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Biochemistry

Interrelationships between Ca^{2+} and adenylate and guanylate cyclases in the control of platelet secretion and aggregation

[prostaglandin E_1/ATP/Ca^{2+}-ionophore (A23,187)/adenosine 3′:5′-cyclic monophosphate/guanosine 3′:5′-cyclic monophosphate]

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ABSTRACT **Ca^{2+} is a powerful inhibitor ($K_i \simeq 16$ μM) of basal and prostaglandin E_1 (PGE_1)-stimulated adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] activity in membranes obtained from homogenized human platelets. Ca^{2+} (but not the ionophore A23,187) decreased V_{max} of the reaction without an effect on the K_s for ATP. Neither ATP nor PGE_1 affected K_i for Ca^{2+}. In *intact* platelets A23,187 induced Ca^{2+} influx and markedly inhibited PGE_1-stimulated rise in adenosine 3′:5′-cyclic monophosphate (cAMP) levels. Guanylate cyclase [GTP pyrophosphate-lyase (cyclizing); EC 4.6.1.2] activity was mainly found in the soluble fraction (>90%). Both soluble and membrane bound enzymes were stimulated by Mn^{2+} and Ca^{2+} and inhibited by Zn^{2+}. Adenylate and guanylate cyclase activity were both present in a membrane fraction which contained Ca^{2+}-activated ATPase activity, and accumulated Ca^{2+} from the medium in the presence of ATP and oxalate. Other evidence indicates that these membranes originated in large part from the dense tubular system of the platelets. It is proposed that concurrent inhibition of adenylate cyclase and stimulation of guanylate cyclase facilitates the direct initiating effect of Ca^{2+} on platelet secretion and aggregation.**

The aggregation of human platelets requires the presence of extracellular Ca^{2+}. Secretion of ADP and serotonin, on the other hand, can be evoked by certain stimuli (e.g., thrombin) in the absence of external Ca^{2+} (1, 2). It has been postulated that part of the large store of intracellular calcium is utilized in the process of excitation-secretion coupling (3–5). Support for this view comes from studies employing the calcium ionophores X537A and A23,187 which increase Ca^{2+} permeability and/or release internal Ca^{2+} stores in other cells or organelles. These ionophores induce platelet aggregation (4–8) as well as secretion of adenine nucleotides (3–6) and serotonin (4–6). Secretion induced by A23,187 could not be completely blocked by Ca^{2+}-chelating agents (4–6) and it was concluded therefore that release of intracellular Ca^{2+} was responsible for initiating the release reaction under those conditions. The site of the intracellular Ca^{2+}-stores involved in the control of the platelet release reaction is not known. Lately attention has been focused on the channels of the dense tubular system (DTS) which may serve a role analogous to that of the sarcoplasmic reticulum in muscle (9, 10). Indeed an ATP-dependent Ca^{2+} sequestering system obtained from homogenized platelets displays properties analogous to the isolated sarcoplasmic reticulum of muscle (11, 12), but its site of origin in the platelet is not known. A recent cytochemical investigation in *intact* platelets has shown that the DTS is an important site for ATPase and adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] activity*.

Abbreviations: PGE_1, prostaglandin E_1; cAMP, adenosine 3′:5′-cyclic monophosphate; DTS, dense tubular system; Bu_2cAMP, dibutyryl adenosine 3′:5′-cyclic monophosphate; cGMP, guanosine 3′:5′-cyclic monophosphate; EGTA, ethylene glycol-*bis*(β-aminoethyl ether)-*N,N′*-tetraacetic acid.

