Vol. 113, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 15, 1983 Pages 598-604

THE CYTOPLASMIC CONCENTRATION OF FREE CALCIUM IN PLATELETS IS CONTROLLED BY STIMULATORS OF CYCLIC AMP PRODUCTION (PGD_2, PGE_1, FORSKOLIN)

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Received May 5, 1983

SUMMARY: Maximal stimulation of platelets with thrombin results in a rapid increase in cytoplasmic Ca^{2+} (from 0.1 μM to 1-3 μM), as measured with the fluorescent intracellular Ca^{2+} indicator Quin-2. Prior addition of the adenylate cyclase stimulators PGD_2, PGE_1 or forskolin inhibited the rise in cytoplasmic Ca^{2+}. When added after the maximal response to thrombin was attained adenylate cyclase stimulators caused a rapid fall of cytoplasmic Ca^{2+} back to the original "resting" level. This effect coincides with the reversal of thrombin-induced, Ca^{2+}-dependent protein phosphorylation, and cytoskeleton assembly. It is suggested that cAMP-dependent reactions maintain low levels of cytoplasmic Ca^{2+} by promoting transport and/or binding of Ca^{2+}.

Calcium is an important second messenger involved in the stimulus-induced activation of platelets to aggregate, secrete and generate biologically potent metabolites of arachadonic acid (1,2). Cyclic AMP, on the other hand, acts to prevent nearly all manifestations of platelet activation (2). The most potent inhibitors of platelet responses are prostaglandins (i.e., PGI_2, PGD_2 and PGE_1) that act through specific receptors to stimulate adenylate cyclase (3), and forskolin which acts directly on the enzyme or through regulatory proteins (4). Other responses to stimulation that can be prevented by prostaglandins that stimulate adenylate cyclase include the assembly of Triton X-100-insoluble cytoskeletons (containing actin, actin-binding protein and myosin), and the phosphorylation of myosin light chain (Mr 20,000) and a Mr 47,000 cytosolic polypeptide (5,6). Furthermore, if cytoskeletal assembly and protein phosphorylation are first allowed to occur normally in response to thrombin they can then be rapidly reversed by the addition of PGD_2 or forskolin (6). The phosphorylation of myosin and the Mr 47,000 polypeptide is under the

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control of Ca^{2+} + calmodulin-dependent myosin light chain kinase (7) and Ca^{2+}-/diglyceride-stimulated, phospholipid-dependent protein kinase C (8) respectively. It appears therefore that cyclic AMP-dependent reactions might affect protein phosphorylation by restricting the amount of cytoplasmic Ca^{2+} available to these enzyme systems. However, direct evidence in support of this mechanism of action is lacking. In addition, other explanations for some of the actions of cyclic AMP are possible; i.e. myosin light chain kinase can be phosphorylated *in vitro* by a cyclic AMP-dependent kinase resulting in a decrease in both V_{max} and affinity of the enzyme for calmodulin (9).

Tsien (10) has developed a fluorescent, selective, Ca^{2+}-chelator probe that permits the assessment of free cytoplasmic Ca^{2+} levels in small cells which are inaccessible by other means. Quin-2 acetyoxymethyl ester readily enters cells where it is hydrolyzed by intracellular esterases to generate the impermeant anionic Ca^{2+} chelator Quin-2 (K_d 0.1 μM). Quin-2 is trapped in the cytoplasm, and shows little or no binding to membranes or mitochondria (11). Upon chelating Ca^{2+} the fluorescence of Quin-2 is enhanced about 5-fold (10,11). Rink *et al.* (12) employing measurements of Quin-2 fluorescence in platelets showed that stimulation by thrombin increased free Ca^{2+} from a resting level of 10^{-7}M up to micromolar concentrations. In this paper we utilize this method and demonstrate that the increase in cytoplasmic Ca^{2+} levels normally brought about by thrombin can be prevented or reversed by the stimulation of adenylate cyclase with PGE_1, PGD_2 or forskolin. These experiments provide the first direct demonstration in intact platelets that cytoplasmic free Ca^{2+} levels are strongly regulated by cAMP-dependent reactions.

