INFLUENCE OF β -LACTAM ANTIBIOTICS ON PLATELETS. II. *IN VITRO* EFFECTS OF SOME β -LACTAM ANTIBIOTICS ON THE BIO-CHEMICAL RESPONSES OF RAT PLATELETS

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The inhibitory mechanism of β -lactam antibiotics on rat platelet activation was studied using carbenicillin (CBPC) as a representative of the antibiotics. CBPC suppressed all thrombin-induced cellular responses, including shape change, secretion and aggregation; however, it only suppressed aggregation of adenosine diphosphate (ADP)-induced responses. This suggested that ADP-binding to its own receptor was not affected by CBPC while thrombin-binding was inhibited. Inhibition of thrombin binding was confirmed using [125 I]thrombin. In the case of ADP-stimulated platelets, fibrinogen-binding, which has an essential role for ADP-induced primary aggregation, was significantly suppressed by CBPC. Increase in a net negative charge of the membrane surface was observed after treatment of platelets with antibiotics and a good correlation was obtained between suppression of the platelet responses and increase in net negative charge of the antibiotics. These findings strongly suggest that the inhibition of ligand binding to their own receptors was due to the increase in the negative charge of the platelet membrane, which was probably caused by the antibiotic bound to the platelet membrane.

Keywords — β -lactam antibiotics; platelet dysfunction; ADP; thrombin; fibrinogen; platelet surface charge

INTRODUCTION

In recent years, attention has been drawn to the fact that continuous administration of β -lactam antibiotics occasionally caused the occurrence of a bleeding diathesis. Bleeding is a serious side effect of drugs and is presumed to be caused by a dysfunction of blood coagulation, platelet aggregation or both.^{1,2)} There have been a number of reports of impaired platelet response to aggregation and release-inducing agents³⁻⁶⁾ and impaired adhesiveness⁷⁾ when penicillin or cephalosporin antibiotics are added to platelet-rich plasma (PRP) *in vitro* or when patients, volunteers³⁻⁵⁾ or experimental animals^{8,9)} received large doses of these β -lactam antibiotics. The reactions shown to be inhibited are adenosine diphosphate (ADP)-induced release primary and secondary phases of aggregation in human citrated PRP and release and aggregation of washed human platelets exposed to collagen, thrombin or ADP. Although the mechanism for dysfunction of the platelets remained obscure, some investigators suggested the effect was secondary to binding of the drug to the platelet membrane resulting in blockage of the aggregation agonist site,¹⁰⁾ which may consist of glycoproteins em-

bedded in the lipid bilayer of the platelet membrane.¹¹⁾ In our previous study, we examined the *in vivo* effects of some β -lactam antibiotics on rat platelet membrane proteins and glycoproteins.¹²⁾ We have now extended our investigation *in vitro* to obtain the precise inhibitory mechanism of β -lactam antibiotics, especially the biochemical responses on rat platelets. Degradation of the polyphosphoinositides by phospholipase C has been proposed to be a primary event in the initial response of the platelets to physiological stimuli,¹³⁾ corresponding to a rapid rise of intracellular Ca^{2+} and phosphorylation of several proteins, which may induce subsequent shape change, secretion and aggregation of the platelets. In this respect, using rat washed platelets, we investigated the inhibitory mechanism of some β -lactam antibiotics for the biochemical responses induced by thrombin or ADP in order to get insight into the relationship between structure and inhibition. The representative β -lactam antibiotics used were some penam derivatives (carbenicillin (CBPC), sulbenicillin (SBPC), penicillin G (PCG) and ampicillin (ABPC)) and cephem or oxacephem derivatives (latamoxef (LMOX), 6315-S, cefoperazone

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(CPZ) and cefotaxime (CTX)). These antibiotics have all been clinically used in the treatment of infectious disease except for 6315-S¹⁴⁾ which is under phase III studies.

