Vol. 120, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 30, 1984 Pages 579-585

CYTOPLASMIC Ca^{2+} IN PLATELETS IS CONTROLLED BY CYCLIC AMP: ANTAGONISM BETWEEN STIMULATORS AND INHIBITORS OF ADENYLATE CYCLASE

George B. Zavoico and Maurice B. Feinstein

Department of Pharmacology, University of Connecticut Health Center, Farmington, CT 06032

Received March 22, 1984

SUMMARY: Activation of platelets by thrombin rapidly increases cytoplasmic free calcium, [Ca2+]i, measured by Quin-2, and induces secretion. Stimulators of adenylate cyclase (i.e. PGI2, PGD2, forskolin) suppressed or reversed the increase of [Ca2+]i. Inhibitors of adenylate cyclase (i.e. epinephrine, ADP), added before or after thrombin, counteracted PGI2, PGD2 and forskolin and thereby increased [Ca2+]i and restored secretion. Responses to epinephrine (via alpha-2 adrenoreceptors) and ADP were independent of extracellular Ca2+, but required maintained occupancy of thrombin receptors and intact cAMP-phosphodiesterase activity. These results indicate that cAMP serves as an inhibitory second-messenger that antagonizes the mobilization of Ca2+, an activator second-messenger.

Thrombin stimulates platelets to change shape, aggregate and secrete. Platelet activation involves two Ca^{2+}-dependent parallel pathways for protein phosphorylation that are important for secretion: i.e. phosphorylation of Mr 20,000 myosin light chains by Ca^{2+}/calmodulin-dependent myosin light chain kinase (1), and phosphorylation of a Mr 47,000 protein by Ca^{2+}/phosphatidylserine-dependent protein kinase C (2). Although increasing Ca^{2+} alone directly causes myosin phosphorylation (3) and secretion (4) in platelets permeabilized by digitonin or an intense electric field, the responses of intact platelets to thrombin are characterized by the mobilization of both Ca^{2+} and 1,2-diacylglycerol. 1,2- Diacylglycerol, that is generated from phosphoinositides, functions as a cooperative second-messenger with Ca^{2+} by greatly increasing the affinity of protein kinase C for Ca^{2+} (5). Furthermore, it has been proposed that the mobilization of Ca^{2+} in many cells is due to the receptor-linked activation of hydrolysis of polyphosphoinositides (6,7), suggesting that both second-messengers could be generated simultaneously.

0006-291X/84 $1.50

Cyclic AMP serves as a second-messenger that mediates the inhibition of most platelet functions. The antithrombotic activity of PGI_2, which is produced by vascular endothelium, is attributable to its potency as a stimulator of adenylate cyclase (9). One mechanism by which cyclic AMP may regulate platelet functions is to suppress the levels of the activator second-messengers Ca^{2+} (10-12) and 1,2-diacylglycerol (13), which in turn could diminish protein phosphorylation. In this paper we show for the first time that epinephrine and ADP, which act on receptors linked to inhibition of adenylate cyclase, can antagonize stimulators of adenylate cyclase (PGI_2, PGD_2, forskolin) and thereby indirectly promote the mobilization of $[Ca^{2+}]i$ and secretion by thrombin.

METHODS: Fresh platelet concentrates obtained from the Connecticut Red Cross Blood Center were washed as previously described (14) and suspended in 10 mM sodium HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 145 mM NaCl, 5.4 mM KCl, 5.5 mM glucose at pH 7.4. These platelets were incubated with 10-15 uM Quin-2/acetoxymethylester at 37°C for 30 min, washed in medium containing fatty acid-free BSA (0.2 mg/ml), and then resuspended in the HEPES-salt buffer with BSA and 1.0 mM CaCl2 and MgCl2. Quin-2 fluorescence was measured in a Perkin-Elmer MPF-2A spectrofluorometer and [Ca2+]i calculated by the calibration procedure of Tsien et al, (15). Secretion of dense granule constituents, Ca2+ and ATP, was measured simultaneously; Ca2+ with a Ca2+-selective electrode (Radiometer) inserted into the temperature-controlled fluorometer cuvet (16), and ATP by withdrawing samples from the cuvet and assaying with luciferin-luciferase in a Chronolog Lumiaggregometer (Chronolog Corp.). For optimal electrode response external Ca2+ was 50 μM when secretion was measured. Many experiments were conducted at 23°C to slow biochemical reactions so as to permit better temporal resolution of these events (data to be published elsewhere), however all effects described in this paper were obtained at either 23°C or 37°C.

