Thrombin-induced phosphoinositide hydrolysis in platelets

Receptor occupancy and desensitization

Evelyn Mei HUANG and Thomas C. DETWILER

Department of Biochemistry, State University of New York Health Science Center at Brooklyn, Brooklyn, NY 11203, U.S.A.

The relationship between occupancy of thrombin receptors on platelets and enhanced phosphoinositide hydrolysis was analysed by examination of the dose–response relationship, the effects of thrombin inhibitors and the contribution of secondary effects. Washed human platelets were labelled with [³H]inositol, and agonist-induced accumulation of labelled inositol phosphates was measured. (1) The dose–response curves and the time courses for α -thrombin- or γ -thrombin-induced accumulation of inositol phosphates were similar to those for dense-granule secretion. (2) Addition of the thrombin inhibitor hirudin to thrombin-activated platelets revealed that the continuous presence of active thrombin was required to maintain the accumulation of labelled inositol phosphates; the total production of inositol phosphates increased with longer periods of exposure to thrombin, reaching a maximum between 5 and 10 min. (3) After activation with thrombin, the ability of a second, greater, addition of thrombin to induce additional phosphoinositide hydrolysis decreased with time; it was absent within 10 min after the first addition. (4) The failure to sustain accumulation of labelled inositol phosphates or to respond to a second addition of thrombin beyond 10 min was not due to depletion of the pool of labelled precursors, because the platelets retained their ability to respond to collagen. (5) Addition of ADP-consuming enzymes decreased sensitivity to thrombin, but inhibition of cyclo-oxygenase with indomethacin did not impair the thrombin-induced hydrolysis of phosphoinositides. (6) It was concluded that thrombin-induced hydrolysis of phosphoinositides (i) has characteristics consistent with mediation by a receptor that is similar to that that triggers dense-granule secretion, (ii) requires continuous presence of active thrombin to be maintained, (iii) is mediated by a receptor that displays thrombin-induced desensitization, and (iv) is only partially enhanced by secondary agents.

INTRODUCTION

Agonist-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate has been postulated to be an important event that mediates receptor activation and cellular responses in many types of cells. The phospholipase C-catalysed breakdown of phosphatidylinositol bisphosphate generates two putative second messengers, diacylglycerol and InsP_3 , which can activate protein kinase C and mobilize intracellular Ca^{2+} respectively (for reviews see Berridge, 1984; Berridge & Irvine, 1984; Nishizuka, 1984).

If the hydrolysis of phosphoinositides (presumably by activation of phospholipase C) is coupled directly to receptors, it would be the obvious parameter for the study of agonist–receptor interactions. It is not clear, however, that the agonist-induced activation of phosphoinositide hydrolysis is mediated directly by the same receptor–effector process that initiates other platelet responses. Phosphoinositide hydrolysis could be only a secondary consequence of platelet activation, or it could involve a separate receptor. Rittenhouse (1984) concluded that phosphoinositide hydrolysis induced by the ionophore A23187 was completely dependent on generation of secondary agents, and Holmsen *et al.* (1981, 1984) concluded that thrombin-induced phosphoinositide hydrolysis was closely correlated with acid hydrolase release, but not with dense-granule secretion. Because acid hydrolase release is a late response in terms of

strength of stimulus and period of exposure to stimulus, and because there is evidence for more than one type of thrombin receptor on platelets (McGowan & Detwiler, 1986), this leaves open the question whether phosphoinositide hydrolysis is coupled directly to receptors responsible for other platelet responses.

Thrombin is the most potent physiological platelet agonist (Berndt & Phillips, 1981). A receptor has not been identified, and the nature of the interaction of thrombin with its putative receptor is not known. One difficulty is the absence of a direct assay. As an assessment of the relationship between occupancy of thrombin receptors and hydrolysis of phosphoinositides, we have examined the thrombin dose–response relationship and the contribution of secondary effects. The thrombin-concentration-dependence and the time course for phosphoinositide hydrolysis were similar to those for activation of other platelet responses, such as secretion. The continuous presence of active thrombin was required to maintain phosphoinositide hydrolysis. Although hydrolysis could be sustained, the thrombin receptor became desensitized.

