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BIOLOGICAL PROPERTIES OF THE VENOM OF THE PAPUAN BLACK SNAKE (*PSEUDECHIS PAPUANUS*): PRESENCE OF A PHOSPHOLIPASE A_2 PLATELET INHIBITOR

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A. S. KAMIGUTI, G. D. LAING, G. M. LOWE, M. ZUZEL, D. A. WARRELL and R. D. G. THEAKSTON. Biological properties of the venom of the Papuan black snake (*Pseudechis papuanus*): presence of a phospholipase A_2 platelet inhibitor. *Toxicon* **32**, 915–925, 1994.—The whole venom of *Pseudechis papuanus*, in addition to its anticoagulant activity, powerfully inhibited platelet aggregation induced by ADP, adrenaline, collagen, ristocetin and thrombin. High levels of phospholipase A_2 (PLA_2) activity were detected. A mild procoagulant activity was also observed. Following exposure of platelets to *P. papuanus* venom, platelet factor 3 (procoagulant platelet phospholipid) showed decreased cofactor activity in factor X activation by Russell's viper, venom suggesting that the venom PLA_2 plays a major role in the inhibition of the coagulation mechanism. *In vivo* rodent assays confirmed the inhibitory effect on platelets and the haemorrhagic and neurotoxic activities. It is possible that PLA_2 is responsible for anticoagulation and that this, combined with the effect on platelet aggregation, a mild procoagulant and a moderately potent haemorrhagin, is responsible for the haemorrhagic diathesis observed in systemically envenomed patients. Polyvalent (Australia–Papua New Guinea) Commonwealth Serum Laboratories antivenom, currently used for clinical treatment of snakebite in Papua New Guinea, proved highly effective against *P. papuanus* venom in rodent and *in vitro* assays, despite the absence of this particular venom from the immunising mixture.

INTRODUCTION

THE PAPUAN black snake, *Pseudechis papuanus*, occurs along the southern coast of New Guinea. Twenty-five years ago it was thought to be the major cause of snakebite in the Papuan region (CAMPBELL, 1967), but more recent observations suggest that it is now uncommon (LALLOO *et al.*, 1994). In the accompanying paper (LALLOO *et al.*, 1994) the clinical and laboratory features of nine cases of proven bites by *P. papuanus* are described, and the literature on bites by this species is critically reviewed. Apart from neurotoxic signs,

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vomiting and lymphadenopathy, some of the patients showed spontaneous systemic haemorrhage and incoagulable blood.

The venom of this species was originally thought to have a procoagulant action resulting in a defibrination syndrome in envenomed patients (CHAMPNESS, 1966; CAMPBELL, 1967), but KAIRE (1964) and CAMPBELL and CHESTERMAN (1972), who carried out *in vivo* experiments in dogs and *in vitro* studies on the whole venom, concluded that this procoagulant was counterbalanced by a powerful heat-stable anticoagulant. The presence of the anticoagulant was confirmed by MARSHALL and HERRMANN (1983) but it is still not identified; MARSHALL and HERRMANN (1989) also reported mild irreversible *in vitro* aggregation of platelets by *P. papuanus* venom. CAMPBELL and CHESTERMAN (1972) considered their experimental findings inconsistent with clinical observations which at that time were subject to doubt about identification of the biting snake. The present study investigates some biological properties of *P. papuanus* venom with the aim of revealing pathophysiological mechanisms for the symptomatology of human envenoming.

MATERIALS AND METHODS

Venom

Lyophilised *P. papuanus* venom of uncertain origin, pooled from an undisclosed number of specimens, was purchased from Sigma Ltd, U.K. When we began the studies, this was the only source of the venom available throughout the world and was the remnants of the stock held by the company. More recently, we obtained a living specimen, 162 cm in length (LALLOO *et al.*, 1994; Fig. 1a,b), confirmed as *P. papuanus* (LALLOO *et al.*, 1994; Fig. 1b; WORRELL, 1972). This specimen was originally captured in 1980 in Bamustu village, Arami River area, Western Province of Papua New Guinea. It has been milked regularly in the herpetarium at the Liverpool School of Tropical Medicine since June 1993. Venom from this snake was freeze-dried and stored at 4°C in a dark bottle. Venom solutions from each source were freshly reconstituted in physiological saline before use.

