Br. J. Pharmac. (1985), **84**, 551–560

Platelet release reaction and aggregation induced by canatoxin, a convulsant protein: evidence for the involvement of the platelet lipoxygenase pathway

C.R. Carlini, J.A. Guimarães[1] & J.M.C. Ribeiro

Department of Biochemistry-ICB, CCS, Universidade Federal do Rio de Janeiro, C.P. 68041, 21.910 Rio de Janeiro, Brazil

1 Canatoxin is a toxic protein isolated from *Canavalia ensiformis* seeds. It induces death preceded by convulsions of spinal cord origin and also produces *in vitro* aggregation of platelets in rabbit, human and guinea-pig plasma. The aggregating effect is dose-dependent at nanomolar concentrations.

2 Rabbit platelets pretreated with canatoxin became refractory to a second exposure to this protein or to collagen, but were still responsive to ADP, Paf-acether or arachidonic acid. [^{14}C]-5-hydroxytryptamine was released from pre-labelled platelets on stimulation with canatoxin.

3 Washed rabbit platelets, but not thrombin-degranulated ones, aggregated on stimulation with canatoxin provided that fibrinogen was added before the toxin.

4 Canatoxin's pro-aggregating activity was inhibited by mepacrine, EDTA, caffeine, prostacyclin, adenosine monophosphate and also by the ADP scavenger system, creatine phosphokinase/creatine phosphate. Furthermore, 3-amino-l-[m-(trifluoromethyl)-phenyl]-2-pyrazoline (BW 755C), eicosatetraynoic acid (ETYA) and nordihydroguaiaretic acid (NDGA) were potent inhibitors of canatoxin-induced aggregation. In contrast, no inhibition was seen with indomethacin.

5 The data indicate that canatoxin is mainly a release-reaction-promoting agent, being devoid of any direct aggregating activity. Thus the aggregation is totally dependent on the release of ADP. Furthermore, canatoxin-induced platelet activation is probably dependent on platelet phospholipase A_2 and lipoxygenase activity but is not dependent on cyclo-oxygenase products or the release of Paf-acether.

Introduction

Canatoxin is a potent convulsant and lethal protein recently isolated from *Canavalia ensiformis* seeds and shown to be distinct from concanavalin A (Carlini & Guimarães, 1981). The highly purified toxin (mol. wt. 180,000, dimeric form) has an LD_{50} of $2\,mg\,kg^{-1}$ (mice, i.p.) and causes death invariably preceded by tonic convulsions of spinal cord origin (Carlini, *et al.*, 1984).

In the last decade blood platelets have been proposed as models for studies on synaptosomes (Sneddon, 1973; Pletscher & Laubscher, 1980). Among several other similarities, it has been demonstrated that the platelet's release reaction is physiologically equivalent to the synaptosome's secretion of neurotransmitters (Stormorken, 1969). In addition, both preparations share common agonists and receptor properties (Boullin & Glenton, 1978; Graf & Pletscher, 1979; Brodde, *et al.*, 1983).

[1]Author for correspondence

We have recently found that intact canatoxin induces platelet aggregation and secretion (Carlini, *et al.*, 1982). This finding may provide further data concerning the relationship between platelets and synaptosomes, while contributing to the elucidation of the action of canatoxin on the central nervous system. In this study, we have investigated the mechanism by which canatoxin activates rabbit platelets resulting in the release reaction and aggregation.

Methods

Platelet-rich plasma and platelet aggregation

Platelet-rich plasma (PRP) was prepared from rabbit or guinea-pig blood after collection by cardiac puncture and from human blood obtained by venipuncture of volunteers free from any drug for at least 10 days. Sodium citrate (0.313% v/v) was added to the blood

samples which were then centrifuged at 180 *g* for 5 min (human and guinea-pig) or 12 min (rabbit) at room temperature and the PRP was separated from erythrocytes. Platelet count in rabbit and guinea-pig PRP was usually 5 to 7 x 10^5 platelets ml^{-1} and 4 to 5 x 10^5 platelets ml^{-1} for human PRP. Platelet aggregation induced by canatoxin and other agents was monitored photometrically by the method of Born & Cross (1963), and the light transmission across the PRP suspension was registered on a chart recorder. PRP aliquots (300 µl) were transferred to siliconized glass cuvettes and stirred (1,100 r.p.m.) at 37°C for 2 or 3 min before the addition of any test drug (maximal volume 30 µl). Controls were run for all diluents. Known inhibitors of platelet aggregation were pre-incubated with PRP for 1 or 2 min at 37°C before the addition of the aggregating agent. All concentrations given are the final values in the PRP suspension.