* L. S. Cutler, M. B. Feinstein, and G. A. Rodan, submitted for publication

Ionophore-induced secretion and aggregation was inhibited (4, 5) by dibutyryl adenosine 3′:5′-cyclic monophosphate (Bu_2 cAMP) and prostaglandin E_1 (PGE_1), the latter a potent stimulator of platelet adenylate cyclase, and these effects were greatly potentiated by the phosphodiesterase inhibitor theophylline (5). Such findings strongly imply that cellular adenosine 3′:5′-cyclic monophosphate (cAMP) can prevent the mobilization of intracellular Ca^{2+}, or block the results of its mobilization. Inhibition of platelet secretion and aggregation by PGE_1 could be partially overcome by increasing the ionophore and/or Ca^{2+} concentrations (5). Thus, increased intracellular Ca^{2+} may in turn overcome the effects of cAMP or may inhibit PGE_1-induced cAMP synthesis. Vigdahl *et al.* (13) have previously shown that platelet adenylate cyclase was inhibited by Ca^{2+}, but the mechanism of inhibition was not studied. While an increase in platelet cAMP leads to inhibition of platelet aggregation and the release reaction (14), a rise in guanosine 3′:5′-cyclic monophosphate (cGMP) has been associated with these platelet functions (15–17). To learn more about the interactions of Ca^{2+} with cyclic nucleotides in the control of platelet function, we studied cyclase activities in various fractions of homogenized cells and in *intact* cells. In addition, we have undertaken to identify sites of localization of platelet adenylate and guanylate cyclases [GTP pyrophosphate-lyase (cyclizing) EC 4.6.1.2].

METHODS AND MATERIALS

Human platelet concentrates obtained from the Connecticut Red Cross Blood Center were employed in these studies. Washed platelets were prepared as described by Miller *et al.* (2).

Membrane preparations containing cyclase activity were obtained by sonication of platelet suspensions and centrifugation for 15 min at 40,000 × *g*, or by homogenization and differential centrifugation according to the method of Robblee *et al.* (12) to obtain fractions sedimenting at 14,000 × *g* and 40,000 × *g*. Samples of the membrane fractions containing 400 μg of protein were incubated at 30° for 5 min according to the method of Solomon *et al.* (18) for the measurement of adenylate cyclase activity. [α-^{32}P]ATP (New England Nuclear) was employed as substrate in the reaction. The recovery of [^{32}P]cAMP was determined by employing [^{3}H]cAMP (New England Nuclear) as a marker. Guanylate cyclase activity was assayed by the method of White and Zenser (19). α-^{32}P-Labeled GTP (New England Nuclear) was employed as the substrate and recovery of [^{32}P]cGMP was determined using [^{3}H]cGMP as a marker. cAMP was measured by the method of Steiner *et al.* (20).

The calcium of the medium was adjusted with a calcium–ethylene glycol-*bis*(β-aminoethyl ether)-*N,N′*-tetraacetic acid (EGTA) buffer system using an apparent binding constant for $CaEGTA^{-2}$ of 4.4×10^6 M^{-1} determined by the method of Briggs and Fleishman (21) for the conditions of the adenylate

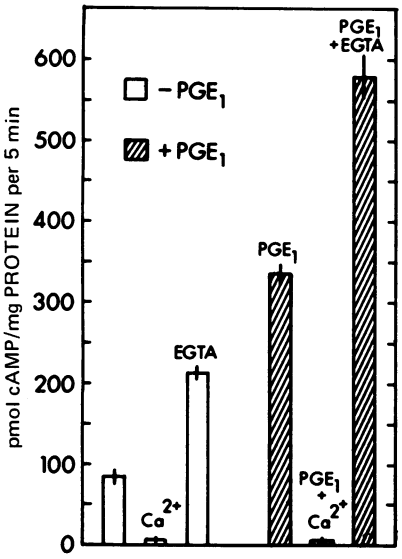

FIG. 1. Effect of Ca^{2+}, EGTA, and PGE_1 on the synthesis of cAMP. A 40,000 × *g* particulate fraction obtained from sonicated platelets was employed. The concentrations used were: Ca^{2+} 1.0 mM, EGTA 1 mM, PGE_1 0.5 μM. The incubation medium was the same as given in Table 1.