MATERIALS AND METHODS

Materials — Carrier-free ^{32}P i and carrier-free Na^{125}I were purchased from the Japan Radioisotope Association (Tokyo). Quin2 acetoxymethyl ester, bovine thrombin, ADP, bovine fibrinogen, prostaglandin I_2 (PGI_2), apyrase, and bovine serum albumin were purchased from Sigma (St. Louis, MO. U.S.A.). The β -lactam antibiotics were purchased from commercial sources; LMOX (Shionogi & Co., Ltd., Osaka), CPZ (Toyama Chemical Co., Ltd., Tokyo), CTX (Hoechst Japan Co., Ltd., Tokyo), CBPC (Fujisawa Pharmaceutical Co., Ltd., Osaka), SBPC (Takeda Chem. Ind. Co., Ltd., Osaka), PCG (Banyu Pharmaceutical Co., Ltd., Tokyo), and ABPC (Toyo Jozo Co., Ltd., Shizuoka). 6315-S was synthesized at Shionogi & Co., Ltd. The structure of the β -lactam antibiotics is shown in Fig. 1.

Preparation of Rat Platelets — Platelets were prepared from fresh rat blood anticoagulated with 0.15 volumes of acid citrate dextrose (85

mM trisodium citrate, 70 mM citric acid, and 110 mM glucose). PRP was obtained by centrifugation at $160 \times g$ for 10 min. To obtain labeled platelets, the PRP was incubated with ^{32}P i (500 $\mu\text{Ci}/\text{ml}$) for 1 h at room temperature. The PRP was mixed with PGI_2 (0.5 $\mu\text{g}/\text{ml}$)¹⁵⁾ and apyrase (25 $\mu\text{g}/\text{ml}$)¹⁶⁾ and layered on 40% bovine serum albumin.¹⁷⁾ Platelets were centrifuged at $1200 \times g$ for 25 min and resuspended in 0.5 ml of resuspension buffer (137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl_2 , 3.8 mM NaH_2PO_4 , 3.8 mM Hepes, 5.6 mM glucose, and 0.035% bovine serum albumin, pH 7.35). Platelets were separated from plasma proteins by gel filtration through a column of Sepharose 2B, and suspended in the resuspension buffer to a final concentration of 5×10^8 cells/ml.

Assay for Aggregation, Shape Change and Secretion — Washed platelets were preincubated for 2 min at 37 °C with 1.5 mM CaCl_2 in the presence or absence of drugs. When platelet shape change was to be examined, 2.5 mM ethyleneglycol-bis-(β -aminoethyl ether)- N, N, N', N' -tetraacetic acid (EGTA) was used instead of CaCl_2 . Then, the platelets were stimulated with 0.4 unit/ml thrombin or 10 μM ADP. Platelet aggregation and shape change were monitored in an

FIG. 1.

aggregometer (Rikadenki Co., Ltd., Tokyo). Adenosine triphosphate (ATP) release was continuously monitored in a lumi-aggregometer (Chrono-log) using a luciferase-luciferin reagent, Chrono-lume (Chrono-log).

Scanning Electron Microscopy — Platelets were fixed in 0.1 M sodium phosphate buffer (pH 7.3) containing 1% glutaraldehyde. Samples were then filtered through a glass fiber filter paper and washed with 0.1 M sodium phosphate buffer (pH 7.3). Filters were dehydrated in a graded series of ethanol, critical point dried in a JEOL drier with liquid CO₂ as the transition fluid and coated with Au in a JEOL ion sputter coater. Platelets were examined in a JEOL JSM-35CF scanning electron microscope at an accelerating voltage of 10–15 kV.

Assay for Phosphoinositide (PI) Turnover — ³²P-Labeled platelets were incubated in an aggregometer with the drug and stimulated as described above. The radioactive lipids were directly extracted from the platelets by the method of Bligh and Dyer¹⁸⁾ and subjected to thin-layer chromatography as reported by Jolles *et al.*¹⁹⁾ The areas corresponding to phosphatidic acid (PtdOH), phosphatidylinositol (PtdIns), phosphatidylinositol 4-monophosphate (PtdIns4P) and phosphatidylinositol 4,5-bis-phosphate (PtdIns4,5P₂) were scraped off and radioactivity was determined.

Assay for Protein Phosphorylation — ³²P-Labeled platelets were incubated with drugs and stimulated as described above in an aggregometer. Radioactive platelet proteins were subjected to sodium dodecyl sulfate (SDS) slab gel electrophoresis carried out by the method of Laemmli.²⁰⁾ The gel was dried on filter paper and then exposed to an X-ray film to prepare an autoradiograph. Phosphorylation of each protein was measured by densitometric tracing of the autoradiograph. Resting phosphorylation level of each protein was subtracted from each value, and the data in each figure were expressed as the maximal phosphorylation of each protein when the sample without the drug was taken as 100%.