The two batches of *P. papuanus* venom (Sigma and that obtained from the specimen held in this laboratory), spotted black snake (*P. guttatus*) venom (obtained from a specimen maintained at the Liverpool School of Tropical Medicine) and king brown or Mulga snake (*P. australis*) venom (purchased from Dr P. MIRTSCHIN, Australia) were compared by running them under non-reducing conditions on 15% SDS-PAGE (LAEMMLI, 1970).

Estimation of venom toxicity

The i.v. lethal toxicity (LD_{50}) of *P. papuanus* venom obtained from our specimen was assessed by injection of various doses of venom in 0.2 ml physiological saline into the tail veins of 18–20 g male TFW strain mice (Tuck and Sons Ltd, U.K.) (THEAKSTON and REID, 1983). To assess the potency of polyvalent Australia–Papua New Guinea antivenom (Commonwealth Serum Laboratories [CSL], Parkville, Australia; batch 08801; expiry 11/91), various amounts of antivenom were mixed with 5 × i.v. LD_{50} venom obtained from our specimen; the mixtures were then incubated at 37°C for 30 min before injection as described above. The median effective dose (ED_{50}) of the antivenom was then calculated as described by LAING *et al.* (1992). It was not possible to carry out this assay using the more realistic s.c. route for injection of venom and i.v. route for injection of antivenom (LAING *et al.*, 1992) because only small amounts of venom were available for testing.

Haemorrhagic activity of the venom

In order to estimate haemorrhagic activity of the venom, male Sprague–Dawley rats (340–360 g) were lightly anaesthetised by halothane/O_2 inhalation. Aliquots of 0.1 ml of physiological saline containing 5–120 μg of venom were injected into the shaved dorsal skin of the animals. After 24 hr they were killed by CO_2 inhalation, the dorsal skin was removed, and the diameter of the lesion was measured on the inner surface of the skin, in two directions at right angles, using calipers and background illumination. Care was taken not to stretch the skin. The mean diameter of the haemorrhagic lesion was calculated for each venom dose and the minimum haemorrhagic dose (MHD) estimated by plotting mean lesion diameter against venom dose and reading-off the dose corresponding to a haemorrhage 10 mm in diameter (THEAKSTON and REID, 1983).

Effects of the venom on the haemostatic mechanism

Male rats (340–390 g) were anaesthetised with an i.p. injection of 0.4 ml/kg of Hypnorm (Janssen Ltd, U.K.: 0.315 mg/ml sentanyl citrate; 10 mg/ml fluanisone) plus 3 mg/kg diazepam (C. P. Pharmaceuticals Ltd, Wrexham,

U.K.). Six rats were injected i.v. into the tarsal vein with doses of venom ranging from 5 to 200 μg in a total volume of 0.2 ml sterile physiological saline, and two were injected s.c. with 100 and 150 μg, respectively. Four control rats were injected with 0.2 ml saline alone (two i.v. and two s.c.). Two hours after venom or control saline injection, the anaesthetised animals were killed by placing them in a CO_2 chamber for 2–3 min. Immediately after death, animals were bled by cardiac puncture; blood was collected into tubes containing trisodium citrate (final concentration 13 mM). Four other rats were bled in a similar manner to provide a pool of normal plasma.

Whole blood clotting was monitored by placing 1 ml of blood in a new, clean, dry glass tube. After 20 min undisturbed at room temperature, the presence or absence of a clot was recorded. Clot quality was also assessed using the 1–5 grading system (Reid *et al.*, 1963). Whole blood platelet counts were performed by phase contrast microscopy (Brecher and Cronkite, 1950) using 1 ml blood containing 10 μl of 10% potassium EDTA as anticoagulant. Red cell counts and haematocrit levels were also measured. Other assays were carried out using plasma obtained after centrifugation of citrated blood (final concentration, 13 mM trisodium citrate) at 2000 *g* for 10 min at 4°C. All citrated samples contained 2.5% v/v of CSL polyvalent antivenom to neutralise any venom present in the specimen. Duplicate fibrinogen assays were carried out using the method of Ratnoff and Menzie (1951). Platelet aggregation (Born and Cross, 1963) was determined in platelet-rich plasma (PRP), which was obtained by centrifugation of citrated blood at 125 *g* for 10 min at room temperature. After the addition of either 6 μM ADP (Sigma Ltd, U.K.) or 0.1 U/ml thrombin (Diagen Ltd, U.K.) or 5 μg/ml collagen (Hormon Chemie, Germany), aggregation was recorded for 5 min in a dual channel aggregometer (Aggregraph Ltd, U.K.) and changes in light transmission were measured. Rates of aggregation were recorded in mm/sec.