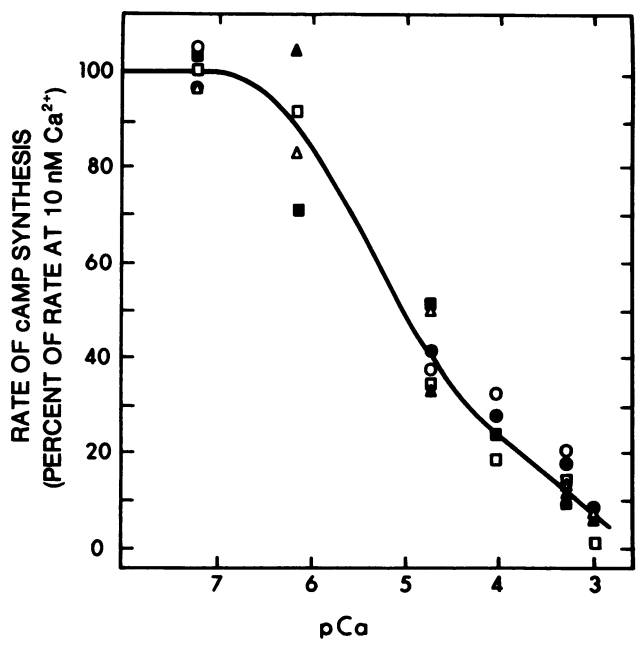

FIG. 2. Adenylate cyclase activity as a function of pCa (negative log of free Ca^{2+} concentration). The rate of cAMP synthesis was normalized to the rate at 10 nM Ca^{2+} at which maximal enzymatic activity was observed. The ATP concentration was 0.1 mM in all experiments. PGE_1 (1 μM) was present in experiments ○, ●, △ only. A23,187 was 5 μM, and present in experiments ○, □ only. ▲, ■, no PGE_1 or A23,187.

cyclase assay. The "free" calcium concentration in the medium was computed by solving the simultaneous equations describing the binding of calcium and magnesium to ATP and EGTA, using the iterative Newton-Raphson procedure.† A23,187 was a gift of Dr. David Wong (Eli Lilly Co.) and prostaglandin E_1 was kindly donated by Dr. J. Pike (Upjohn Co.).

RESULTS

As seen in Fig. 1 the presence of even trace amounts of a bivalent cation was strongly inhibitory to platelet adenylate cyclase since addition of EGTA, in the absence of added Ca^{2+}, increased cAMP formation by 1.7- to 2.4-fold. The inhibition of adenylate cyclase by added Ca^{2+} was independent of the presence of PGE_1 or A23,187 but was a function of the free Ca^{2+} concentration (Fig. 2 and Table 1). The apparent K_i for Ca^{2+} inhibition was 16 μM both in the presence and absence of the ionophore or PGE_1. Thus, inhibition is not *competitive* with respect to PGE_1. PGE_1 at 1 μM caused a 10-fold increase in the maximal velocity of adenylate cyclase activity. A23,187 had no effect on the V_{max} of either the basal or PGE_1-stimulated enzyme. These results indicate that the effects of the ionophore on intact platelets (i.e., see below) are most likely a result of Ca^{2+}-translocation. To further investigate the interaction of calcium with adenylate cyclase, we studied the enzyme activity as a function of both Ca^{2+} and ATP concentration. These experiments were carried out on the PGE_1-stimulated enzyme. K_s and V_{max} were determined by fitting the data to the Michaelis-Menten equation in its hyperbolic form by a numerical iterative procedure. It is shown in Table 2 (A) that with increasing Ca^{2+} concentrations a progressive fall in V_{max} was observed. On the other hand, the K_s for ATP was essentially unchanged at Ca^{2+} concentrations between 67 nM and 0.5 mM, a range over which an 87% fall in V_{max} occurred. At higher Ca^{2+} concentration, K_s for ATP decreased. Furthermore, the K_i for Ca^{2+} did not change significantly over a 200-fold range of ATP concentration [Table 2 (B)]. In conjunction with the previous data, this indicates that the interactions of the enzyme with ATP and Ca^{2+} are independent of each other over a wide range of concentrations. A Hill plot of the effect of Ca^{2+} (Fig. 3) yielded a value of 0.3, which suggests negative cooperativity with respect to the binding of Ca^{2+} to the inhibitory site.

In order to determine if Ca^{2+} could inhibit cyclic AMP synthesis in *intact platelets*, we employed PGE_1 to stimulate adenylate cyclase activity in washed cells. The cAMP level rose from 3 pmol/mg of platelet protein to 11.2 pmol/mg of protein

Table 1. The effects of A23,187, PGE_1, and calcium on platelet adenylate cyclase activity

Assay conditions	V_{max} (nmol of cAMP/ mg of protein per 5 min)	K_i Ca^{2+} × 10^5 M
Control	0.009 (±0.002)	1.87 (±0.43)
+ A23,187	0.011 (±0.002)	1.40 (±0.33)
+ PGE_1	0.098 (±0.017)	1.61 (±0.40)
+ PGE_1 + A23,187	0.106 (±0.011)	1.16 (±0.15)

