Stimulus-Secretion Coupling in Platelets. Effects of Drugs on Secretion of Adenosine 5′-Triphosphate†

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ABSTRACT: The mechanism of stimulus-secretion coupling in platelets was investigated by observing the effects of drugs on the kinetics on ATP secretion induced by either thrombin or the divalent cation ionophore A23187. The actual secretion is the same with either of these agents, since the rate constants and activation energies of secretion are the same and since drugs that affect the final, enzyme-independent steps of thrombin-induced secretion have the same effect on ionophore-induced secretion. Drugs that affect early steps of thrombin-induced secretion have no effect on ionophore-induced secretion. Drugs that act through cAMP (PGE_1, theophylline, dibutyryl-cAMP) slow an early step in the mechanism of thrombin-induced secretion and completely block at higher levels, with the required concentration of inhibitor dependent on thrombin concentration. The inhibition of rate appears to be all-or-none, with no intermediate rates observed. By replacing thrombin with trypsin, which makes it possible to observe a complete change in rate-determining step from an enzyme-dependent to an enzyme-independent platelet step, it was found that these drugs slow the rate only when the enzyme-independent step is rate determining. These drugs have no effect on A23187-induced secretion. It was concluded that cAMP inhibits at a step after the enzyme step but before the final step by interfering with transmission of the stimulus-secretion coupling signal. Disruption of microfilament function by cytochalasin B (10 μM) accelerates the rate of secretion induced by either thrombin or ionophore. The microtubule agents colchicine, vinblastine, and vincristine had effects only at concentrations above those usually considered necessary for the specific inhibition of microtubule function. Drugs that inhibit prostaglandin synthesis (aspirin, indomethacin, eicosatetraynoic acid), drugs that block ATP production (antimycin A, deoxyglucose), or several other drugs previously reported to inhibit platelet function had no effect on secretion.

Stimulation of platelets by such diverse agents as thrombin, collagen, and ADP leads to a variety of important responses (for reviews see Marcus, 1969; Mustard and Packham, 1970), but there is little known about the mechanism of stimulation or about how the initial stimulus is coupled to the responses. Secretion, the release of a specific pool of chemical substances that include 5-hydroxytryptamine, adenine nucleotides, and calcium (for review see Holmsen et al., 1969b), has a key and central position in the overall response of platelets to stimulation. With the recent development of a sensitive and quantitative method for continuously following the time course of secretion (Detwiler and Feinman, 1973b), new approaches to detailed analysis of the process became possible. This paper describes an investigation into the mechanism of thrombin-induced secretion by platelets using drugs to modify different parts of the overall process. The purpose was to identify individual steps involved in coupling stimulation to secretion and to place them in the approximate order of occurrence.

Materials and Methods

Washed human platelets were prepared as previously described (Detwiler and Feinman, 1973a). Bovine thrombin was purified from Parke-Davis topical thrombin by the method of Glover and Shaw (1971).

Secretion was followed by the luminescence produced by the reaction of released ATP with firefly lantern extracts as previously described (Detwiler and Feinman, 1973b). Reactions were in siliconized cuvets in a final volume of 1.0 ml. Unless noted otherwise, reactions were at 24° and drugs were added 1 min before starting the reaction by addition of 5 μl of a secretion inducer, either thrombin (usually about 3×10^{-8} M final concentration) or A23187 (usually 1 μM final concentration). Platelet concentrations were about 2×10^8/ml.

The progress-time curves obtained (for example, see Figure 2) are quantitated (Detwiler and Feinman, 1973a,b) as (i) the time to the inflection point, t_i, which is an inverse function of the rate of initial steps of the reaction (formation of P* in reaction 1); (ii) the first-order rate constant for the final steps of the reaction, k_2, which is calculated from the slope of a log plot of the progress-time curve; and (iii) yield, the final amount of ATP released. Typical control values for these parameters are: t_i, 10 sec; k_2, 0.030 sec^{-1}; yield, 2.5 nmol/10^8 platelets. Since there is some variation in these values with different platelet preparations, results are usually expressed as percent of controls.

