). For example, small increases in the phosphorylation of P47 and P20 caused by thrombin were associated with a proportionately much larger enhancement of secretion. Little phosphorylation of P47 occurred during equilibration with Ca^{2+} buffers at 0°C but, with a pCa of 5, though not 6, complete phosphorylation of P20 occurred at this step (Fig. 6). However, no secretion of [^{14}C]serotonin was observed until the platelets were warmed to 25°C. Protein phosphorylation in the absence of secre-

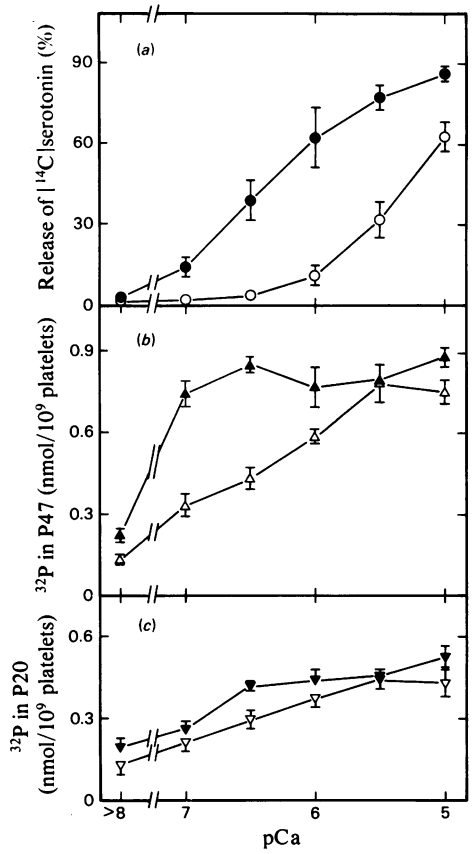

Fig. 5. *Relationship between the release of [^{14}C]serotonin from permeabilized platelets by different Ca^{2+}_{free} concentrations with and without thrombin and the phosphorylation of P47 and P20*

Release of platelet [^{14}C]serotonin and the incorporation of ^{32}P from [γ-^{32}P]ATP into P47 and P20 were determined in parallel incubations of samples from the same suspension of permeabilized platelets. Half of the suspension was preincubated at 0°C with [γ-^{32}P]ATP and samples without and with [γ-^{32}P]ATP were then incubated with Ca^{2+} buffers, first for 15min at 0°C without thrombin and then for a further 10min at 25°C in the absence (open symbols) or presence (filled symbols) of 2 units of thrombin/ml. Other details are given in the Experimental section. (*a*) Release of [^{14}C]serotonin (○ ●); (*b*) ^{32}P found in P47 (△ ▲); (*c*) ^{32}P found in P20 (▽ ▼). Values shown are means ± S.E.M. from three experiments with different preparations of permeabilized platelets. In paired *t* tests, the increases in phosphorylation of P47 caused by thrombin were significant ($2P < 0.05$) at pCa values of 6.5 and 5.0 and highly significant ($2P < 0.01$) at a pCa of 7.0, whereas the increases in phosphorylation of P20 were significant at pCa values of >8 and 6.5 and highly significant at a pCa of 6.0

tion has previously been observed in intact platelets exposed to thrombin in the cold, but in the latter case P47 rather than P20 was phosphorylated (Wallace & Bensusan, 1982).