The velocity (V_{max} ± SEM) of cAMP synthesis was determined in an incubation mixture containing: Tris·HCl 25 mM at pH 7.6, $MgCl_2$ 5 mM, cAMP 1 mM, dithiothreitol 1 mM, ATP 0.1 mM, [α-^{32}P]ATP 3 × 10^6 cpm per tube, creatine phosphate 19 mM, creatine kinase 0.85 mg/ml. The calcium concentration was varied from 0.32 μM to 1 mM. The K_i (±SEM) for calcium was calculated by plotting $1/V$ versus $[Ca^{2+}]$ under each set of conditions. PGE_1 and A23,187 were present at 1 μM and 5 μM, respectively. The reactions were linear with time (up to 15 min) and with protein concentration in the range employed.

† The other stability constants used were: $CaATP^{-2} = 9.12 \times 10^3\ M^{-1}$, $MgATP^{-2} = 1.74 \times 10^4\ M^{-1}$, and $MgEGTA^{-2} = 10^2\ M^{-1}$ (22). Over the range of "free" calcium concentrations of 6.7×10^{-8} M–9.9×10^{-4} M, the other constituents of the assay mixture were calculated to change as follows: $MgATP^{-2}$ 9.9–9.0×10^{-5} M; $CaATP^{-2}$ 6.8×10^{-10}–9.5×10^{-6} M; Mg^{+2} 4.6–4.9×10^{-3} M; ATP not associated with Ca^{2+} or Mg^{2+} 1.2–1.05×10^{-6} M.

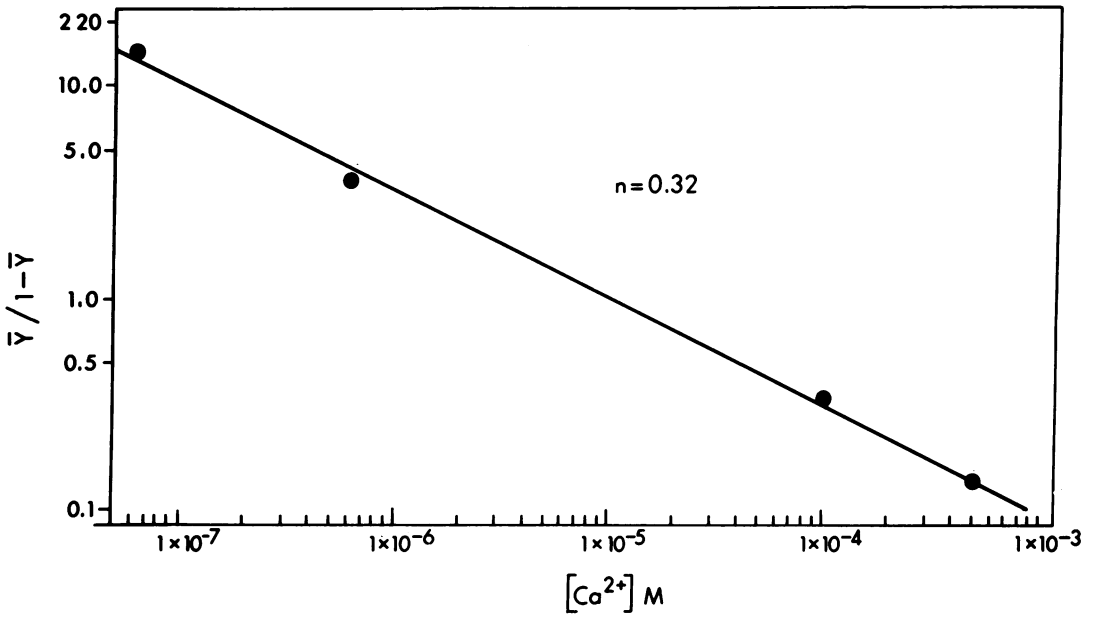

FIG. 3. Inhibition of PGE_1-stimulated adenylate cyclase activity by Ca^{2+}. Hill plot. $[Ca^{2+}]$ is the free calcium ion concentration in equilibrium with CaEGTA. $\bar{Y}$ represents the fraction of maximal activity observed.