Lactate dehydrogenase was assayed by the method of Bergmeyer et al. (1965) but with NADH oxidation measured fluorometrically instead of spectrophotometrically for greater sensitivity. Calibration was by addition of known concentrations of NADH.

Drug solutions were prepared daily and stored in ice. Concentrations were such that addition of 5 μl to the reaction volume of 1 ml gave the desired concentration; addition of 5 μl of solvent alone had no effect on the reaction. A23187 (a gift of Dr. R. Hosley, Lilly), PGE_1 (courtesy of Dr. J. Pike, Upjohn Company), and cytochalasin B (Impe-

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[1] Abbreviations used are: PGE_1, prostaglandin E_1; TYA, 5,8,11,14-eicosatetraynoic acid; cAMP, adenosine 3′:5′-cyclic monophosphate; dibutyryl-cAMP, N^6,$O^{2'}$-dibutyryl-cAMP.

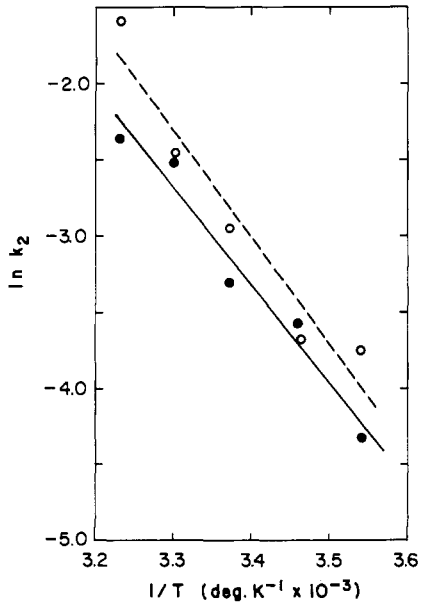

FIGURE 1: Arrhenius plot showing dependence of the rate of secretion on temperature. Platelets were added to reaction mixtures at various temperatures and allowed to equilibrate for 2 min before initiating reactions with 3×10^{-8} *M* thrombin (O- - -O) or 1 μM A23187 (●—●). Values for k_2 were obtained as described under Materials and Methods.

rial Chemical Industries, England) were dissolved in absolute ethanol; gliclazide (provided by Dr. Derome-Tremblay, Les Laboratoires Servier, France), indomethacin (Merck, Sharpe, and Dohme), TYA (a gift of Dr. W. E. Scott, Hoffmann-La Roche), and antimycin A (Sigma) were dissolved in dimethyl sulfoxide; and acetylsalicylic acid (Matheson Coleman and Bell) was dissolved in dimethylformamide. The following drugs were made in the buffered saline of the reaction medium (54 m*M* NaCl-45 m*M* sodium phosphate-10 m*M* Tris (pH 7.4)) and added as required (up to 50 μl) to give the final volume of 1.0 ml: colchicine (Calbiochem); S2574 (courtesy of Dr. Derome-Tremblay); theophylline (Nutritional Biochemicals); cAMP and dibutyryl-cAMP (Sigma); chlorpromazine hydrochloride (provided by Dr. A. M. Moore, Parke-Davis); 2-deoxy-D-glucose (Mann); phenol, methylguanidine, *p*-hydroxyphenylacetic acid, *m*-hydroxyphenylacetic acid, and guanidinosuccinic acid (courtesy of Dr. H. J. Carroll, Downstate Medical Center).

