

# Human Platelet Secretion and Aggregation Induced by Calcium Ionophores

## *Inhibition by PGE<sub>1</sub> and Dibutyryl Cyclic AMP*

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**ABSTRACT** Ca<sup>2+</sup>, Mg<sup>2+</sup>-ionophores X537A and A23,187 (10<sup>-7</sup>-10<sup>-6</sup> M) induced the release of adenine nucleotides adenosine diphosphate (ADP, adenosine triphosphate (ATP), serotonin,  $\beta$ -glucuronidase, Ca<sup>2+</sup>, and Mg<sup>2+</sup> from washed human platelets. Enzymes present in the cytoplasm or mitochondria, and Zn<sup>2+</sup> were not released. The rate of ATP and Ca<sup>2+</sup> release measured by firefly lantern extract and murexide dye, respectively, was equivalent to that produced by the physiological stimulant thrombin. Ionophore-induced release of ADP, and serotonin was substantially (~60%) but not completely inhibited by EGTA, EDTA, and high extracellular Mg<sup>2+</sup>, without significant reduction of Ca<sup>2+</sup> release. The ionophore-induced release reaction is therefore partly dependent upon uptake of extracellular Ca<sup>2+</sup> (demonstrated using <sup>45</sup>Ca), but also occurs to a significant extent due to release into the cytoplasm of intracellular Ca<sup>2+</sup>. The ionophore-induced release reaction and aggregation of platelets could be blocked by prostaglandin E<sub>1</sub> (PGE<sub>1</sub>) or dibutyryl cyclic AMP. The effects of PGE<sub>1</sub>, and N<sup>6</sup>,O<sup>2</sup>-dibutyryl adenosine 3':5'-cyclic monophosphoric acid (dibutyryl cAMP) were synergistically potentiated by the phosphodiesterase inhibitor theophylline. It is proposed that Ca<sup>2+</sup> is the physiological trigger for platelet secretion and aggregation and that its intracellular effects are strongly modulated by adenosine 3':5'-cyclic monophosphoric acid (cyclic AMP).

### INTRODUCTION

As an essential feature of their role in hemostasis platelets undergo a physiological secretory process known as the "release reaction" in which the contents of certain intracellular granules are expelled into the surrounding medium. This occurs without damage or destruction to platelets since cytoplasmic constituents are not released (Holmsen and Day, 1970). Platelet secretion and aggregation are induced physiologically by low concentrations of the blood-clotting enzyme, thrombin, or by platelet interaction with subendothelial connective tissue components, especially collagen. Platelet aggregation may

be impaired by exposure to chemical substances which prevent platelet secretion (Mustard and Packham, 1970), such as aspirin (acetylsalicylic acid), indomethacin, and prostaglandin  $E_1$  ( $PGE_1$ ). Genetic defects in the release reaction, or in the storage pool of adenine nucleotides and serotonin, are associated with impaired aggregation and bleeding tendencies (Weiss, 1972; Hardisty and Mills, 1972). Platelet aggregation is also dependent upon the presence of certain protein factors present in plasma (Cross, 1964; Mustard et al., 1972; Bang et al., 1972) or released from within the platelet (Miller et al., 1975).

Although human platelet aggregation requires the presence of  $Ca^{2+}$  the release reaction induced by some agents, such as thrombin, occurs unimpaired in the absence of  $Ca^{2+}$  in the medium (Morse et al., 1965; Miller et al., 1975). Since the requirement for  $Ca^{2+}$  in cellular secretory processes appears to be so universal (Rubin, 1970), it is logical to suspect that an intracellular pool of calcium may be mobilized in such instances where a requirement of extracellular  $Ca^{2+}$  cannot be demonstrated. The blood platelet contains much stored calcium, a large proportion of which is released during the secretory process (Mürer, 1969; Detwiler and Feinman, 1973; Miller et al., 1975), but no influx of  $Ca^{2+}$  preceding thrombin-induced secretion has been observed (Robblee et al., 1973 *b*). The hypothesis that  $Ca^{2+}$  serves as a trigger for the secretory process would be substantially enhanced if it could be shown that intracellular injection of  $Ca^{2+}$  initiated the event. However, the very small size of platelets precludes this approach. A means of selectively altering  $Ca^{2+}$  permeability could provide the same sort of information. Recently two naturally occurring ionophorous substances (X537A and A23,187) have been described which act as highly mobile divalent cation carriers (Pressman, 1973) and thereby transport  $Ca^{2+}$  and  $Mg^{2+}$  down their thermodynamic activity gradients in various cell organelles such as mitochondria and sarcoplasmic reticulum (Reed and Lardy, 1972; Scarpa et al., 1972; Entman et al., 1972). We have employed these ionophores to study the role of  $Ca^{2+}$  in the platelet release reaction and the ensuing platelet aggregation. The results indicate that  $Ca^{2+}$  is the normal physiological trigger for the secretory and aggregation processes in platelets, and that its effects are strongly modulated by adenosine 3':5'-cyclic monophosphoric acid (cyclic AMP) within the cells.

#### METHODS

##### *Platelet Preparations*

Four different types of platelet preparations were utilized: (*a*) platelet-rich plasma (PRP) obtained from freshly drawn human blood, (*b*) human platelet concentrates obtained from the Connecticut Red Cross Blood Center, Farmington, Conn. (*c*) washed human platelets suspended in a buffered physiological salt solution, and (*d*) washed human platelets suspended in a buffered physiological salt solution containing serum albumin and low concentrations of potato apyrase.

PRP was prepared from freshly drawn blood anticoagulated with 3.8% (wt/vol) trisodium citrate solution at a concentration of 1 vol to 9 vol of blood, or with acid-citrate-dextrose (ACD) solution (1:6 vol/vol) of Aster and Jandl (1964). Centrifugation at room temperature in plastic tubes for 15 min at 120 *g* yielded PRP. Washed platelets were prepared from Red Cross platelet concentrates anticoagulated with ACD (Miller et al., 1975) and were suspended in a medium containing 137 mM NaCl, 2.7 mM KCl, 0.2% (wt/vol) dextrose, and pH adjusted to 7.5 with 25 mM Tris-HCl. In an alternate procedure the wash medium and all subsequent solutions contained 4 mg/ml bovine serum albumin (Cohn fraction V, Sigma Chemical Co., St. Louis, Mo.) and 50  $\mu$ g/ml potato apyrase (Type II, Sigma), a medium in which platelets maintain their normal discoid shape (Mustard et al., 1972). Platelet concentrates in ACD-plasma were also employed after dilution three- to sixfold with the Tris-buffered physiological salt solution containing albumin and apyrase described above.

Protein was determined by a biuret method (Gornall et al., 1949) in concentrated washed platelet suspensions which were then adjusted to a concentration of 1.0-mg platelet protein/ml by dilution with the buffered salt solution. Platelet counts were made with a Technicon Platelet Counting System (Technicon Instruments Corp., Tarrytown, N. Y.). A value of  $1.8 \pm 0.2$  (mean  $\pm$  SE) mg protein per  $10^9$  platelets was found, in close agreement with Holmsen and Day (1970).

#### *Platelet Aggregation*

Platelet aggregation was studied by the turbidimetric method of Born (1962), utilizing a Turner 350 spectrophotometer (G. K. Turner Associates, Palo Alto, Calif.) modified to provide continuous stirring and maintenance of constant temperature (37°C) as previously described by Miller et al. (1975). Aggregation results in an increase in the light transmittance of platelet suspensions. The supernatants resulting from complete centrifugation of formed elements (3,000 *g*, 10 min) were used to set 100% transmittance. Zero transmittance was set with a blocking filter in the light path.