within 1 min after addition of PGE_1. When A23,187 was added 25 sec after PGE_1, the cAMP level rapidly fell by 3–4 pmol/mg of protein (Fig. 4). When the ionophore was added 60 sec prior to PGE_1, the cAMP level rose only 2.1 pmol/mg of protein—as compared to a rise of 8.0 pmol/mg of protein for PGE_1 alone. After 2–3 min, the level of cAMP in A23,187 pretreated cells was only 1.2–1.6 pmol/mg of protein above the control level, whereas with PGE_1 alone cAMP was still 6.1–6.7 pmol/mg of protein higher than the control. Thus, the ionophore inhibited the PGE_1-induced rise in cAMP by 75–80%. In the absence of PGE_1 (control platelets) A23,187 (1 μM) together with 1.0 mM extracellular calcium had no significant effect on the level of cAMP. The uptake of calcium induced by 1 μM A23,187, as measured with ^{45}Ca and corrected for surface binding, was found to be about 1 nmol/mg of platelet protein in 60 sec (5). Assuming a platelet volume of 6.2 μm^3, and uniform distribution of this calcium in the cytoplasm, we calculate that an intracellular concentration of approximately 0.4 mM would be attained. At this level of Ca^{2+}, the adenylate cyclase activity in isolated platelet membranes was inhibited about 85–90%.

Table 2. (*A*) The effect of calcium on the kinetics of PGE_1-stimulated platelet adenylate cyclase activity

Calcium (M)	K_s ATP $\times 10^4$ M	V_{max} (nmol of cAMP/ mg of protein per 5 min)
6.7×10^{-8}	1.21 (±0.24)	0.379
6.3×10^{-7}	0.90 (±0.20)	0.296
1.75×10^{-5}	1.34 (±0.26)	0.236
1.0×10^{-4}	1.10 (±0.12)	0.106
5.0×10^{-4}	1.10 (±0.18)	0.050
1.0×10^{-3}	0.59 (±0.24)	0.028

(*B*) Effect of ATP on the apparent dissociation constant (K_i) for calcium

ATP (M)	K_i $Ca^{2+} \times 10^5$ M
5×10^{-6}	2.17 (±0.73)
1×10^{-5}	1.62 (±0.76)
5×10^{-5}	1.80 (±0.37)
1×10^{-4}	1.46 (±0.21)

(*A*) At each concentration of Ca^{2+}, the velocity of cAMP synthesis was determined as a function of ATP concentration, which varied from 5 μM to 0.1 mM. V_{max} and K_s (±SEM) for ATP were determined by a plot of $1/V$ versus 1/[ATP]. The correlation coefficients for these linear plots ranged from 0.992 to 0.994 except at 1 mM Ca^{2+} which was 0.865. The incubation mixture was the same as in Table 1.

(*B*) The K_i (±SEM) for calcium was calculated by plotting $1/V$ versus $[Ca^{2+}]$ from the experiments of Table 2 (A) for each ATP concentration.

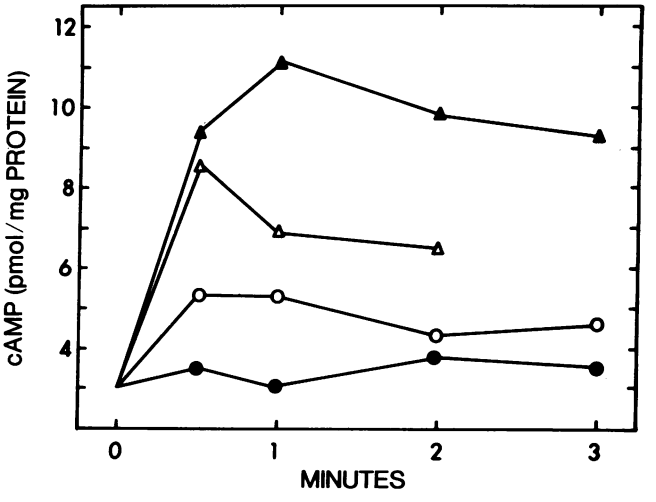

FIG. 4. Effect of A23,187 on PGE_1-induced increase in platelet cAMP levels. The external Ca^{2+} concentration was 1 mM. (●) Washed human platelets (1 mg of protein per ml) exposed at time zero to A23,187 (1 μM) alone. In the other experiments (○, △, ▲), PGE_1 (0.5 μM) was added to platelets at time zero and A23,187 was added 25 sec after PGE_1 (△) or (○) 60 sec prior to PGE_1. The reaction was stopped by boiling. Aliquots of the platelet suspensions, taken at the times indicated, were analyzed for cAMP. Each point is the mean (±SEM) of duplicate samples of two to three experiments.