Previous results with intact platelets have suggested that phosphorylation of both P47 and P20 is required for platelet degranulation (Lyons *et al.*, 1975; Haslam & Lynham, 1977; Haslam *et al.*, 1979, 1980; Kawahara *et al.*, 1980; Yamanishi *et al.*, 1983; Kaibuchi *et al.*, 1983). Our present results are consistent with this view but expose significant discrepancies between the relative extents of protein phosphorylation and secretion. A partial explanation could arise from recent evidence that phosphorylation of both of the P-light chains of myosin may be required for enhancement of actin-activated ATPase activity, at least in smooth muscle (Persechini & Hartshorne, 1982), and that P47 undergoes multisite phosphorylation (Imaoka *et al.*, 1983), so that in both cases functional modification could depend on the last phosphate residue added. In this context, calculation does suggest that both P20 and P47 become highly phosphorylated in permeabilized platelets incubated with Ca^{2+} ions. Thus, assuming as a first approximation that 5% of platelet protein is myosin (Daniel *et al.*, 1981) and 0.25% is P47 (Imaoka *et al.*, 1983), myosin incorporated 2 phosphate residues/mol and P47 7 phosphate residues/mol. However, the fact remains that at certain pCa values, determined by whether or not thrombin was present, maximal phosphorylation of both P47 and P20 was observed in association with submaximal release of [^{14}C]serotonin. It is therefore likely that the mechanism of secretion also involves other Ca^{2+} and thrombin-dependent reactions that take place either in parallel with or subsequent to protein phosphorylation.

1,2-Diacylglycerol formation in relation to the release of [^{14}C]serotonin

Preliminary experiments with intact platelets labelled with [^{3}H]arachidonic acid and suspended in Ca^{2+}-free Tyrode's solution containing 0.35% (w/v) albumin showed that incubation with thrombin (2 units/ml) at 25°C caused a 5.2±0.5-fold increase in [^{3}H]1,2-diacylglycerol after 30s (mean ±S.E.M., three experiments). This [^{3}H]1,2-diacylglycerol had almost disappeared by 2min. Several workers have previously observed similar transient increases in 1,2-diacylglycerol in intact platelets stimulated with thrombin (e.g. Rittenhouse-Simmons, 1979, 1981; Kawahara *et al.*, 1980). In permeabilized platelets, thrombin also briefly stimulated [^{3}H]1,2-diacylglycerol formation, but the pattern observed differed in that no removal of the compound was detected within 5min (Fig. 7). Although thrombin caused little or no increase in

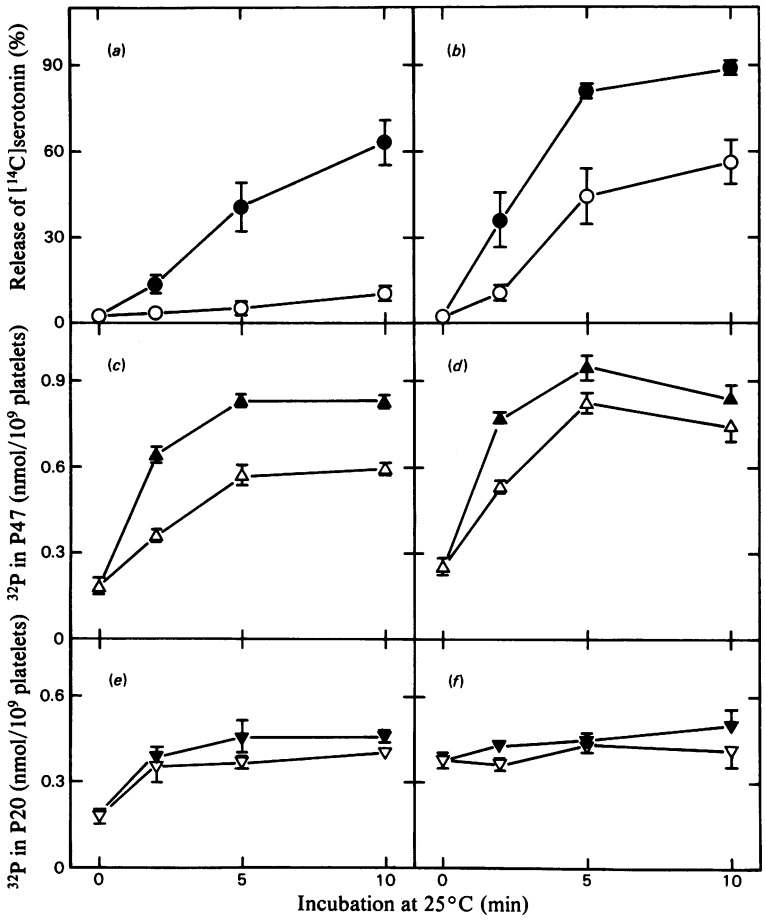

Fig. 6. *Time course of the release of* [^{14}C]*serotonin from permeabilized platelets by* Ca^{2+} *with and without thrombin in relation to the phosphorylation of P47 and P20*