Assay for Ca²⁺ Mobilization — PRP was mixed with PGI₂ (0.5 μg/ml) and apyrase (25 μg/ml) and centrifuged at 1200 × *g* for 15 min. The platelet pellet was resuspended in the resuspension buffer and incubated for 20 min at 25 °C with 10 μM quin2 acetoxymethyl ester. The suspension was mixed with PGI₂ and apyrase

and layered on 40% bovine serum albumin. The quin2-loaded platelets were obtained by gel filtration and suspended in the resuspension buffer to a final concentration of 1 × 10⁸ cells/ml. The quin2-loaded platelets were preincubated for 2 min at 37 °C with 1 mM CaCl₂ while stirring in the presence or absence of 10 mM CBPC. Then, 0.4 unit/ml thrombin or 10 μM ADP was added. The fluorescence was measured using excitation and emission wavelengths of 339 and 500nm, respectively. Cytoplasmic Ca²⁺ concentration was calculated as previously described.²¹⁾

Assay for Fibrinogen Binding — Bovine fibrinogen was radiolabeled using carrier-free Na¹²⁵I by the chloramine T method²²⁾ and specific activity of 5 × 10⁶ cpm/mg was obtained. Aliquots of 0.7 ml platelet suspensions were incubated with 5 μg/ml labeled fibrinogen and 10 μM ADP in the presence or absence of drugs. After 20-min incubation with ADP, triplicate samples of 200 μl were layered onto 0.5-ml volumes of 20% (w/v) sucrose in the resuspension buffer and immediately centrifuged at 12000 × *g* for 2 min.¹⁶⁾ The ¹²⁵I radioactivity in the platelet pellets was measured in a gamma counter. Non-specific binding to platelets was measured by incubating [¹²⁵I]fibrinogen with the platelets in the absence of ADP. ADP-induced specific binding of fibrinogen to platelets was calculated by subtracting the non-specific binding from the total value.

Assay for Thrombin Binding — Bovine thrombin (Sigma) was further purified by the method of Kang and Triantaphyllopoulos²³⁾ and radiolabeled by the chloramine T method. Specific activity of 8 × 10⁶ cpm/unit was obtained. Samples of 0.7 ml platelet suspensions were incubated with 0.1 unit/ml labeled thrombin in the presence or absence of drugs. After 20-min incubation with thrombin, ¹²⁵I bound to the platelets was separated from free ¹²⁵I and measured as described in the previous assay for fibrinogen binding. Non-specific binding to platelets was measured by incubating [¹²⁵I]thrombin with platelets in the presence of an excess (100-fold) of unlabeled thrombin. Specific binding of thrombin to platelets was calculated by subtracting the non-specific binding from the total value.

Assay for Surface Charge ²⁴⁾ — 2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS, 5 μM) was added to a platelet suspension. The fluorescence was measured at 37 °C in a spectrofluorometer

using excitation and emission wavelengths of 323 and 433 nm, respectively. After 2-min preincubation with TNS, drugs were added to the suspension and the relative fluorescence change was determined.

RESULTS

Inhibition of thrombin or ADP-induced

platelet aggregation by several β -lactam antibiotics were compared (Figs. 2 and 3). All the drugs used except for ABPC and 6315-S significantly inhibited the aggregation at concentrations of 2 to 7 mM. The order of inhibition by the drugs was the same for thrombin- and ADP-induced aggregation. Among penam derivatives (PCG, ABPC, SBPC and CBPC), the degree of

FIG. 2. Effect of β -Lactam Antibiotics on Thrombin-Induced Aggregation

Washed platelets were preincubated for 2 min with the drugs in the presence of 1.5 mM CaCl_2 and stimulated with 0.4 unit/ml of thrombin. Aggregation was measured as maximal light transmission and expressed with the aggregation without drugs at 100%. Data are mean values of three experiments. A, cephem and oxacephem derivatives; 6315-S ($\circ$), CTX ($\square$), CPZ ($\triangle$), and LMOX ($\bullet$). B, penam derivatives; ABPC ($\circ$), PCG ($\triangle$), CBPC ($\bullet$), and SBPC ($\square$).