METHODS: Platelet concentrates freshly obtained from the Connecticut Red Cross Blood Center were washed as previously described (6) and then suspended in a solution containing 25 mM Tris-HCl (pH 7.4), 135 mM NaCl, 5.4 mM KCl, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.35 g/l fatty acid-free bovine serum albumin, and 1.0 g/l dextrose. The platelets were incubated at 37°C for 30-75 min with 10 μM Quin-2 acetoxymethyl ester (Lancaster Synthesis Ltd, Eastgate, England) and then either washed or diluted 30-fold into the suspending medium. Fluorescence was measured in a model 8000S SLM

photon-counting spectrofluorometer. The platelet suspensions were maintained at 37°C and were magnetically stirred. Solutions of human thrombin (2500U/mg), PGD_2, PGE_1, theophylline (Sigma Chem. Co.) and forskolin (Calbiochem) were added with microsyringes through a small hole in the cover of the cuvet chamber. Fluorescence excitation was at 339 nm and emission was measured every 2 sec at 492 nm. The digital output from the fluorometer was led into a Hewlett-Packard model 85 computer for analysis and the data was printed on a Hewlett-Packard X-Y recorder. Protein phosphorylation was measured as described previously (6).

RESULTS AND DISCUSSION: The addition of 0.5-1.0 U/ml of thrombin to platelets pre-loaded with Quin-2 caused a rapid increase in fluorescence (fig. 1). Cytoplasmic calcium was estimated to increase from about 0.1 μM to at least 0.5-1.0 μM (12). Pretreatment of platelets with PGD_2, PGE_1 or forskolin inhibited both the rate and magnitude of the increase in cytoplasmic Ca^{2+} (fig. 1). This effect was potentiated by the phosphodiesterase inhibitor

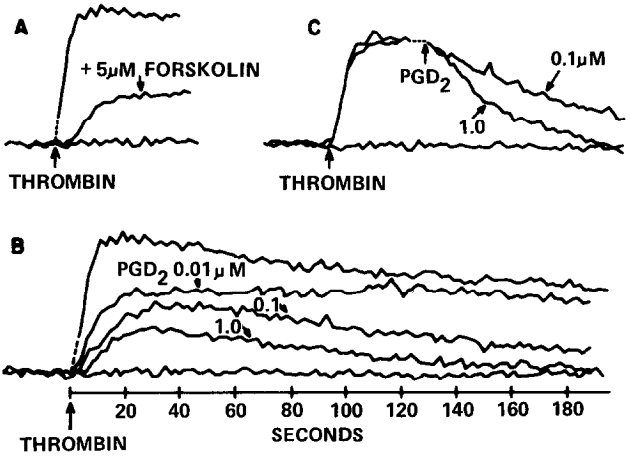

Fig. 1. Fluorescence measurements in platelets pre-loaded with Quin-2 and stimulated with thrombin (1.0 U/ml). In response to thrombin cytoplasmic peak Ca^{2+} levels (increased fluorescence) were attained within 10 sec., with no discernible initial lag phase. (A) Platelets (~$0.7X10^8$/ml); upper trace, control response to thrombin; middle trace, thrombin added after 2 min. pre-treatment with 5 μM forskolin (half-maximal stimulation of adenylate cyclase occurs at about 3 μM forskolin, maximum effect occurs at about 100 μM, ref. 4); lowest trace, control not stimulated by thrombin. (B) Upper trace, platelets stimulated by thrombin; lowest trace, unstimulated; other traces are from platelets pre-treated for 2 min. prior to thrombin with 0.01, 0.1 and 1.0 micromolar PGD_2. (C) Platelets were first stimulated with thrombin and then PGD_2 at 0.1 or 1.0 μM was added 35 sec. later. 10^{-6}M PGD_2 reduced Ca^{2+} back to control level (lowest trace) in 60 sec. PGD_2 produces half-maximal stimulation of platelet membrane adenylate cyclase at 0.4 μM (24). Although the stimulation of cAMP production in intact platelets by PGD_2 is partially inhibited by pre-treatment with thrombin (6), 1 μM PGD_2 completely reversed both the elevated Ca^{2+} levels and myosin phosphorylation induced by 1U/ml thrombin (fig. 3, and ref. 6).