Results and Discussion

The experiments described here were designed and analyzed with reference to a simple reaction model postulated on the basis of kinetic studies (Martin et al., 1975), as shown in reaction 1 where T represents thrombin, R the

$$\begin{array}{l} T + R \overset{K}{\rightleftharpoons} TR \overset{k_{cat}}{\rightleftharpoons} TR^0 \\ \qquad\qquad\qquad\quad \searrow^{k_1} \\ \qquad\qquad\qquad\quad P \xrightarrow{k_1} P^* \xrightarrow{k_2} \text{secretion} \end{array} \quad (1)$$

thrombin receptor, P the platelet, and P* a kinetically distinguishable but undefined intermediate form of the platelet. K is a dissociation constant, k_{cat} is the rate constant for the thrombin-catalyzed step, k_1 is a rate constant for a thrombin independent platelet step, and k_2 the rate constant for the final observed step, probably the actual release. With reference to the terminology of Holmsen et al. (1969b), formation of TR^0 would be "initiation", TR^0 to P* would be "transmission", and the k_2 step would be "extrusion". For routine evaluation of drugs, secretion was induced by just enough thrombin, usually about 3×10^{-8} *M*, to make the thrombin catalysis step rapid enough so that the k_1 step becomes rate determining for the formation of P*. At this thrombin concentration, any change in either K, k_{cat}, or k_1 would result in a change in t_i.

Secretion Induced by the Ionophore A23187. The divalent cation ionophore A23187 induces secretion of ATP with a progress-time curve and a yield that are nearly identical with those for thrombin-induced secretion (Feinman and Detwiler, 1974) and it was therefore proposed that A23187 acts at an intermediate step in the coupling of thrombin stimulation to secretion, with the final steps identical for the two agents. To test this hypothesis, the temperature dependence of k_2, the rate constant for the final step in the process, was studied for both thrombin and ionophore-induced secretion (Figure 1); the activation energies were found to be 13.4 kcal/mol for thrombin, and 13.5 kcal/mol for ionophore-induced secretion (average of two separate determinations for each), further evidence that they represent the same process. To confirm that the observed release was due to specific secretion and not to lysis of platelets, release was induced by either thrombin or A23187 and levels of extracellular lactate dehydrogenase were determined using a sensitive fluorometric enzyme assay. Five minutes after addition of 6×10^{-8} *M* thrombin or 2 μM A23187, lactate dehydrogenase activity in the supernatant solution after centrifugation of either thrombin or A23187-treated platelets was less than 5% of total lactate dehydrogenase activity of sonicated platelets, indicating essentially no lysis.

Drugs that Elevate Intracellular cAMP. cAMP is a well-known regulator of many cellular processes (for reviews see Robison et al., 1971; Pastan and Perlman, 1971) and it has been shown to affect platelet function (for reviews see Salzman, 1972; Mills, 1974), although its mode of action in platelets is not known.

We studied the effects of cAMP on thrombin-induced secretion by addition of PGE_1, an adenyl cyclase activator (Wolfe and Shulman, 1969; Moskowitz et al., 1971; Salzman and Levine, 1971; Brodie et al., 1972), theophylline, which inhibits platelet phosphodiesterase (Mills and Smith, 1971), or by addition of dibutyryl-cAMP. Although other mechanisms cannot be completely excluded, the major effect of these drugs is almost certainly to increase the level of intracellular cAMP, since it has been established that platelet cAMP levels are elevated by PGE_1 (Vigdahl et al., 1969; Mills and Smith, 1971; McDonald and Stuart, 1973), and by PGE_1 in synergism with theophylline and other phosphodiesterase inhibitors (Cole et al., 1971; Haslam, 1973; Mills and Smith, 1971).

Two effects of these drugs were observed: low doses increased t_i with little effect on yield, while slightly higher levels also decreased yield. Examples of these effects are shown in Figure 2 for PGE_1. Similar results were obtained with theophylline, with a combination of PGE_1 and theophylline, or with dibutyryl-cAMP. These effects are shown quantitatively in Figure 3. Figure 3A shows (i) a tenfold increase in t_i before any appreciable decrease in yield and (ii) that the effects of theophylline and PGE_1 are synergistic, as would be expected in a cAMP-dependent process. While platelets incubated with cAMP for periods of up to 30 min responded normally, incubation with dibutyryl-cAMP, which is presumed to cross membranes more readily due to