[^{14}C]-5-hydroxytryptamine-labelled platelets and release reaction

[^{14}C]-5HT-labelled platelets were prepared as follows: rabbit platelets were separated from rabbit PRP by centrifugation at 1,200 *g* for 15 min at room temperature. The platelets were then suspended in the first washing liquid (a modified Tyrode solution) described by Ardlie *et al.*, 1970, containing 0.1 µCi ml^{-1} of [^{14}C]-5-HT. After 30 min incubation, the washing procedure of Ardlie *et al.*, 1970, was followed. The labelled platelets were suspended in their own original platelet-poor plasma (PPP) and the volume was adjusted to contain 6 x 10^5 platelets ml^{-1}. Release of [^{14}C]-5-HT by pre-labelled platelets during canatoxin-induced aggregation was measured by counting the released [^{14}C]-5-HT in the supernatant. To do this, 5.4 ml of PPP containing washed pre-labelled platelets were stirred at 37°C for 3 min. At this time, 2 groups of samples (300 µl, in triplicate) were taken as controls. Canatoxin (2.0 µM) was then added to the remaining suspension which was stirred and aliquots were withdrawn at 60, 120, 180 and 300 s after the addition of the toxin. The platelets were immediately mixed with 12 µl formaldehyde 40% v/v (final concentration 1.5%) in order to stop the release reaction and to prevent any re-uptake of [^{14}C]-5-HT (Costa & Murphy, 1975). After centrifugation for 5 min at 3,000 *g*, aliquots (200 µl) of the clear platelet-free supernatants were counted in a Beckman Liquid Scintillation Counter. To measure the total platelet [^{14}C]-5-HT content the same procedure was used except that a 200 µl sample of the whole pre-labelled platelet suspension was counted without centrifugation (control 1). Corrections were made for dilution after canatoxin addition and for any free [^{14}C]-5-HT present in the medium (control 2) before the addition of the toxin.

Thrombin-degranulated platelets

Thrombin-degranulated rabbit platelets were prepared by the method of Kinlough-Rathbone *et al.*, (1975). Suspensions of thrombin-degranulated platelets and control platelets (similarly washed but not exposed to thrombin) were made to contain 750,000 platelets ml^{-1}, and kept at 37°C prior to use.

Materials

Canatoxin was obtained from mature *Canavalia ensiformis* seeds as described by Carlini & Guimarães, 1981. The highly purified and stabilized toxin of mol. wt = 180,000 (Carlini *et al.*, 1984; Carlini & Guimarães, unpublished observations) had an LD_{50} of 2.0 mg kg^{-1} (mice, i.p.). The purified toxin was usually concentrated under nitrogen pressure (1.0 kgf cm^{-2}) inside Visking dialysis tubing to a final concentration of 5 to 8 mg ml^{-1} in 25 mM Tris HCl buffer, pH 7.5.

Solutions Paf-acether (platelet-aggregating-factor:1-O-alkyl-2-acetyl-sn-glycero-phosphocholine) was dissolved in Tyrode solution containing bovine serum albumin (BSA) to prevent adsorption to glass; ADP and AMP were dissolved in 1.0 M Tris (free base) and the pH was adjusted to 6.0–6.5 with 5.0 N HCl. Arachidonic acid and eicosatetraynoic acid (ETYA) were converted to their K salts and dissolved in 0.2 M Tris HCl, pH 8.2. Indomethacin was first dissolved in 0.1 M Na_2CO_3 then diluted with 0.15 M NaCl and finally adjusted to pH 6.0 with 5.0 N HCl. Prostacyclin (sodium salt, PGI_2) was dissolved in 0.1 M Tris (free base). Nordihydroguaiaretic acid (NDGA) was prepared as a 10% ethanol-water solution. All other solutions were prepared in 0.15 M NaCl.

Reagents Mepacrine (quinacrine HCl), bovine tendon collagen, arachidonic acid, prostaglandin E_1 (PGE_1), soybean trypsin inhibitor, potato apyrase, heparin, creatine phosphokinase, creatine phosphate, adenosine diphosphate (ADP), adenosine monophosphate (AMP), nordihydroguaiaretic acid and bovine serum albumin (BSA) were purchased from Sigma Chemical Company, U.S.A.; ethylenediaminetetracetic acid (EDTA) from British Drug House, England; formaldehyde from Carlo Erba, Italy; caffeine from Merck Darmstadt, Germany. Human fibrinogen and thrombin were gifts from Dr G. Murano, Biological Bureau of Standards, NIH, Bethesda Md, U.S.A. Human plasmin was from the American National Red Cross, Bethesda, Md, U.S.A. Paf-acether was kindly provided by Dr B. Vargaftig, Institut Pasteur, Paris, France. Prostacyclin (sodium salt), eicosatetraynoic acid and indomethacin were gifts from Dr F. Ubatuba, Universidade de Brasília, Brazil. 3-Amino-l-[*m*-(trifluoromethyl)-phenyl]-2-py-

razoline (BW 755C) was a kind gift from Dr Thomas Maack, Cornell University Medical School, New York, U.S.A. 3-[^{14}C]-5-hydroxytryptamine (5-HT; creatinine sulphate complex), 0.2 mCi mg^{-1}, was purchased from New England Nuclear Co., Boston, Ma, U.S.A. All other reagents used were of analytical grade.

