Biochem. J. (1984) **222**, 833–836
Printed in Great Britain

Effects of prostaglandin I_2 and forskolin on the secretion from platelets evoked at basal concentrations of cytoplasmic free calcium by thrombin, collagen, phorbol ester and exogenous diacylglycerol

Timothy J. RINK and Ana SANCHEZ*
Physiological Laboratory, Downing Street, Cambridge CB2 3EG, U.K.

(*Received 29 June 1984/Accepted 24 July 1984*)

Cytoplasmic free calcium ($[Ca^{2+}]_i$) and secretion of ATP were measured in quin2-loaded human platelets. In certain conditions thrombin and collagen cause secretion while $[Ca^{2+}]_i$ remains at basal concentrations, a response attributed to activation of protein kinase by diacylglycerol formed by hydrolysis of inositol lipids. This secretion evoked by thrombin could be totally suppressed by prostaglandin I_2 or forskolin, as expected from the known ability of cyclic AMP to inhibit phospholipase C. The secretory response evoked by collagen at basal $[Ca^{2+}]_i$ and that evoked by exogenous diacylglycerol or phorbol ester, direct activators of protein kinase-C, were much less affected by these inhibitors, suggesting that thrombin and collagen may promote formation of diacylglycerol by different mechanisms.

There is increasing evidence that the immediate breakdown products of inositol lipids, diacylglycerol and inositol trisphosphate, have important second-messenger functions [see, e.g., Nishizuka (1984) and Berridge (1984)]. In blood platelets, the activation of protein kinase-C by diacylglycerol appears to play a key role in stimulus–secretion coupling, and activation of this pathway can trigger secretory exocytosis even when $[Ca^{2+}]_i$ remains at the resting value (Rink *et al.*, 1983). For instance, direct activation of protein kinase-C by TPA (Castagna *et al.*, 1982) or oleoylacetylglycerol, stimulates secretion without raising $[Ca^{2+}]_i$. In certain experimental conditions, thrombin and collagen can also stimulate secretion while $[Ca^{2+}]_i$ remains at basal values, an effect which we have attributed to stimulus-dependent formation of diacylglycerol.

Agents that increase cyclic AMP (Haslam *et al.*, 1980; Feinstein *et al.*, 1981), such as PGI_2 or the diterpene compound forskolin (Siegl *et al.*, 1982), inhibit platelet responses to a wide range of agonists. A major effect of cyclic AMP seems to be to suppress the generation of intracellular second messages. We have found that PGI_2 and forskolin inhibit agonist-induced Ca^{2+} influx and release from internal stores (Rink & Smith, 1983; Hallam *et al.*, 1983), and it is known that cyclic AMP inhibits the hydrolysis of inositol lipids (see, e.g., Rittenhouse, 1982), thus shutting off the supply of diacylglycerol and inositol phosphates. It might therefore be expected that elevation of cyclic AMP concentrations would inhibit the secretion evoked by collagen and thrombin that we attribute to formation of diacylglycerol. This paper reports tests of this expectation and of the effect of PGI_2 and forskolin on the response to direct activation of protein kinase-C by TPA and oleoylacetylglycerol.

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic free calcium; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PGI_2, prostaglandin I_2 (prostacyclin).

* Present address: Departamento de Fisiologia y Bioquimica, Facultad de Medicina, Vallodolid, Spain.

Methods

Human platelets were prepared and loaded with about 1 mM-quin 2 as previously described (Rink *et al.*, 1983). The cells were suspended at a cell density near 1.4×10^8 per ml in a physiological saline containing 145 mM-NaCl, 5 mM-KCl, 1 mM-$MgSO_4$, 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 10 mM-dextrose, pH 7.4 at 37°C; 1 mM-EGTA or 1 mM-$CaCl_2$ was added as required. All the experiments reported here were carried out at 37°C, and unless stated otherwise, cells were pretreated with 100 μM-aspirin to block cyclo-oxygenase and prevent formation of thromboxane. Quin 2 fluorescence was monitored in a continuously stirred suspension, and the signals were calibrated in terms of

$[Ca^{2+}]_i$ essentially as previously described (Tsien *et al.*, 1982). Secretion from dense granules was monitored in parallel experiments, by measuring the release of ATP with luciferin–luciferase, in a Chronolog lumi-aggrometer. Oleoylacetylglycerol and TPA were added from stock solutions in dimethyl sulphoxide. PGI_2 (200nM), forskolin (20μM) and isobutylmethylxanthine (10μM) were added from stock solutions in methanol. Isobutylmethylxanthine was added, together with the PGI_2 or forskolin, to inhibit cyclic AMP phosphodiesterase. The concentration of vehicle did not exceed 0.2% (v/v). Cyclic AMP was measured in samples extracted by mixing the platelet suspension with an equal volume of 10% trichloroacetic acid. The supernatants were then extracted three times with diethyl ether, and the extracts were freeze-dried and resuspended in 50mM-Tris/EDTA, pH7.4. The cyclic AMP was then measured by radioimmunoassay (Amersham), and expressed as pmol/10^8 cells.