FIG. 3. Effect of β -Lactam Antibiotics on ADP-Induced Aggregation

Washed platelets were preincubated as in Fig. 2 and stimulated with 10 μM ADP. Aggregation was measured and expressed as in Fig. 2. Data are mean values of three experiments. A, cephem and oxacephem derivatives; 6315-S ($\circ$), CTX ($\square$), CPZ ($\triangle$), and LMOX ($\bullet$). B, penam derivatives; ABPC ($\circ$), PCG ($\triangle$), CBPC ($\bullet$), and SBPC ($\square$).

inhibition appeared to correlate well to the net negative charge of the drugs. However, we could not find such good correlation with cephem and oxacephem derivatives, probably due to the complex structure of these drugs.

In order to ascertain at which step platelet activation was inhibited, we studied the effects of drugs on several biochemical or physiological responses observed by ADP or thrombin stimulation. In the following experiments, we used CBPC as a representative of β -lactam antibiotics on account of its potent inhibitory action against rat washed-platelets and its frequent use by other investigators to study the effect on platelet function.^{3,4,8-10} An initial reaction of platelet activation induced by agonist binding may depend on the PI breakdown.¹³ Rapid PI breakdown was observed with regard to PtdOH formation (*ca.* 400% increase) on addition of thrombin to rat washed platelets as previously reported on human platelets.²⁵ PI breakdown was completely blocked by the presence of 7 mM CBPC (Fig. 4B). In contrast, rat platelets showed a slight increase of [32 P]PtdOH (*ca.*

20–30%) upon stimulation with ADP, which is in agreement with the results seen with human platelets.²⁶ The maximum formation of [32 P]PtdOH induced by ADP was reached at 10 s (data not shown) and this increase was unaffected by CBPC (Fig. 4A).

In parallel with PI breakdown, the substantial role of cytoplasmic ionized calcium elevation has been widely accepted as a signal of transduction through platelet activation.^{21,27} Inositol triphosphate, one of the products of PI turnover, is supposed to play an essential role for Ca^{2+} mobilization, especially in thrombin-stimulated platelets,²⁸⁻³⁰ whereas it is still uncertain what acts as the trigger for Ca^{2+} mobilization for ADP-stimulated platelets.²⁶ As shown in Fig. 5, the elevation of a cytoplasmic ionized calcium concentration induced by thrombin was depressed by CBPC, yet the elevation induced by ADP was not affected.

Two platelet proteins, myosin light chain (MLC) and 47 kDa protein, are known to be phosphorylated during platelet activation.^{31,32} The former is phosphorylated by

FIG. 4. Effect of CBPC on PI Turnover

(a) shows the effect of CBPC on [32 P]PtdOH formation by stimulation of 32 P-labeled platelets with 10 μ M ADP for 30 s (A) or with 0.4 unit/ml thrombin for 60 s (B). (b) shows the change of inositol lipids by stimulation of 32 P-labeled platelets with 0.4 unit/ml thrombin in the presence (A) or absence (B) of 7.2 mM CBPC. ●, PtdInsP₂; ○, PtdInsP; ▲, PtdIns. Data are mean values of duplicate experiments.

FIG. 5. Effect of CBPC on Intracellular Ca^{2+} Mobilization

Quin2-loaded platelets were preincubated for 2 min without (A, C) or with 10 mM CBPC (B, D), and stimulated with 0.4 unit/ml thrombin (A, B) or with 10 μM ADP (C, D). Data are representatives of three experiments.

MLC kinase³¹⁾ which is activated by Ca^{2+} and the latter is phosphorylated by protein kinase C which is activated by both Ca^{2+} and 1,2-diacylglycerol,³²⁾ another product of PI breakdown. It is accepted that MLC phosphorylation plays an important role in the platelet shape change, aggregation and secretion.^{33,34)} However phosphorylation of 47 kDa protein is implicated to be suppressive for platelet responses.³⁵⁾ As shown in Fig. 6, phosphorylation of both proteins induced by thrombin was inhibited by CBPC, while that induced by ADP was unaffected.