Experiments were carried out as described in the legend to Fig. 5, except that only two Ca^{2+} buffers were used, giving pCa values of either 6 (*a*, *c*, *e*) or 5 (*b*, *d*, *f*). The period of incubation at 25°C in the absence (open symbols) or presence (filled symbols) of 2 units of thrombin/ml was varied. (*a*, *b*) Release of [^{14}C]serotonin (○ ●); (*c*, *d*) ^{32}P found in P47 (△ ▲); (*e*, *f*) ^{32}P found in P20 (▽ ▼). Values shown are means±S.E.M. from three experiments.

[^{3}H]1,2-diacylglycerol in the absence of any added Ca^{2+}, this stimulus enhanced formation of the compound at a pCa of 7 and was even more effective at a pCa of 6 (Figs. 7 and 8). Ca^{2+} alone also caused some [^{3}H]1,2-diacylglycerol formation, particularly at the higher of these concentrations. As a result, incubation with thrombin (2 units/ml) for 5min at a pCa of 6 increased the amount of [^{3}H]1,2-diacylglycerol in permeabilized platelets to a level comparable with that observed 30s after addition of thrombin to the intact platelets. The potentiation by thrombin of the release of [^{14}C]serotonin correlated better with its effect on the accumulation of [^{3}H]1,2-diacylglycerol (Figs. 7 and 8) than with its action on the phosphorylation of P47, which was maximal at a pCa of 7 (Fig. 5). Thus, it is possible that the 1,2-diacylglycerol formed at a pCa of 7 in the presence of thrombin is sufficient to stimulate complete phosphorylation of P47 by Ca^{2+}-activated, phospholipid-dependent protein kinase and that larger amounts affect mainly the initial rate of this reaction and perhaps other processes involved in secretion. In this context, 1,2-diacylglycerol has been reported to facilitate membrane fusion (Allan & Michell, 1975). As Ca^{2+}-activated phospholipid-dependent protein kinase can phosphorylate the P-light chains of platelet myosin (Naka *et al.*, 1983), the small increases in phosphorylation of P20 caused by thrombin may also be attributable to 1,2-diacylglycerol formation. A report (Knight & Baker, 1983) that addition of phorbol ester, which acts as a 1,2-diacylglycerol analogue, can enhance the sensitivity to Ca^{2+} ions of the secretion of catechol-

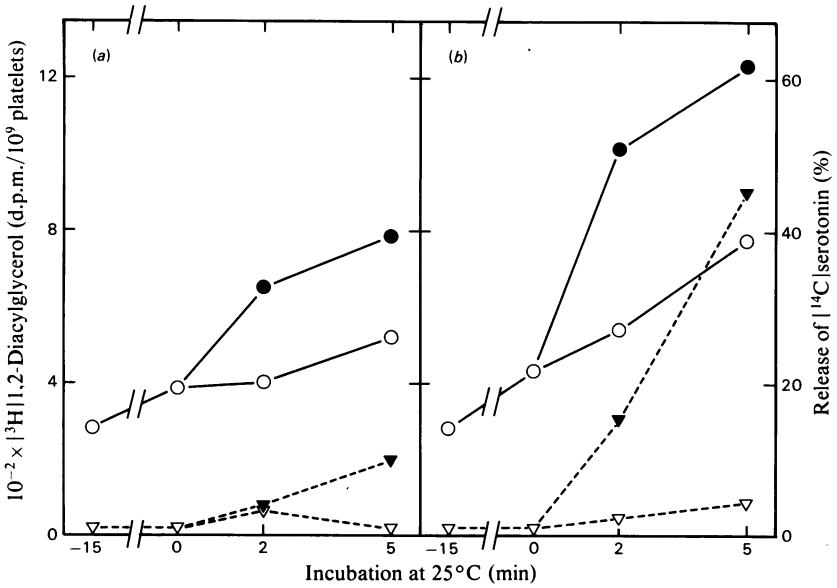

Fig. 7. *Time course of the effects of Ca^{2+} and thrombin on the formation of [^{3}H]1,2-diacylglycerol in permeabilized platelets; relationship to the release of [^{14}C]serotonin*