#### *Platelet Release Reaction*

**SEROTININ** [ $3\text{'-}^{14}\text{C}$ ]Serotonin creatinine sulfate (Amersham/Searle Corp., Arlington Heights, Ill.) with a specific activity of 57 mCi/mmol was added at a concentration of 0.02  $\mu$ Ci/ml to the platelet wash medium and uptake was allowed to proceed for 20 min at 20–23°C. The normal washing procedure was then resumed providing essentially complete removal of any extracellular [ $3\text{'-}^{14}\text{C}$ ]serotonin. Aliquots of cell suspensions and supernatants were dissolved in Aquasol (New England Nuclear, Boston, Mass.) and the radioactivity measured in a scintillation counter. [ $^{14}\text{C}$ ]Serotonin was also incubated with PRP for 30–45 min at 37°C. The radioactivity in the platelets was calculated as the difference between the total amount in PRP and that present in an aliquot of platelet-free plasma obtained after centrifugation of PRP at 5,000 *g* for 5 min. Under the prevailing conditions 85–90% of [ $3\text{'-}^{14}\text{C}$ ]serotonin was taken up into the platelets during the incubation period. Platelets were incubated with ionophores in siliconized test tubes at 37°C for the appropriate time and then placed in an ice bath for 5 min. The amount of additional radioactivity released into the

platelet-free supernatant was determined after centrifugation (3,000 *g*) at 0°C for 5 min. Controls which did not receive ionophores were treated in the same way.

**ADENINE NUCLEOTIDES** ADP was measured by the NADH<sub>2</sub>-pyruvate kinase-lactate dehydrogenase coupled enzyme system (Miller et al., 1975). The rate of ATP release was determined by the method of Detwiler and Feinman (1973). The light output due to reaction of released ATP with firefly lantern extract in the platelet suspension medium was measured in a Perkin-Elmer 356 spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) set in the energy mode and with 900 V supplied to the photomultiplier tube (Miller et al., 1975).

**CALCIUM, MAGNESIUM, AND ZINC ASSAYS** Platelet suspensions (1.0 mg total platelet protein) were heated in Vycor test tubes overnight at 180°C with 0.5 ml of concentrated nitric and 70 % perchloric acids, 1:1 (vol/vol). The resulting dry ash was dissolved in 1.0 ml of 0.05 N HCl containing 0.01 M LaCl<sub>3</sub>. Calcium, magnesium, and zinc were determined by atomic absorption spectrophotometry. The calculations for cellular cation content were corrected for the amount of each cation in the extracellular fluid obtained by centrifugation of platelet suspensions at 3,000 *g*. The extracellular fluid and cation standards in platelet buffer were adjusted to contain 0.01 M LaCl<sub>3</sub> and 0.05 N HCl before analysis. The rate of calcium release was also determined by the murexide dye method of Ohnishi and Ebashi (1963) as modified for platelet suspensions by Miller et al. (1975). All glassware was washed in concentrated sulfuric acid saturated with sodium nitrate to reduce calcium contamination.

**<sup>45</sup>Ca UPTAKE** The uptake of calcium was measured in washed platelets by the method of Mürer and Holme (1970) employing <sup>45</sup>Ca as a tracer. The suspension medium contained 1.0-mg platelet protein/ml and 1.0 mM calcium chloride<sup>1</sup> with a specific activity of  $2.9 \times 10^6$  cpm/ $\mu$ mol. Under these conditions release of platelet calcium into the medium could decrease the specific activity by only about 5 %. After the appropriate incubation period with or without A23,187 the cells were rapidly separated from the medium by centrifuging at 8,000 *g* (Eppendorf Microcentrifuge, model 3200) through a sucrose gradient at 0°C for 30 s. A duplicate aliquot of cells was centrifuged through sucrose containing 0.1 mM EGTA to remove superficially bound calcium. The platelet pellets were washed three times with ice-cold 0.25 M sucrose, or 0.25 M sucrose + 0.1 mM EGTA, dissolved in 1 % Triton X-100 and Aquasol and the radioactivity determined by liquid scintillation. Contamination of the platelet pellets with extracellular fluid was assessed by using [<sup>14</sup>C]inulin ( $7.7 \times 10^6$  cpm/ml) as a marker. The corrections of <sup>45</sup>Ca uptake amounted to an average of only 0.004 % and were therefore insignificant.

**ENZYME ASSAYS** Beta glucuronidase was assayed by the method of Fishman et al. (1965) with phenolphthalein glucuronide as the substrate. One unit of enzyme activity

<sup>1</sup> Calcium chloride was present at a concentration of 1 mM rather than 10–20  $\mu$ M so as to avoid large changes in its specific activity due to the increase of about 50  $\mu$ M calcium in the medium expected as a result of the A23187-induced release reaction (see Table I).

produced 1  $\mu\text{mol}$  of phenolphthalein per minute at 37°C. Lactic dehydrogenase was measured by the method of Bergmeyer et al. (1965). Malate dehydrogenase activity was assayed by measuring the rate of NADH<sub>2</sub> oxidation accompanying the reduction of oxaloacetate to malate (Bergmeyer and Bernt, 1965). Pyruvate kinase activity was assayed in the presence of ADP, phosphoenolpyruvate, lactate dehydrogenase, and NADH<sub>2</sub> (Beisenherz et al., 1953). All pyridine nucleotide-linked enzyme assays were performed at 25°C in a Perkin-Elmer 356 dual-beam spectrophotometer at 345 nm. One unit of enzyme results in an initial rate of NADH<sub>2</sub> oxidation of 1  $\mu\text{mol}/\text{min}$  at 25°C.

A23,187 and X537A were generously supplied by Dr. David Wong of Eli Lilly Co., Indianapolis, Ind. and Dr. Julius Berger of Hoffman-LaRoche, Nutley, N.J. PGE<sub>1</sub> was a gift of Upjohn Co., North Haven, Conn. Thrombin (250 U/mg) and dibutyryl cyclic AMP were obtained from Sigma Chemical Co. Hirudin (2,400 Anti-Thrombin [AT] U/mg) was a gift from Dr. D. Bagdy of the Research Institute for Pharmaceutical Chemistry, Budapest, Hungary.

## RESULTS

### *Ca<sup>2+</sup>, Mg<sup>2+</sup> Release from Washed Platelets*

The incubation of washed human platelets with either one of the ionophores (1–5  $\mu\text{M}$ ) in a medium without added divalent cations resulted in a rapid loss of a substantial portion of cellular calcium and magnesium into the incubation medium. The initial extracellular calcium and magnesium concentrations ranged from 10–20  $\mu\text{M}$ . The amount of calcium released from washed platelets in 5 min at 37°C by either A23,187 or X537A was about 67% of the total platelet content (Fig. 1). The same fraction of the total platelet calcium was released by thrombin (0.5 U/ml), or by cell lysis due to repeated freezing and thawing. A23,187 also released as much magnesium (69%) as freeze-thaw-induced platelet lysis. Less magnesium (39%) was released by X537A, and very little (7.3%) by thrombin (Fig. 1). The latter finding indicates that only a small proportion, if any, of the cellular magnesium content is present in the secretory granules of human platelets since under these conditions thrombin caused maximal release of platelet nucleotides and 5 serotonin. Human platelets also contain a substantial amount of zinc ( $19.2 \pm 0.9$  nmol/mg protein, seven experiments), 35% of which was released upon cellular lysis. However, neither the ionophores (17 experiments) nor thrombin (4 experiments) produced any detectable release of this cation. The relationship between A23,187 concentration and release of cellular calcium and magnesium is shown in Fig. 2. The threshold for release was at about  $5 \times 10^{-8}$  M and a plateau in the response was reached at  $1 \times 10^{-6}$  M. The concentration-response curves for the two cations are for the most part superimposable. Approximately 2 mol of calcium were released for every mole of magnesium. This is somewhat greater than their molar ratios within the cell ( $\text{Ca}/\text{Mg} =$

