PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **182**, 34–42 (1986)

Platelet Aggregation Caused by Carybdea Rastonii Toxins (CrTX-I, II, and III) Obtained from a Jellyfish, *Carybdea rastonii* (42305)

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Abstract. The pharmacological mechanisms of platelet aggregation induced by highly toxic proteins (CrTX-I, CrTX-II, and CrTX-III) obtained from tentacles of a jellyfish, *Carybdea rastonii,* were investigated. When the partially purified toxin (pCrTX) and CrTXs were added to the citrated platelet-rich plasma (PRP), aggregation was produced in a concentration-dependent manner. The activity of CrTXs was approximately 100 times more potent than pCrTX. The CrTXs-induced aggregation was little affected by indomethacin and quinacrine at concentrations sufficient to inhibit arachidonic acid- and collagen-induced aggregation. The CrTXs-induced aggregation in washed platelets was significantly augmented in the presence of Ca^{2+}. The pretreatment with verapamil failed to modify this augmentation of aggregation. The concentration of cytoplasmic-free calcium ($[Ca^{2+}]_i$) of platelets was increased by CrTXs at the same concentrations that produced aggregation. This effect of CrTXs was again little affected by verapamil. CrTXs at the same concentrations as those that produced aggregation and increased $[Ca^{2+}]_i$ caused depolarization of platelets, which was unchanged after pretreatment with sodium or potassium transport inhibitors. CrTX-I significantly increased the ^{22}Na flux into platelets and this effect of CrTX-I was unaffected by tetrodotoxin. The CrTX-I-induced aggregation, depolarization, and increase in $[Ca^{2+}]_i$ were all significantly attenuated in the low Na^+ medium. These results suggest that CrTXs cause a massive depolarization by increasing cation permeability and this generalized depolarization permits an inward movement of Ca^{2+} down its electrochemical gradient which, in turn, triggers platelet aggregation.

During the summer months, one of the species of box jellyfish, *Carybdea rastonii,* is commonly present in sea waters along the coast of Japan. The nematocyst of this jellyfish is capable of producing severe cutaneous pain, erythema, wheeling, and hemorrhagic skin lesions in humans who accidentally come into contact with its tentacles. The physiological and toxicological effects such as lethal toxicity on mammals, hemolytic activity, dermatonecrotic activity, and cardiotoxic effect of various coelenterate toxins have been well documented (1–6), particularly those derived from the Portuguese man-of-war (*Physalia physalis*), the sea wasp (*Chironex fleckeri*), and the sea nettle (*Chrysaora quinquecirrha*). The toxin (pCrTX) partially purified from tentacles of *C. rastonii* also possesses lethal toxicity, hemolytic activity, dermatonecrotic activity, and cardiotoxic effect in mammals (7, 8).

As to the mechanisms of action of these toxins, Larsen and Lane (9) have demonstrated that *P. physalis* toxin increases the permeability of frog skin to sodium, but not to active sodium transport. Warnick *et al.* (10) suggested that the sea nettle toxin depolarized nerve and muscle membranes by a sodium-dependent, tetrodotoxin-insensitive mechanism which secondarily increased Ca^{2+} influx. In addition, it has been reported (11) that sea nettle venom (SNLF) causes formation of cation channel in cell membrane and the initial effects of such channels would be a sodium ion flux and cell depolarization leading to cardiotoxic effects. According to Burnett and Calton (12) and Burnett *et al.* (13), calcium transport has been found to be also important for jellyfish toxins, since verapamil, a calcium antagonist, is effective in delaying death in mice after intravenous challenge with sea nettle (*C. quinquecirrha*) or Portuguese man-of-war (*P. physalis*) crude venom.

Purification and characterization of the toxin of crude venom have been hindered by their instability. However, Tamkun and Hessinger (14) have reported that a glycoprotein purified from the Portuguese man-of-war possesses potent lethal toxicity and hemolytic ac-

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tivity. Also, Guar *et al.* (15) tried to purify the toxic substance by preparing monoclonal antibodies against the sea nettle venom mouse lethal factor. Cobbs *et al.* (11) using the same monoclonal antibody, isolated proteins in one tentacle type. Furthermore, Kelman *et al.* (16) succeeded in isolating three lethal proteins from another sea nettle tentacle type. We recently also found highly toxic proteins from *C. rastonii* and named them CrTX-I, CrTX-II, and CrTX-III (17). Although these three proteins possess physiological and toxicological effects similar to those of pCrTX, the detailed pharmacological properties are unknown. Since pCrTX has been found to produce platelet aggregation (18), the present experiments were undertaken to investigate the pharmacological mechanisms of CrTXs-induced platelet aggregation in connection with ion transport. Here, we suggest that CrTXs cause a massive depolarization by increasing cation permeability indiscriminately and that this generalized depolarization permits an inward movement of calcium down its electrochemical gradient which, in turn, triggers platelet aggregation.