Platelets were labelled with [^{3}H]arachidonic acid and [^{14}C]serotonin, permeabilized and isolated, as described in the Experimental section. Samples of suspension (0.52×10^9 platelets) were incubated in Ca^{2+} buffers giving pCa values of 7 (*a*) or 6 (*b*), first for 15 min at 0°C and then for the periods indicated at 25°C, either in the absence (open symbols) or presence (filled symbols) of 2 units of thrombin/ml. The accumulation of [^{3}H]1,2-diacylglycerol (○ ●) and release of [^{14}C]serotonin (▼ ▽) were then measured. Results obtained from samples of suspension (at 0°C) to which no Ca^{2+} was added are plotted at −15 min. All values are means of duplicate determinations in the same experiment.

amine from permeabilized adrenal medullary cells, is consistent with our evidence that 1,2-diacylglycerol formation is reponsible for the potentiation of secretion from permeabilized platelets by thrombin. However, Rink *et al.* (1983) have recently found that, in intact platelets, either phorbol ester or 1-oleoyl-2-acetylglycerol can cause a slow but substantial secretion of platelet granule constituents in the absence of any increase in intracellular [Ca^{2+}_{free}] measurable with quin 2. The latter results differ in emphasis from those obtained earlier with low concentrations of the same compounds, when effective secretion required the simultaneous presence of a low concentration of Ca^{2+} ionophore (Yamanishi *et al.*, 1983; Kaibuchi *et al.*, 1983). Our experiments, in which only a limited release of granule [^{14}C]serotonin was observed at a pCa of 7, even in the presence of a high concentration of thrombin, also favour the view that synergistic interactions between the effects of 1,2-diacylglycerol formation and Ca^{2+} mobilization may be required for an optimal secretory response under physiological conditions.

Because a pCa of 7 corresponds to the intracellular [Ca^{2+}_{free}] found in unstimulated intact platelets (Rink *et al.*, 1982), our results provide the first direct evidence that thrombin can increase 1,2-diacylglycerol formation without any increase in intracellular Ca^{2+} concentration, presumably as a result of the receptor-activated hydrolysis of phosphatidylinositol (Rittenhouse-Simmons, 1979; Bell & Majerus, 1980) or, more probably, phosphatidylinositol 4,5-bisphosphate (Agranoff *et al.*, 1983; Lapetina, 1983). This conclusion is consistent with evidence that the thrombin-induced breakdown of the latter compound in platelets is insensitive to inhibitors of Ca^{2+} mobilization (Billah & Lapetina, 1982*a*) and is in accord with the general hypothesis that hydrolysis of phosphoinositides to 1,2-diacylglycerol can be a primary receptor effect and therefore a potential cause rather than a result of Ca^{2+} mobilization (Michell, 1975; Michell *et al.*, 1981; Berridge, 1981). However, our experiments also show that an increase in [Ca^{2+}_{free}] from 0.1 μM to 1.0 μM, which is similar to that believed to occur in intact platelets stimulated by thrombin (Rink *et al.*, 1982), enhances the 1,2-diacylglycerol formation attributable to the direct action of thrombin. Thus, mobilization of Ca^{2+} ions in intact platelets would be expected to lead to a rapid acceleration of receptor-initiated 1,2-diacylglycerol formation.