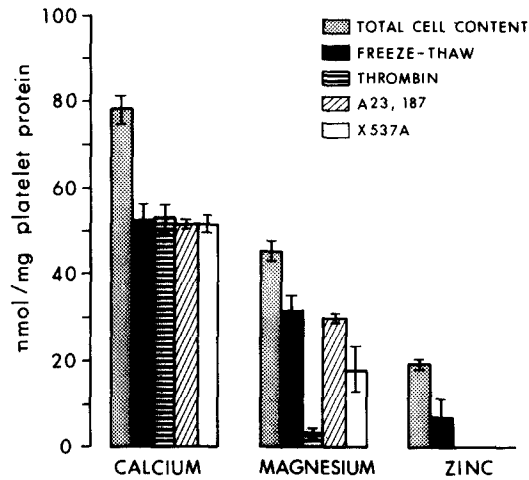


FIGURE 1. Release of calcium, magnesium, and zinc from platelets. Washed human platelets incubated in a buffered salt solution at 37°C with A23,187 (1.6  $\mu$ M), X537A (5  $\mu$ M), or thrombin (0.5 U/ml) for 10 min. Freeze-thaw platelets were frozen in a dry ice-ethanol bath and then thawed three times. Values are means  $\pm$  standard errors (vertical bars) for 11–21 experiments.

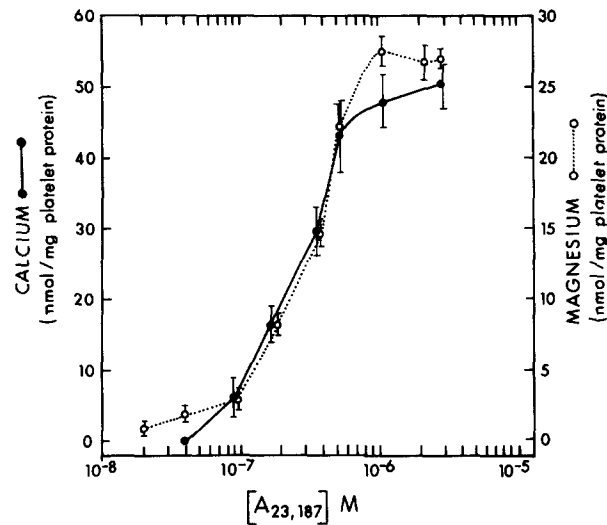


FIGURE 2. Release of calcium and magnesium from washed human platelets in a buffered salt solution, as a function of A23,187 concentration. Temperature 37°C, incubation time 10 min. Values are means  $\pm$  standard errors for 8–12 experiments for each point.

1.73). The rate of  $\text{Ca}^{2+}$  release (Fig. 3 A) measured at 23° by the murexide method was found to coincide with the rate of release induced by thrombin (Detwiler and Feinman, 1973; Miller et al., 1975). The  $\text{K}^+$  ionophores nigericin and valinomycin (1–5  $\mu$ M) did not induce release of platelet calcium

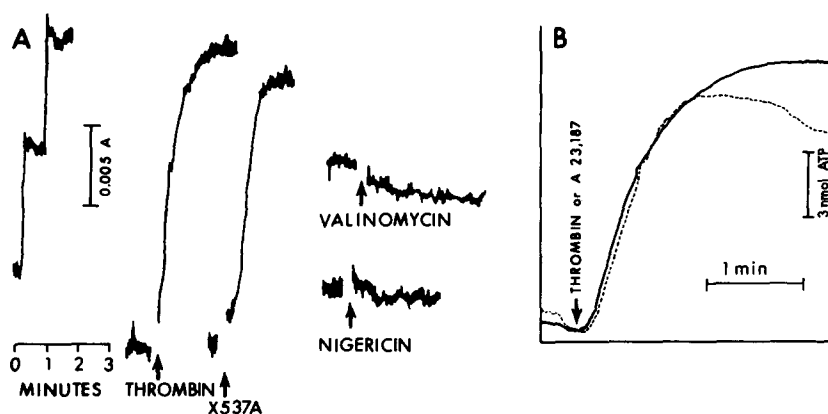


FIGURE 3. (A) Spectrophotometer measurement of calcium release from washed human platelets. Nigericin ( $2 \mu\text{M}$ ), valinomycin ( $2 \mu\text{M}$ ), A23,187 ( $1.6 \mu\text{M}$ ), X537A ( $5 \mu\text{M}$ ), or thrombin ( $0.5 \text{ U/ml}$ ) added to  $3.0 \text{ ml}$  of platelet buffer containing  $250 \mu\text{M}$  murexide and  $1 \text{ mg}$  platelet protein/ml. Calibration responses are  $25 \mu\text{M}$  additions of calcium. Temperature  $23^\circ\text{C}$ . (B) Release of platelet ATP measured as light emission with a crude firefly lantern extract (Detwiler and Feinman, 1973) in the incubation medium. Thrombin  $0.5 \text{ U/ml}$  (—), A23,187,  $1 \mu\text{M}$  (---). Temperature  $23^\circ\text{C}$ .

(Fig. 3 A) or adenine nucleotide from washed platelets, nor did they have any effect on platelet aggregation.

**CALCIUM UPTAKE** The effect of A23,187 on  $^{45}\text{Ca}$  uptake in washed platelets was determined over a 5-min period at  $37^\circ\text{C}$ . The  $^{45}\text{Ca}$  uptake reached a maximum within  $60 \text{ s}^2$  after addition of the ionophore ( $2 \mu\text{M}$ ), and was 65–90% complete within 45 s. The radioactivity at 60 s increased from a control level of 1,370 ( $\pm 326$ ) cpm/mg platelet protein to 11,531 ( $\pm 336$ ) cpm/mg in five experiments. EGTA-washed platelets at 60 s contained 453 ( $\pm 89$ ) and 3,311 ( $\pm 646$ ) cpm/mg, respectively. Background was 10 cpm. The ionophore-induced apparent calcium uptake calculated from the specific activity of  $\text{Ca}^{2+}$ , and corrected for bound calcium removed by EGTA, was  $0.97 (\pm 0.03) \text{ nmol calcium/mg platelet protein}$ .<sup>3</sup> It cannot be determined from the radioactivity data alone to what extent the calcium uptake represents increased net uptake or self-exchange.

#### *Release of Adenine Nucleotides and Serotonin from Washed Platelets*

A23,187 ( $1.6 \mu\text{M}$ ) released 72% of the radioactive serotonin and 47% of the ADP in 5 min at  $37^\circ\text{C}$  (Table I). The rate and total amount of ATP release at  $25^\circ\text{C}$  induced by thrombin and A23,187 are nearly identical (Fig. 3 B). The same observation was made recently by Feinman and Detwiler (1974).

<sup>2</sup> Samples taken at 30 s after addition of ionophore, plus 30 s centrifugation time.

<sup>3</sup> At an initial extracellular calcium level of  $25 \mu\text{M}$  a net calcium uptake of  $0.04 \text{ nmol/mg}$  in 1 min was found. This was calculated assuming an average extracellular calcium level of  $50 \mu\text{M}$  as a result of the concurrent release of platelet calcium.