Materials and Methods. *Preparation of CrTX-I, CrTX-II, and CrTX-III.* The preparation of CrTX-I, CrTX-II, and CrTX-III is detailed in the previous report (17). In brief, aliquots of the lyophilized tentacles were sonicated in 50 m*M* sodium acetate (pH 6.0). The suspension was then centrifuged at 6000*g* for 30 min. The supernatant was separated and treated with 0 to 40% ammonium sulfate for 60 min. Centrifugation at 6000*g* for 30 min was carried out to obtain supernatant, which was again treated with 40 to 60% ammonium sulfate for 60 min. The precipitate was separated after centrifugation at 6000*g* for 30 min and dissolved in 50 m*M* sodium acetate (pH 6.0). The solution was then dialyzed by means of Amicon YM-2 to obtain pCrTX. All the procedures described above were carried out at 4°C. The pCrTX was further chromatographed (HPLC, Tri Rotar, SR-2, Nihonbunko) on a CM-2SW column (7 × 600 mm, Toyo Soda). Three active fractions which were monitored by hemolytic activity could be obtained at 0.35, 0.45, and 0.55 *M* NaCl. These fractions were then separated and concentrated by Centricon-10 (Amicon). Finally, the concentrated fractions were rechromatographed on a G-3000SW column. These three active fractions were named CrTX-I, CrTX-II, and CrTX-III (17). The hemolytic activity of CrTXs disappeared after treatments with acid (pH < 4.0), alkaline (pH > 8.0), trypsin, and chymotrypsin, or by heating (50°C). Thus, CrTXs appeared to be protein in nature. However, trypsin, chymotrypsin, leucine aminopeptidase, alkaline phosphatase, and phospholipase A_2 activities of CrTXs were very low (17): Sodium dodecyl sulfate (SDS)–polyacrylamide gels (PAGE) of CrTX-I, CrTX-II, and CrTX-III did not stain with dansyl hydrazine, indicating that CrTXs are not glycoproteins. In addition, since the electrophoresis profile on the SDS–polyacrylamide gels of CrTXs was unchanged in the presence of 2-mercaptoethanol, CrTXs do not contain disulfide bonds (17). Apparent molecular weight which was determined by means of SDS–PAGE (19) was 49K for CrTX-I, 100K for CrTX-II, and 51K for CrTX-III. The half-lives of CrTX-I, CrTX-II, and CrTX-III (1 μg/ml each, 4°C, pH 6.0) in hemolytic activity were determined to be 4.0, 8.0, and 12.0 hr, respectively. Whereas, the hemolytic activity of pCrTX conducted on 1 μg/ml did not decrease for at least 2 weeks when stored at 4°C. The minimum lethal doses (iv) of CrTX-I, CrTX-II, and CrTX-III were 3.5, 3.6, and 3.0 ng/g body wt in mice, respectively, as compared to an iv LD_{50} of 127 ng/g of pCrTX.

pCrTX was diluted to appropriate concentrations by physiological saline just prior to use. CrTXs were prepared from pCrTX just prior to use. The concentrations of pCrTX and CrTXs were expressed as gram protein per milliliter. Protein was assayed by the Lowry method (20) using bovine serum albumin (BSA) as a standard.

Platelet aggregation study. Rabbit blood was collected into 1/10 vol of 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation at 90*g* for 15 min at room temperature, and platelet-poor plasma (PPP) was obtained by further centrifugation at 1000*g* for 15 min. Platelet aggregation was studied by the turbidometric method of Born (21) utilizing a dual sample aggregation meter (Sienco, DP-247E) which was modified to provide continuous stirring and to maintain constant temperature (37°C). The standard reaction mixtures consisted of 297 μl of citrated PRP or washed platelets (WP) and 3 μl of pCrTX, CrTXs, or known aggregating agents. When the effects of various agents on aggregations were tested, 3 μl of the solution of agents or vehicle (control) was added 3 min prior to the aggregating agents. The extent of aggregation was evaluated by the maximum change of light transmission expressed as a percentage, taking the difference between light transmission for PRP (or WP) and PPP (or Hepes buffer) as a value of 100%. Percentage of inhibition of aggregation by the test agents was calculated by dividing the percentage of aggregation by that observed in the control run, then multiplying by 100. All individual tests were carried out in rapid succession (within 3 hr of preparation) to avoid potential errors which might be due to alterations in the sensitivity of the platelets toward aggregating agents during storage at room temperature. The number of platelets was determined with the aid of a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