EXPERIMENTAL

Materials

Purified human α - and γ -thrombin were gifts from Dr. John W. Fenton II, Division of Laboratories and

Fig. 1. Thrombin concentration-dependence and time course for thrombin-induced accumulation of labelled inositol phosphates

Platelets were prelabelled with [^3H]inositol and were activated with various concentrations of α -thrombin (●) or γ -thrombin (□) for 1 min or with 45 nM-thrombin for various times. Labelled inositol phosphates were extracted and analysed as described under 'Methods'. The data (representative of three similar experiments) are expressed as radioactivity (d.p.m./ml of platelet suspension) above the zero-time values, which were: InsP, 5208; InsP₂, 632; InsP₃, 176. Dense-granule secretion (upper panels) was measured as ATP released under the same conditions.

Research, New York State Department of Health, Albany, NY, U.S.A. Phosphocreatine, creatine kinase, indomethacin and hirudin were from Sigma, St. Louis, MO, U.S.A.; 1 unit of hirudin/ml was able to block the activation of platelets by 10 nM- α -thrombin. Collagen was from Hormon, München 45, West Germany; *myo*-[2- ^3H (n)]inositol was from American Radiolabeled Chemicals, St. Louis, MO, U.S.A.; and the ion-exchange resin AG1-X8 (200–400 mesh, formate form) was from Bio-Rad, Richmond, CA, U.S.A. A luciferin-luciferase reagent (Chronolume) was from Chronolog, Havertown, PA, U.S.A.

Methods

Human platelets were prepared from 200 ml of blood collected into acid/citrate/dextrose (Aster & Jandl, 1964) anticoagulant by venipuncture of healthy donors who had not taken any drugs for at least 2 weeks. Platelet-rich plasma was obtained by centrifugation of the blood at 300 *g* for 20 min. After addition of 0.3 μM -prostaglandin E₁, platelets were collected by centrifugation at 1000 *g*

for 20 min. The platelets were suspended in 10 ml of a modified Ca²⁺-free Tyrode-Hepes buffer (134 mM-NaCl/12 mM-NaHCO₃/3 mM-KCl/0.34 mM-sodium phosphate/2 mM-MgCl₂/5 mM-Hepes/5 mM-glucose/10 mM-citrate/1 mM-EGTA, adjusted to pH 7.4) containing 75 μCi of [^3H]inositol/ml. After incubation for 2 h at 37 °C, the platelets were collected by centrifugation (1000 *g* for 20 min) and resuspended in the above buffer without citrate and EGTA but with 2 mg of albumin/ml. This final suspension contained about 5×10^8 platelets/ml.

Activation of platelets with thrombin or collagen was carried out with 1 ml of platelet suspension in a 5 ml cuvette at 37 °C. At the indicated times, reactions were stopped by addition of an equal volume of 10% (w/v) trichloroacetic acid to the cuvette, so that each time point was from a separate sample. For short periods of incubation (2 min or less), samples were stirred with a 3 mm magnet-driven Teflon bar. For incubation periods longer than 2 min, stirring was discontinued after an initial mixing of agonist.

Fig. 2. Effects of hirudin on α -thrombin-induced accumulation of labelled inositol phosphates

The experiments were as described for Fig. 1. [^3H]Inositol-labelled platelets were activated with 15 nM- α -thrombin (●—●), and 10 units of hirudin/ml was added after either 30 s or 1, 2 or 5 min (○---○). The data (typical of four similar experiments) are expressed as radioactivity (d.p.m./ml of platelet suspension) above zero-time values, which were: InsP, 7368; InsP₂, 712; InsP₃, 188. As a control, hirudin was added before thrombin (○—○).

Labelled inositol phosphates were extracted with 10% trichloroacetic acid and separated on AG1-X8 anion-exchange columns. The procedures were essentially the same as those used by Downes & Michell (1981); details have been described (Huang & Detwiler, 1986a).

Dense-granule secretion was monitored by a continuous recording of the concentration of extracellular ATP measured by the luminescence produced by its reaction with luciferin and luciferase (Detwiler & Feinman, 1973b). The measurements were made with a Lumi-aggregometer (Chronolog). Details of this procedure have been described (Charo *et al.*, 1977).