TABLE I  
EFFECTS OF EGTA, EDTA, AND MAGNESIUM ON A23,187-INDUCED RELEASE OF  
ADP, Ca<sup>2+</sup>, AND Mg<sup>2+</sup>

	ADP	Calcium	Magnesium
Total Cell Content*	14.25 (±0.4)	78.2 (±3.2)	45.4 (±2.3)
Released by:			
A23,187 (1.6 μM)	6.72 (±0.6)	51.3 (±1.6)	29.7 (±1.0)
A23,187 + EGTA (1 mM)	2.71 (±0.4)	48.7 (±3.2)	19.5 (±1.1)
A23,187 + EDTA (1 mM)	2.90 (±1.3)	45.3 (±2.4)	26.1 (±1.1)
A23,187 + Mg <sup>2+</sup> (1.3 mM)	1.56	78.2 (±8.5)	—
	3.12		

\* Concentrations (mean ± SE, for 6–14 experiments) are expressed in nanomoles per milligram of platelet protein. Washed platelets were incubated for 10 min at 37°C with A23,187. EGTA, EDTA, or Mg<sup>2+</sup> were added 2 min before A23,187. The supernatant solutions were analyzed as described in Methods.

#### *Release of Cellular Enzymes*

The total cellular activity of lactate dehydrogenase and pyruvate kinase was found in the 40,000 *g* (30 min) supernatant after disruption of the platelets by sonication. About 80% of lactate dehydrogenase, 43% of pyruvate kinase, and 65% of malate dehydrogenase activity was found in the soluble supernatant after centrifugation (10,000 *g*) of three-times freeze-thaw lysed cells. The incubation medium of untreated cells usually contained no more than 0.5% of each of the enzyme activities of the sonicated cell suspension. After exposure of platelets to 0.5 U/ml thrombin the enzyme activity appearing in the medium amounted only to about 0.6–1.7% of the total cellular content. A23,187 or X537A (1–2 μM) slightly increased the levels of these enzymes appearing in the incubation medium (no added protein present), to about an additional 2% in the case of lactate dehydrogenase and 1–1.5% for the other enzymes (Fig. 4). Thus, these enzymes which are essentially retained within platelets during the release reaction elicited by thrombin are also not released to any substantial extent by the ionophore. The β-glucuronidase activity of washed human platelets lysed by 0.5% Triton X-100 hydrolyzed phenolphthalein glucuronide at a rate of 1.3 nmol/min/10<sup>8</sup> cells. β-Glucuronidase, located in the lysosomal-like α granules of the platelet, was released to the extent of about 10% of its total cellular activity by exposure to 1.0 U/ml thrombin (Mills et al., 1968), and in our experiments 8–11% was released by a 10-min exposure to 0.7–1.4 μM A23,187 at 37°C.

In the presence of added albumin the concentration of X537A or A23,187 had to be increased severalfold to elicit the release reaction. This was due to binding of the ionophores to albumin, which was demonstrated by fluorescence spectroscopy. No significant differences in enzyme release were noted in



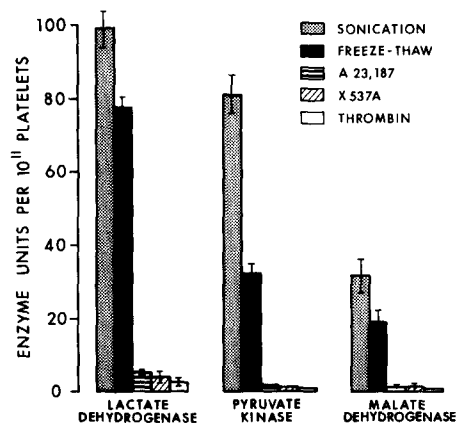


FIGURE 4.

FIGURE 4. Release of platelet enzymes. Washed human platelets incubated in a buffered salt solution at 37°C with X537A (5  $\mu$ M), A23,187 (1.6  $\mu$ M), or thrombin (0.5 U/ml) for 10 min. The enzyme content of the supernatant solution was measured after centrifugation at 3,000 *g* for 10 min. Total enzyme content was measured in sonicated (30 s at 70 W, Branson Sonifier) platelet suspensions. The values (mean  $\pm$  SE) given are corrected for the amount of enzyme activity present in the medium before any treatment. Number of experiments: lactate dehydrogenase, 9; pyruvate kinase, 6; malate dehydrogenase, 5.

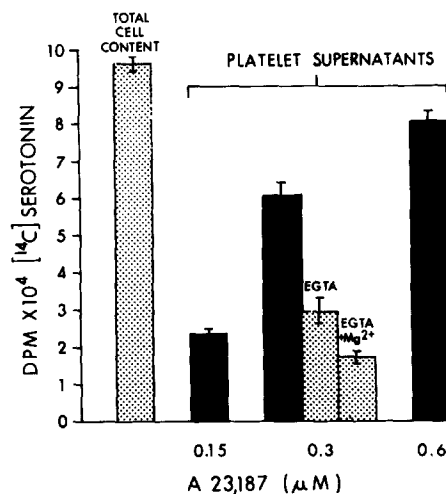


FIGURE 5.

FIGURE 5. Effect of EGTA and EGTA + Mg<sup>2+</sup> on A23,187-induced release of platelet [<sup>14</sup>C]serotonin. Platelet suspensions incubated at 37°C for 10 min with varying concentrations of A23,187. No calcium or magnesium added. EGTA (1 mM) or EGTA + Mg<sup>2+</sup> (1 mM) added 2 min before the ionophore. Values are means  $\pm$  SE for eight experiments.

the presence of albumin except for somewhat lower initial levels of the enzymes in the extracellular fluid of untreated cells. Presumably this was due to better preservation of the platelets under these conditions.

#### *Effects of EGTA and EDTA on the Release Reaction*

With 1 mM EGTA in the suspension medium (no added divalent cations) the release of calcium from washed platelets by the ionophore was  $94.8 \pm 6.4\%$  (11 experiments) of that in the absence of the Ca<sup>2+</sup> chelator (Table I). However, Mg<sup>2+</sup> release was decreased 34.2%. EDTA (1 mM) reduced Ca<sup>2+</sup> release by only 11.7%. Ca<sup>2+</sup> release induced by A23,187 and X537A is therefore apparently not dependent upon initial influx of extracellular Ca<sup>2+</sup> or Mg<sup>2+</sup> into the platelets. However, in the presence of 1.3 mM extracellular Mg<sup>2+</sup> the ionophore released essentially all of the platelet calcium. EGTA or EDTA (1 mM) reduced the release of ADP by A23,187 (1.6  $\mu$ M) from 6.7 ( $\pm$ 0.6) nmol/mg protein to 2.7 ( $\pm$ 0.39) nmol/mg protein (six experiments, Table I). The addition of 1.3 mM magnesium to the incubation medium containing no

added calcium also reduced ADP release by the ionophore to 1.56 and 3.1 nmol/mg protein in two experiments. [ $^{14}\text{C}$ ]Serotonin release from washed platelets or platelets in diluted plasma was also substantially, but not completely blocked by EGTA or EDTA. When the calcium concentration (no EGTA) of diluted plasma was raised from an initial level of 0.7 mM to 1.7 mM, [ $^{14}\text{C}$ ]serotonin release increased by about 30%. In washed platelets 1 mM  $\text{Mg}^{2+}$  + EGTA inhibited serotonin release to a greater extent than EGTA alone (Fig. 5). In the presence of 1–3.3 mM EGTA or EDTA the release of serotonin from platelets in plasma, induced by 2.8  $\mu\text{M}$  A23,187, was decreased by 59–71% (eight experiments).