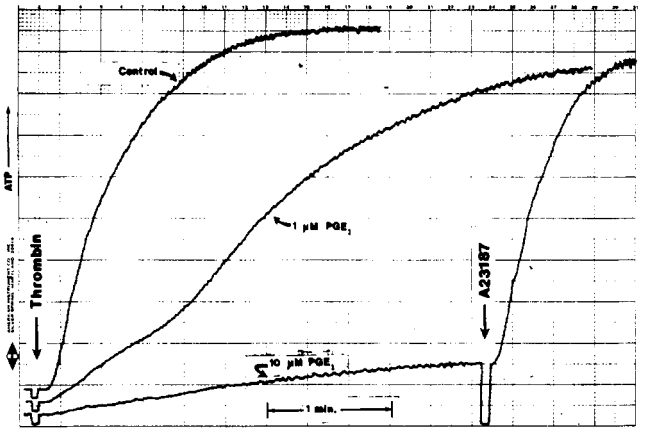

FIGURE 2: Effect of PGE_1 on thrombin- and A23187-induced secretion of ATP. PGE_1 was added 1 min before thrombin. Full scale deflection represents 5 μM ATP.

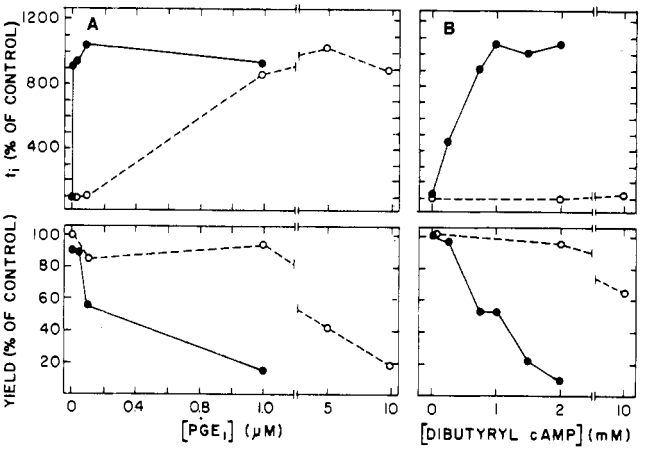

FIGURE 3: Effect of drugs that elevate cAMP on the thrombin-induced secretion of ATP. Thrombin was 3×10^{-8} M. (●—●) All reactions included 250 μM theophylline; (O- - -O) no theophylline; (A) PGE_1 (drugs were added 1 min before thrombin); (B) dibutyryl-cAMP, (drugs were added 20 min before thrombin). With only 1 min prior incubation with up to 10 mM dibutytyl-cAMP, there was essentially no effect.

its more lipophilic character, increased t_i, and decreased yield (Figure 3B). Gliclazide and S2574 (1–10 mM), both of which activate adenylyl cyclase (pharmacological files, Les Laboratoires Servier, France), produced the same effects on PGE_1, and were also synergistic with theophylline. None of these agents, even at concentrations above those needed for complete inhibition of the thrombin-induced reaction, had any effect on A23187-induced secretion (Figure 2).

Significantly, the inhibition of both yield and t_i by a fixed concentration of PGE_1 (or of other drugs that increase platelet cAMP) could be overcome by increasing the amount of thrombin added. Conversely, the amount of PGE_1 required for a certain degree of inhibition depended on the concentration of thrombin used to stimulate. These relationships were, however, difficult to analyze because of a peculiar characteristic of partially inhibited curves; we observed no intermediate value of t_i between the control and the fully inhibited values. Instead, at less than complete inhibition the progress–time curve appeared to consist of a curve due to partial secretion with a normal t_i, superimposed on a curve of secretion with a very long t_i (this may be seen by careful examination of the early part of the curve with a long t_i in Figure 2). If the concentration of inhibitor was decreased or the level of thrombin increased, the contribution of the normal t_i component appeared to increase and a curve without a distinct inflection was obtained. It thus appears that the platelets existed in either of two forms, normal or inhibited (very long t_i), and that the ratio of these forms was determined by the levels of inhibitor and thrombin.