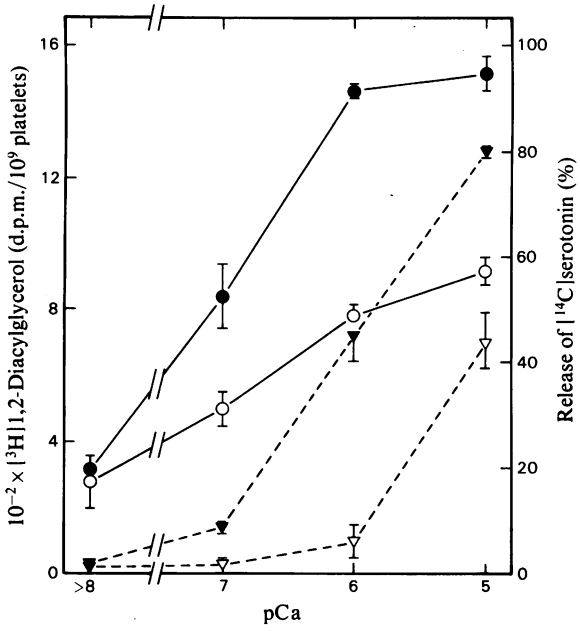

Fig. 8. *Effects of thrombin on the formation of [^{3}H]1,2-diacylglycerol in permeabilized platelets equilibrated with different Ca^{2+}_{free} concentrations; relationship to the release of [^{14}C]serotonin*

Platelets were labelled with [^{3}H]arachidonic acid and [^{14}C]serotonin, permeabilized and isolated, as described in the Experimental section. Samples of suspension (0.45×10^9 platelets) were incubated for 15min at 0°C with the Ca^{2+} buffers indicated and then for 5min at 25°C in the absence or presence of thrombin (2 units/ml). The accumulation of [^{3}H]1,2-diacylglycerol (○ ●) and release of [^{14}C]serotonin (▽ ▼) were then measured: open symbols, without thrombin; filled symbols, with thrombin. All values are means ± S.E.M. from three identical incubation mixtures. The increases in [^{3}H]1,2-diacylglycerol caused by addition of thrombin at pCa values of 7, 6 and 5 were statistically significant ($2P < 0.05$; Student's t test).

Such a mechanism could readily account for the well-documented observation that thrombin is far more effective than ionophore A23187 in promoting the formation of 1,2-diacylglycerol in intact platelets (Rittenhouse-Simmons, 1981; Billah & Lapetina, 1982*b*). Our results indicate that permeabilized platelets may prove useful for analysing aspects of receptor action, as well as for identifying the intracellular effects of Ca^{2+} ions and other second messengers.

Note added in proof

A recent report that, in addition to thrombin, 1-oleoyl-2-acetylglycerol increases the sensitivity of the secretion of serotonin from permeabilized platelets to Ca^{2+} ions [Knight, D. E. & Scrutton, M. C. (1984) *Nature (London)* **309**, 66–68] supports our conclusion that the action of thrombin is mediated by the receptor-activated formation of 1,2-diacylglycerol.

This work was supported by a grant (MT 5626) from the Medical Research Council of Canada. We also thank Mr. Edward Padgett, who built the high-voltage electric discharge apparatus, for his invaluable help.

References

Agranoff, B. W., Murthy, P. & Seguin, E. B. (1983) *J. Biol. Chem.* **258**, 2076–2078

Allan, D. & Michell, R. H. (1975) *Nature (London)* **258**, 348–349

Aster, J. H. & Jandl, J. H. (1964) *J. Clin. Invest.* **43**, 843–855

Bell, R. L. & Majerus, P. W. (1980) *J. Biol. Chem.* **255**, 1790–1792

Bergmeyer, H.-U., Bernt, E. & Hess, B. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 736–743, Academic Press, New York

Berridge, M. J. (1981) *Mol. Cell. Endocrinol.* **24**, 115–140

Billah, M. M. & Lapetina, E. G. (1982*a*) *Biochem. Biophys. Res. Commun.* **109**, 217–222

Billah, M. M. & Lapetina, E. G. (1982*b*) *J. Biol. Chem.* **257**, 11856–11859

Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917

Daniel, J. L., Molish, I. R. & Holmsen, H. (1981) *J. Biol. Chem.* **256**, 7510–7514

Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505

Feinman, R. D. & Detwiler, T. C. (1974) *Nature (London)* **249**, 172–173

Feinstein, M. B. (1978) in *Calcium in Drug Action* (Weiss, G. B., ed.), pp. 197–239, Plenum, New York