Results

(I) *Canatoxin-induced platelet aggregation*

Rabbit, guinea-pig and human platelet-rich plasma suspensions undergo aggregation when exposed to nM concentrations of canatoxin. The aggregating effect is dose-dependent (Figure 1) and a full aggregation response comparable to that obtained with 10 μM ADP in the same assay conditions was produced by 0.5 to 1.0 μM canatoxin in rabbit platelets. The latter dose is equivalent to the amount of toxic protein present in the blood of a mouse injected i.v. with one LD_{50} (see Methods). The sensitivity of different platelet preparations to canatoxin showed some variability. A lag-time before the initiation of the aggregating response was observed for platelets of the three species and it was inversely proportional to the dose tested (Figure 1). For rabbit platelets, maximal aggregation to higher doses of canatoxin (0.5 to 2.0 μM) occurred 2 to 3 min after the addition to PRP. Thereafter, rabbit platelets deaggregated independently of canatoxin concentration and stirring velocity. Complete reversal of aggregation in rabbit PRP was usually achieved within 5 to 6 min (Figure 1). Reversal of platelet aggregation in guinea-pig and human PRP was not usually seen, even at the lowest dose of canatoxin used. Platelet shape change (decreased light transmission) was seen in some preparations before the onset of aggregation but only with higher doses of canatoxin.

(II) *Effect of canatoxin pretreatment on rabbit platelet aggregation*

Rabbit platelets become refractory to a second exposure to canatoxin (Figure 2). In addition, toxin-treated platelets did not re-aggregate to collagen stimulation (0.3 mg ml^{-1}, final concentration). On the other hand, arachidonic acid (0.5 mM) and other direct inducers of aggregation, such as ADP (20 μM) or Paf-acether (3.0 μM), were able to elicit aggregation in platelets previously exposed to 2.0 μM canatoxin (Figure 2).

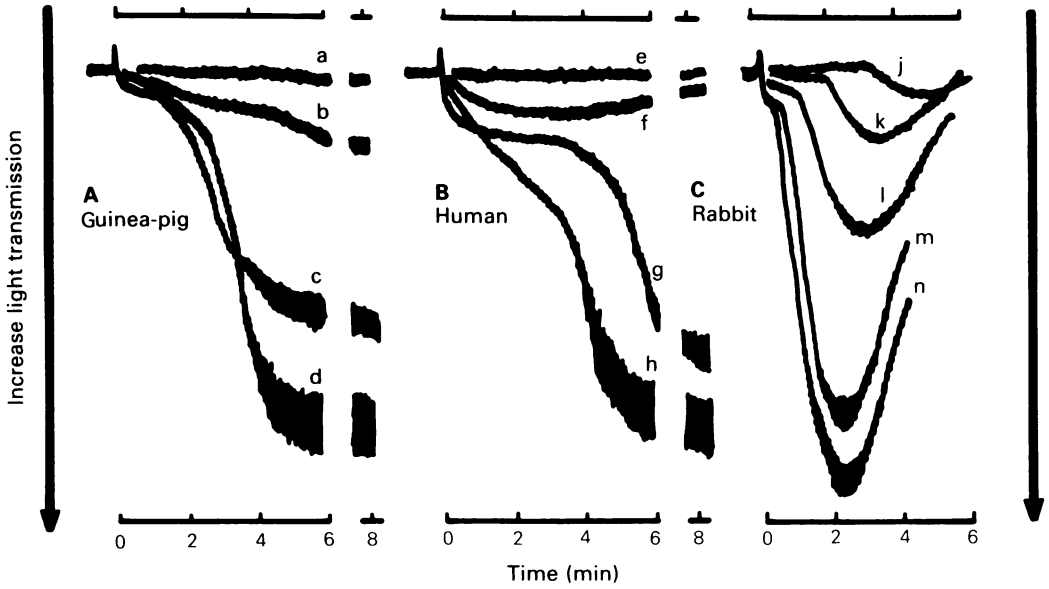

Figure 1 Canatoxin-induced platelet aggregation. Platelet-rich plasma (PRP; 300 μl) was transferred to siliconized glass cuvettes which were stirred (1,100 r.p.m.) at 37°C for 2 min before addition of canatoxin at 0 min. Light transmission across the PRP suspension was monitored photometrically before and after the addition of canatoxin and the individually obtained tracings were superimposed. (A) Guinea-pig platelets plus (a) 200 nM; (b) 400 nM; (c) 600 nM and (d) 1.2 μM canatoxin. (B) Human platelets plus (e) 200 nM; (f) 400 nM; (g) 1.2 μM and (h) 1.7 μM canatoxin. (C) Rabbit platelets plus (j)19 nM; (k) 60 nM; (l) 160 nM; (m) 400 nM and (n) 1.0 μM canatoxin. One of 4 experiments for each type of platelet is shown.