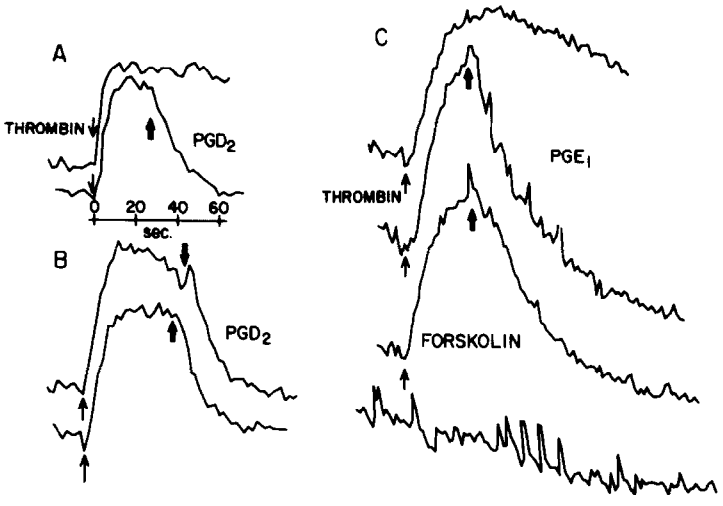

Fig. 2. Fluorescence measurements in Quin-2 loaded platelets stimulated with 0.5 U/ml thrombin. (A) Upper trace, thrombin alone; lower trace 1.0 μM PGD_2 + 1.0 mM theophylline was added 30 sec. after thrombin. Cytoplasmic Ca^{2+} returned to control level in 30 sec. Myosin phosphorylation was decreased by 80% in 45 sec. by the same concentrations of PGD_2 and theophylline (fig. 3). (B) Same as in (A); upper trace, PGD_2 3 μM; lower trace PGD_2 0.3 μM + 0.3 mM theophylline. (C) traces from top to bottom: thrombin alone; PGE_1 (1.0 μM) + 1.0 mM theophylline added 30 sec. after thrombin; forskolin 75 μM; unstimulated control. PGE_1 produces half-maximal stimulation of platelet membrane adenylate cyclase at 0.9 μM (24).

theophylline. When PGD_2, PGE_1 or forskolin were added after the maximum response to thrombin was attained, the elevated cytoplasmic Ca^{2+} levels were rapidly reduced towards normal resting levels (fig. 1,2). Under the same conditions both thrombin-induced protein phosphorylation (fig.3), and cytoskeleton assembly (6) were reversed concurrently by PGD_2 or forskolin. These effects were also potentiated by theophylline.

These experiments provide the first direct demonstration that stimulators of adenylate cyclase activity (i.e. PGD_2, PGE_1, forskolin) can regulate the levels of cytoplasmic Ca^{2+} in intact platelets. Much evidence indicates that these agents block platelet responses by stimulating the generation of cyclic AMP (21), and the potentiation of their effects on cytoplasmic Ca^{2+} levels by theophylline is consistent with this hypothesis. The ability of prostaglandins and forskolin to suppress the rise in cytoplasmic Ca^{2+} that is normally caused by thrombin provides a logical basis for understanding