Gel-filtered platelets (GFP). GFP were prepared by a modification of the method of Tangen *et al.* (22), using Sepharose 2B (Pharmacia Chemicals), and were suspended in Hepes buffer. The Hepes buffer contained a final concentration of 145 m*M* NaCl, 1 m*M* $MgCl_2 \cdot 6H_2O$, 5 m*M* KCl, 1 m*M* $CaCl_2 \cdot 2H_2O$, 5 m*M* dextrose, 0.5 m*M* Na_2HPO_4, 0.35% BSA, and 10 m*M* Hepes (pH 7.55 at room temperature). Ca^{2+}-free medium was prepared by omitting $CaCl_2$ from the buffer. For the low Na^+ solution, 72.5 m*M* NaCl was replaced with 72.5 m*M* choline chloride.

Washed platelets (WP). WPs were prepared as follows: PRP were centrifuged at 1000*g* for 10 min at room temperature, then the platelet pellet was suspended in Hepes buffer. The suspension was again centrifuged at 1000*g* for 10 min. Finally, the pellet was resuspended in Hepes buffer or low Na^+ buffer to a desired platelet count. Centrifugation was carried out using plastic tubes containing 50% BSA in the bottom layer.

Determination of platelet membrane potential. The membrane potential of platelets was determined using a membrane potential probe, 3,3′-dipropyl-2,2′-thiadicarbocyanine iodide (DiS) according to Friedhoff and Sonenberg (23). In brief, GFP were suspended in Hepes buffer in a platelet count of 55,000/μl. Six microliters of 0.5 m*M* DiS solution in absolute ethanol was added to a quartz cuvette containing 1.5 ml of platelet suspension. Five min after addition of DiS, various concentrations of CrTXs were added for desired periods. All the procedures were carried out at 37°C. In order to test the effects of agents on the CrTXs-induced depolarization, the agents were added 3 min prior to CrTXs. Fluorescence was measured using a Hitachi MPF-3 spectrofluorometer. Excitation was at 620 nm and emission was measured at 670 nm. Band widths for both were 10 nm. A change in fluorescence of 1 U is equivalent to a change of 0.951 mV membrane potential (23).

^{22}Na-flux study. WPs were suspended in Hepes buffer in a population of 4 to 5 × 10^5/μl and incubated with $^{22}NaCl$ (0.5 μCi/ml) and ^{125}I-labeled human serum albumin (0.2 μCi/ml) at 37°C for 0, 2, 4, 6, and 10 min in the presence of CrTX-I or vehicle (control). Platelets from 1 ml of WP suspension were sedimented through silicon oil (SH550/SH200 = 5/1, Toray) by centrifugation at 12000*g* for 1 min immediately after termination of the incubation. The maximum speed was reached within 15 sec which separated the platelets from the buffer and pelleted them beneath the silicon oil layer. The platelet pellet as well as samples of platelet-free buffer was prepared for radioactivity determinations according to Feinberg *et al.* (24). Radioactivities of ^{22}Na and ^{125}I were measured simultaneously in a gamma counter (Packard 800C) for 10 min. Crossover of ^{125}I to the ^{22}Na channel was negligible, while that of ^{22}Na to the ^{125}I channel was calculated to be 5.19 ± 0.06% ($n = 10$). The volume of buffer trapped with the sedimented platelets was estimated from the ^{125}I present in the pellet. The pellet ^{22}Na radioactivity was determined as the difference between the total pellet ^{22}Na radioactivity and ^{22}Na in the trapped buffer. The amount of ^{22}Na presumed to have entered into the platelets was expressed as a "space" (μl/10^8 platelets), which was calculated as the ratio between ^{22}Na in the pellet and ^{22}Na in the platelet-free buffer (24).