Coagulant activity of the venom

Coagulant activity was investigated by mixing *in vitro* 1 ml of either human whole blood, human citrated plasma or 2 mg/ml bovine fibrinogen solution (Diagen Ltd, U.K.) with 4–500 μg/ml venom at 37°C. The clotting time was recorded and compared with the clotting obtained with bovine thrombin (10 U/ml; Diagen Ltd, U.K.).

The possibility that the prolongation of the clotting time was a consequence of degradation of fibrinogen was also investigated. Fibrinogenolytic activity was estimated in duplicate by incubating 200 μl of either fibrinogen or plasma with 50 μg of venom at 37°C for 5 min. One hundred microlitres of thrombin (100 U/ml) was added and the clotting time recorded. The clots thus formed were washed to remove all soluble proteins and the amount of fibrin was estimated using the method of Ratnoff and Menzie (1951).

Fibrinolytic activity was assessed using fibrin plates. Human fibrinogen (Kabi, Sweden) was dissolved to 10 mg/ml in 20 mM Tris buffer containing 10 mM $CaCl_2$, pH 7.3. A fibrin clot was produced by adding 1 U/ml bovine thrombin followed by gentle thorough mixing. Thirty minutes' incubation were allowed for the formation of fibirin; then 30 μl samples of venom solutions containing 0.97–125 μg were pipetted onto the surface of the plate (three samples/7 cm plate). After 18 hr of incubation at 37°C, the areas of lysis produced by the samples were measured.

Effect of venom on platelets

The effect of the venom on platelet aggregation was assessed as described earlier using 1 ml of human platelet-rich plasma (PRP) diluted to the desired platelet concentration with Hank's medium containing 13 mM trisodium citrate. Venom in various concentrations was incubated with platelets for 5 min. In some experiments, venom was treated with either a final concentration of 160 μM phenylmethylsulphonyl fluoride (PMSF) to inhibit venom serine proteases, or 5 mM final concentration of ethylenediamine tetraacetic acid (EDTA), to inhibit metalloproteases, before the incubation with PRP. Following incubation, a final concentration of 6 μM of either ADP or adrenaline (Sigma Ltd, U.K.) or 0.5 mg/ml (final concentration) of ristocetin (Lundbeck Ltd, U.K.) or 5 μg/ml (final concentration) of collagen was added and the aggregation recorded for 5 min.

Since platelet aggregation and secretion are accompanied by the exposure of procoagulant phospholipid, we verified whether this phospholipid can act as a cofactor in the activation of factor X in the presence of *P. papuanus* venom. Therefore, platelet factor 3 availability was estimated (Hardisty and Hutton, 1966), using *D. russelii* venom (DRV, Thailand) at a 1:1000 dilution. Human PRP was stimulated with 20 μM ADP for 20 min in the presence or absence of 5 μg/ml *P. papuanus* venom (5 min). Platelet aggregation was monitored throughout this period and 100 μl of platelet suspensions were taken at 0, 2, 5, 10, 15 and 20 min intervals, and mixed with 100 μl of DRV and 15 mM $CaCl_2$ at 37°C. Clotting times were immediately recorded. A similar experiment was carried out using 5 μg/ml collagen instead of ADP, except that initially PRP alone was stimulated with collagen; then the aggregated platelets were removed from aliquots of platelet suspensions by 30 sec centrifugation at 11,000 *g*. The supernatant, containing released PF3, was then incubated with or without *P. papuanus* venom and then subjected to the clotting assay with DRV as described above.