#### Platelet Aggregation

**WASHED PLATELETS** Both A23,187 and X537A produced an increase in light transmittance (Fig. 6) when added to washed platelet suspensions at concentrations of 0.4–0.8  $\mu\text{M}$ . This effect was almost entirely eliminated by EGTA or EDTA, and was enhanced by the addition of calcium above the 10–20  $\mu\text{M}$  level normally present.  $\text{Ca}^{2+}$ , but not  $\text{Mg}^{2+}$ , reversed the inhibitory effect of

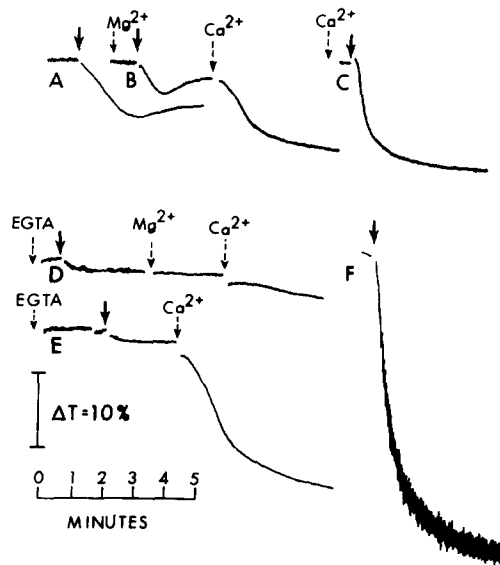


FIGURE 6. Effects of EGTA,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and plasma on aggregation of washed human platelet suspensions. Initial calcium and magnesium concentrations in the extracellular fluid were 10–20  $\mu\text{M}$ . A23,187 (1.6  $\mu\text{M}$ ) added at times indicated by  $\downarrow$ . (A) A23,187 alone. (B)  $\text{MgCl}_2$  (1.3 mM),  $\text{CaCl}_2$  (1.3 mM). (C)  $\text{CaCl}_2$  (1.3 mM). (D) EGTA (0.7 mM),  $\text{MgCl}_2$  (1.3 mM),  $\text{CaCl}_2$  (1.3 mM). (E) Same experiment as D only  $\text{CaCl}_2$  (1.3 mM), rather than  $\text{Mg}^{2+}$ , was added after the ionophore. Note prior addition of  $\text{Mg}^{2+}$  (1.3 mM) in D decreases response to subsequent addition of  $\text{Ca}^{2+}$ . (F) 0.5 vol of platelet-free plasma added to 2.5 vol of washed platelet suspension before addition of A23,187. Temperature 37°C.

EGTA or EDTA.  $Mg^{2+}$  also tended to oppose the effect of  $Ca^{2+}$  (Fig. 6). The transmittance changes induced by the ionophores were never as great as that produced by thrombin, and large aggregates visible by eye were not usually seen. After addition of 15% plasma (platelet free) to the suspending medium the ionophore produced an aggregation pattern like that of thrombin (Fig. 6 F) and large aggregates were easily visible by eye. The effect of plasma was not prevented by hirudin (25 AT U/ml) and was therefore not due to generation of thrombin. Gel-filtered platelets (Tangen et al., 1971) were induced to secrete maximally without aggregation by 5-min exposure to thrombin (0.5 U/ml) in the presence of 1.0 CTA (Committee on Thrombolytic Agents) U/ml plasmin (Miller et al., 1975). Such platelets, which lost 65% of their total adenine nucleotide content and 86% of their [ $^{14}C$ ]serotonin, were separated from the incubation medium by a second filtration through Sepharose-2B gel. Platelets treated in this way no longer aggregated with thrombin or collagen, but did aggregate normally in the presence of A23,187 (an effect potentiated by the addition of plasma) without the release of any additional detectable ADP.

**PLATELETS IN PLASMA** The addition of  $Ca^{2+}$  ionophores to platelet-rich plasma, or platelet concentrates diluted with buffered physiological saline solution containing albumin and apyrase, resulted in rapid aggregation. Maximal aggregation was usually produced within 2 min by concentrations of 1.5–7  $\mu M$  A23,187. Ionophore-induced aggregation of platelets in plasma required  $Ca^{2+}$  in the medium as in the case of collagen-, thrombin-, or epinephrine-induced aggregation.

*Inhibition of Platelet Release Reaction and Aggregation by PGE<sub>1</sub> and Dibutyryl Cyclic AMP*

The release of [ $^{14}C$ ]serotonin by A23,187 was prevented by PGE<sub>1</sub> and dibutyryl cAMP, an effect enhanced by theophylline (Fig. 7). Low concentrations of PGE<sub>1</sub> substantially reduced the extent of A23,187-induced aggregation of platelets in plasma (Figs. 8–10). Marked inhibition of aggregation was produced by 1–2-min preincubation of platelets with PGE<sub>1</sub> before the addition of the ionophore. Theophylline at a concentration of  $8.3 \times 10^{-4}$  M usually had little or no inhibitory effect by itself, but resulted in a 10-fold potentiation of the effect of PGE<sub>1</sub> (Figs. 8, 9). Increasing the ionophore or  $Ca^{2+}$  concentration tended to partially reverse the effect of PGE<sub>1</sub>. But the degree of reversal decreased as the PGE<sub>1</sub> concentration increased, and it was much less when theophylline was present as well (Figs. 9, 10).

The ionophores, like other aggregating agents, cause an initial decrease of light transmittance and an abolition of spontaneous transmittance oscillations. These effects are indicative of a change in platelet shape from discoid to sphere (Born, 1970). The ionophore-induced shape change was not blocked by PGE<sub>1</sub> or PGE<sub>1</sub> + theophylline (Figs. 8–10). When platelet aggregation was

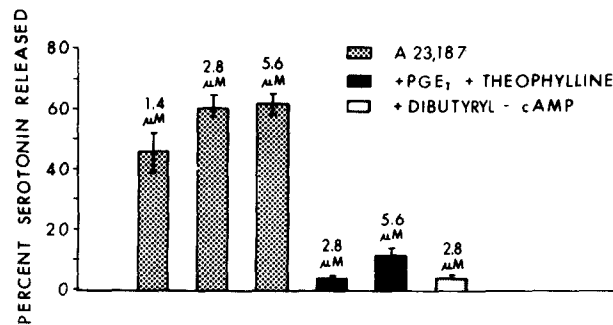


FIGURE 7. Release of [<sup>14</sup>C]serotonin from platelets in plasma by A23,187. A23,187 concentrations are given above the bars which represent the percent of total radioactive serotonin release (eight experiments, mean  $\pm$  SE) in 5 min. PGE<sub>1</sub> ( $4.6 \times 10^{-8}$  M) + theophylline (0.83 mM) added 2 min before A23,187. Dibutyl cAMP (0.7 mM) added 60 min before A23,187. Controls incubated for 60 min exhibited normal ionophore-induced release of [<sup>14</sup>C]serotonin. Temperature 37°C.