Results and discussion

Fig. 1(*a*) shows that thrombin evoked a substantial secretion from quin 2-loaded platelets incubated in Ca^{2+}-free medium containing 1mM-EGTA. Measured $[Ca^{2+}]_i$ rose from 60nM to only 150nM, close to the normal basal value for cells incubated with 1mM external Ca^{2+}, and far below the micromolar concentrations needed for $[Ca^{2+}]_i$ to stimulate secretion (Rink *et al.*, 1982; Knight *et al.*, 1982). The elevation of $[Ca^{2+}]_i$ is attributed to discharge of residual internal Ca^{2+} stores. This secretion was suppressed by increasing cyclic AMP with either PGI_2 or forskolin, as shown in Fig. 1(*b*) and seen in nine similar trials. The quin 2 signal was also decreased by some 80%, as previously observed (Rink & Smith, 1983).

Fig. 1(*c*) shows the secretory response typically produced by collagen, when formation of thromboxane is prevented by inhibition of cyclo-oxygenase. Under the condition of our experiments there is little or no increase in $[Ca^{2+}]_i$ even in the presence of normal (1mM) external Ca^{2+} (Rink *et al.*, 1983; Hallam *et al.*, 1983). Fig. 1(*d*) shows the unexpected result that application of forskolin sufficient to suppress completely the secretory response to thrombin is relatively ineffective against collagen, the secretion being decreased by only 20%. A similar result was obtained with PGI_2. The $[Ca^{2+}]_i$ records were not discernibly affected by increasing cyclic AMP. (Suppression of secretion evoked by thrombin and persistence of that evoked by collagen was also seen with cells not loaded with quin 2.)

One possible explanation for the ineffectiveness of forskolin and PGI_2 in decreasing the response to collagen could have been that collagen somehow reversed the rise in cyclic AMP. However, the measured increase in cyclic AMP, produced by forskolin, was not affected by collagen, although it was decreased by some 30% by thrombin, as shown in Fig. 2. Nor did it seem likely that collagen was generally preventing effects of cyclic AMP in these quin 2-loaded cells, for instance by inhibiting cyclic AMP-dependent protein kinase. We found that forskolin or PGI_2 did affect the responses evoked by collagen in platelets not treated with cyclo-oxygenase inhibitors, suppressing the rise in $[Ca^{2+}]_i$ and decreasing the secretory response, as shown in Figs. 1(*e*) and 1(*f*). Indeed, the responses to collagen are very similar in platelets with increased cyclic AMP and those in which cyclo-oxygenase is blocked by aspirin (cf. Figs. 1*e* and 1*f*), as expected from the known ability of increased cyclic AMP to inhibit cyclo-oxygenase (Malmsten *et al.*, 1976).

If the responses to collagen, shown in Fig. 1, are mediated by diacylglycerol, the failure of PGI_2 and forskolin to suppress the secretion implies that cyclic AMP does not severely affect protein kinase-C or subsequent steps leading to secretion. The response to exogenous diacylglycerol or TPA should likewise be relatively immune to the influence of cyclic AMP, and this was indeed the case. Application of 200nM-PGI_2 or 20μM-forskolin, with 10μM-isobutylmethylxanthine, produced only a slight slowing and decrease in extent of secretion. In one series of tests, we measured the release of ATP from quin 2-loaded platelets in Ca^{2+}-free medium 5min after adding 20nM-TPA or 60μg of oleoylacetylglycerol/ml. The secretion evoked by TPA from PGI_2-treated cells was $70 \pm 3.7\%$ (S.E.M.; $n = 8$) of that from paired controls. PGI_2 had a somewhat greater effect on the response to oleoylacetylglycerol, the secretion being decreased to $43 \pm 3\%$ (S.E.M.; $n = 8$) that of paired controls. The reason for this difference between TPA and oleoylacetylglycerol is not clear, but the main point is that sufficient PGI_2 to suppress completely the response to thrombin left a sizeable response to TPA and oleoylacetylglycerol. Similar results were obtained with forskolin; the response to TPA was $75 \pm 4\%$ ($n = 3$) that of controls, and the response to oleoylacetylglycerol $63 \pm 9.5\%$ ($n = 4$) that of the controls. These results fit well with the findings by Knight & Scrutton (1984) in platelets made permeable to Ca^{2+} buffers by high-voltage electric discharge. They found that thrombin, TPA and oleoylacetylglycerol increased the Ca^{2+}-sensitivity of the secretory process and that cyclic AMP inhibited this effect of thrombin, but had much less influence on this effect of TPA and oleoylacetylglycerol. It was suggested that cyclic AMP was inhibiting thrombin-stimulated