Determination of phospholipase A_2 (PLA_2) activity

For estimation of PLA_2 activity, 0.2 μCi of tritiated phosphatidylcholine (L-3-phosphatidylcholine, 1-stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H] arachidonyl; Amersham International), was added to 200 μmoles cold phosphatidylcholine, dissolved in chloroform in an Eppendorf tube. The solvent was removed under a stream of nitrogen at 60°C. The phospholipids were resuspended to 1 ml in a HEPES buffer [20 mM HEPES, 2 mM $CaCl_2$, pH 7.4,

0.5% fatty acid free bovine serum albumin (BSA) and 0.01% Triton X-100]. The total counts present were 148,600 ± 12,700 cpm. The reaction was initiated by the addition of 3 μg/ml of either *P. papuanus* venom or PLA_2 from *Crotalus durissus terrificus* venom (Sigma). The tubes were incubated at 37°C for 30 min, then placed on ice and 40 μg of unlabelled arachidonate (free acid, 1 mg/ml in methanol. Sigma) was added. The extracts were then transferred to glass tubes containing 2 ml of cold methanol and were incubated at 4°C for 30 min prior to further extraction of phospholipids by the addition of 2 ml of chloroform and 1 ml of water. To give a clear separation of phases, the tubes were centrifuged at 1500 *g* for 10 min at 4°C. The lower organic phase was removed and evaporated under a stream of nitrogen at 60°C. The extract was resuspended to 60 μl of chloroform:methanol (2:1) and 15 μl was spotted onto a glass silica gel 60 TLC plate (Merck). The plate was developed in hexane:diethyl ether:methanol:acetic acid (90:20:3:2). The spots were visualised by immersing it in 150 ml of Coomassie Blue solution (0.03% Coomassie Blue R, 25% methanol and 100 mM NaCl) (NAKAMURA and HANDA, 1984). The phosphatidylcholine fraction remained at the origin, while arachidonic acid had an R_f value of 0.19. Areas corresponding to phosphatidylcholine and arachidonate were scraped and counted.

RESULTS

Venoms from all *Pseudechis* spp. studied showed similar protein bands when separated on SDS-PAGE. However, the band patterns of the two *P. papuanus* sources were more similar to each other than to the other two species studied (Fig. 1).

In vivo *effects of the venom*

The i.v. LD_{50} of *P. papuanus* venom was 10.4 μg/mouse (95% confidence limits, 8.3–12.6 μg/mouse). CSL polyvalent antivenom proved to be highly effective in neutralising the venom in the standard mouse model (Table 1). The main cause of death in rats

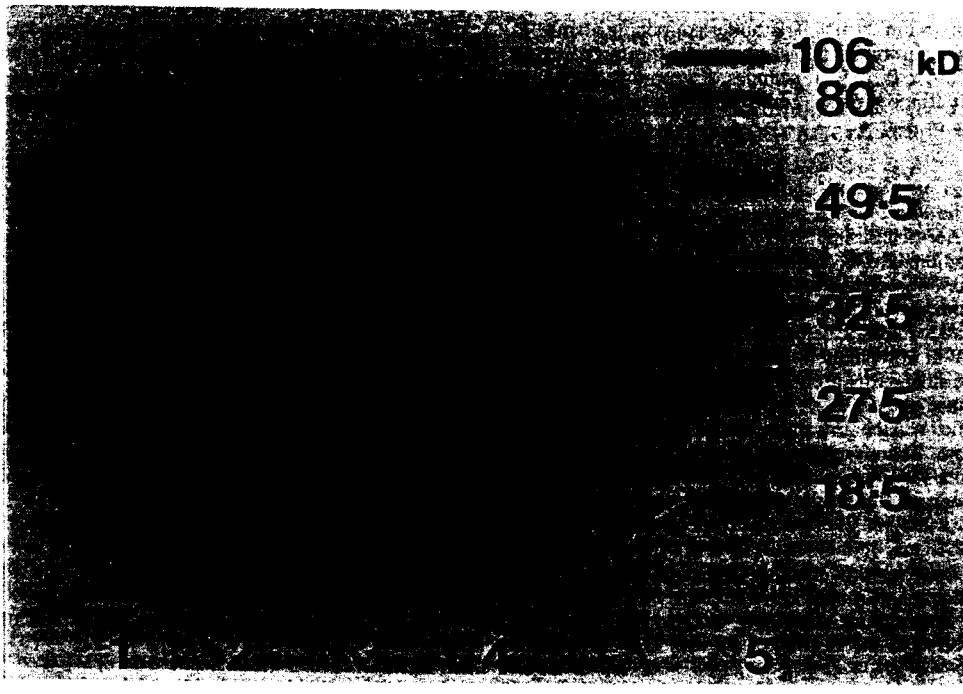

FIG. 1. SDS-PAGE GELS OF COMMERCIAL AND RECENTLY ACQUIRED *Pseudechis papuanus* VENOM. Lane 1, *P. guttatus* venom (from specimen maintained at the Liverpool School of Tropical Medicine; Lane 2, *P. australis* venom (commercial source); Lane 3, *P. papuanus* venom obtained from a recently acquired specimen; Lane 4, commercial *Pseudechis papuanus* venom; Lane 5, mol. wt markers.