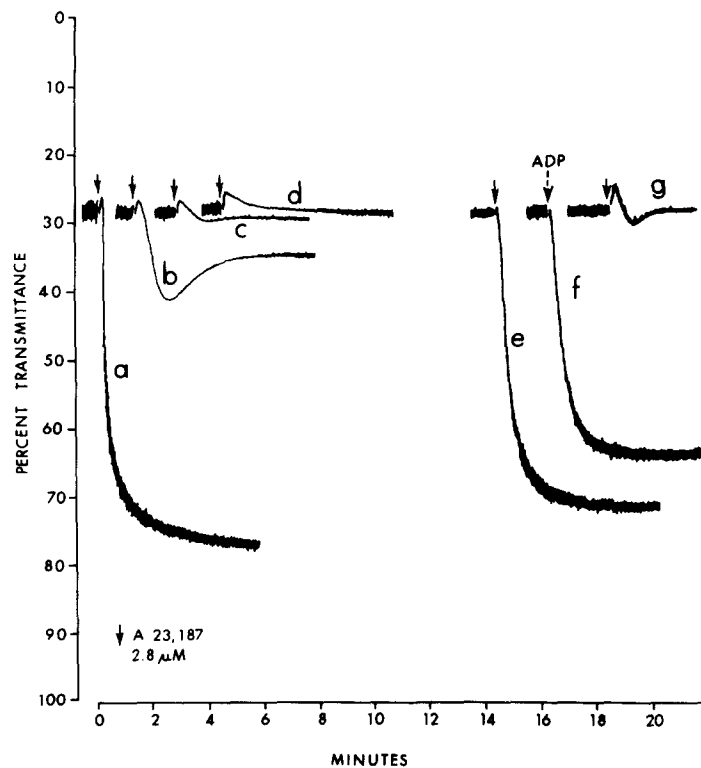


FIGURE 8. Inhibition of platelet aggregation by PGE<sub>1</sub>. A23,187 added at the arrows ( $\downarrow$ ). (a-d) Diluted platelet concentrate ( $1.39 \times 10^8$  platelets/ml). PGE<sub>1</sub> and theophylline addition, were made 2 min before A23,187. (a) Control. (b) PGE<sub>1</sub>  $4.6 \times 10^{-8}$  M. (c) PGE<sub>1</sub>  $4.6 \times 10^{-7}$  M. (d) +PGE<sub>1</sub>  $4.6 \times 10^{-8}$  M + theophylline  $8.3 \times 10^{-4}$  M. (e-f) Platelet-rich plasma treated with A23,187 or ADP (10  $\mu$ M). (g)  $4.6 \times 10^{-7}$  M PGE<sub>1</sub> added 2 min before the ionophore.

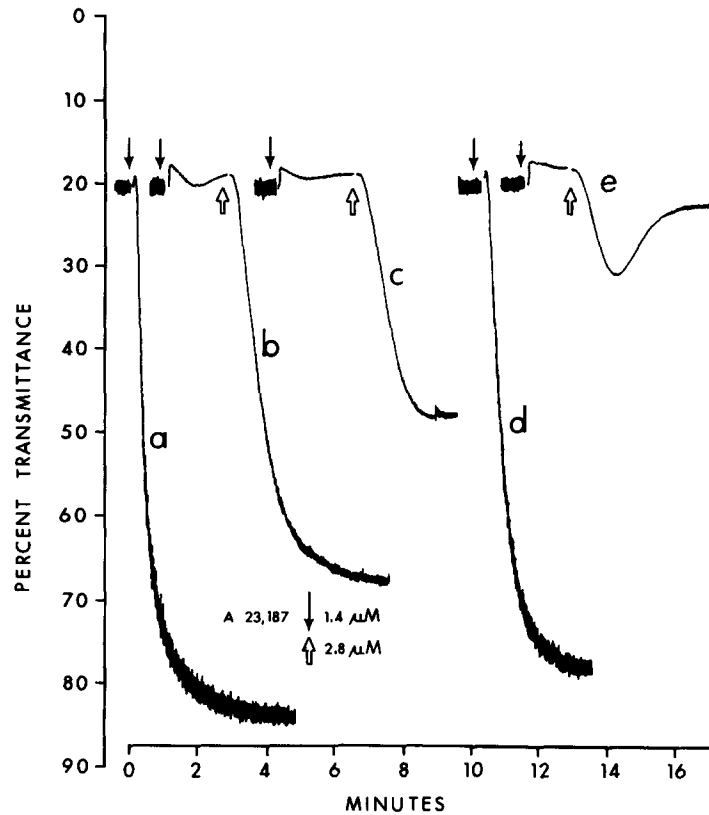


FIGURE 9. Effect of ionophore concentration on PGE<sub>1</sub> inhibition of platelet aggregation. Diluted platelet concentrate ( $2.8 \times 10^8$  platelets/ml). (a) Control. (b) PGE<sub>1</sub>  $4.6 \times 10^{-8}$  M. (c) PGE<sub>1</sub>  $4.6 \times 10^{-7}$  M. (d) Theophylline  $8.3 \times 10^{-4}$  M. (e) PGE<sub>1</sub>  $4.6 \times 10^{-7}$  M + theophylline  $8.3 \times 10^{-4}$  M. PGE<sub>1</sub> and theophylline were added 2 min before the first addition of A23,187 ( $\downarrow$ ).

completely inhibited the spontaneous oscillations gradually increased to normal again after several minutes (Figs. 8 *d*, 10 *f*). Thus, under these conditions the ionophore-induced shape change was spontaneously reversible.

Dibutyryl cAMP also strongly inhibited ionophore-induced platelet aggregation. The effect was dependent upon both the concentration of cyclic nucleotide and the time of incubation, due to its slow accumulation within platelets (Salzman and Levine, 1971). Incubation with cyclic nucleotide for 60 min greatly increased the inhibition of ionophore-induced aggregation (Fig. 11), whereas controls were unaffected by such prolonged incubation at 37°C. Theophylline also potentiated the inhibitory action of dibutyryl cAMP so that striking effects were produced at nucleotide concentrations as low as  $3 \times 10^{-5}$  M (Fig. 11). Inhibition of ionophore-induced platelet aggregation by dibutyryl cAMP plus theophylline was only weakly reversed by increasing Ca<sup>2+</sup> or ionophore concentrations.