RESULTS

Thrombin-induced phosphoinositide hydrolysis and dense-granule secretion

As a preliminary study of the relationship between phosphoinositide hydrolysis and other parameters of platelet activation, the accumulation of labelled inositol phosphates was compared with dense-granule secretion, a readily quantifiable platelet response to activation. Each

response showed a similar α -thrombin concentration-dependence and a similar time course (Fig. 1, continuous lines). Similar comparisons also were made with γ -thrombin (Fig. 1, broken lines), which has a different dose-response curve and a different and distinctive time course of platelet activation (Tam *et al.*, 1980; McGowan & Detwiler, 1986). The shift in dose-response curve and the lag in the time course were observed for γ -thrombin-induced hydrolysis of phosphoinositides as well as for dense-granule secretion. Thus the agonist-induced hydrolysis of phosphoinositides (an event postulated to be coupled directly to the receptor) approximately parallels in time and concentration-dependence the agonist-induced dense-granule secretion (presumably a final consequence of platelet activation).

Reversal by hirudin of α -thrombin-induced phosphoinositide hydrolysis

Platelet responses have different requirements for continuous occupancy of thrombin receptors. Final responses, such as aggregation and secretion, continue after brief exposure to thrombin (Detwiler & Feinman, 1973a), whereas some metabolic responses have been reported to require the continuous presence of active thrombin (Holmsen *et al.*, 1981, 1984; E. B. McGowan, H. Xu, E. Becker & T. C. Detwiler, unpublished work). For example, thrombin-induced incorporation of [^{32}P]phosphate into phosphatidic acid, presumably at least in part a reflection of increased phosphoinositide hydrolysis, ceased when thrombin was inhibited (Holmsen *et al.*, 1981, 1984). We tested the requirement for continuous occupancy of the thrombin receptor by observing the effects of addition of an excess of hirudin, a high-affinity inhibitor that causes the immediate dissociation of thrombin from saturable sites on platelets (Tam & Detwiler, 1978; Tam *et al.*, 1979). The effects of hirudin on the thrombin-induced accumulation of inositol phosphates are shown in Fig. 2. Hirudin had a somewhat different effect on each of the intermediates; it caused a return toward the basal amount of InsP₃, a slight decrease in the amount of labelled InsP₂ and a slowed accumulation of InsP. This demonstrates the need for the continuous presence of active thrombin to sustain phosphoinositide hydrolysis.

Because the measured accumulation of labelled inositol phosphates reflects a steady-state value rather than a total amount produced, it is not possible to define the relationship between the period of receptor occupancy and the magnitude of the response from the data in Fig. 2. We therefore did similar experiments with platelets that had been incubated with Li⁺, which we have shown to inhibit InsP phosphatase in platelets, effectively trapping all inositol phosphates as InsP (Huang & Detwiler, 1986a). By exposure of platelets to thrombin for various times before addition of hirudin, we were thus able to determine the cumulative amount of labelled phosphoinositides hydrolysed as a function of time of receptor occupancy (Fig. 3). Accumulation of labelled inositol phosphates increased with time of exposure to active thrombin, approaching a plateau by 5 min.

Desensitization of thrombin-induced hydrolysis of phosphoinositides

The decreasing phosphoinositide response with longer periods of exposure to the stimulus (Figs. 2 and 3)

Fig. 3. Relationship between increasing periods of exposure to active thrombin and the total accumulation of labelled inositol phosphates

The experiments were similar to those described for Fig. 2, except that hydrolysis of InsP was blocked by Li^+ , so that the final amount of labelled InsP represents the sum of labelled InsP, InsP_2 and InsP_3 produced in response to thrombin. $[^3\text{H}]\text{Inositol}$ -labelled platelets were incubated for 90 min at 37°C with 10 mM-LiCl. At zero time, 15 nM- α -thrombin was added, and the increase in labelled InsP was measured for 30 min; 10 units of hirudin/ml was added at various times to stop further action of thrombin. In (a) the time courses for the accumulation of InsP are shown for thrombin alone (●) or for thrombin followed by hirudin after $\frac{1}{2}$, 1, 2, 5, 10 or 15 min (○). In (b) labelled InsP at 15 min (▲) or 30 min (△) is shown as a function of the period of time between addition of thrombin and hirudin.

suggests either that the cell has become 'desensitized' to the stimulus or that the response has been exhausted. We tested this by comparison of the responses to thrombin or collagen added to control or to thrombin-pretreated platelets (Fig. 4). The pretreated platelets failed to show an increased accumulation of labelled inositol phosphates in response to a second addition of thrombin (Fig. 4, closed symbols). The response to collagen (Fig. 4, open symbols) was, however, greater with the pretreated platelets than with control platelets. Thus the pretreated platelets retained a pool of labelled phosphoinositides and a potentially active phospholipase C, but they were selectively desensitized to thrombin. This desensitization is better quantified in Fig. 5, which shows the additional response to a second dose of thrombin. The additional response decreased with time before the second addition; it was absent by 10 min. Thus the failure to sustain a continuous increase in labelled inositol phosphates during longer periods of exposure to thrombin (Figs. 2 and 3) was in part due to the selective desensitization of platelets to thrombin.