RESULTS AND DISCUSSION: In keeping with their ability to increase platelet cAMP levels, pretreatment of platelets with PGI_2, PGD_2 and forskolin inhibited the rate and extent of rise of $[Ca^{2+}]i$ and the secretion brought about by thrombin. Dibutyryl cAMP exerted the same effects. PGI_2 was by far the most potent antagonist, in accord with its relative ability to stimulate adenylate cyclase: I_{50} for inhibition of Ca^{2+} mobilization vs 1.0 U/ml thrombin was 0.5-1.0 nM; 95 percent inhibition was obtained at 10 nM PGI_2 (Fig. 1). When platelets were first stimulated with thrombin, the addition of PGI_2 (or PGD_2, or forskolin) at the time that $[Ca^{2+}]i$ reached its peak caused the elevated $[Ca^{2+}]i$ (about 1-3 μM) to rapidly

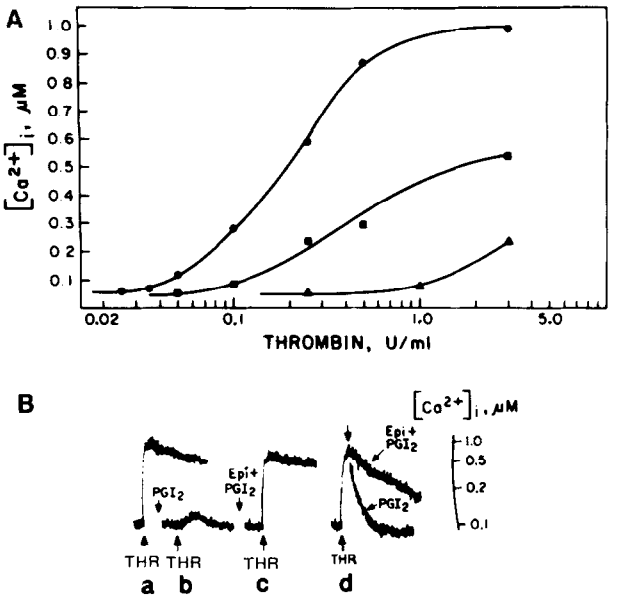

Figure 1. A. Dose-response relationship for inhibition of calcium mobilization by PGI2, and the antagonism of PGI2 by epinephrine. $(Ca^{2+})i$ was measured in Quin-2 loaded platelets (10^8 /ml) at 23°C; ●, control, ■ + 1 nM PGI2, ▲ + 10nM PGI2. In the presence of 1 μM epinephrine the responses with PGI2 were shifted back to the control curve. B. Antagonism of PGI2 by epinephrine: (a) increase of platelet $(Ca^{2+})i$ in response to 1.0 U/ml thrombin (THR), (b) inhibition by 10 nM PGI2, (c) antagonism of PGI2 by 1 μM epinephrine (Epi), (d) 10 nM PGI2 added at peak of response to thrombin causes rapid fall of $(Ca^{2+})i$; pretreatment with epinephrine prevents accelerated resequestration of Ca^{2+} caused by PGI2.

fall back to the normal pre-stimulus level of 50-100 nM (Fig. 1B,d); an effect that is accompanied by protein dephosphorylation (14). The mechanism for enhancement of Ca^{2+} resequestration is not known, but could be due to cAMP-dependent stimulation of Ca^{2+} transport and/or to termination of the formation or action of a mediator of Ca^{2+} mobilization; i.e. inositol triphosphate (7).