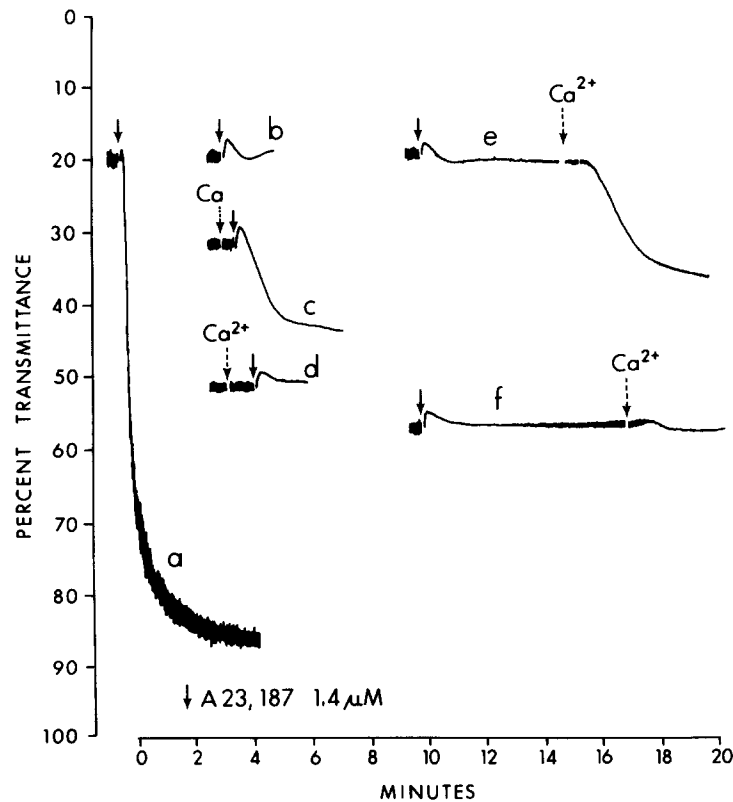


FIGURE 10. Effect of  $\text{Ca}^{2+}$  on the inhibition by  $\text{PGE}_1$  of A23,187-induced aggregation. Diluted platelet concentrate ( $3.0 \times 10^8$  platelets/ml). A23,187 added at the arrows. (a) Control, calcium concentration of the extracellular fluid was 0.7 mM. (b)  $\text{PGE}_1$   $4.6 \times 10^{-8}$  M. (c)  $\text{PGE}_1$   $4.6 \times 10^{-8}$  M. (d)  $\text{PGE}_1$   $4.6 \times 10^{-8}$  M + theophylline  $8.3 \times 10^{-4}$  M. (e)  $\text{PGE}_1$   $4.6 \times 10^{-8}$  M. (f)  $\text{PGE}_1$   $4.6 \times 10^{-8}$  M + theophylline  $8.3 \times 10^{-4}$  M.  $\text{PGE}_1$  and theophylline were added 2 min before A23,187. An additional 1.0 mM  $\text{CaCl}_2$  was added as indicated to increase the extracellular calcium concentration to 1.7 mM.

#### *Other Inhibitors of Platelet Release Reaction and Aggregation*

*N*-ethyl maleimide NEM ( $6.7 \times 10^{-5}$  M to  $2.7 \times 10^{-4}$  M) was an effective inhibitor of A23,187-induced calcium release from washed platelets, and [ $^{14}\text{C}$ ]serotonin release and aggregation in platelets in diluted plasma. Aspirin (1mM) and indomethacin ( $10^{-4}$  M) had a weak inhibitory effect which was only apparent at low concentrations of A23,187 which did not produce full aggregation. This lack of effect was in marked contrast to their total inhibition of low concentrations of collagen (30  $\mu\text{g}$ ) which induced aggregation equivalent in degree to that evoked by 5  $\mu\text{M}$  A23,187. It suggests that ionophore-induced platelet aggregation occurs independent of intracellular prostaglandin synthesis.

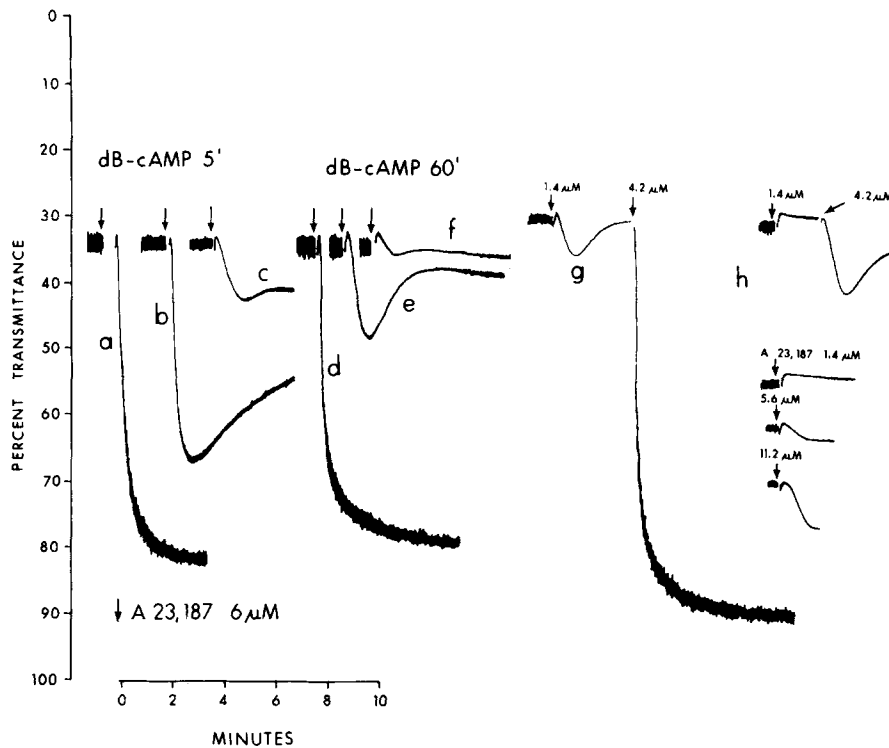


FIGURE 11. Inhibition of ionophore-induced aggregation by dibutyryl cAMP. (a-f): A23,187 ( $6 \mu\text{M}$ ) added at arrows ( $\downarrow$ ) to diluted platelet concentrates ( $1.6 \times 10^8$  platelets/ml). (a)  $1 \times 10^{-4}$  M dB-cAMP 5 min before A23,187. (b) dB-cAMP  $3.3 \times 10^{-4}$  M. (c) dB-cAMP  $6.6 \times 10^{-4}$  M. (d) Control response of platelets preincubated at  $37^\circ\text{C}$  for 60 min. (e) dB-cAMP  $3 \times 10^{-5}$  M. (f) dB-cAMP  $8 \times 10^{-5}$  + theophylline  $8.3 \times 10^{-4}$  M. (g-h): Diluted platelet concentrate ( $2.5 \times 10^8$  platelets/ml). (g) Control platelet aggregation. (h) Upper-addition A23,187 ( $\downarrow$ ) 30 min after incubation with  $8 \times 10^{-5}$  M dB-cAMP lower 30 min after incubation with dB-cAMP  $8 \times 10^{-5}$  M + theophylline  $8.3 \times 10^{-4}$  M.

#### DISCUSSION

The  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  ionophores A23,187 and X537A elicit a pattern of secretion from human platelets which is in most respects indistinguishable from the normal physiological release reaction. The release of adenine nucleotides (ADP and ATP), serotonin, calcium, and  $\beta$ -glucuronidase was equivalent in quantity and time-course to that produced by thrombin. Furthermore, the release process was selective in that cytoplasmic constituents such as lactate and malate dehydrogenases, pyruvate kinase, and zinc, which escape from lysed platelets, were not released by the ionophores. A23,187 also increased

the uptake, or exchange, of calcium (measured as  $^{45}\text{Ca}$ ) in platelets. The partial dependence of ionophore-induced secretion on extracellular  $\text{Ca}^{2+}$  was demonstrated by two observations: the inhibition by EGTA of ADP and serotonin release, and the increased release of serotonin due to elevation of extracellular  $\text{Ca}^{2+}$ . However, a significant degree of platelet secretion persisted in the presence of EGTA or EDTA concentrations greatly in excess of the extracellular divalent cation concentration. Ionophore-induced secretion is therefore different, in the degree of its dependency on extracellular  $\text{Ca}^{2+}$  from that elicited by thrombin or collagen. The release reaction due to thrombin is not inhibited by EGTA in human platelets (Morse et al., 1965; Miller et al., 1975) and presumably depends upon mobilization of intracellular calcium, whereas we found the release of serotonin by collagen to be entirely abolished by EGTA. A greater extracellular  $\text{Ca}^{2+}$  dependency, for the thrombin-induced release reaction is observed in pig platelets (Kinlough-Rathbone et al., 1973). The large loss of cellular magnesium which accompanies the ionophore-induced release reaction does not occur in platelets stimulated by thrombin. The ionophores also act as mobile  $\text{Mg}^{2+}$  carriers (Reed and Lardy, 1972; Pressman, 1973) which undoubtedly accounts for the large magnesium loss which is not directly related to the secretory process.