Since the first effect of these drugs was to increase t_i, their point of action in reaction 1 must be before formation of P*. Further definition of this site of action requires detailed study of the kinetics under some modifying condition, but the experiments are restricted by the above limitations on quantitation of t_i in partially inhibited reactions. This quantitative problem is not as pronounced with dibutyryl-cAMP, but the very long incubations required make this a less satisfactory drug. The approach we used was to observe the effects of PGE_1 when different steps of the reaction were rate determining. The rate-determining step can be changed by decreasing the concentration of enzyme, making formation of TR^0 rate determining, but this technique is severely limited because at low thrombin yield is also decreased, to such an extent that it is very difficult to accurately follow the reactions. We therefore made use of an interesting aspect of the reaction of trypsin with platelets. Thrombin- and trypsin-induced secretions are very similar, but at low trypsin t_i can be very long without a decrease in yield (Martin et al., 1975). We therefore studied the effects of PGE_1 on secretion induced by trypsin. At low levels of trypsin, where the formation of TR^0 in reaction 1 is very slow, 1 μM PGE_1 caused 50% inhibition of yield but essentially no further increase in the already long t_i. At higher levels of trypsin, where the control t_i was short (i.e., the k_1 step was rate determining), 1 μM PGE_1 led to both a decreased yield and increased t_i. Thus, PGE_1 affects the rate of the initial steps only when the rate-determining step is *after* the formation of TR^0, placing the site of action between TR^0 and P* in reaction 1.

The inhibition of yield at higher levels of these agents may simply reflect an extension of the same action that caused an increase in t_i, since there can be a relationship between rate of the early steps and final yield (Martin et al., 1975). However, the fact that the *maximum* t_i is observed with concentrations of PGE_1 too low to affect yield suggests that there may be two different mechanisms of action of cAMP, or two separate pools. Indeed, several researchers have suggested multiple pools of cAMP (Salzman, 1972; McDonald and Stuart, 1973; Dechavanne and Lagarde, 1974), and two forms of human platelet phosphodiesterase have been reported (Amer and Mayol, 1973).

Drugs that Affect Microfilaments and Microtubules. Platelets contain both microtubules and microfilaments (White, 1971), structures with roles in many cellular processes involving motility or shape changes (for reviews, see Wessells et al., 1971; Allison, 1973; Olmstead and Borisy, 1973; Wessells et al., 1973).

Cytochalasin B, which disrupts microfilament systems (Wessells et al., 1971), produced a three- to fivefold increase in k_2 for both thrombin and ionophore-induced secretion (Figure 4), with the maximum effect observed with an incubation period of only a few seconds and a concentration of less than 10 μM. The effect of cytochalasin B on secretion has been tested with many other cells (for review see Allison, 1973); in some, cytochalasin B inhibits, while in others, it increases secretion. The most obvious explanation

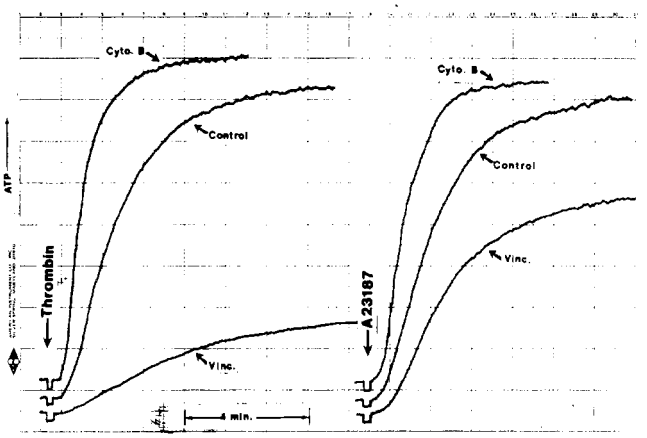

FIGURE 4: Effects of cytochalasin B and vincristine on thrombin- and A23187-induced secretion. Cyto. B, 10 μM cytochalasin B; Vinc., 1 mM vincristine. Full scale deflection represents 5 μM ATP.

for the increased rate of secretion, as observed with platelets, is that movement of secretory granules for exocytotic secretion is facilitated by depolymerization of a mesh of microfilaments that confers internal structure and shape to the cell. However, there is no evidence for this mechanism nor is there definitive evidence for secretion by exocytosis in platelets.