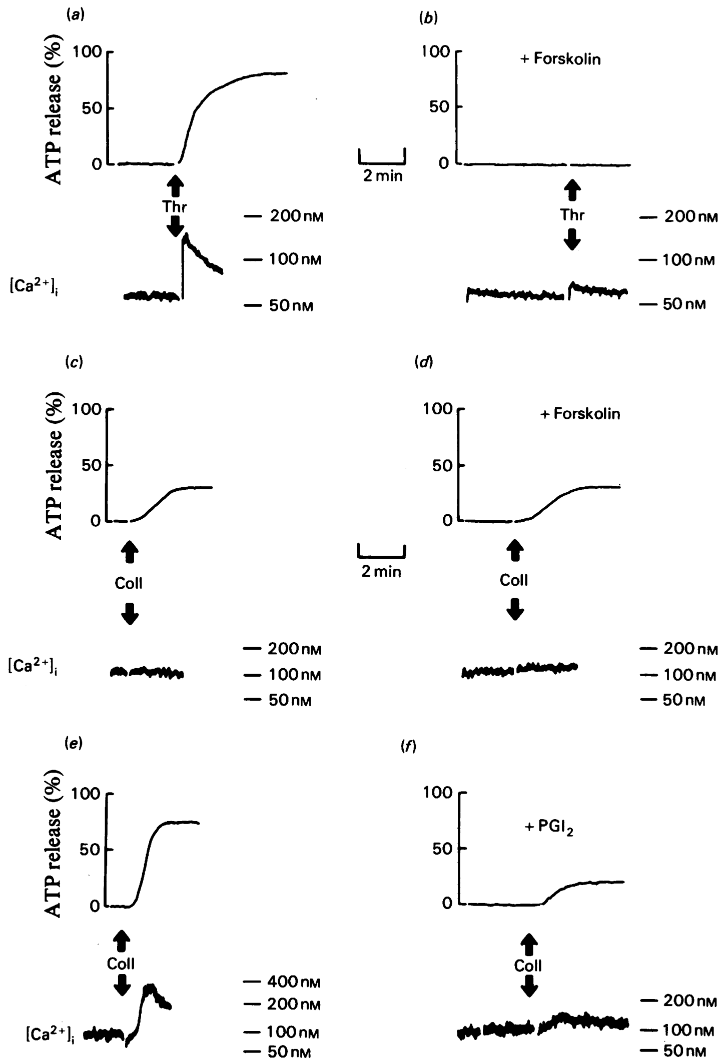

Fig. 1. *Effects of forskolin and* PGI_2 *on responses to thrombin and collagen*
The upper traces show release of ATP, expressed as a percentage of the maximal releasable by thrombin; the lower traces show $[Ca^{2+}]_i$ reported by intracellular quin2. Thrombin (Thr; 0.5 unit/ml) or collagen (Coll; 10 μg/ml) was added as indicated. In (*a*) and (*b*) the medium contained 1 mM-EGTA and no added Ca^{2+}. In (*c*), (*d*), (*e*) and (*f*), 1 mM-$CaCl_2$ was added. The cells shown in (*e*) and (*f*) were not preincubated with aspirin. Forskolin or PGI_2 was added 2 min before the agonist.

formation of diacylglycerol. Cyclic AMP was without effect on the basic Ca^{2+}-secretion relation, again suggesting that the major influence is on the generation of second messages rather than their intracellular actions.

If one works from the premise that the secretory responses produced by thrombin and collagen at basal $[Ca^{2+}]_i$ involve activation of protein kinase-C by diacylglycerol, then it appears that cyclic AMP inhibits the requisite formation of diacylglycerol when it is stimulated by thrombin, and not when it is stimulated by collagen. Some support for this proposal is given by an experiment on platelets preincubated with [^{3}H]arachidonate, reported by Kaibuchi *et al.* (1983) in their Table 1. The increased labelling of diacylglycerol produced by thrombin was more inhibited by a prostaglandin-evoked increase in cyclic AMP than was that produced by collagen. If the production of diacylglycerol is all from inositol lipids, these results imply that the process of phosphoinositide breakdown may be different for these two different agonists.