Cytoplasmic-free calcium in platelets. Cytoplasmic-free calcium ($[Ca^{2+}]_i$) was measured using the intracellularly trapped fluorescent indicator quin 2 according to Rink *et al.* (25) and Tsien *et al.* (26). In brief, WP suspended in Hepes buffer (pH 7.55) were incubated for 30 min at 30°C with 15 μM quin 2 acetoxymethylester (quin 2/AM)/1 × 10^5 platelets. After loading, the cells were centrifuged at 1000*g* for 3 min and resuspended in fresh Hepes buffer containing 1 m*M* Ca^{2+}. The loaded platelet suspension was preincubated for 5 min at 37°C and then treated with various concentrations of CrTXs for 10 min. In order to test the effect of verapamil, the agent was added 3 min prior to CrTXs. When the effect of low Na^+ was examined, the loaded platelets were suspended in the low Na^+ Hepes buffer. Fluorescence was recorded in a Hitachi MPF-3 spectrofluorometer. Excitation was at 339 nm with a 4 nm slit and emission was measured at 492 nm with a 10 nm slit. The signals were calibrated by releasing quin 2 from the cells with 50 m*M* digitonin at the end of the experiments (F_{max}) and chelating extracellular calcium by 2 m*M* EDTA (F_{min}). From these fluorescence levels of F_{max} and F_{min}, the $[Ca^{2+}]_i$ corresponding to fluorescence F from trapped dye was calculated by the following equation (26): $[Ca^{2+}]_i\ (nM) = 115 \times (F - F_{min})/(F_{max} - F)$. Stock solutions of quin 2/AM were made up at 15 m*M* in dry dimethylsulfoxide (DMSO) and kept desiccated at −80°C until required. The final concentration of DMSO from the quin 2/AM stock did not exceed 0.5% (v/v). All solutions were made with water purified to >15 MΩ-cm by a Milli Q system (Millipore Corp., Bedford, Mass.).

Chemicals. The following chemicals were used for these experiments: $^{22}NaCl$ (sp act: 282.4 mCi/mg, New England Nuclear, Boston, Mass.), ^{125}I-labeled human serum albumin (sp act: 0.036 mCi/mg, Green Cross, Osaka), collagen (Collagen Reagent Horm, Hormon-Chemie, Münchem, GMBH), indomethacin and digitonin (Merck), verapamil hydrochloride (Shiratori Pharmaceutical Co.), quin 2/AM (2-[(2-amino-5-methylphenoxy)methyl]-6-methoxy-8-aminoquinoline-*N,N,N′,N′*-tetraacetic acid tetraacetoxymethyl ester, Wako Pure Chemicals), DiS (3,3′-dipropyl-2,2′-thiadicarbocyanine iodide, Nihon Kankoshikiso Kenkyusho). Arachidonic acid (AA), quinacrine, tetrodotoxin (TTX), 4-aminopyridine (4AP), tetraethylammonium chloride (TEA), procaine hydrochloride, and valinomycin were all from Sigma. Because indomethacin and quin 2/AM were insoluble in water, they were dissolved in DMSO, which was present in a final concentration of 0.5% or less in all experiments with these agents, and this concentration had no effect on any parameters studied.

Statistical analysis. Results shown in the text, table, and figures are expressed as means ± SE. For statistical evaluation, data were analyzed by Student's *t* test.

Results. When pCrTX and CrTXs were added to the citrated PRP, aggregation was produced in a concentration-dependent manner. The concentration-aggregation curves for pCrTX and CrTXs are shown in Fig. 1. The EC_{50}s (concentration which produced 50% aggregation) obtained from regression curves for CrTX-I, CrTX-II, and CrTX-III were 2.2, 2.2, and 2.3 × 10^{-9} g/ml, respectively, as compared to that of 1.8 × 10^{-7} g/ml for pCrTX. In this respect, the activity of CrTXs was approximately 100 times more potent than that of pCrTX.

The 3 × 10^{-9} g/ml CrTXs-induced aggregations were only slightly inhibited by indomethacin (3 μM) and quinacrine (100 μM) at concentrations sufficient to inhibit AA- and collagen-induced aggregation (Table I).