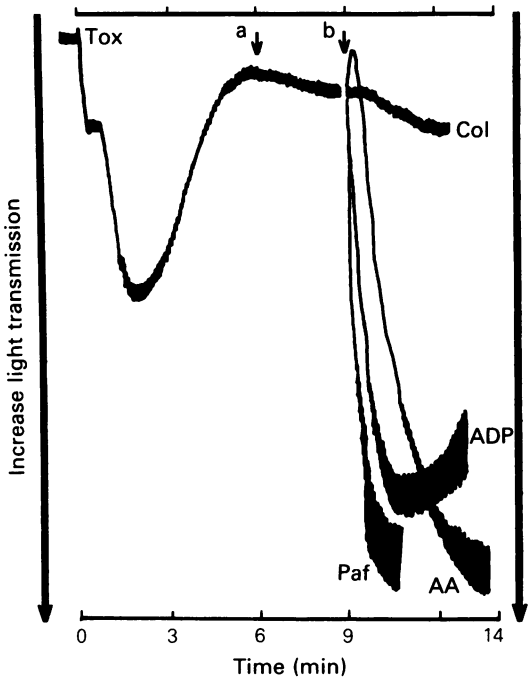

Figure 2 Effect of a second aggregating agent on canatoxin-treated rabbit platelets. Test conditions were as described in the legend to Figure 1. Rabbit PRP was stirred for 2 min at 37°C before the first addition of 2.0 μM canatoxin (Tox) at 0 min. After complete reversal of the aggregation, platelets were exposed again to 2.0 μM canatoxin at 6 min, (arrow a). The same platelet suspension was finally challenged with 0.3 mg ml^{-1} collagen (Col); 20 μM ADP; 500 μM arachidonic acid (AA) or 3.0 μM Paf-acether (Paf) or 2.0 μM canatoxin at 9 min (arrow b). The experiment was repeated three times with similar results.

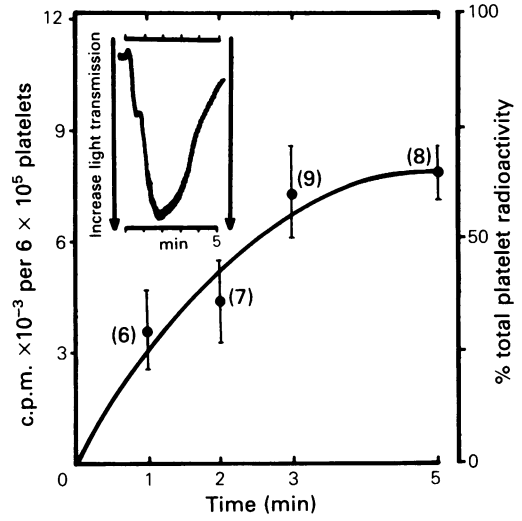

Figure 3 [^{14}C]-5-hydroxytryptamine (5-HT) release reaction induced by canatoxin. Washed rabbit platelets were pre-labelled with [^{14}C]-5-HT and re-suspended in their own PPP. The release reaction was stopped by formaldehyde 1, 2, 3 and 5 min after the addition of 2.0 μM canatoxin and the radioactivity released to the supernatant was determined (see Methods for detailed procedure). Results are expressed as mean c.p.m. released per 6 × 10^5 platelets and as % of the total platelet radioactivity content (content = 12,305 ± 2,976 c.p.m., n = 12). The number of experiments is also indicated for each point (number in parentheses) and vertical lines show s.e. mean. Inset: Typical experiment (similar to Figure 1) showing the aggregation of [^{14}C]-5-HT labelled platelets by 2.0 μM canatoxin.

(III) *Release reaction induced by canatoxin*

[^{14}C]-5-HT pre-labelled rabbit platelets suspended in rabbit PPP retained the ability to aggregate upon stimulation with canatoxin. Figure 3 shows the amount of [^{14}C]-5-HT released into the supernatant during aggregation expressed both as c.p.m. and percentage of the total radioactivity present in the labelled platelets. It can be seen that 31% of total [^{14}C]-5-HT taken up by the platelets was released within the first minute after challenge with the toxin. At this time aggregation was nearly 30% of the maximal response (Figure 3, inset), but the platelets had already released about half of the total radioactivity unbound by canatoxin. When aggregation was maximally developed (2 to 3 min; Figure 3, inset), the release reaction reached a plateau equivalent to 60–65% of the total [^{14}C]-5-HT content of the platelet. After complete reversal of aggregation at 5 min [^{14}C]-5-HT release remained at the plateau level.

(IV) *Effect of canatoxin on thrombin-degranulated platelets*

The response of thrombin degranulated platelets to canatoxin was compared with their response to Paf-acether and thrombin (Figure 4). Degranulated platelets suspended in Tyrode-BSA were aggregated by Paf-acether (100 nM) as well as by ADP (10 μM) in the presence of 1 mg ml^{-1} human fibrinogen. In contrast, there was no response to thrombin (1.3 u ml^{-1}) or canatoxin (2.4 μM), even in the presence of added fibrinogen. Similarly, collagen (0.3 mg ml^{-1}) did not stimulate these platelets. Rabbit control platelets (washed but not degranulated) responded to all the above agents. In this case prior addition of fibrinogen (1 mg ml^{-1}) to the suspension medium of the platelets was necessary for the aggregation induced by ADP, Paf-acether and canatoxin. Only thrombin and collagen were able to induce aggregation of the