As an initial physiological expression, shape change occurs prior to aggregation. Stimulation of the platelets by physiological agonists in the presence of 2.5 mM EGTA showed no response except for the shape change observed by a decrease in light transmission.³³⁾ As expected from the results in Fig. 6, thrombin-induced shape change was inhibited by CBPC; however, ADP-

FIG. 6. Effect of CBPC on Protein Phosphorylation
 ^{32}P -Labelled platelets were preincubated for 2 min with CBPC and stimulated with 10 μM ADP for 10 s (○) or with 0.4 unit/ml thrombin for 30 s (●). Phosphorylation of 47 kDa protein (A) and 20 kDa protein (B) are presented. Phosphorylation was measured and expressed as described under Materials and Methods. Data are mean values of duplicate experiments.

induced shape change was unaffected (Fig. 7). The effects of CBPC on the platelet shape change were further examined by means of scanning electron microscopy (Fig. 8). Platelets were discoid prior to agonist addition (Fig. 8A) but by addition of agonists in the presence of EGTA, the shape changed to spheroid with pseudopodia (Fig. 8B and D). Only thrombin-induced shape change was inhibited by CBPC (Fig. 8C and E), an observation which corresponded well to the data obtained by the aggregometer.

Together with platelet aggregation, a release of granular content occurs by the stimulation with thrombin. As shown in Fig. 9, thrombin-induced ATP release was inhibited by CBPC. ADP (up to 10 μM) did not cause any apprecia-

FIG. 7. *Effect of CBPC on Platelet Shape Change*
Washed platelets were preincubated for 2 min with CBPC in the presence of 2.5 mM EGTA. Then, the platelets were stimulated with 0.4 units/ml thrombin (●) or 10 μ M ADP (○). Platelet shape change was measured as the initial rate of decrease in light transmission and expressed with the shape change without CBPC at 100%.

FIG. 9. *Effect of CBPC on Secretion*
Washed platelets were preincubated for 2 min with CBPC and stimulated with 0.4 unit/ml thrombin. Released ATP was monitored as described under Materials and Methods and maximal level of released ATP was measured.

FIG. 8. *Scanning Electron Microscopy of Shape Changed Platelets*

Washed platelets were treated as in Fig. 7 and fixed with glutaraldehyde. (A) shows control platelets. (B) and (C) show platelets fixed 30 s after the addition of 0.4 unit/ml thrombin, and (D) and (E) show the platelets fixed 30 s after the addition of 10 μ M ADP, in the absence (B, D) and presence (C, E) of 10 mM CBPC.

FIG. 10. Effect of CBPC on Binding of $[^{125}\text{I}]$ Fibrinogen or $[^{125}\text{I}]$ Thrombin to Platelets (A) shows the fibrinogen binding and (B) shows the thrombin binding. Data are expressed with the bound ^{125}I without CBPC (5900 cpm/ 10^9 cells for fibrinogen and 15000 cpm/ 10^9 cells for thrombin, respectively) at 100%. Data are mean values of triplicate experiments.

FIG. 11. Comparison of the Effects of β -Lactam Antibiotics on the Binding of Fibrinogen or Thrombin to the Platelets

(A) shows the inhibition of $[^{125}\text{I}]$ thrombin binding by 3 mM CBPC (a), ABPC (b), PCG (c), LMOX (d), CTX (e), and 6315-S (f). (B) shows the inhibition of $[^{125}\text{I}]$ fibrinogen binding by 5 mM CBPC (a), PCG (b), and LMOX (c). Data are mean $\pm$ S.E. (triplicate experiments).

ble ATP release (data not shown).

In ADP-stimulated platelets, among the physiological responses only aggregation was inhibited by CBPC suggesting that all responses except for aggregation were not directly affected by the drug. Fibrinogen binding to the platelets via glycoprotein IIb-IIIa complex has been es-

tablished to be essential for ADP-induced primary aggregation.³⁶⁾ Then, the effect of CBPC on ADP-induced fibrinogen binding was examined by $[^{125}\text{I}]$ fibrinogen and it was found that CBPC significantly inhibited its binding to the platelets in a concentration dependent manner (Fig. 10B). On the other hand, all responses were

inhibited by CBPC in thrombin-stimulated platelets, suggesting that an inhibition of agonist binding to the platelets may result in depression of all platelet responses. This assumption was confirmed by the data represented in Fig. 10A.