If the hypothesis that cAMP controls $[Ca^{2+}]i$ is correct then inhibitors of adenylate cyclase, such as epinephrine and ADP, should exert significant effects on calcium mobilization. Epinephrine and ADP can reduce the elevated concentrations of cyclic AMP caused by adenylate cyclase stimulants in intact platelets (17). They also inhibit the basal and prostaglandin-stimulated adenylate cyclase activity in platelet membrane fractions (18, 19). Both antagonists act through specific receptors coupled to guanine nucleotide-binding proteins (Ni) that inhibit the enzyme (18-20).

Epinephrine and ADP by themselves had little or no effect on $[Ca^{2+}]i$ when added to washed platelets; occasionally a transient rise of about 50 nM, without secretion, was observed. However, epinephrine (or ADP, data not shown for the sake of brevity) prevented the inhibition of Ca^{2+} mobilization by the adenylate cyclase stimulants in the thrombin-stimulated platelets (Figs. 1B, a-c). The ability of PGI_2 to rapidly restore elevated $[Ca^{2+}]i$ to its basal level after stimulation by thrombin was also counteracted by epinephrine and ADP (Fig. 1B,d). Epinephrine and ADP were unable to reverse the effects of dibutyryl cyclic AMP, presumably because the cyclic nucleotide analog can bypass adenylate cyclase to directly activate cyclic AMP-dependent kinases and thereby inhibit the mobilization of Ca^{2+}. In another type of experiment, platelets were first incubated with PGI_2 or PGD_2 to prevent Ca^{2+} mobilization and secretion by thrombin. The subsequent addition of epinephrine (0.01-1.0 uM) or ADP (0.1-1.0 uM) caused an increase in $[Ca^{2+}]i$ to levels ranging from 300 to 700 nM in platelets from more than 15 different donors (Fig. 2). The rate and magnitude of the rise of $[Ca^{2+}]i$ were substantially less than the response of untreated plate-

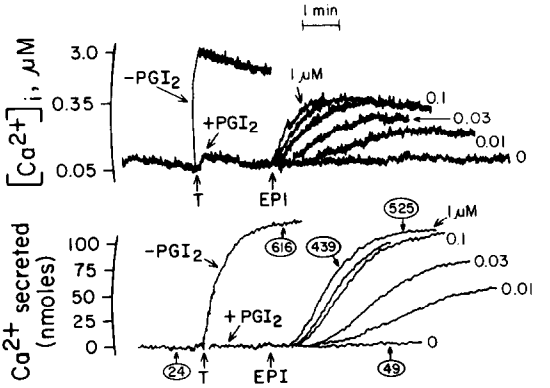

Figure 2. Epinephrine added post-stimulation reverses PGI2-induced inhibition of Ca2+ mobilization and secretion. Upper traces, (Ca2+)i; lower traces, secretion of calcium. Quin-2 fluorescence and secretion were measured simultaneously (see methods). Values for ATP secreted (pmoles in 0.1 ml aliquot) are shown as enclosed numbers at specific time points (arrows) along Ca2+ secretion traces. Control responses to 1 U/ml thrombin (T): -PGI2. Both (Ca2+)i mobilization and secretion were blocked by 10 nM PGI2: +PGI2. (Ca2+)i mobilization and secretion were restored as a function of epinephrine (EPI) concentrations from 0.01 to 1.0 μM. Epinephrine concentrations are indicated at right edge of traces. Platelets 1 x 10^8 per ml, temperature 23°C.

lets to the same concentration of thrombin. This was probably due in part to the onset of desensitization to thrombin (21), which effects Ca^{2+} mobilization (data not shown), by the time epinephrine or ADP were added. Nevertheless, the rise in $[Ca^{2+}]i$ was accompanied by secretion of Ca^{2+} and ATP. The rate and extent of these responses were a function of the concentration of the adenylate cyclase inhibitors (Fig. 2).* The effective concentrations of epinephrine (30-100 nM) were below those that directly cause aggregation and secretion by platelets in plasma. Secretion never occurred in the absence of mobilization of $[Ca^{2+}]_i$. However, the concentrations of $[Ca^{2+}]i$ attained were less than those that evoke secretion in electrically permeabilized platelets (4), indicating that other stimulatory factors were produced concurrently; i.e. diacylglycerol (2, 10).