Ionophores may release stored amines from cells by a nonexocytotic mechanism (Thoa et al., 1974), attributable to their ability to complex some organic amines such as norepinephrine and epinephrine (Pressman, 1973). We have found (Feinstein and Fraser, unpublished observations) that both X537A and A23,187 can transport serotonin from water into an immiscible organic solvent. A23,187 was much weaker than X537A in this respect, and its ability to transport serotonin was inhibited by  $\text{Ca}^{2+}$  ( $50 \mu\text{M}$ ) but was not affected by  $\text{PGE}_1$  or dibutyryl cAMP. Serotonin release from platelets by A23,187 therefore has the characteristics of the physiological release process and not a direct transport of the amine by the ionophore. Further experiments will be necessary to determine to what extent, if any, X537A acts as a carrier to transport serotonin out of platelets. However, neither the release of adenine nucleotides, nor the aggregation of platelets induced by the ionophores occurs secondarily as a result of serotonin release. This conclusion is based on our observation (unpublished experiments) that nigericin releases serotonin from platelets, but not  $\text{Ca}^{2+}$  or adenine nucleotides, nor does this ionophore induce platelet aggregation.

The release of serotonin and ADP by A23,187 was reduced by EGTA, EDTA, or  $\text{Mg}^{2+}$  without a significant decrease in the amount of  $\text{Ca}^{2+}$  released. This may indicate that a fraction of platelet calcium originates from sources other than the secretory granules, or that the ionophore may have gained access to the granule membranes and thereby selectively transported intragranular calcium out of the cell. In this case it must be assumed that release of



$\text{Ca}^{2+}$  from the secretory granules into the cytoplasm could occur without incurring the full release reaction. Further work will be required to identify the intracellular sources of ionophore-releasable  $\text{Ca}^{2+}$ . Detwiler and Feinman (1973) reported that the release of  $\text{Ca}^{2+}$  from thrombin-stimulated platelets preceded in time the release of ATP, and Steiner and Tateishi (1974) observed that epinephrine and collagen released the same amount of serotonin as thrombin, but much less calcium. These findings also argue for the existence of extragranular pools of calcium which may be involved in stimulus-secretion coupling. Extragranular sources of calcium may include mitochondria or the ATP-dependent calcium-accumulating vesicles which have been isolated from disrupted platelets (Statland et al., 1969; Robblee et al., 1973 *a*). The latter has properties which are analogous in many ways to the calcium "pump" of sarcoplasmic reticulum (SR), but has not as yet been positively identified with any specific anatomic structure. The system of so-called dense tubules seen in high magnification electron micrographs of platelets are intimately associated with the surface-connecting open canicular system (White, 1973). The close apposition of these membranes has been likened to the T tubule, SR association in muscle. The lipophilic ionophore molecules would be expected to readily gain access to the membranes of the dense tubular system via the system of open canals. If the dense tubular system comprises a calcium sink for the cell, then the ionophores might be able to discharge a portion of the accumulated  $\text{Ca}^{2+}$  into the cytoplasm. A23,187 and X537A cause the release of  $\text{Ca}^{2+}$  previously transported into SR fragments from skeletal or cardiac muscle (Scarpa et al., 1972; Entman et al., 1972; Pressman, 1973). Ionophore-mediated transport of intracellularly sequestered  $\text{Ca}^{2+}$  into the cytoplasm probably serves as the trigger for that fraction of the platelet release reaction which is independent of extracellular calcium.

A functional analogy with muscle is further indicated by the presence in platelets of contractile proteins which have the properties of myosin, actin, tropomyosin, and a protein factor conferring  $\text{Ca}^{2+}$  sensitivity (Thorens et al., 1973). Activation of the  $\text{Ca}^{2+}$ -dependent contractile system has been proposed to be involved in the morphological changes of platelets, induced by various stimuli (White, 1971), leading to secretion and aggregation.  $\text{Mg}^{2+}$ -ATPase activity of platelet actomyosin is low compared to  $\text{Ca}^{2+}$ -ATPase activity (Abramowitz et al., 1972) which may account for the inhibition of ionophore-induced secretion by a high  $\text{Mg}^{2+}/\text{Ca}^{2+}$  ratio in the medium. Under such conditions the essentially complete depletion of cellular  $\text{Ca}^{2+}$  (see Table I) would be highly unfavorable for ATPase activity. The mechanism of action of NEM as an inhibitor of platelet secretion and aggregation may also be related to the platelet contractile proteins since their ATPase activity and superprecipitation has been reported to be inhibited by this agent (Booyse and Rafelson, 1972).

Inhibition of platelet aggregation by PGE<sub>1</sub> and dibutyryl cAMP could in itself effect the ionophore-induced release reaction since Packham et al. (1973) reported that cellular aggregation enhances secretion from rabbit platelets. However, we find that the time-course of the release evoked by ionophores precedes the time-course of the aggregation process. Moreover, cellular aggregation is not necessarily an absolute prerequisite for the release reaction in human platelets since thrombin-induced secretion occurs unimpeded in the presence of EGTA or plasmin, both of which prevent aggregation (Miller et al., 1975). Additional study will be required to determine the extent to which cell-cell contact influences the ionophore-induced release reaction.

Holmsen (1974) has proposed that shape change, the release reaction and aggregation are all manifestations of one basic ATP-requiring reaction (i.e., cellular contraction) initiated by Ca<sup>2+</sup> released from membrane vesicles. These platelet functions are viewed as being a function of stimulus intensity and in parallel with each other, rather than in series. The role of Ca<sup>2+</sup> as the initiator of all of these processes is substantiated by the results obtained with Ca<sup>2+</sup> ionophores. Furthermore our findings are consistent with Holmsen's view in that ionophore-induced secretion and aggregation can occur independently of each other under certain experimental conditions. The latter case (aggregation without release) was found to occur in response to A23,187 in gel-filtered platelets previously exhausted of their releasable pool of ADP.

Several facts link the effect of PGE<sub>1</sub> to cAMP metabolism. PGE<sub>1</sub> is a powerful stimulant of platelet adenyl cyclase activity (Haslam, 1973), and increases intraplatelet levels of cyclic 3',5'-AMP (Butcher et al., 1967; Viggdahl et al., 1969). Elevated cAMP levels in the platelet are well correlated with PGE<sub>1</sub> inhibition of aggregation (Viggdahl et al., 1969). Both PGE<sub>1</sub>-induced cAMP synthesis and inhibition of platelet aggregation and secretion are potentiated by phosphodiesterase inhibitors (Ball et al. 1970; Marquis et al., 1969; Salzman et al., 1970). The ability of both PGE<sub>1</sub> and dibutyryl cAMP to inhibit secretion and aggregation due to Ca<sup>2+</sup>-ionophores indicates that high cellular concentrations of cAMP probably modify passive or active cellular Ca<sup>2+</sup> transport, or some Ca<sup>2+</sup>-dependent processes which may involve contractile proteins. Alternatively, cAMP inhibits an essential process beyond the Ca<sup>2+</sup>-requiring steps. Further studies on the complex interrelationships between Ca<sup>2+</sup> and cyclic nucleotides should provide much greater insight into normal physiological processes in the platelet.

*Note Added in Proof* Since the presentation of this manuscript for publication two papers have appeared concerning X537A and A23,187 effects on platelets (Massini, P. and E. F. Luscher. 1974. *Biochim. Biophys. Acta.* **372**:109, and White, J. G., Gundu H. R. Rao, and Jonathan M. Gerrard. 1974. *Am. J. Pathol.* **77**:135). Our results are, for the most part, in excellent agreement with the above studies.

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