To determine whether microtubules are involved in platelet secretion, we tested colchicine, vinblastine, and vincristine, drugs that bind microtubule protein (Owellen et al., 1972). Vincristine (1 mM) decreased k_2 by 57 and 41% and yield by 70 and 32% for secretion induced by 3×10^{-8} M thrombin or 0.5 μM A23187, respectively; t_i was not affected (Figure 4). Higher concentrations of either secretion inducer overcame these effects. Incubation of platelets for one minute with low concentrations of colchicine or vinblastine had no effect on thrombin-induced secretion, but with either concentrations above 1 mM or longer periods of incubation, these agents themselves caused a slow release that would have masked any effect on the thrombin reaction. The high concentrations of these drugs required for any effect requires cautious interpretation, for they cannot be considered specific microtubule agents at these concentrations. White (1968) found that while less than 1 mM colchicine or vinblastine caused the microtubules of some platelets to disappear, higher concentrations caused many platelets to become completely disrupted, so that the slow release of ATP we observed does not show a role for microtubules in secretion. It is also significant that these experiments were done with platelets that had been prepared at 4° (they had been at 4° for from 2 to 4 hr when used) and that there was no break in the Arrhenius plot (Figure 1). Since microtubules are cold labile and are not usually observed in platelets that have been cooled (Behnke, 1967; White and Krivit, 1967; Behnke, 1970), and since inhibition of secretion was observed only with very high concentrations of vincristine, it is doubtful that intact microtubules play any essential role in thrombin-induced secretion, at least in cooled platelets as were used here.

Drugs that Inhibit Prostaglandin Synthesis. Stimulated platelets rapidly synthesize prostaglandins, which may serve as intracellular messengers in stimulus–response coupling (for review, see Smith et al., 1974). Prostaglandin synthesis in platelets is inhibited by aspirin and indomethacin (Smith and Willis, 1971), and by TYA (Willis et al., 1974). Platelets incubated with aspirin (1 mM), indomethacin (1 mM), or TYA (1.4 mM) in the usual manner, or in platelet rich plasma for 1 hr before subjecting platelets to the usual washing procedure, showed normal thrombin-induced secretion, confirming a previous report by Smith and Willis (1971) and indicating that prostaglandin synthesis has no direct function in coupling thrombin stimulation to secretion. Increasing concentration of aspirin had no effect until 5 mM, where secretion was completely blocked, but this is substantially above the level required to inhibit prostaglandin synthesis (Smith and Willis, 1971).

Drugs that Affect Energy Metabolism. To assess the role of ATP production in the secretory response, respiration was inhibited by antimycin A and glycolysis by deoxyglucose. It should be noted that the secretable pool of ATP is distinct from the metabolic pool (Holmsen, 1967; Ireland, 1967; Holmsen et al., 1969a) so that inhibition of ATP synthesis would have no direct effect on the secretory pool of ATP. It has also been shown that deoxyglucose, in addition to inhibiting platelet glycolysis, utilizes a large amount of metabolic ATP by its phosphorylation (Detwiler, 1971). Antimycin A concentrations below 0.01 mg/ml had no effect on secretion. Higher doses caused slow release of ATP but had no apparent effect on the thrombin-induced secretion of the ATP still present in the platelets at the time of stimulation. Incubation with 5 mM deoxyglucose for up to 45 min or preparation of platelet suspensions with deoxyglucose instead of glucose also had no effect on thrombin-induced secretion, nor did a combination of antimycin and deoxyglucose have any effect. This combination would be expected to inhibit any process with a requirement for a substantial supply of ATP. The lack of effect suggests either that there is only a slight requirement for ATP for the process or that there is a separate pool of ATP used as an energy source for secretion, as suggested by Holmsen et al. (1969a). Murer (1970) has reported that these two inhibitors do inhibit release; there is no obvious explanation for this discrepancy.