The ability of epinephrine (or ADP) to increase $[Ca^{2+}]i$, was unaffected by the absence of Ca^{2+} in the medium, indicating that internal Ca^{2+} was being mobilized. However, the adenylate cyclase inhibitors were ineffective in the presence of theophylline (Fig. 3A,B). Although ADP and epinephrine can reduce cAMP levels elevated by prostaglandins, the presence of a phosphodiesterase inhibitor slows the rate of fall and the new steady-state attained remains well above the basal level (17). Thus, intact activity of cAMP-phosphodiesterase is necessary for epinephrine and ADP to reduce cAMP to levels that would permit mobilization of Ca^{2+} by thrombin. The response to epinephrine and ADP also depended upon the occupancy of thrombin receptors, since they could not increase $[Ca^{2+}]_i$ if thrombin was first removed by hirudin (Fig. 3B). This result implies that the mechanisms for mobilizing Ca^{2+}, although inhibited by cAMP-dependent reactions, remained potentially operant for some time, as long as thrombin remained associated with its receptors. The rise of $[Ca^{2+}]i$ could then occur when the antagonism imposed by cAMP was lifted.

*Epinephrine has also been shown to reverse the inhibition of arachidonic acid-induced cytoskeleton assembly and phosphorylation of myosin and Mr 47,000 protein caused by PGI2 (22). It is likely that these effects are in part related to the ability of epinephrine to reverse the inhibition of [Ca2+]i mobilization by PGI2.

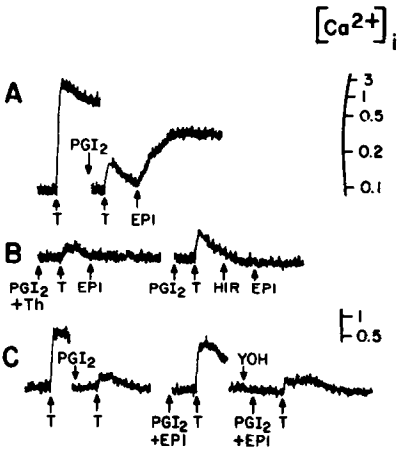

Figure 3. Antagonism of PGI2 by epinephrine. A. Control Ca2+ mobilization by 1 u/ml thrombin (T), inhibition of Ca2+ mobilization by 10 nM PGI2 and its reversal by 1 μM epinephrine (EPI). B. Epinephrine is inneffective in the presence of 0.5 mM theophylline (theo), or 3 U/ml hirudin (hir). C. Inhibition of thrombin by PGI2 is reversed by 1 μM EPI, but effect of EPI is blocked by 0.1 μM yohimbine (YOH).

Alpha-adrenergic receptors mediate two types of physiological responses; i.e., the alpha-2 subtype causes inhibition of adenylate cyclase (23), and the alpha-1 adrenoreceptors promote the mobilization of $[Ca^{2+}]i$ (24) and increased turnover of phosphoinositides (23). All of the effects of epinephrine on platelet $[Ca^{2+}]i$ were blocked by the alpha-2 adrenoreceptor antagonist yohimbine, but not by 10 uM corynanthine (Fig. 3C) which is a highly selective alpha-1 antagonist (25). Although alpha-2 adrenoreceptors have not previously been linked to the mobilization of Ca^{2+} these results in platelets indicate that they can do so indirectly in cells in which cyclic AMP suppresses $[Ca^{2+}]i$.

The rate of cyclic AMP production represents the net effects of the various receptors on the stimulatory (Ns) and inhibitory (Ni) GTP-binding protein regulators of adenylate cyclase activity (18,26,27). This dynamic balance between the opposing forces acting on the enzyme was clearly reflected in the changes in $[Ca^{2+}]i$ levels in stimulated platelets. Variations in platelet $[Ca^{2+}]i$ caused by this interplay between PGI_2 and various physiological inhibitors of adenylate cyclase (28) could be an important factor

determining platelet reactivity and the antithrombotic efficacy of PGI_2 produced by vascular endothelium in vivo (29).