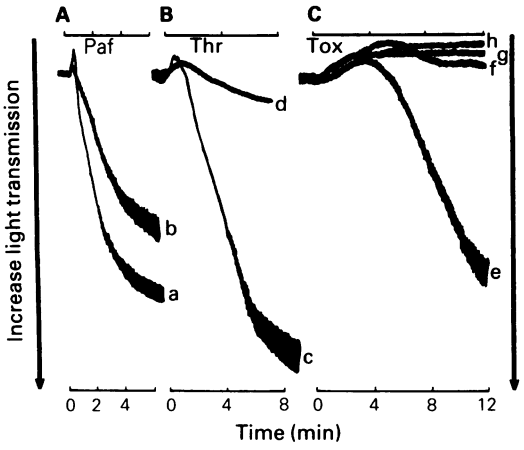

Figure 4 Effect of canatoxin on washed thrombin-degranulated rabbit platelets. Thrombin-degranulated washed platelets and their respective washed but not degranulated control platelets were prepared as described in Methods. Both suspensions were stirred for 3 min at 37°C before addition of the aggregating agent at 0 min. When indicated, 1 mg ml^{-1} human fibrinogen was added before the 3 minute pre-incubation period. (A) Aggregation induced by 100 nM Paf-acether (Paf) in the presence of fibrinogen in control (a) and degranulated (b) platelets. (B) Aggregation induced by 1.3 u ml^{-1} thrombin (Thr) in control (c) and in degranulated (d) platelets. (C) Aggregation induced by 2.4 μM canatoxin (Tox) in control platelets plus fibrinogen (e); degranulated platelets plus fibrinogen (f); control platelets without fibrinogen (g); control platelets plus fibrinogen pre-incubated 1 min with 30 μl of creatine phosphokinase/creatine phosphate system (h; see text). One of three similar experiments is shown.

control platelets in the absence of human fibrinogen. Additionally, it was observed that aggregation induced by canatoxin in plasma-free washed control platelets was not reversible, even 15 min after its initiation. This result contrasts with that seen in rabbit platelet-rich plasma (Figure 1) in which complete reversal of aggregation was observed within 5 to 6 min.

(V) *Effect of inhibitors on the pro-aggregating activity of canatoxin*

Several known inhibitors of platelet aggregation were tested in order to characterize the mechanism of action of canatoxin. Figures 5 and 6 as well as Tables 1, 2 and 3 illustrate the results. Mepacrine, a phospholipase A_2 inhibitor, at μM concentrations blocked the pro-aggregating activity of canatoxin (Figure 5). This inhibitory effect was independent of the toxin concentration. Indomethacin, a known cyclo-oxygenase inhibitor, completely blocked the aggregation produced by 0.5 mM arachidonic acid but had little effect on canatoxin-induced aggregation (Figure 5). Actually, some potentiation by indomethacin (150 μM) was observed when smaller doses of canatoxin (0.2 to 0.5 μM) were used (results not shown). In contrast, eicosatetraynoic acid, which is able to block both the lipoxygenase and cyclo-oxygenase pathways in platelets, was a potent inhibitor of canatoxin-induced aggregation with an IC_{50} of 19 μM (Figures 5 and 6). Complete blockade of aggregation induced by 3.5 μM canatoxin was achieved with 60 μM ETYA (Figure 5 and Table 1). In the same platelets the effect of 0.1 mM arachidonic acid was blocked at a 10 fold lower concentration of ETYA as expected (Hamberg & Samuelsson, 1974), while that of Paf-acether (50 nM) was not affected even at the highest concentration used (Table 1). The compound BW 755C, a known in-

Table 1 Comparison of the inhibitory effects of eicosatetraynoic acid (ETYA), BW 755C and nordihydroguaiaretic acid (NDGA) on platelet aggregation induced by canatoxin, arachidonic acid and Paf-acether

Aggregation inducer	*Inhibitors** ETYA (μM)		BW 755C (μg ml^{-1})		NDGA (mM)	
	IC_{50}	100% inhibition	IC_{50}	100% inhibition	IC_{50}	100% inhibition
Canatoxin (3.5 μM)	19.0	60.0	56.0	280.0	0.52	1.1
Arachidonic acid (100 μM)	–	6.0	–	28.0	–	–
Paf-acether (50 nM)	–	>60.0	–	>280.0	–	>1.1

*Inhibition was calculated as percentage of control aggregation produced by each aggregating agent. ETYA and BW 755C were pre-incubated with PRP at 37°C for 2 min before addition of the aggregating agent; NDGA was pre-incubated with PRP for 2 min at 37°C (for the IC_{50} calculation, see Figure 6) or for 1 h at 25°C (to obtain 100% inhibition).