We have no way of knowing how much intracellular stored Ca^{2+} was released into the platelet cytoplasm.

The other cyclic nucleotide believed to play an important role in platelet aggregation is cGMP (15–17). Although most of the platelet guanylate cyclase activity was recovered in the supernatant fraction after centrifugation of sonicated cells for 1 hr at 49,000 *g*, some activity (<10%) was found to be associtted with the particulate fraction. Both the soluble and membrane bound enzymes required Mn^{2+} for maximum activity. The stimulatory effect of 3 mM Mn^{2+} was completely abolished by 1.0 mM Zn^{2+}. Triton X-100 did not activate either form of the platelet enzyme, which was unlike its effects on the guanylate cyclase in certain other cells (24). More importantly perhaps from the physiological standpoint, Ca^{2+} alone, in the absence of Mn^{2+}, was able to stimulate both the soluble and membrane-associated enzymes (Fig. 5).

Because of the proposed role of cellular membranes in the control of platelet Ca^{2+} concentrations, we fractionated homogenized platelets according to the method of Robblee *et al.* (12) and determined the distribution of adenylate and guanylate cyclase activity. The 40,000 *g* pellet (fraction II) contained 35–60% of the total platelet PGE_1-stimulated adenylate cyclase activity and 70% of the membrane-associated guanylate cyclase. The adenylate cyclase activity was inhibited by Ca^{2+}, as described above, while that of guanylate cyclase was stimulated by Ca^{2+} (Fig. 5). This membrane fraction also contained ATPase activity which was stimulated by Ca^{2+} and oxalate, not affected by 0.5 mM azide, and strongly inhibited by 10 μM mersalyl. In the presence of 1 mM K^+ oxalate and 0.1 mM ATP‡ the 40,000 × *g* membrane fraction incubated according to Robblee *et al.* (12) accumulated about 37 nmol of calcium per mg of protein in 60 min.

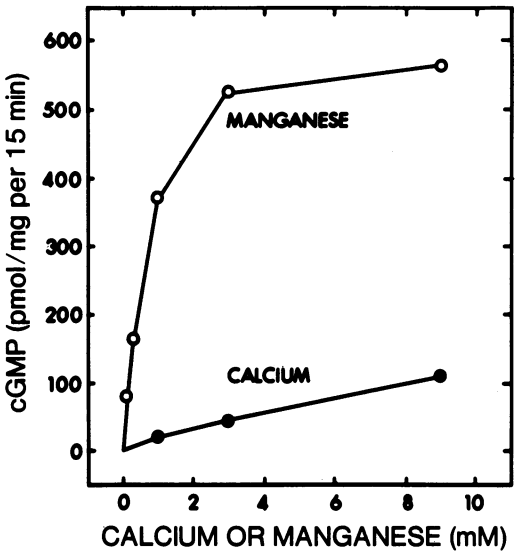

FIG. 5. Effect of Mn^{2+} and Ca^{2+} on membrane guanylate cyclase activity. The 40,000 × *g* pellet was employed after removal of supernatant and 14,000 × *g* pellet fractions (12). The mixture was incubated at 30° and contained 10 mM Tris·HCl at pH 7.6, 2 mM cGMP, 1 mM dithiothreitol, 0.1 mM GTP, [^{32}P]GTP 1 × 10^6 cpm, 10 mM phosphocreatine, and 0.85 mg/ml of creatine phosphokinase.

DISCUSSION

Our results indicate that Ca^{2+} is a powerful inhibitor of platelet adenylate cyclase activity. Assuming Michaelis-Menten kinetics, the effect of Ca^{2+} is mainly on the V_{max} of both the basal and PGE_1-stimulated activity. The substrate (ATP) affinity for the enzyme is not affected by Ca^{2+}, nor is the Ca^{2+} binding affinity affected by either ATP or PGE_1. In these respects the kinetic behavior of the enzyme corresponds quite well to the properties of the adenylate cyclase described in turkey erythrocyte ghosts (25). The Hill plot of our data suggests negative cooperativity for the Ca^{2+} effect, which is opposite to the conclusion of Steer and Levitzki (26). However, the latter plotted $\bar{Y}/1-\bar{Y}$ against the total *added* calcium concentration rather than the calculated "free" Ca^{2+} concentration.