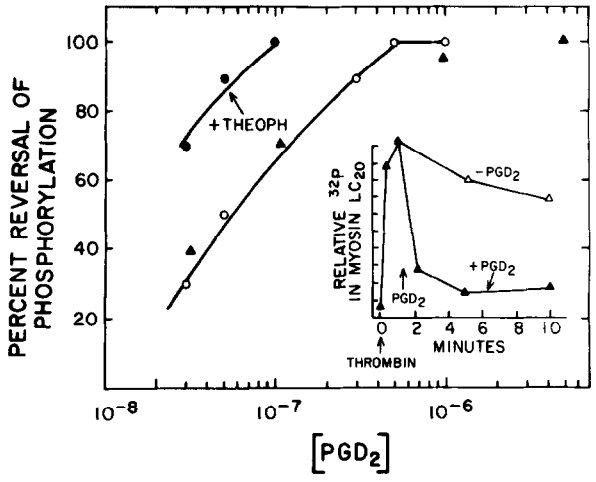

Fig. 3. Reversal of protein phosphorylation as a function of concentration of PGD_2 (+ 1.0 mM theophylline). Platelets were stimulated with 1.0 U/ml thrombin. PGD_2 (± theophylline) was added 75 sec. later. Protein phosphorylation was measured after 5 min. as described in reference 6: myosin Mr 20,000 light chain (LC_{20}) ○, ●; Mr 47,000 polypeptide ▲. Open symbols - theophylline, closed symbols + theophylline 1.0 mM. Insert: Time course of the reversal of myosin LC_{20} phosphorylation by PGD_2 (1.0 μM) plus theophylline. Thrombin concentration was 1.0 U/ml.

their capacity to prevent platelet activation, since many of the biochemical steps in the activation process are either stimulated by, or dependent upon, Ca^{2+} (1,2). These findings are also in agreement with previous work demonstrating that PGI_2 (prostacyclin) inhibited the fall in chlortetracycline fluorescence (13,14) that is believed to measure the release of membrane-bound Ca^{2+} by agonists. Further work is necessary to determine how the rise in cytoplasmic Ca^{2+} is inhibited by cyclic AMP. It is plausible that some crucial initial biochemical reaction necessary for the intracellular release of Ca^{2+}, or its influx from the medium is inhibited. Although recent work suggests the involvement of polyphosphoinositide metabolism in the mobilization of intracellular calcium (15,16) this has not been conclusively established. The likelihood that cyclic AMP acts primarily through stimulation of Ca^{2+} transport, and/or calcium-binding (13) is suggested by the fact that the prostaglandins and forskolin can stimulate processes that rapidly remove from the cytoplasm the Ca^{2+} that previously had been released by thrombin (fig. 1,2). Furthermore, the lowering of cytoplasmic Ca^{2+} by

cyclase-stimulators closely corresponds in both time course and dose-response to their ability to reverse thrombin-induced protein phosphorylation (fig. 3, and ref.6) and cytoskeleton assembly (6). However, in the case of myosin dephosphorylation concurrent cyclic AMP-mediated inhibition of myosin light chain kinase (9) cannot be excluded as a contributory factor.

The striking fall in cytoplasmic Ca^{2+} that is brought about by prostaglandins and forskolin in thrombin-stimulated platelets may be related to the ability of cyclic AMP-dependent protein kinase to stimulate the uptake of Ca^{2+} (18-20), and the phosphorylation of certain proteins (17,19,20,21,6), by a platelet membrane fraction believed to be largely derived from the dense tubular system (DTS). The DTS, which anatomically resembles the smooth endoplasmic reticulum of embryonic muscle (1), contains crucial elements for the regulation of Ca^{2+} transport; i.e. both Ca^{2+}/Mg^{2+} ATPase and prostaglandin-stimulated adenylate cyclase (22,23). Thus the DTS may function in a manner analogous to the sarcoplasmic reticulum of muscle, playing an important role in both activation by platelet aggregating agents (1), as well as in the recovery from stimulation. In addition, mitochondria and the Ca^{2+}/Mg^{2+}-ATPase activity present in the membranes of the surface-connected open canalicular system (22,23), may also play a role in the regulation of cytoplasmic Ca^{2+}, that is yet to be fully defined.

Acknowledgement: This work was supported by NIH Research Grants HL18937 and AI13734.

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