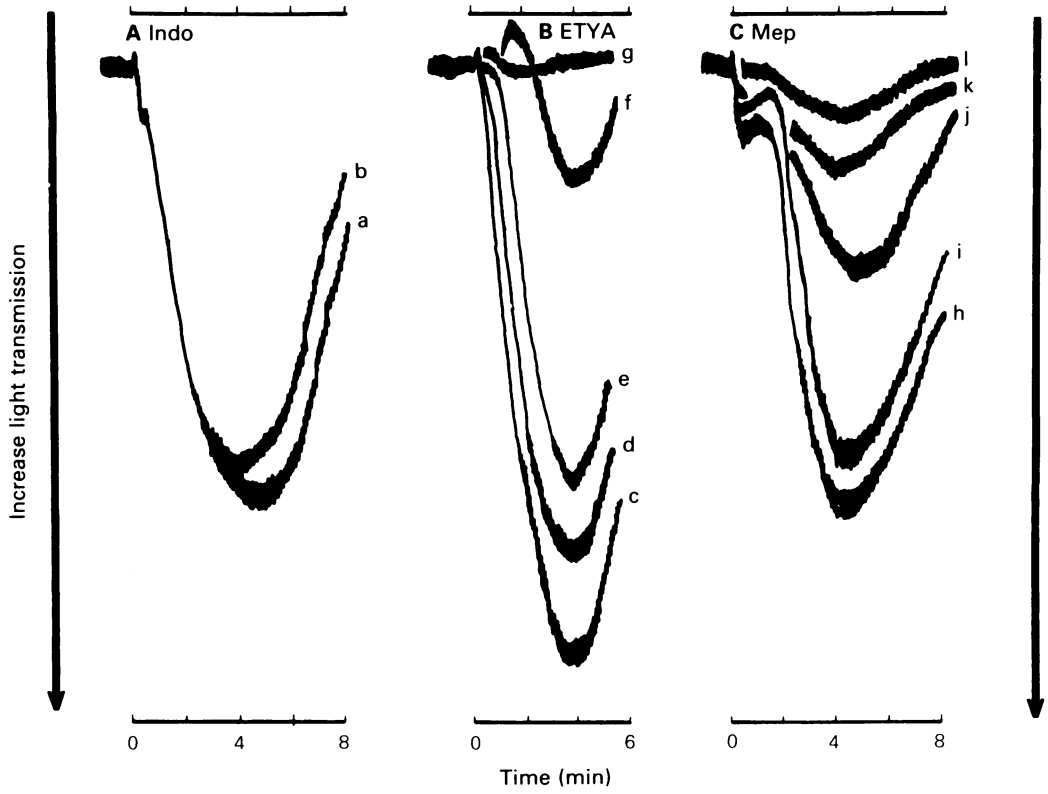

Figure 5 Effects of indomethacin (Indo), eicosatetraynoic acid (ETYA) and mepacrine (Mep) on canatoxin's pro-aggregating activity. Test conditions were as described in the legend to Figure 1. The inhibitors were pre-incubated with rabbit PRP at 37°C for 1 or 2 min before addition of the aggregating agent. (A) Aggregation induced by (a) canatoxin (1.7 μM) in control PRP and (b) canatoxin in the presence of 150 μM indomethacin. (B) Aggregation induced by (c) canatoxin, (3.5 μM) in control PRP and (d) canatoxin plus ETYA 6.0 μM; (e) 15 μM ETYA; (f) 30 μM ETYA; (g) 60 μM ETYA. (C) Aggregation by (h) canatoxin (1.7 μM) in control PRP and (i) in the presence of 1.0 μM mepacrine; (j) 3.3 μM mepacrine; (k) 25 μM mepacrine and (l) 100 μM mepacrine. One of three similar experiments for each inhibitor is shown.

hibitor of both cyclo-oxygenase and lipoxygenase, also inhibited (IC_{50}56 μg ml^{-1}) the effects induced by 3.5 μM canatoxin (Figure 6 and Table 1). Table 1 shows, in addition, that the lipoxygenase-specific inhibitor, nordihydroguaiaretic acid (NDGA), was able to abolish the aggregation induced by canatoxin (3.5 μM) in platelets pre-incubated with this inhibitor (1.1 mM) for 1 h at room temperature. These platelets, however, remained responsive to Paf-acether (Table 1). Shorter incubation periods of platelets with NDGA (2 min at 37°C) resulted in partial inhibition of canatoxin-induced aggregation. Under these conditions, inhibition of aggregation was not potentiated by simultaneous pretreatment of rabbit platelets with 150 μM indomethacin plus NDGA. The estimated IC_{50} for NDGA was 0.52 mM (Figure 6). The ADP scavenger system creatine phosphokinase/creatine phosphate (CPK/CP) was also used to study the role of ADP as a mediator of canatoxin pro-aggregating activity. Table 2 shows that the ADP scavenger abolished the aggregation produced by 1.5–3.5 μM canatoxin, the inhibitory effect being linear to the logarithm of the CPK/CP concentration. A similar action of CPK/CP was also obtained in washed rabbit platelets aggregated upon stimulation by canatoxin (see Figure 4 h). As expected the platelet's responses to 5.0 or 50.0 nM Paf-acether were not affected at the maximal CPK/CP concentration used (Cazenave *et al.*, 1979).

Other potential inhibitors of platelet aggregation were also studied. Table 3 shows that EDTA, AMP, caffeine and prostacyclin inhibited the pro-aggregating activity of canatoxin in rabbit PRP.