Role of secondary agonists in thrombin-induced phosphoinositide hydrolysis

Activation of platelets leads to the release of agents, such as ADP and endoperoxide/thromboxane, that can act as platelet agonists (for review, see Huang & Detwiler, 1986b). Rittenhouse (1984) reported that activation of phospholipase C in platelets activated with ionophore A23187 was entirely dependent on such secondary agonists. We therefore considered the possibility that part of the thrombin-induced phosphoinositide hydrolysis resulted from secondary effects rather than from direct coupling to a thrombin receptor.

ADP neither induced appreciable increases in inositol phosphates nor potentiated the thrombin-induced accumulation of labelled inositol phosphates, but inclusion of the ADP-scavenger enzyme systems, phosphocreatine/creatin kinase (Fig. 6) or apyrase, decreased the sensitivity of platelets to thrombin-induced phosphoinositide hydrolysis. Endoperoxide/thromboxane mimics have been reported to elicit a weak phosphoinositide response in platelets (Watson *et al.*, 1985), but inhibition of platelet endoperoxide/thromboxane synthesis by indomethacin had no effect on thrombin-induced accumulation of labelled inositol phosphates (Fig. 6). We noted, however, that addition of hirudin to thrombin-activated platelets caused a more immediate arrest of accumulation of InsP_3 in the presence of the cyclo-oxygenase inhibitor (results not shown), revealing a slight contribution of endoperoxide/thromboxane to the overall thrombin-induced accumulation of labelled inositol phosphates. We conclude that these secondary agonists, ADP and endoperoxide/thromboxane, are not essential for, but may enhance, thrombin-induced hydrolysis of phosphoinositides.

DISCUSSION

There is suggestive evidence that in many types of cells the activation of phospholipase C, leading to the production of InsP_3 and diacylglycerol, mediates cellular responses to various agonists (for reviews see Berridge, 1984; Berridge & Irvine, 1984; Nishizuka, 1984). Phospholipase C appears to be coupled to the receptors by a GTP-binding protein (Cockcroft & Gomperts, 1985; Haslam & Davidson, 1985; Litosch *et al.*, 1985; Uhing *et al.*, 1986; Straub & Gershengorn, 1986).

Fig. 4. Effect of pretreatment of platelets with thrombin on thrombin- and collagen-induced accumulation of labelled inositol phosphates

The experiments were as described for Fig. 1. [^3H]Inositol-labelled platelets were incubated at 37 °C for 20 min with no addition (control) or with 15 nM- α -thrombin (thrombin-pretreated). Increases in labelled inositol phosphates were measured after activation with an additional 30 nM- α -thrombin (●) or 25 μg of collagen/ml (◇). The zero time on the graphs is the time of the second addition of agonist. The data (representative of four similar experiments) are expressed as radioactivity (d.p.m./ml of platelet suspension) above the zero-time values, which were, for control and thrombin-pretreated platelets respectively: InsP, 3592 and 8438; InsP₂, 732 and 1280; InsP₃, 200 and 339.

Phospholipase C-catalysed hydrolysis of phosphoinositides may thus represent the immediate receptor-linked response of cells to agonists. There is some evidence consistent with such a crucial role for phospholipase C in platelets.

Thrombin is a good agonist for induction of the phosphoinositide response in platelets. In this study we have analysed the relationship between the thrombin-receptor reaction and the phosphoinositide response. If phosphoinositide hydrolysis is coupled directly to receptors and their effectors, it should be a good parameter for studies of the agonist-receptor interaction. It is necessary to establish, however, that thrombin-induced phosphoinositide hydrolysis shows a dose-dependence and a time course consistent with such a direct mechanism.