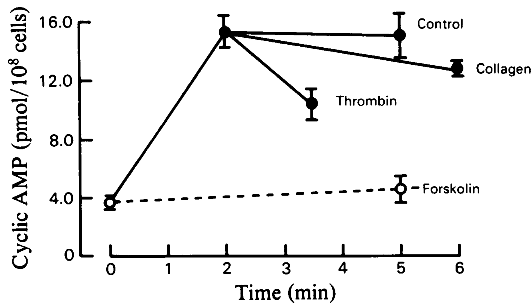

Fig. 2. *Effect of thrombin and collagen on the cyclic AMP increases produced by forskolin*

Forskolin (20 μM) together with 10 μM-isobutylmethylxanthine was added at zero time, just after the initial samples were taken. Another sample was taken at 2 min, and then either no further additions were made, or collagen (10 μg/ml) or thrombin (0.5 unit/ml) was added as indicated. The incubation conditions were as for Fig. 1. The bars indicate the S.E.M. for three determinations for each of control, with and without forskolin, collagen stimulation and thrombin stimulation. ○, Values for cells without addition of inhibitor or agonist; ●, values for cells which forskolin was added.

Thrombin appears to promote hydrolysis of phosphatidylinositol bisphosphate (Agranoff *et al.*, 1983), the reaction currently favoured as the receptor-mediated step in numerous instances of stimulated phosphoinositide turnover (Michell, 1982). Could it be that collagen actually promotes the hydrolysis of phosphatidylinositol? Speculating further along these lines, might cyclic AMP cause inhibition of one or both of the kinases that are required to maintain the relevant pool of phosphatidylinositol bisphosphate and so decrease the substrate for thrombin-mediated stimulation of phospholipase C, while leaving phosphatidylinositol available for collagen-stimulated breakdown? Some support for an effect of cyclic AMP on synthesis of phosphatidylinositol bisphosphate comes from experiments by Billah & Lapetina (1983), which show that the initial fall in phosphatidylinositol bisphosphate evoked by platelet-activating factor was not blocked by PGI_2, but that the subsequent increase was. This general notion has one other attractive feature: it offers an explanation for the failure of collagen to discharge internal Ca^{2+}. If inositol trisphosphate, the water-soluble product of the hydrolysis of phosphatidylinositol bisphosphate, is a signal for internal Ca^{2+} release, as it seems to be in pancreatic (Streb *et al.*, 1983) and other cell types (Berridge, 1984), then collagen would not make the signal, but thrombin would. However, it has yet to be shown in platelets that inositol trisphosphate is the mediator of internal Ca^{2+} release. It is also worth bearing in mind the possibility that inositol lipids may not be the only source of diacylglycerol.

This work was funded by the S.E.R.C. and Ciba-Geigy. We thank Alec Simpson for technical assistance, Dr. R. Y. Tsien for a gift of oleoylacetylglycerol, Dr. J. L. Gordon for a gift of PGI_2 and Dr. T. J. Hallam for helpful discussion. A. S. held a long-term E.M.B.O. fellowship.

References

Agranoff, B. W., Murthy, P. & Seguin, E. B. (1983) *J. Biol. Chem.* **258**, 2076–2078
Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
Billah, M. M. & Lapetina, E. G. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 965–968
Castagna, M., Takai, Y., Kaibuchi, K., Sano, K. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851
Feinstein, M. B., Rodan, G. A. & Cutler, L. S. (1981) in *Platelets in Biology and Pathology* (Gordon, J. L., ed.), pp. 437–472, Elsevier/North-Holland, Amsterdam
Hallam, T. J., Rink, T. J. & Sanchez, A. (1983) *J. Physiol. (London)* **343**, 97*P*–98*P*
Haslam, R. J., Salama, S. E., Fox, J. E. B., Lynham, J. A. & Davidson, M. M. L. (1980) in *Platelets* (Rotman, A., Meyer, F. A., Gitler, C. & Silberg, A., eds.), pp. 213–231, John Wiley, Chichester
Kaibuchi, K., Takai, Y., Sawakmura, M., Hoshijima, M., Fujikura, T. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 6701–6704
Knight, D. E. & Scrutton, M. C. (1984) *Nature (London)* **309**, 66–68
Knight, D. E., Hallam, T. J. & Scrutton, M. C. (1982) *Nature (London)* **296**, 256–257
Malmsten, C., Granstrom, E. & Samuelsson, B. (1976) *Biochem. Biophys. Res. Commun.* **68**, 569–576
Michell, R. H. (1982) *Cell Calcium* **3**, 285–294
Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
Rink, T. J. & Smith, S. W. (1983) *J. Physiol. (London)* **338**, 66*P*–67*P*
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, S. E. (1982) *Cell Calcium* **3**, 311–322
Siegl, A. M., Daly, J. W. & Smith, J. B. (1982) *Mol. Pharmacol.* **21**, 680–687
Streb, H. D., Berridge, M. J., Irvine, R. F. & Schulz, I. (1983) *Nature (London)* **306**, 67–69
Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–334