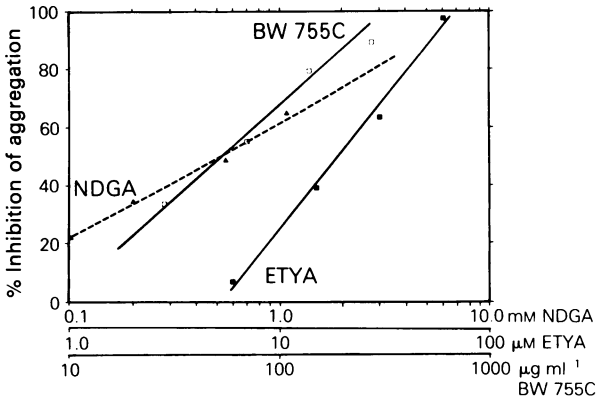

Figure 6 Dose-dependent inhibitory effects of eicosatetraynoic acid (ETYA), nordihydroguaiaretic acid (NDGA) and BW 755C on canatoxin-induced aggregation. The inhibitors were pre-incubated with rabbit PRP for 2 min at 37°c before addition of 3.5 μM canatoxin. Control platelet aggregation was performed in the presence of the respective diluents. Each point represents the mean of three experiments. The IC_{50} values were estimated from the semi-log plot of percentage inhibition versus concentration of drug.

(VI) *Lipoxygenase activity of the canatoxin preparation*

In order to determine whether canatoxin could act alone to promote arachidonate lipoxygenation (Bills *et al.*, 1976; Taylor & Morris, 1983), the toxic protein (2.0 μM) was incubated with 0.5 mM archidonic acid in Tyrode solution (pH 8.5) at 37°C. Absorbance at 234 nm was monitored spectrophotometrically as described by Nugteren, (1982). After incubation for two hours, no increase at 234 nm absorbence was found indicating that canatoxin does not catalyze hydroxyperoxidation of arachidonic acid.

Table 3 Inhibitory effects of EDTA, AMP, caffeine and prostacyclin on platelet aggregation induced by canatoxin

Inhibitor	*Concentration* (mM)	*Inhibitory effect** *Mean* (%)	*Range*
EDTA	5.0	90	87–100
AMP	5.0	80	74–92
Caffeine	5.0	98	91–100
Prostacyclin	0.005	88	78–94

*Inhibition was calculated as percentage of the aggregation produced by 2.0 μM canatoxin in the presence of each inhibitor's solvent. Inhibitors were pre-incubated with PRP at 37°C for 1 min before addition of canatoxin. At least three experiments were carried out for each drug.

Discussion

The present findings indicate that canatoxin, a potent convulsant and lethal neurotoxin protein (Carlini & Guimarães, 1981), is able to activate platelets leading to the release reaction and aggregation. The pro-aggregating effect is dose-dependent and occurs at the same protein concentration range assumed to be present in the blood of an animal injected with one LD_{50} unit of canatoxin.

Whether the *in vitro* platelet activation induced by canatoxin is related to its *in vivo* toxic effects is not known. The symptoms exhibited by mice injected i.p. or i.v. with canatoxin have some similarities with those described for convulxin (Prado-Franceschi *et al.*, 1981) and also for tetanus toxin (Habermann, 1978).

Convulxin, a neurotoxic protein extracted from the venom of the snake *Crotalus durissus*, induces convulsions and flaccid paralysis in cats (Prado-Franceschi *et*

Table 2 Inhibition of the aggregating activity of canatoxin by the ADP scavanger system creatinephosphokinase/creatine phosphate (CPK/CP)

Aggregation inducer	*Concentration*	*CPK/CP** (μl)	*Inhibitory effect* *Mean* (%)	*Range*
Canatoxin	1.5 μM	1.5	13.5	11.0–16.0
		3.2	29.9	16.0–43.0
		7.	64.8	62.0–68.0
		15.0	74.8	71.0–77.0
		30.0	100.0	–
	3.5 μM	30.0	100.0	–
ADP	20.0 μM	15.0	100.0	–

*CPK/CP: creatinephosphokinase (9.4 mg ml^{-1}) and creatine phosphate (12.8 mg ml^{-1}). The mixture was added to the PRP suspension at 37°C, 2 min before addition of the aggregating agent. Results shown are mean of three experiments.