TABLE 1. SOME BIOLOGICAL PROPERTIES OF *Pseudechis papuanus* VENOM (VENOM FROM LIVE SPECIMEN ONLY USED UNLESS OTHERWISE INDICATED)

Assay	Result
Minimum haemorrhagic dose (MHD)	60 μg/rat†
Median lethal dose (i.v. LD_{50}) mice	10.4 μg/18–20 g mouse (8.3–12.6 μg/mouse)*
Median effective dose (i.v. ED_{50}) mice, against CSL antivenom	8.5 μg/18–20 g mouse (7.7–9.3 μg/mouse)*
Coagulant activity on plasma	Trace activity only at very high concentrations (500 μg/ml)‡
Coagulant activity on fibrinogen	Nil‡
Anticoagulant activity on whole blood	Intense: 100 μg venom added to 1 ml whole blood prevented clotting completely. Inhibition of pathways involving phospholipid‡
Fibrin plate lysis	Nil‡
Fibrinogenolytic activity	Nil‡

*95% Confidence limits in parentheses.
†MHD of Sigma venom was 45 μg/rat.
‡Same result with Sigma venom.

injected with 20–200 μg *P. papuanus* venom i.v. was sudden respiratory arrest preceded by cyanosis, suggesting the action of a powerful venom neurotoxin (Table 2). In anaesthetised rats the rapidity of onset of the respiratory distress was directly related to the venom dose (Table 2).

The venom was moderately haemorrhagic when injected intradermally into rats (Table 1), with MHDs ranging from 46 μg/rat (Sigma venom source) to 60 μg/rat (recently acquired specimen) (each result was the mean of two assays).

When lethal or sublethal doses were applied by i.v. or s.c. routes, there was no change in the platelet count, red blood cell numbers or haematocrit up to 2 hr after injection of venom (Table 2). Pigmented urine, which may have been due to the presence of either haemoglobin or myoglobin, was observed in rats treated with doses greater than 20 μg *P. papuanus* venom i.v. The nature of the pigment was not investigated in this study. Table 3 shows the rates of platelet aggregation induced by ADP, thrombin and collagen in PRP from envenomed rats. Following i.v. injection, there was a pronounced decrease in the response of platelets to the three agonists.

TABLE 2. HAEMATOLOGICAL AND COAGULATION DISTURBANCES IN RATS INJECTED I.V. AND S.C. WITH *Pseudechis papuanus* VENOM (SIGMA)

No. of rats	Venom (μg)	Route	Clot† qual	RBC ($\times 10^6$/mm^3)	Ht (%)	Platelets ($\times 10^9$/litre)	Fibrinogen (mg/ml)	Time to death (min)
2	0	i.v.	1	6.9	34.5	625	2.32	—
1	5	i.v.	1	6.2	30.0	570	2.07	—
1	10	i.v.	1	7.1	34.0	650	2.09	120*
1	20	i.v.	1	6.4	31.0	600	2.03	98
1	50	i.v.	1	9.7	48.0	750	1.42	62
1	100	i.v.	1	7.8	40.0	630	1.42	45
1	200	i.v.	1	8.5	43.0	720	1.41	26
2	0	s.c.	1	6.7	33.0	600	2.12	—
1	100	s.c.	1	6.5	31.0	590	2.05	—
1	150	s.c.	1	6.6	33.0	610	1.89	—

*Animal was killed at the point of death.
†Grade 1 = normal clot.