When CrTXs (10^{-9} to 10^{-8} g/ml) were added to WP suspended in Ca^{2+}-free Hepes buffer, slight but concentration-dependent aggregation was produced, whereas the CrTXs-induced aggregation was significantly augmented in the presence of 1 m*M* Ca^{2+} (Fig. 2). It is well known that in platelets calcium ion plays a significant role in a variety of phys-

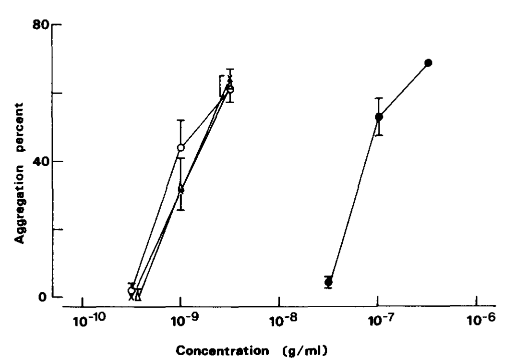

FIG. 1. Concentration–aggregation curves for pCrTX and CrTXs in citrated PRP. ●: pCrTX, ○: CrTX-I, ×: CrTX-II, △: CrTX-III. Each point represents the mean of four to six observations and vertical bars show SE.

TABLE I. EFFECTS OF INDOMETHACIN AND QUINACRINE ON AGGREGATION INDUCED BY ARACHIDONIC ACID (AA), COLLAGEN, AND CrTXs

Aggregating stimuli	Indomethacin (3 μM)	Quinacrine (100 μM)
AA (125 μM)	94.1 ± 3.8 (6)	94.0 ± 3.1 (6)
Collagen (1 × 10^{-5} g/ml)	94.7 ± 5.3 (4)	100 (7)
CrTX-I (3 × 10^{-9} g/ml)	3.8 ± 0.9 (4)	13.5 ± 1.1 (4)
CrTX-II (3 × 10^{-9} g/ml)	3.5 ± 1.2 (4)	18.5 ± 5.5 (4)
CrTX-III (3 × 10^{-9} g/ml)	4.0 ± 0.9 (4)	12.9 ± 1.1 (4)

Note. Results are given as the percentage mean inhibition ± SE. Figures in parentheses indicate the number of observations.

iological functions. Thus, we examined whether or not $[Ca^{2+}]_i$ was increased when CrTXs produced aggregation. As can be seen in Fig. 3, the $[Ca^{2+}]_i$ was increased by CrTXs at the same concentrations that produced aggregation. When 10^{-9} g/ml of CrTX-I, CrTX-II, and CrTX-III was applied to WP, the $[Ca^{2+}]_i$ increased from the basal level of 200.4 ± 33.7, 147.5 ± 33.4, and 120.2 ± 20.2 nM to 610.0 ± 158.7, 840.1 ± 159.9, and 275.5 ± 37.7 nM, respectively. The increase in $[Ca^{2+}]_i$ caused by 10^{-9} g/ml of CrTXs was little affected by pretreatment with verapamil at the relatively high concentration of 100 μM (Fig. 4b). In addition, the augmented aggregation caused by 10^{-9} g/ml CrTXs in the presence of 1 mM Ca^{2+} was also little affected by verapamil (Fig. 4a). Neither diltiazem (100 μM) nor nifedipine (10 μM) affected the increase in $[Ca^{2+}]_i$ and the augmentation of aggregation (data not shown). Figure 5 shows the time course of platelet membrane depolarization after addition of 3 × 10^{-9} g/ml of CrTXs. The time course coincided well with that of platelet aggregation. In addition, CrTXs at the same concentrations that produced aggregation and increased the $[Ca^{2+}]_i$ caused membrane depolarization. The magnitude of depolarization was 25.2 ± 2.2 mV by 3 × 10^{-9} g/ml CrTX-I, 25.5 ± 1.6 mV by 3 × 10^{-9} g/ml CrTX-II, and 26.0 ± 2.2 mV by 3 × 10^{-9} g/ml CrTX-

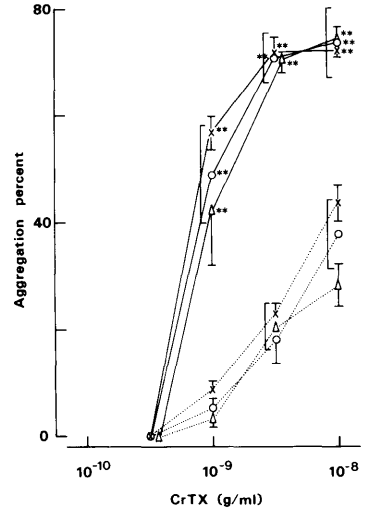

FIG. 2. Potentiation of aggregating activities of CrTXs in the presence of Ca^{2+} in washed platelets. —: Responses in the presence of 1 mM Ca^{2+}, ---: responses in the absence of Ca^{2+}, O: CrTX-I, ×: CrTX-II, Δ: CrTX-III. Each point represents the mean of four to five observations and vertical bars show SE. **$P < 0.01$ vs aggregation percentage in the absence of Ca^{2+}.