al., 1981). Convulxin was shown to aggregate platelets through a pathway independent of cyclo-oxygenase products or ADP released by aggregating platelets, with concomitant Paf-acether liberation (Vargaftig, *et al.*, 1980b; 1981; 1983). The latter effect could not explain, however, the convulsant property of this toxin. Whether or not canatoxin activates rabbit platelets by any of the known pathways of platelet aggregation was investigated. The activation mechanism is independent of the cyclo-oxygenase products, prostaglandin endoperoxides and thromboxane A_2, since the enzyme inhibitor indomethacin (Vargaftig & Zirinis, 1973; Bills *et al.*, 1976) had no effect on canatoxin-induced platelet aggregation. On the other hand, ETYA and BW 755C, known to be inhibitors of both platelet lipoxygenase and cyclo-oxygenase (Hamberg & Samuelsson, 1974; Higgs *et al.*, 1979; Higgs & Vane, 1983), were potent inhibitors of canatoxin-induced aggregation. These results suggest an involvement of the platelet lipoxygenase in mediating the aggregation induced by canatoxin, independent of cyclo-oxygenase products. This hypothesis was further reinforced with NDGA, a lipoxygenase-specific blocker (Hamberg, 1976; Higgs & Vane, 1983; Cerletti *et al.*, 1983), which inhibited the canatoxin-induced aggregation. Although these lipoxygenase blockers could be also exerting an as yet unsuspected lipoxygenase-independent effect, their action seemed to be selective for canatoxin, since aggregation by Paf-acether was not prevented by any of these agents. The inhibitory effect of mepacrine, a phospholipase A_2 inhibitor (Vargaftig & Dao, 1972; Blackwell *et al.*, 1978), is suggestive of an involvement of the Paf-acether pathway (Cazenave *et al.*, 1979; Vargaftig *et al.*, 1981) as in the aggregation stimulated by thrombin or by the calcium ionophore A23 187 (Lapetina *et al.*, 1978; Vargaftig *et al.*, 1980a). However, the aggregation induced by canatoxin was totally dependent on the released ADP since the scavenger system CPK/CP (Cazenave *et al.*, 1979) blocked, in a dose-related manner, the pro-aggregating effect of either low or high doses of canatoxin (Table 2). In addition, it was observed that thrombin-degranulated platelets are insensitive to canatoxin, confirming that the toxic protein depends on the release of ADP to produce aggregation. These data also indicate that canatoxin has no direct aggregating properties, such as those observed for Paf-acether or ADP. In view of these findings it seems clear to us that the Paf-acether pathway is not involved in the activation of platelets by this toxic protein.

Once activated by canatoxin, rabbit platelets become refractory to a second exposure to canatoxin or collagen. However, these platelets are still able to respond to directly acting aggregating agents such as ADP, Paf-acether and arachidonic acid. The data also indicate that platelets are not being lysed by the toxic protein. On the other hand, the ability of canatoxin to induce the release reaction was demonstrated using [^{14}C]-5-HT pre-labelled rabbit platelets. The results suggest that canatoxin primarily induces the release reaction and that aggregation is secondary to the release of ADP. If this is so, then the toxin-induced aggregation would be expected to be dependent on the presence of fibrinogen (Mustard *et al.*, 1978).

Release reaction and/or aggregation induced by canatoxin seem to be modulated by cyclic AMP mediation, since prostacyclin and caffeine (Mustard & Packham, 1970; Gorman *et al.*, 1977) are inhibitors of canatoxin-induced aggregation. In addition EDTA and also AMP, a blocker of ADP-induced aggregation (Mustard & Packham, 1970), inhibited the effects of canatoxin in rabbit platelets (Table 3). These and other specific metabolic inhibitors of platelet activation (mepacrine, ETYA, BW 755C, NDGA and the CPK/CP system) indicated that clumping of platelets by canatoxin is true aggregation and not just particle agglutination (Jenkins *et al.*, 1971; Kattlove & Gomez, 1975; Kirby & Mills, 1975).

Disaggregation of rabbit platelets activated by canatoxin was observed (Figure 1), despite the fact that a release reaction had occurred, while aggregation of human and guinea-pig platelets were shown to be irreversible. Although rabbit platelets are known to dis-aggregate more easily than human platelets (Kinlough-Rathbone *et al.*, 1983), the reasons for this unexpected reversal of aggregation in rabbit PRP are not completely clear. The observed reversal may be related to the presence of some factor(s) present in rabbit plasma, since irreversible aggregation was seen when washed rabbit platelets were suspended in Tyrode-BSA solution containing fibrinogen. Production and transient accumulation of an inhibitory metabolite such as some arachidonic acid lipoxygenase products (Aharony *et al.*, 1982) and prostaglandin (Watanabe *et al.*, 1982) or the inhibitory chondroitin sulphate released during aggregation as described by Nader *et al.*, 1981, could be alternative explanations for the reversal of aggregation induced by canatoxin in rabbit platelet-rich plasma.

Altogether, the present data strongly suggest that canatoxin is primarily an inducer of a release reaction but has no direct platelet aggregating properties. Since the toxin is devoid of phospholipase A_2-like activity and haemagglutinating properties (Carlini *et al.*, 1984), as well as of any arachidonate lipoxygenase activity, the biochemical pathway by which canatoxin activates platelets seems to be distinct from all those previously characterized. The results are, however, indicative of an involvement of the platelet lipoxygenase. Although the direct product of platelet arachidonate lipoxygenase, 12-hydroperoxyeicosatetraenoic acid, is known to inhibit platelet functions (Aharony *et al.*, 1982; Croset & Lagarde, 1983), there are other

studies indicating that platelet lipoxygenase may play a significant role in aggregation (Dutilh *et al.*, 1981; Nishigawa *et al.*, 1983). This seems to be the case for canatoxin where an as yet poorly characterized lipoxygenase product(s) may be mediating its aggregating activity. Further investigation of platelet activation induced by canatoxin may help in the elucidation of its action on the nerve endings. Moreover, canatoxin seems to be a valuable pharmacological tool for understanding the platelet itself.

C.R.C. receives a graduate fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brasília. This work was subsidised by Financiadora de Estudos e Projetos, FINEP, 43.83.0052 and CNPq.

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(Received August 15, 1984.
Accepted October 8, 1984.)