By employing the divalent cation ionophore A23,187 it was possible to demonstrate that the influx of Ca^{2+} was associated with a marked fall in PGE_1-stimulated cAMP levels in *intact* platelets. From the observed calcium uptake values it was calculated that the cytoplasmic calcium concentrations could have attained levels which produce about 90% inhibition of adenylate cyclase activity. It is important to note that other platelet aggregating agents (ADP, epinephrine) also produce a fall in PGE_1-stimulated cAMP levels (26), even in the presence of phosphodiesterase inhibitors. They, therefore, appear to inhibit the rate of synthesis of cAMP rather than accelerate its breakdown. Furthermore, the similar response to A23,187 suggests that the effects of ADP and epinephrine on cAMP levels are mediated by Ca^{2+} rather than being caused by a direct effect of the aggregating agents on the enzyme. If so, the degree of fall of cAMP levels may actually be a reflection of the intracellular free Ca^{2+} concentration.

While it is clear, therefore, that Ca^{2+} is an important regulator of platelet adenylate cyclase activity, it is also evident that the effects of Ca^{2+} are in turn overcome by conditions which elevate the intracellular cAMP levels (4, 5). This could result from any one of a number of possible mechanisms such as: (*a*) inhibition of Ca^{2+} influx or of release from internal Ca^{2+} stores, (*b*) inhibition of Ca^{2+} binding to its site(s) of action, or (*c*) inhibition of the subsequent processes initiated by interaction of Ca^{2+} with its receptor site(s). No evidence exists as yet to indicate if cAMP affects any of these processes. In cardiac muscle, it appears that cAMP can stimulate the Ca^{2+}-pump of the sarcoplasmic reticulum by activating protein kinases (29). Such a mechanism would act to lower cytoplasmic Ca^{2+} levels. A membrane system, morphologically analogous to the sarcoplasmic reticulum of muscle, is found in platelets, i.e., the dense tubular system. Recent cytochemical evidence has demonstrated that both adenylate cyclase and ATPase activities exist in this structure*. We have also found that the membrane fraction isolated from platelets which is active as a Ca^{2+}-pump is high in adenylate cyclase activity. This membrane fraction contained a high concentration of vesicles having a somewhat electron-dense interior, resembled that of the DTS in intact cells, and stained cytochemically for adenylate cyclase activity (L. S. Cutler, personal communication). Thus, there is good reason to believe that the DTS may function as a Ca^{2+}-pump analogous to that of muscle sarcoplasmic reticulum (11, 12) and that its adenylate cyclase activity may modulate the pump's activity. If the DTS is indeed the seat of such a Ca^{2+}-sequestering activity, it may also function as an important source for the release of internal Ca^{2+} into the cytoplasm in response to stimulation by aggregating agents acting at the cell surface. The inhibitory effect of Ca^{2+} on adenylate cyclase activity provides a powerful mechanism for positive feedback, and facilitates the action of Ca^{2+} on platelet secretion and aggregation.

Recent studies in platelets have revealed that cyclic GMP levels rise in response to aggregating agents (15–17). In certain

‡ A creatine phosphate-creatine phosphokinase system for regenerating ATP was present.

other cells, i.e., leukocytes (27) A23,187 can also induce a rise in cGMP levels, and indicates that this nucleotide is also under some control by Ca^{2+}. Most of the platelet guanylate cyclase is found in the soluble fraction of disrupted platelets. We found that Ca^{2+} alone could stimulate enzyme activity, albeit much less than Mn^{2+}. Both soluble and membrane forms of the enzyme responded to Ca^{2+} but not to thrombin. The levels of Ca^{2+} required were high (1–10 mM) and it is not evident that Ca^{2+} can serve as an activator of the enzyme *in vivo* under physiological conditions. Additional work will be necessary to evaluate the significance of this response. Furthermore, the ultimate effect of cGMP itself is unknown. However, it is highly significant that both cyclases are present in the same membrane fraction which contains a Ca^{2+}-activated ATPase, and which most likely functions as an intracellular Ca^{2+}-"sink", as well as a source of internal Ca^{2+} involved in excitation-secretion coupling. Further elucidation of the interactions between Ca^{2+} and cyclic nucleotide synthesizing enzymes, as well as the effects of the cyclic nucleotides themselves on calcium actions and intracellular translocation, are central to understanding the cellular processes which are the basis for the ultimate hemostatic functions of platelets.

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