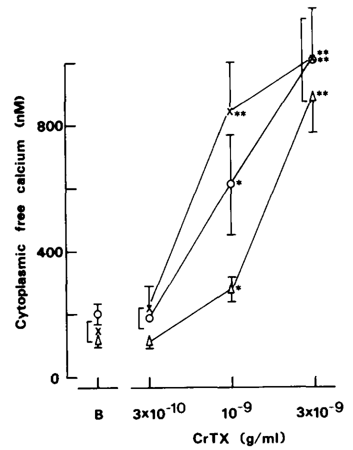

FIG. 3. Increase in cytoplasmic-free calcium induced by CrTXs in washed platelets. O: CrTX-I, ×: CrTX-II, Δ: CrTX-III, B: basal level of cytoplasmic-free calcium without CrTXs, *$P < 0.05$ and **$P < 0.01$ vs basal level (B). Each point represents the mean of four observations and vertical bars show SE.

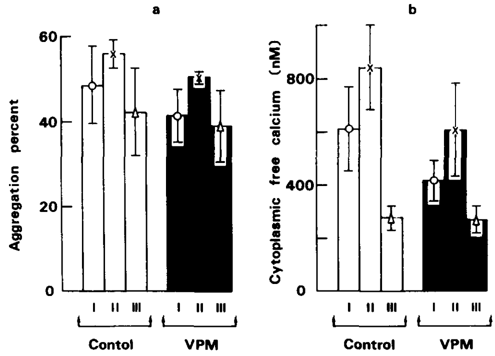

FIG. 4. Effect of verapamil on CrTXs-induced aggregation (a) and increase in cytoplasmic-free calcium concentration (b) in washed platelets. □: Control responses, ■: responses in the presence of 100 μ*M* verapamil (VPM), I(○): 10^{-9} g/ml CrTX-I, II(×): 10^{-9} g/ml CrTX-II, III(△): 10^{-9} g/ml CrTX-III. Each experiment consists of four observations and vertical bars show SE.

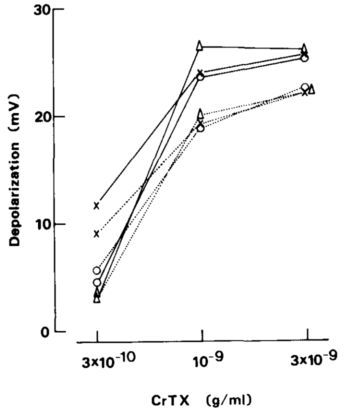

FIG. 6. Concentration–depolarization curves for CrTXs in the presence or absence of Ca^{2+} in gel-filtered platelets. —: Responses in the presence of 1 m*M* Ca^{2+}, ---: responses in the absence of Ca^{2+}. ○: CrTX-I, ×: CrTX-II, △: CrTX-III. Each point represents the mean of four observations. Concentration of CrTX-I, II, and III (g/ml).

III 6 min after addition. The degree of depolarization caused by CrTXs in Ca^{2+}-free medium was not significantly different from that in the medium containing 1 m*M* Ca^{2+} (Fig. 6). As shown in Fig. 7, CrTX-I at concentration of 3×10^{-9} g/ml significantly increased the ^{22}Na space in WP. The time course of the increase in ^{22}Na space also coincided well with that of aggregation and membrane depolarization. The ^{22}Na space 2 min after application of CrTX-I was approximately 4.5 times higher (0.3917 ± 0.0631 μl/10^8 platelets) than that in the control (0.0868 ± 0.0317 μl/10^8 platelets) without CrTX-I. The pretreatment with 1 μ*M* TTX failed to modify the increase in the ^{22}Na space caused by 3×10^{-9} g/ml CrTX-I (data not shown).