Other Drugs Tested. The following uremic toxins, which inhibit platelet aggregation (Horowitz et al., 1970; Rabiner and Molinas, 1970), had no effect on thrombin-induced secretion after incubation with platelets for 15 min: phenol (5 mM), methylguanidine (1 mM), *p*- or *m*-hydroxyphenylacetic acid (1 mM), and guanidinosuccinic acid (1 mM). The membrane active drugs desmethylimipramine and chlorpromazine, which inhibit platelet aggregation (Mills and Roberts, 1967), were also tested; concentrations below 250 μM had no effect on secretion while higher concentrations caused a slow release without thrombin stimulation.

Conclusions

These experiments were based in part on the assumption that the divalent cation ionophore A23187 induces the same secretory process as thrombin. The evidence for this is that comparison of secretion induced by these two agents shows that the amounts of ATP released are identical, the rate constants for release are identical, the energies of activation for release are identical, and they are each activated to the same extent by cytochalasin B and inhibited to the same extent by vincristine. It thus seems almost certain that the k_2 step in reaction 1, which is the extrusion process of Holmsen et al. (1969b), is the same for secretion induced by either of these agents. In contrast, the initial steps with thrombin and A23187 are not the same, since with the higher levels of A23187 t_i is appreciably shorter than with saturating levels of thrombin. Thus, A23187 must by-pass

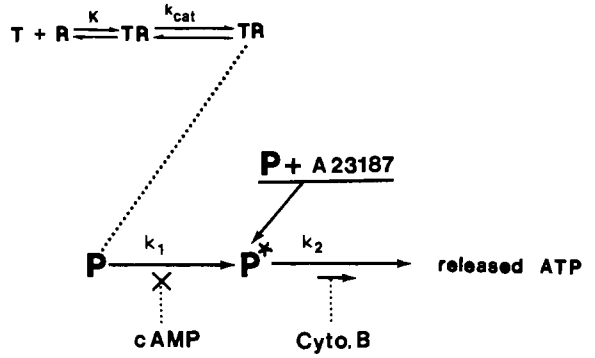

FIGURE 5: Proposed model of thrombin-induced secretion showing the points of action of cAMP, cytochalasin B, and A23187. This model distinguishes between the thrombin-receptor reaction and the thrombin-independent platelet processes. The X indicates inhibition and the → stimulation. Symbols and constants are defined in the text for reaction 1.

the rate-limiting thrombin step, which is the k_1 step at saturating levels of thrombin, and enter the sequence at P*, as shown in Figure 5. The mechanism of A23187 is presumed, but not proved, to involve an intracellular Ca^{2+} flux (Feinman and Detwiler, 1974).

We concluded that cAMP inhibited the k_1 step and, as shown in Figure 5, this is consistent with its failure to inhibit A23187-induced secretion. This inhibition can be overcome by increasing the concentration of thrombin, but since the effect is not at steps that directly involve thrombin the mechanism is not competitive. Thus it is as if cAMP regulates the amplitude of the transmission of a thrombin-dependent signal. One of the more significant aspects of cAMP inhibition is that it modifies that part of the overall reaction, the coupling of initiation to extrusion, that is most difficult to investigate experimentally by other means. It is thus a valuable experimental tool.

Cytochalasin B clearly modifies the k_2 step, accelerating secretion induced by either thrombin or A23187. Since this drug is rather specific for microfilaments, which are identifiable and isolatable entities, this effect presents an ideal opportunity for correlative studies of structure, function, and chemistry. No convincing evidence was found of a role for microtubules.

Prostaglandin synthesis is not essential in coupling thrombin stimulation to secretion. Since prostaglandin synthesis is essential for thrombin-induced aggregation (Willis et al., 1974), at least some steps in coupling thrombin stimulation to secretion and aggregation are different. To further clarify the role of prostaglandin synthesis, it would be of interest to determine whether A23187 can initiate prostaglandin synthesis and whether cAMP inhibits thrombin-induced prostaglandin synthesis.