TABLE 3. PLATELET AGGREGATION RATES OF PLATELET-RICH PLASMA (PRP) FROM RATS INJECTED WITH *Pseudechis papuanus* VENOM (SIGMA)

Dose	Aggregation rates (mm/sec) ADP	Thrombin	Collagen	Platelets (PRP) ($\times 10^9$/ml)
Control ($n = 3$)	2.00	2.00	2.03	473
5 μg i.v.	2.00	0.60	0.40	570
10 μg i.v.	0.80	0.10	0.30	450
20 μg i.v.	0.40	0.40	0.30	400
50 μg i.v.	0.50	0.60	0.80	290
100 μg i.v.	0.60	0	0.20	270
200 μg i.v.	1.20	1.00	0.30	200
100 μg s.c.	1.60	2.30	0.30	400
150 μg s.c.	2.00	1.10	1.00	400

Final ADP concentration was 6 μM; thrombin, 0.1 units/ml; collagen, 5 μg/ml.

In vitro *activity of the venom*

When whole venom was added to citrated human plasma or fibrinogen solution, no clot formed except at very high venom concentrations in plasma only (Table 1). The addition of venom to whole blood completely prevented clotting at 100 μg venom/ml blood (Table 1), confirming the results of CAMPBELL and CHESTERMAN (1972).

Fibrinogen incubated with the venom was clottable with thrombin, thus demonstrating that *P. papuanus* venom did not possess fibrinogenolytic activity. Also, the venom caused no fibrinolysis (Table 1). Thus, the lack of a coagulant effect of the venom on either plasma or fibrinogen was not due to the lysis of the substrate required for fibrin clot formation.

When mixed with human PRP, the venom reduced the rate and extent of platelet aggregation induced by ADP, adrenaline, collagen and ristocetin, and also prevented the secondary aggregation with all four agonists (Fig. 2). The maximum response decreased

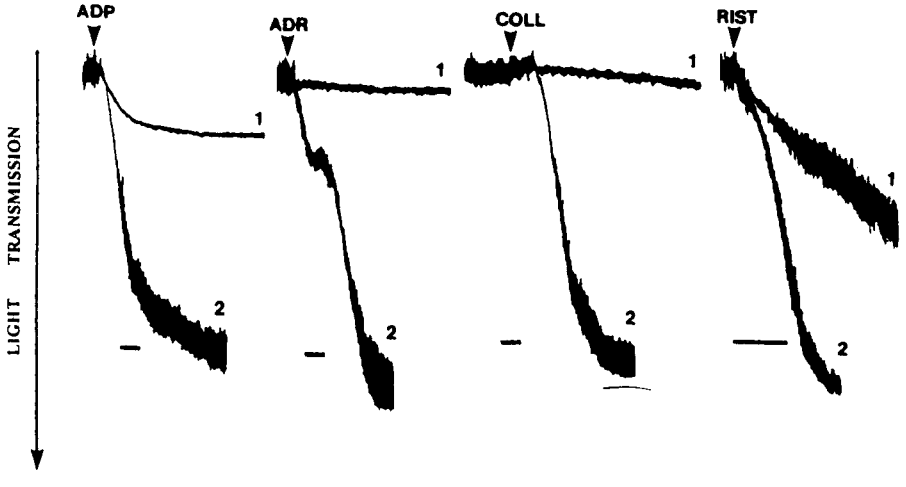

FIG. 2. EFFECTS OF *Pseudechis papuanus* VENOM ON HUMAN PLATELET-RICH PLASMA. Venom (10 μg/ml) was incubated with platelet-rich plasma (PRP) for 5 min at 37°C and aggregation was induced by either 6 μM ADP or adrenaline (ADR), or 5 μg/ml collagen (COLL), or 0.5 mg/ml ristocetin (RIST). Trace 1 represents PRP incubated with venom and trace 2 with phosphate-buffered saline. Results were recorded from three independent experiments. Bars represent 1 min.

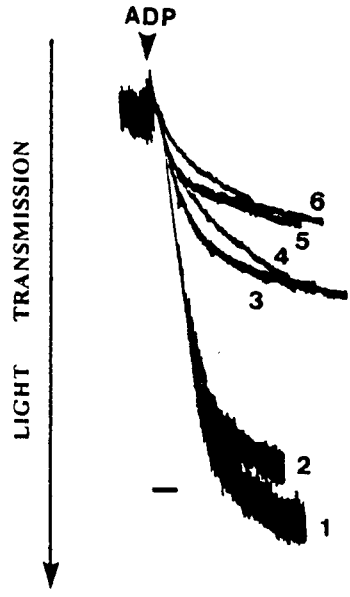

FIG. 3. DOSE–RESPONSE OF *Pseudechis papuanus* VENOM ON AGGREGATION OF HUMAN PLATELET-RICH PLASMA (PRP) INDUCED BY ADP.
Trace 1, control; 2, 0.25 μg/ml; 3, 0.5 μg/ml; 4, 1.0 μg/ml; 5, 2 μg/ml; 6, 5 μg/ml. Platelet aggregation was induced by 6 μM ADP. A single plasma donor was used for this experiment. Bar represents 1 min.