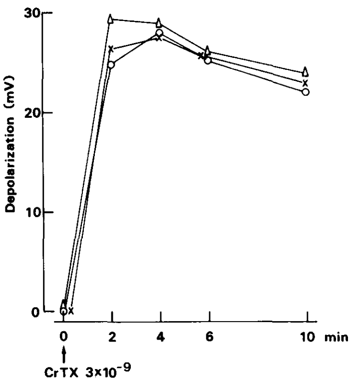

FIG. 5. Time course of platelet membrane depolarization after addition of CrTXs in gel-filtered platelets. The arrow indicates the time of addition of 3×10^{-9} g/ml CrTXs. ○: CrTX-I, ×: CrTX-II, △: CrTX-III. Each point represents the mean of four observations.

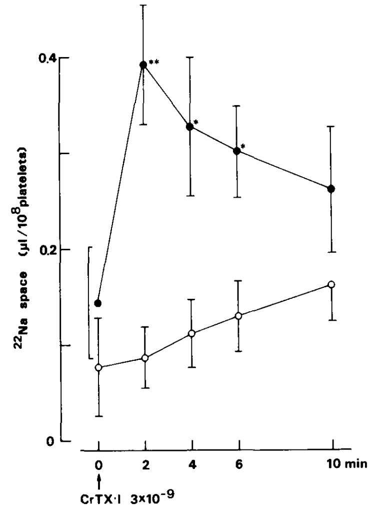

FIG. 7. Time course of increase in ^{22}Na space caused by CrTX-I in washed platelets. Washed platelets were incubated in the presence (●) or absence (○) of 3×10^{-9} g/ml CrTX-I. Each point represents the mean of six observations and vertical bars show SE. *$P < 0.05$ and **$P < 0.01$ vs each time control (○). The arrow shows the time of addition of 3×10^{-9} g/ml CrTX-I.

The CrTXs-induced depolarization of GFP and aggregation of the citrated PRP were little affected by sodium transport inhibitors such as TTX (1 *μM*) and procaine (50 *μM*), or potassium transport inhibitors such as 4AP (300 *μM*) and TEA (10 m*M*) (data not shown). On the other hand, CrTX-I-induced aggregation as well as depolarization and increase in the $[Ca^{2+}]_i$ was all significantly attenuated in the low Na^+ medium (Figs. 8a, b, c).

Discussion. The aggregation caused by CrTX-I, II, and III was only slightly attenuated