In order to obtain further confirmation for the proposal that the depression of thrombin-induced and ADP-induced platelet aggregation is caused by the inhibition of thrombin as well as fibrinogen binding to the platelets, comparison of the inhibitory actions against ligand binding were performed using several β -lactam antibiotics. The order of inhibition of thrombin binding (Fig. 11A) agreed well with that of aggregation as shown in Fig. 2. A similar result was also obtained in ADP activation (Figs. 11B and 2). These results strongly suggested that the inhibitory mechanism of β -lactam antibiotics depends substantially on blockage of initial agonist binding or secondary fibrinogen binding to their own receptors on the platelets and not on direct effects for signal transduction.

Since β -lactam antibiotics generally contain carboxyl groups within the molecules and can interact hydrophobically with phospholipid bilayers of the platelets,³⁷⁾ this interaction may result in an increased net negative charge of the platelets. Inhibition of platelet aggregation seemed to correlate well to the net negative charge of the drugs, especially among penem derivatives (Fig. 2). Hence, we examined the effects of some β -lactam antibiotics on the membrane surface charge by a fluorescent reagent TNS, which is a probe for measurement of membrane surface charge.²⁴⁾ Good correlations were observed between decrease of TNS fluorescence (Fig. 12) and inhibition of platelet aggregation by the respective antibiotics (Fig. 2). These alterations of

FIG. 12. Effects of β -Lactam Antibiotics on TNS Fluorescence

The relative fluorescence in the presence of ABPC (○), 6315-S (□), CBPC (△), LMOX (●), and PCG (▲) is shown. Data are expressed with the fluorescence without antibiotics at 100%.

net negative charge of the platelets would probably affect the ligand binding to their own receptors.

DISCUSSION

The inhibitory action of CBPC, which was used as a representative β -lactam antibiotic, against platelet activation was found to be much different between ADP and thrombin. Shattil and coworkers presented data from *in vitro* studies that indicated CBPC impaired the ADP receptor on the platelet surface membrane from the observation of reduced incorporation of an ADP affinity label, 5'-*p*-fluorosulfonyl

TABLE I. Effect of CBPC on Platelet Responses Induced by Thrombin or ADP

Response	Stimulus	
	Thrombin	ADP
PI turnover	+	—
Ca ²⁺ mobilization	+	—
Protein phosphorylation	+	—
Shape change	+	—
Aggregation	+	+
ATP release	+	N.D.

+, inhibited by CBPC. —, not inhibited by CBPC. N.D., not detected.

[^3H]adenosine, into its binding protein in the platelet membrane.¹⁰⁾ However, their experiments were based on extremely high concentrations of the antibiotic (36–180 mM). These high concentrations of the drug will nonspecifically affect the platelet membrane and may result in perturbation for the initial agonist binding to its receptor. Therefore, we studied the action of antibiotics at the minimum concentrations (2–5 mM) sufficient to inhibit aggregation of the washed platelets in response to ADP and thrombin. The inhibitory mechanism of CBPC against platelet activation was clearly demonstrated to differ on respective agonists as summarized in Table I. CBPC suppressed all the processes for platelet activation induced by thrombin, while the biochemical responses corresponding to ADP stimulation were unaffected except for aggregation. These data probably reflect the differences of agonist binding to the platelets between ADP and thrombin. A positive correlation was observed between the inhibition of thrombin binding and interference platelet responses by antibiotics as shown in Fig. 12. On the other hand, CBPC did not suppress an increase of intracellular Ca^{2+} induced by ADP, which is thought to be an initial step for subsequent signal transduction leading to platelet shape change.^{31,33)} Yet, it was clearly demonstrated in Fig. 10 that CBPC inhibited the binding of fibrinogen to the platelets. The essential role for this secondary fibrinogen binding to the platelets *via* GPIIb–IIIa complex has been well established for ADP stimulation to complete primary aggregation.³⁶⁾ Therefore, it is proposed that the inhibitory mechanism of antibiotics on thrombin-induced platelet activation is suppression of agonist binding and that by ADP is the impairment of secondary fibrinogen binding to the platelets, as shown in Fig. 13.