within 1 min after incubation with the venom (data not shown). The inhibitory effect of the venom was dose dependent (Fig. 3). Aggregation inhibition was not affected by treatment of the venom with 160 μM PMSF or 5 mM EDTA (data not shown). Similar inhibitory effects were also observed when rat PRP was treated with *P. papuanus* venom

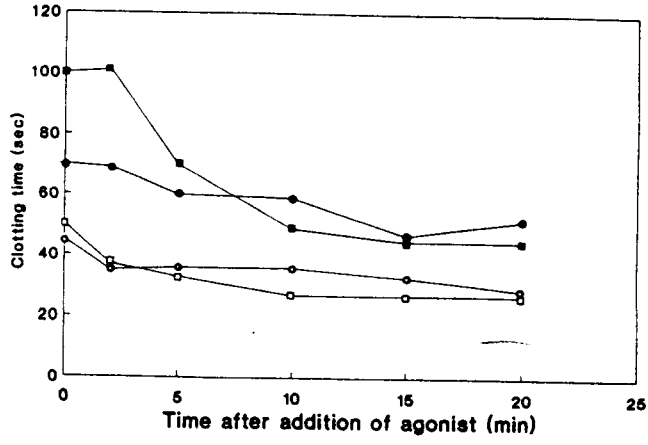

FIG. 4. PLATELET FACTOR 3 (PHOSPHOLIPID) AVAILABILITY.
PRP in either the absence (white circle) or presence (black circle) of 5 μg/ml venom was treated with 20 μM ADP. Aliquots were taken at indicated times after addition of agonist, and clotting times recorded with *Daboia russelli* venom and $CaCl_2$. PRP was also treated with 5 μg/ml collagen and aliquots taken at the indicated times were centrifuged and the clotting times recorded as above in the absence (white square) or presence (black square) of venom. Results represent the means of duplicate assays.

(data not shown). These results are in agreement with the findings of low aggregation rates in PRP for envenomed rats shown in Table 3. Thus, these results demonstrate that *P. papuanus* venom inhibits platelet aggregation induced by various agonists. The inhibition is dose related with complete abolition of the secondary phase (Fig. 2).

The activity of platelet factor 3 in inducing clotting with DRV was diminished as demonstrated by increasing clotting times after the addition of *P. papuanus* venom either to the PRP or to the supernatant of collagen-stimulated PRP (Fig. 4).

The PLA_2 activity of the venom was 2022 μmole/mg/min, compared with the activity of *C. d. terrificus* venom PLA_2 activity of 187 μmole/mg/min as determined by the assay used.

DISCUSSION

In this study we demonstrated that the venom of *P. papuanus* effectively inhibits clot formation and platelet aggregation. The platelet inhibition was not due to either serine proteases or metalloproteinases which may also be present in the venom.

The batch of venom purchased from a commercial source as *P. papuanus* venom and that obtained from our own specimen are so similar in their protein patterns on SDS-PAGE and in their biological properties that they are highly likely to be from the same species. *Pseudechis papuanus* venom is slightly more toxic than that of the Malaysian cobra, *Naja sumatrana*, and about three times less toxic than that of the Australian taipan (*Oxyuranus scutellatus scutellatus*) (THEAKSTON and REID, 1983); venoms from both these species are known to contain powerful neurotoxins. The CSL polyvalent antivenom (Australia–New Guinea), in which *P. papuanus* is not now used in the immunising mixture (THEAKSTON and WARRELL, 1991), effectively neutralised *P. papuanus* venom in the standard animal model.

Pseudechis papuanus venom has high PLA_2 activity, comparable to that of *P. colletti* venom (WEINSTEIN *et al.*, 1992) and other *Pseudechis* species (DOERY and PEARSON, 1961). Basic snake venom PLA_2s are associated with presynaptically acting neurotoxins (KINI and IWANAGA, 1986); therefore, it is possible that the PLA_2 activity of *P. papuanus* venom may play a major role in its toxicity.