ACKNOWLEDGEMENT This work was supported by NIH grant HL 18937.

References

1. Hathaway, D.R. and Adelstein, R.S. (1979) Proc. Natl. Acad. Sci. USA 76, 1653-1657.
2. Kaibuchi, K., Sano, K., Hoshijima, M., Takai, Y. and Nishizuka, Y. (1982) Cell Calcium 3, 323-335.
3. Daniel, J.C., Purdon, A.D. and Molish, I.R. (1982) Fed. Proc. 41, 1118.
4. Knight, D.E. and Scrutton, M.B. (1980) Thrombos. Res. 20, 437-446.
5. Takai, Y., Kishimoto, A., Kawahara, Y., Minakuchi, R., Sano, K., Kikkawa, Y., Mori, T., Yu, B., Kaibuchi, K., and Nishizuka, Y. (1981) Adv. Cyclic Nucl. Res. 14, 301-313.
6. Downes, P. and Michell, R.H. (1982) Cell Calcium 3, 467-502.
7. Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) Nature 306, 67-68.
8. Feinstein, M.B. (1982) In: Progr. in Hemostasis and Thrombosis V.6 (ed. Spaet, T.H.) pp. 25-61, Grune and Stratton, Inc.
9. Tateson, J.E., Moncada, S. and Vane, J.R. (1977) Prostaglandins 13, 389-397.
10. Rink, R.J. and Smith, S.W. (1983) J. Physiol. 338, 66-67P.
11. Feinstein, M.B., Egan, J.J., Sha'afi, R.I. and White, J. (1983) Biochem. Biophys. Res. Commun. 113, 598-604.
12. Yamanishi, J., Kawahara, Y. and Fukuzaki, H. (1983) Thrombosis Res. 32, 183-188.
13. Imai, A., Hattori, H., Takahashi, M. and Nozawa, Y. (1983) Biochem. Biophys. Res. Commun. 112, 693-700.
14. Feinstein, M.B., Egan, J.J. and Opas, E.E. (1983) J. Biol. Chem. 258, 1260-1267.
15. Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) J. Cell Biol. 94, 325-334.
16. Feinstein, M.B. (1980) Biochem. Biophys. Res. Commun. 93, 593-600.
17. Mills, D.C.B. (1974) In: Platelets and Thrombosis (ed. Sherry, S. and Scriabine, A.) pp.45-67, University Park Press, Baltimore.
18. Jakobs, K.H., Aktories, K. and Schultz (1981) Adv. Cyclic Nucl. Res. 14, 173-187.
19. Cooper, D.M.F. and Rodbell, M. (1979) Nature 282, 517-518.
20. Smith, S.K. and Limbird, L.E. (1982) J. Biol. Chem. 257, 10471-10478.
21. McGowan, E.B. and Detwiler, T.C. (1983) Thrombosis Res. 31, 297-304.
22. Cox, A.C., Carroll, R.C., White, J.G. and Rao, G.H.R., J. Cell Biol. (1984) 98, 8-15.
23. Fain, J.N. and Garcia-Sainz, J.A. (1980) Life Sci. 26, 1183-1194.
24. Charest, R., Blackmore, P.F., Berthon, B. and Exton, J.H. (1983) J. Biol. Chem. 258, 8769-8773.
25. Weitzell, R., Tanaka, T. and Starke, K. (1979) Nauyn-Schmiedeberg's Arch Pharmacol. 308, 127-136.
26. Murayama, T. and Ui, M. (1983) J. Biol. Chem. 258, 3319-3326.
27. Rodbell, M. (1980) Nature 284, 17-22.
28. Gorman, R.R., Fitzpatrick, F.A. and Miller, D.V. (1978) Adv. Cyclic Nucl. Res. 9, 597-609.
29. Gryglewski, R.J., Bunting, S., Monsada, S. Flower, R.J. and Vane, J.R. (1976) Prostaglandins 12, 685-713.