Our analysis of these data is based on two premises. First, we consider that there is a consistent relationship between the concentration of thrombin and the occupation of putative thrombin receptors. The equilibrium binding of thrombin to platelets has, in fact, been demonstrated (Martin *et al.*, 1976), but it has not been proved that this binding is to a functional receptor (see Berndt & Phillips, 1981; Detwiler & McGowan, 1985). Second, we consider that the accumulation of labelled inositol phosphates in platelets pre-labelled with inositol is at least a semi-quantitative measure of the extent of

phosphoinositide hydrolysis. There is, of course, no evidence that the phosphoinositides are uniformly labelled, and it is unlikely that the specific radioactivity remains constant over the longer test periods. The alternative parameters are less satisfactory. Incorporation of [^{32}P]phosphate into ATP and then into phosphatidic acid may reflect processes in addition to phosphorylation of diacylglycerol arising from hydrolysis of phosphoinositides, and there may be competing fates of diacylglycerol. The measurement of mass of inositol phosphates by available methods is insufficiently sensitive; it requires very high concentration of platelets, and it restricts the number of measurements.

The dose-response relationships and the time courses of responses indicate that the thrombin-induced hydrolysis of phosphoinositides is mediated by a thrombin-platelet interaction that is similar to that for other platelet responses to thrombin (Fig. 1). There is no evidence for an essential involvement of secondary agents (Fig. 6). The shift of dose-response curves to the right by ADP-consuming enzymes suggests that ADP may enhance the effect of thrombin, but it should be noted that there is evidence that inhibition of platelet activation by ADP-removing enzymes may be due to effects other than removal of secreted ADP (Huang & Detwiler, 1980; Nunn & Chamberlain, 1983). Furthermore, the dose-response curves themselves and, especi-

Fig. 5. Time course of the thrombin-induced desensitization to a second addition of thrombin

The experiments were as described for Fig. 1. The accumulation of labelled inositol phosphates was measured after activation of [^3H]inositol-labelled platelets with ($\bullet$) 15 nM- or ($\circ$) 45 nM- α -thrombin. To other suspensions, 15 nM-thrombin was added first, followed by a second addition of 30 nM after $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 5, 10 or 15 min. The increment in labelled inositol phosphates after the second addition is plotted at the top of each panel. The data (representative of three similar experiments) are expressed as increments in radioactivity (d.p.m./ml of platelet suspension). For the first thrombin additions (at zero time), the values are increments above the zero-time values, which were: InsP, 3184; InsP₂, 452; InsP₃, 124. For the second additions of thrombin, the increments are above the values at the time of the second addition.

ally, the requirement for the continuous presence of active thrombin are inconsistent with an appreciable positive feedback. The data are thus consistent with a direct action of a thrombin receptor on phosphoinositide metabolism and with a central role for phosphoinositide hydrolysis in stimulus-response coupling. Experiments of this type have the potential to disprove, but not to prove, a causal role for an event; it will be necessary to show with a specific inhibitor that phospholipase C is essential for other responses.

The agonist-induced hydrolysis of labelled phosphoinositides was not sustained, even with continuous

Fig. 6. Effects of phosphocreatine/creatine kinase (CP/CPK) and indomethacin on thrombin-induced accumulation of labelled inositol phosphates

The experiments were as described for Fig. 1. Platelet suspensions were incubated for 30 s with no addition (control, $\bullet$), with 30 μM -indomethacin ($\blacktriangle$), with CP/CPK ($\circ$; 5 mM and 30 units/ml respectively) or with indomethacin plus CP/CPK ($\triangle$). The indomethacin stock solution was in ethanol, and each sample contained an equal amount (4 μl /ml) of ethanol. α -Thrombin was added, and after 1 min labelled inositol phosphates were measured as described under 'Methods'. This is typical of two identical experiments, and it is consistent with other experiments in which apyrase was used in place of CP/CPK and in which CP/CPK was added at different times. The data are expressed as radioactivity (d.p.m./ml of platelet suspension) above zero-time values, which were: InsP, 1949; InsP₂, 571; InsP₃, 213.

presence of the stimulus (e.g. Figs. 2 and 5). There are several possible explanations for such an observation. It could represent dilution of label in the phosphoinositide pool rather than a real change in rate of hydrolysis, or it could represent depletion of the phosphoinositide pool or irreversible inactivation of phospholipase C. Our results demonstrate that the labelled precursor pool was still present and phospholipase C was still potentially active, because the desensitized platelets were still able to respond to a different agonist (Fig. 4). Such agonist-specific desensitization of platelets by exposure to the agonist is well established (O'Brien, 1962; Evans & Gordon, 1974; Holme & Holmsen, 1975; Shuman *et al.*, 1978; Hallam *et al.*, 1982; Pollock & MacIntyre, 1986). It is, of course, possible that each type of receptor utilizes a separate pool of coupling proteins (GTP-binding proteins), of phospholipase C or of phosphoinositides.