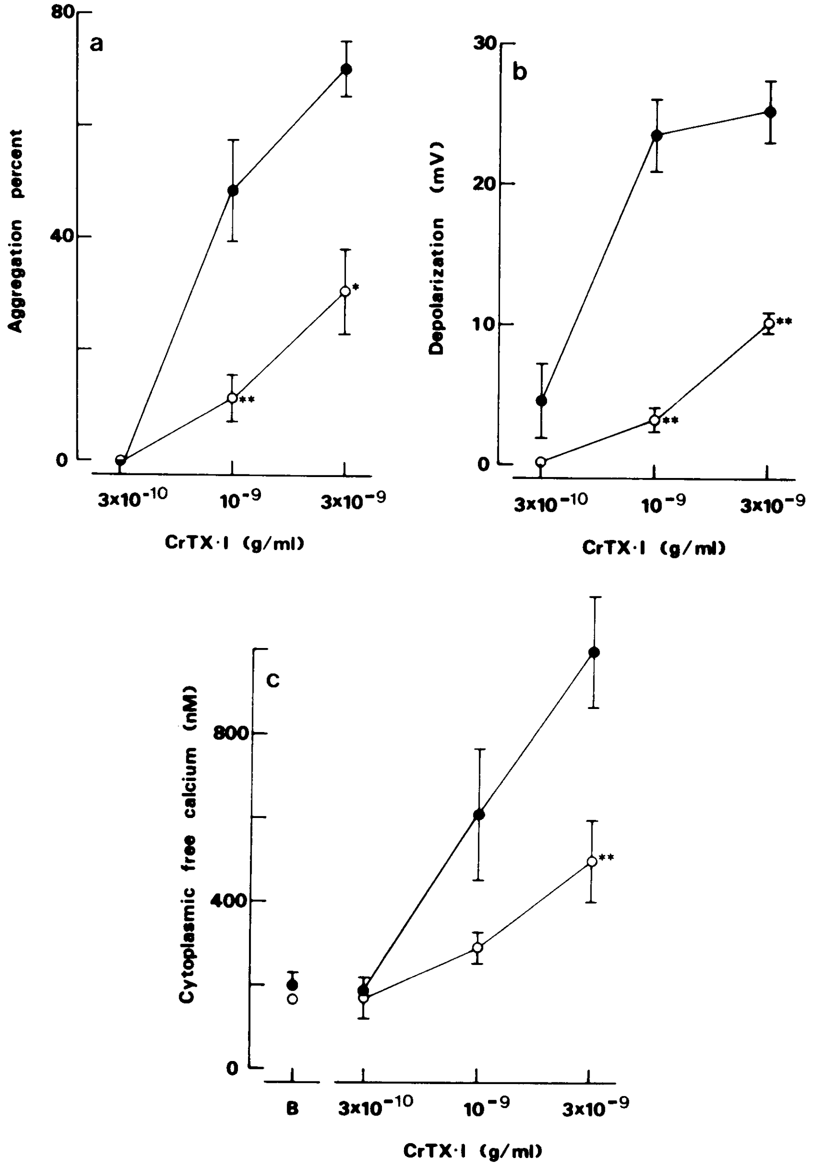

FIG. 8. Inhibitory effect of low Na^+ on CrTX-I-induced aggregation (a), depolarization (b), and increase in cytoplasmic-free calcium concentration (c). ●: Responses in normal Hepes buffer, ○: responses in the low Na^+ medium, B: basal level of cytoplasmic-free calcium without CrTX-I. Each point represents the mean of four observations and vertical bars show SE. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs each value in the normal Hepes buffer (○).

by a cyclooxygenase inhibitor, indomethacin, and a phospholipase A_2 inhibitor, quinacrine, at concentrations sufficient to inhibit collagen- and AA-induced aggregation. Thus, it appears that the pharmacological properties of the CrTXs-induced aggregation may be different from those of collagen and AA, and that AA metabolism may not be involved in the aggregation.

It is well established that transmembrane movement of ions down their electrochemical gradient is the basis of the excitability of many cell types including platelets and that platelets maintain transmembrane gradient for the cations, Na^+, K^+, and Ca^{2+}. Indeed, dye fluorescence studies (27) and $[^{14}C]$methylamine distribution (28) have shown that platelets maintain a membrane potential. In the present experiments, dye (DiS) fluorescence studies revealed that CrTXs produced concentration-dependent depolarization of platelets at the same concentrations as those that produced aggregation. Support for the speculation that the inward movement of Na^+ is involved in the activation process of platelets is provided by the findings that CrTX-I significantly increased the movement of ^{22}Na into platelets and that the aggregation and depolarization induced by CrTX-I were markedly attenuated in the low Na^+ medium. However, it appears that active transport of ions is not involved, but rather results from increased membrane permeability, since the depolarization and aggregation caused by CrTXs were little affected by sodium transport inhibitors such as TTX (29) and procaine (30, 31), or potassium transport inhibitors such as 4AP (32) and TEA (33). In addition, TTX failed to modify the increase in the movement of ^{22}Na into platelets induced by CrTX-I.

On the other hand, CrTXs-induced aggregation was markedly augmented in the presence of Ca^{2+}, although slight aggregation was produced by the toxins in Ca^{2+}-free medium. In contrast, CrTXs caused the same degree of the depolarization in the presence or absence of Ca^{2+}. Furthermore, CrTXs at the same concentrations that produced aggregation and depolarization markedly increased the $[Ca^{2+}]_i$ in WP in the normal Hepes buffer. However, CrTX-I-induced increase in the $[Ca^{2+}]_i$ was greatly attenuated in the low Na^+ medium. All these results lead us to postulate that an increase in the $[Ca^{2+}]_i$ is brought about by the depolarization resulting from indiscriminate increase in the cation permeability, which plays a primary role for the activation mechanism leading to platelet aggregation. The augmentation of aggregation by Ca^{2+} and increase in the $[Ca^{2+}]_i$ were little affected by a calcium channel blocker, verapamil (34). It seems, therefore, likely that the increases in the $[Ca^{2+}]_i$ and platelet aggregation caused by CrTXs do not result from activation of the potential-dependent calcium channel (34). Thus, the inward movement of Ca^{2+} down its electrochemical gradient might be involved in the activation of platelets by CrTXs. However, further experiments are necessary to find precise mechanisms of CrTXs on platelets. Evaluation of the problem of how the mechanisms of platelet aggregation with CrTXs relate to produce such physiological and toxicological effects as lethal toxicity on mammals, hemolytic activity, dermatonecrotic activity, and a cardiotoxic effect is in progress.

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Received October 10, 1985. P.S.E.B.M. 1986, Vol. 182.
Accepted January 21, 1986.