Membrane components of rat platelets which bind ADP and thrombin have not yet been characterized. Thus, receptors for these agonists appear to include several proteins or glycoproteins that are presumably embedded within the membrane lipid bilayer as predicted from human platelets.¹¹⁾ β -Lactam antibiotics might interfere with the binding of agonists or other ligands to their receptors by interacting directly with these membrane proteins or by interaction with the lipid milieu in which these proteins are embedded. No information is available as to

FIG. 13. *Proposed Inhibitory Mechanism of β -Lactam Antibiotics on the Platelet Activation*

In the case of thrombin stimulation, membrane-bound β -lactam antibiotic which is represented by CBPC in the scheme inhibits the thrombin binding to the receptor by increasing the negative charge of the membrane surface. Therefore, all the responses are suppressed. On the other hand, ADP binding to the receptor is not inhibited and intracellular signal transduction is not affected by CBPC and platelet shape change is fully induced. However, fibrinogen binding is inhibited, therefore ADP-induced primary aggregation is suppressed.

whether these antibiotics bind to the platelets. However, if they do bind to the platelet membrane, their binding affinity must be very low because gel filtration of platelets reverses their inhibitory effects almost completely. The degree of inhibition by antibiotics seems to depend on the net negative charge of the drugs, especially penicillin analogues (Figs. 2 and 3), and also the

inhibitory action of antibiotics seems to correspond well with an increase in net negative charge of the platelets as shown in Fig. 12. One of the possibilities for the increased negative charge of the platelets treated with antibiotics may be due to the binding of the drugs to the platelet surface. Drugs which contain carboxyl groups within the molecules may interact hydrophobically with phospholipid bilayers of the platelets, as previously shown for penicillin on an artificial phospholipid bilayer. Platelets carry a net negative surface charge which has been shown to be a factor in platelet adhesion and aggregation.^{38,39)} Thus, modification in the surface negative charge has been proposed to trigger the electrostatic interactions between platelets and alter functional responses, although the relationship between these parameters have not been completely resolved. The same results were reported for the inhibitory action of fatty acids against platelet aggregation which correlated well with increase in platelet surface negative charge.²⁴⁾ Recently, Iida and coworkers⁴⁰⁾ showed that the electrophoretic mobility (E.P.M.) of platelets is increased by injection of latamoxef in rabbit as well as by incubation *in vitro* PRP with the same drug and they found ADP-induced primary aggregation to be negatively correlated with E.P.M. These results are not only consistent with primary aggregation which depends on initial cell-cell contacts but also with an increase in cell surface negative charge resulting in alteration of initial agonist binding or secondary fibrinogen binding.

In the present investigations, we first compared the effects of CBPC on primary aggregation in response to ADP and thrombin, and further clarified the differences of inhibitory action of other β -lactam antibiotics to each agonist. However, further study is necessary on the differences between *in vitro* and *in vivo* observations on the inhibition of platelet function by these drugs. First, these antibiotics inhibit platelets immediately *in vitro*, whereas they appear to prolong the bleeding time only after 12–24 h in the case of parenteral administration.^{4,41)} Second, the peak serum concentration of the respective antibiotic (<1 mM) is several-fold lower than that necessary to inhibit platelet aggregation and secretion *in vitro*. Therefore, the inhibition by drugs *in vivo*, may be the result of either a prolonged contact of platelets at lower concen-

trations of antibiotics or a metabolite of the antibiotic. White considers platelets to be the most sensitive of all cells in circulating blood to chemical and physical agents.⁴²⁾ Therefore it is equally important to appreciate that the influences exerted on the platelets are not confined to their cellular infrastructure or its immediate vicinity, and it may be that the platelet is readily affected by remote stimuli through chemical or physical signals or feedback. From this point of view, we must successively consider the effect of antibiotics on the platelets. Third, our data are based on rat platelets, and some functional differences are well known to exist among animal species.⁴³⁾ However, a good parallelism was observed between human and rat washed platelets with regard to degree and order of inhibition of each antibiotic (data not shown). We emphasize here that it should be possible to use rat platelets instead of human in the confined experiment we performed, as rat platelets are advantageous in respect to conventional availability as well as homogeneity.

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