The lack of inhibition of secretion by inhibitors of ATP synthesis indicates that secretion requires little, if any, ATP. There is no a priori requirement that secretion be an energy driven process and it is, in fact, conceivable that energy be required to maintain the platelet in the nonsecretory state.

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Linkage and Assembly of Polymeric IgA Immunoglobulins†

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ABSTRACT: The intersubunit linkage of polymeric IgA immunoglobulins was determined from studies of the products of reductive and cyanogen bromide cleavage. Under conditions of limited dithioerythritol reduction tetramer IgA molecules were cleaved to yield two monomers and a J chain containing dimer. The stability of the dimer and the conservation of the J chain disulfides indicated that the J chain joins two monomer subunits. Evidence confirming the J chain dimer clasp was obtained from the depolymerization of tetramer and dimer IgA by cyanogen bromide treatment. The cleavage studies also showed that (a) the S–S bonds directly joining the other subunits are located at the same penultimate α chain half-cystines that constitute the site of J chain attachment and (b) during limited reduction the monomer–monomer bonds undergo interchange to release subunits without a concomitant generation of α chain thiols. These linkage data provide strong support for the assembly of IgA and IgM polymers by sequential disulfide exchanges beginning with the formation of a J chain containing dimer.

Many of the structural features of the polymeric immunoglobulins have been determined. The polymers are known to be composed of IgG-like monomers and a single J chain linked by disulfide bonds to form closed, planar molecules (Metzger, 1970; Cebra and Small, 1967; Halpern and Koshland, 1970, 1973). The size of the polymers is a function of the particular heavy chain present in the monomeric subunits. IgM, which contains a μ-type heavy chain, is almost invariably a pentamer, while IgA, which contains an α-type heavy chain, is polydisperse, dimers and tetramers being the most common forms.

The linkage between the monomers and J chain and the mechanism of their polymerization are less well understood. Analyses of the products of limited IgM reduction have indicated that the J chain is located as a disulfide clasp between two of the monomers while the remaining subunits are joined by direct S–S bonds. On the basis of these results, the assembly of IgM was postulated to proceed by a series of sequential disulfide exchanges beginning with the formation of a J-containing dimer (Chapuis and Koshland, 1974). However, proof of the postulated mechanism requires more precise information concerning the intersubunit bonds. For example, the available data do not exclude a monomer linkage for J chain. It is possible that the J chain is joined to a single subunit rather than to two subunits, by insertion in an intrasubunit μ–μ bond. Moreover, the available data do not establish the exact location of the monomer–monomer bonds. A half-cystine in the C_H3 domain of the μ chain is alkylated after limited IgM reduction (Beale and Feinstein, 1969; Frangione et al., 1971; Putnam et al., 1973), but it is not clear whether the residue is directly involved in intersubunit bonding or whether it is modified as a result of disulfide interchange during reductive cleavage.

Studies of a human IgA_1 myeloma protein were undertaken to resolve these linkage questions. The polydisperse IgA had the advantage over pentamer IgM that native dimers as well as tetramers could be isolated and used in analyzing the intersubunit structure. In addition, the α_1 chains contain a limited number of methionine residues located at strategic positions for distinguishing inter- and intrasubunit bonds. The present paper describes the combined application of reductive and cyanogen bromide cleavage to determine the mechanism of IgA polymerization.

Materials and Methods

Preparation of Tetramer IgA. A human myeloma IgA (Hol) was isolated from serum by repeated precipitation with 18% Na_2SO_4 in the presence of 10^{-3} *M* EDTA and 10^{-2} *M* iodoacetamide. After dialysis to remove Na_2SO_4 the preparation was filtered through a column of Sepharose 6B equilibrated with Tris–saline buffer (0.02 *M* Tris-HCl (pH 8.0)–0.15 *M* NaCl–10^{-3} *M* EDTA–0.01% NaN_3). The major protein peak in the eluate consisted exclusively of IgA as judged by immunoelectrophoresis against a polyvalent anti-human serum antibody. The ascending portion

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