Haslam, R. J. & Lynham, J. A. (1977) *Biochem. Biophys. Res. Commun.* **77**, 714–722

Haslam, R. J., Lynham, J. A. & Fox, J. E. B. (1979) *Biochem. J.* **178**, 397–406

Haslam, R. J., Salama, S. E., Fox, J. E. B., Lynham, J. A. & Davidson, M. M. L. (1980) in *Platelets: Cellular Response Mechanisms and their Biological Significance* (Rotman, A., Meyer, F. A., Gitler, C. & Silberberg, A., eds.), pp. 213–231, Wiley, Chichester

Imaoka, T., Lynham, J. A. & Haslam, R. J. (1983) *J. Biol. Chem.* **258**, 11404–11414

Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 6701–6704

Kawahara, Y., Takai, Y., Minakuchi, R., Sano, K. & Nishizuka, Y. (1980) *Biochem. Biophys. Res. Commun.* **97**, 309–317

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. & Nishizuka, Y. (1980) *J. Biol. Chem.* **255**, 2273–2276

Knight, D. E. (1981) in *Techniques in the Life Sciences: Techniques in Cellular Physiology—Part 1, P113*, pp. 1–20, Elsevier/North Holland, Ireland

Knight, D. E. & Baker, P. F. (1983) *FEBS Lett.* **160**, 98–100

Knight, D. E. & Scrutton, M. C. (1980) *Thromb. Res.* **20**, 437–446
Knight, D. E. & Scrutton, M. C. (1983) *Thromb. Haemostasis* **50**, 93
Knight, D. E., Hallam, T. J. & Scrutton, M. C. (1982) *Nature (London)* **296**, 256–257
Lapetina, E. G. (1983) *Life Sci.* **32**, 2069–2082
Lüscher, E. F., Massini, P. & Kaiser-Glanzmann, R. (1980) in *Platelets: Cellular Response Mechanisms and their Biological Significance* (Rotman, A., Meyer, F. A., Gitler, C. & Silberberg, A., eds.), pp. 67–77, Wiley, Chichester
Lyons, R. M., Stanford, N. & Majerus, P. W. (1975) *J. Clin. Invest.* **56**, 924–936
Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
Michell, R. H., Kirk, C. J., Jones, L. M., Downes, C. P. & Creba, J. A. (1981) *Philos. Trans. R. Soc. London Ser. B.* **296**, 123–137
Molnar, J. & Lorand, L. (1961) *Arch. Biochem. Biophys.* **93**, 353–363
Naka, M., Nishikawa, M., Adelstein, R. S. & Hidaka, H. (1983) *Nature (London)* **306**, 490–492
Persechini, A. & Hartshorne, D. J. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 2868–2872
Rink, T. J., Smith, S. W. & Tsien, R. Y. (1982) *FEBS Lett.* **148**, 21–26
Rink, T. J., Sanchez, A. & Hallam, T. J. (1983) *Nature (London)* **305**, 317–319
Rittenhouse-Simmons, S. (1979) *J. Clin. Invest.* **63**, 580–587
Rittenhouse-Simmons, S. (1981) *J. Biol. Chem.* **256**, 4153–4155
Sano, K., Takai, Y., Yamanishi, J. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 2010–2013
Takai, Y., Minakuchi, R., Kikkawa, U., Sano, K., Kaibuchi, K., Yu, B., Matsubara, T. & Nishizuka, Y. (1982) *Progr. Brain Res.* **56**, 287–301
Wallace, W. C. & Bensusan, H. B. (1980) *J. Biol. Chem.* **255**, 1932–1937
Wallace, W. C. & Bensusan, H. B. (1982) *Biochim. Biophys. Acta* **721**, 164–171
White, J. G., Rao, G. H. R. & Gerrard, J. M. (1974) *Am. J. Pathol.* **77**, 135–149
Yamanishi, J., Takai, Y., Kaibuchi, K., Sano, K., Castagna, M. & Nishizuka, Y. (1983) *Biochem. Biophys. Res. Commun.* **112**, 778–786