Snake venom PLA_2s are also implicated in haemostatic alterations by inhibiting both coagulation (EVANS *et al.*, 1980; BOFFA *et al.*, 1980; BOFFA and BOFFA, 1976; ROSENBERG, 1986) and platelet aggregation (HUANG *et al.*, 1984; LI *et al.*, 1986). Whether or not the PLA_2 enzymatic activity is required for the expression of both inhibitory activities is still unclear. Phospholipid hydrolysis seems to be essential for the development of the anticoagulant effect (VERHEIJ *et al.*, 1980), but this is controversial (CONDREA *et al.*, 1981).

CAMPBELL and CHESTERMAN (1972) demonstrated anticoagulation with *P. papuanus* venom in all clotting tests except the thrombin time test. This was confirmed both in this study and by MARSHALL and HERRMANN (1983). Here, we show that the procoagulant phospholipid released from platelets, in the presence of *P. papuanus* venom, has diminished capacity to induce clot formation; whether this is due to the phospholipid hydrolysis by the venom PLA_2 remains to be investigated. We also showed that, at high *in vitro* concentrations, the venom possesses a mild procoagulant activity which is, at lower concentrations, fully counterbalanced by the powerful anticoagulant effect. However, blood of envenomed animals always clotted within 20 min of withdrawal and the appearance of the clots was normal.

The inhibition of platelet function by *P. papuanus* venom could be associated with the presence of a PLA_2 component. Venom PLA_2s have been shown to affect platelet aggregation either by exhibiting a biphasic effect consisting of initial aggregation by arachidonic acid release followed by inhibition (Class A), or by having a monophasic inhibitory effect only (Class B) (OUYANG and HUANG, 1984). The platelet aggregation in the presence of *P. papuanus* venom is strongly reduced and monophasic; these effects are similar to the ones obtained with the purified PLA_2 from the venom of the Australian copperhead snake, *Austrelaps superba* (YUAN *et al.*, 1993). However, the mechanism of platelet inhibition by venom PLA_2 is not well understood (KINI and EVANS, 1990; YUAN *et al.* (1993).

The relationship between PLA_2 enzymatic activity and its pharmacological effects is also not fully understood. It has been shown that heat inactivation of the enzyme does not produce a comparable diminution in its inhibitory effect on platelet aggregation (KAIRE, 1964; LI *et al.*, 1985). Neurotoxins with low PLA_2 activity, such as β-bungarotoxin, have been shown to bind with high affinity to synaptic plasma membranes (OTHMAN *et al.*, 1982; UENO and ROSENBERG, 1990). Therefore, it appears that many of the pharmacological effects of venom PLA_2 may not be associated with its catalytic site.

We confirm the findings of CAMPBELL and CHESTERMAN (1972) that the venom is neither intrinsically fibrinolytic nor fibrinogenolytic. Fibrinogenolysis has been frequently associated with the presence of haemorrhagic metalloproteases in snake venoms (MARKLAND, 1991). The haemorrhagic activity of *P. papuanus* venom is relatively weak compared with some viperine venoms, but is comparable to that of Iranian *Echis sochureki* (saw-scaled or carpet viper) and *Calloselasma rhodostoma* (Malayan pit viper) (THEAKSTON and REID, 1983), both of which are clinically haemorrhagic. The active principle may, like the haemorrhagins of certain viper venoms, be a zinc metalloprotease containing a disintegrin or disintegrin-like domain (PAINE *et al.*, 1992). CAMPBELL (1967) also suggested that *P. papuanus* venom contained a haemorrhagin. However, the presence of a metalloproteinase in an elapid venom is very unusual; it is possible that PLA_2 could be responsible for some haemorrhage due to capillary damage. This aspect requires further investigation.

The present study shows that the haemorrhagic diathesis which occurs in patients bitten by *P. papuanus* (LALLOO *et al.*, 1994) could be caused by a powerful anticoagulant and a platelet inhibitor, combined with a weak haemorrhagin. Whether *P. papuanus* PLA_2 is the main agent responsible for the interference with haemostasis, as well as being the main lethal neurotoxic component of the venom, requires further investigation.

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