Desensitization of platelets to thrombin has been

reported previously (Reimers *et al.*, 1973; Shuman *et al.*, 1978; Hallam *et al.*, 1982; McGowan & Detwiler, 1983), but there is little information about the mechanism. The phenomenon has been difficult to study, because the parameters of platelet activation that have been used (aggregation and dense-granule secretion) do not require the continuous action of thrombin and cannot be repeated. This has necessitated rather complex experimental systems or very low (sub-threshold) concentrations of thrombin in order to demonstrate the desensitization. A potentially sustainable response that requires continuous stimulation, as demonstrated here for thrombin-induced phosphoinositide hydrolysis, permits a more direct and quantitative assessment of desensitization.

Desensitization of cells to a sustained stimulus has been observed with many types of cells, but in no case is the mechanism fully understood. Sibley *et al.* (1984a) demonstrated that desensitization of the β -receptor of turkey erythrocytes involved phosphorylation of the receptor. Phorbol esters, the tumour promoters that activate protein kinase C, were also shown to cause phosphorylation and desensitization (Sibley *et al.*, 1984b), and α_1 -receptors, which stimulate phosphoinositide hydrolysis, are phosphorylated and desensitized by incubation with phorbol esters (Leeb-Lundberg *et al.*, 1985). It has been reported that incubation of platelets with phorbol esters or oleoylacetyl glycerol can inhibit their responses to thrombin (MacIntyre *et al.*, 1985; Rittenhouse & Sasson, 1985; Watson & Lapetina, 1985; Zavoico *et al.*, 1985; but see Krishnamurthi *et al.*, 1986), but this is not specific for thrombin (MacIntyre *et al.*, 1985; Watson & Lapetina, 1985). Although there is no evidence of phosphorylation of platelet receptors by protein kinase C, Katada *et al.* (1985) demonstrated protein kinase C-catalysed phosphorylation of the 40 kDa α -subunit of the inhibitory GTP-binding protein of the adenylate cyclase system in platelet membranes. Although GTP-binding proteins appear to mediate receptor activation of phosphoinositide hydrolysis (Cockcroft & Gomperts, 1985; Haslam & Davidson, 1985; Litosch *et al.*, 1985; Uhing *et al.*, 1986; Straub & Gershengorn, 1986), it is not clear whether they are the same GTP-binding proteins as those of the adenylate cyclase system. With platelets, there is no evidence for an agonist-specific phosphorylation, nor has phosphorylation of receptors or GTP-binding proteins been demonstrated in response to platelet agonists.

This work was supported by grants HL 16355 and HL 17729 from the National Heart, Lung and Blood Institute, U.S. Department of Health and Human Services. We are grateful for the skilled technical assistance of Mr. Ching Chang.

REFERENCES

- Aster, R. H. & Jandl, J. H. (1964) *J. Clin. Invest.* **43**, 843–856
- Berndt, M. C. & Phillips, D. R. (1981) in *Platelets in Biology and Pathology-2* (Gordon, J., ed.), pp. 43–75, Elsevier/North-Holland, Amsterdam
- Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Charo, I., Feinman, R. D. & Detwiler, T. C. (1977) *J. Clin. Invest.* **60**, 866–873
- Cockcroft, S. & Gomperts, B. D. (1985) *Nature (London)* **314**, 534–536
- Detwiler, T. C. & Feinman, R. D. (1973a) *Biochemistry* **12**, 282–289
- Detwiler, T. C. & Feinman, R. D. (1973b) *Biochemistry* **12**, 2462–2468
- Detwiler, T. C. & McGowan, E. B. (1985) in *Mechanisms of Stimulus-Response Coupling in Platelets* (Westwick, J., Scully, M. F., MacIntyre, D. E. & Kakkar, V. V., eds.), pp. 15–28, Plenum, New York
- Downes, C. P. & Michell, R. H. (1981) *Biochem. J.* **198**, 133–140
- Evans, R. J. & Gordon, J. L. (1974) *Br. J. Pharmacol.* **51**, 123P
- Hallam, T. J., Ruggles, P. A., Scrutton, M. C. & Wallis, R. B. (1982) *Thromb. Haemostasis* **47**, 278–284
- Haslam, R. J. & Davidson, M. M. L. (1985) *Nature (London)* **313**, 821–822
- Holme, S. & Holmsen, H. (1975) *Scand. J. Haematol.* **15**, 96–103
- Holmsen, H., Dangelmaier, C. A. & Holmsen, H.-K. (1981) *J. Biol. Chem.* **256**, 9393–9396
- Holmsen, H., Dangelmaier, C. A. & Rongved, S. (1984) *Biochem. J.* **222**, 157–167
- Huang, E. M. & Detwiler, T. C. (1980) *J. Lab. Clin. Med.* **95**, 59–68
- Huang, E. M. & Detwiler, T. C. (1986a) *Biochem. J.* **236**, 895–901
- Huang, E. M. & Detwiler, T. C. (1986b) in *Biochemistry of Platelets* (Phillips, D. R. & Shuman, M. A., eds.), pp. 1–68, Academic Press, New York
- Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. & Jacobs, K. H. (1985) *Eur. J. Biochem.* **151**, 431–437
- Krishnamurthi, S., Joseph, S. K. & Kakkar, V. V. (1986) *FEBS Lett.* **196**, 365–369
- Leeb-Lundberg, L. M. F., Cotecchia, S., Lomasney, J. W., DeBernardis, J. F., Lefkowitz, R. J. & Caron, M. G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5651–5655
- Litosch, I., Wallis, C. & Fain, J. N. (1985) *J. Biol. Chem.* **260**, 5464–5471
- MacIntyre, D. E., McNicol, A. & Drummond, A. H. (1985) *FEBS Lett.* **180**, 160–164
- Martin, B. M., Wasiewski, W. W., Fenton, J. W., II & Detwiler, T. C. (1976) *Biochemistry* **12**, 4886–4893
- McGowan, E. B. & Detwiler, T. C. (1983) *Thromb. Res.* **31**, 297–304
- McGowan, E. B. & Detwiler, T. C. (1986) *J. Biol. Chem.* **261**, 739–746
- Nishizuka, Y. (1984) *Science* **225**, 1365–1370
- Nunn, B. & Chamberlain, P. D. (1983) *Thromb. Res.* **30**, 19–26
- O'Brien, J. R. (1962) *J. Clin. Pathol.* **15**, 452–455
- Pollock, W. K. & MacIntyre, D. E. (1986) *Biochem. J.* **234**, 67–73
- Reimers, H. J., Packham, M. A., Kinlough-Rathbone, R. L. & Mustard, J. F. (1973) *Br. J. Haematol.* **25**, 675–689
- Rittenhouse, S. E. (1984) *Biochem. J.* **222**, 103–110
- Rittenhouse, S. E. & Sasson, J. P. (1985) *J. Biol. Chem.* **260**, 8657–8660
- Shuman, M. A., Botney, M. & Fenton, J. W., II (1978) *J. Clin. Invest.* **63**, 1211–1218
- Sibley, D. R., Peters, J. R., Nambi, P., Caron, M. G. & Lefkowitz, R. J. (1984a) *J. Biol. Chem.* **259**, 9742–9749
- Sibley, D. R., Nambi, P., Peters, J. R. & Lefkowitz, R. J. (1984b) *Biochem. Biophys. Res. Commun.* **121**, 973–979
- Straub, R. E. & Gershengorn, M. C. (1986) *J. Biol. Chem.* **261**, 2712–2717
- Tam, W. S. & Detwiler, T. C. (1978) *Biochim. Biophys. Acta* **543**, 194–201
- Tam, W. S., Fenton, J. W., II & Detwiler, T. C. (1979) *J. Biol. Chem.* **254**, 8723–8725
- Tam, W. S., Fenton, J. W., II & Detwiler, T. C. (1980) *J. Biol. Chem.* **255**, 6626–6632

Uhing, R. J., Prpic, V., Jiang, H. & Exton, J. H. (1986) *J. Biol. Chem.* **261**, 2140–2146
Watson, S. P. & Lapetina, E. G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2623–2626

Watson, S. P., Reep, B., McConnell, R. J. & Lapetina, E. G. (1985) *Biochem. J.* **226**, 831–837
Zavoico, G. B., Halenda, S. P., Sha'afi, R. I. & Feinstein, M. B. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3859–3862

Received 3 June 1986/1 September 1986; accepted 26 September 1986