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Roles of PMN Leucocytes, Platelets and Some Mediators in Rat Hind-paw Oedema Induced by Two Phospholipase A_2 Enzymes From *Trimeresurus mucrosquamatus* Venom

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Abstract—Two phospholipase A_2 (PLA_2) enzymes, $TMVPLA_2$ I and $TMVPLA_2$ II, isolated from *Trimerusurus mucrosquamatus* venom induced rat hind-paw oedema. Recovered myeloperoxidase activity increased within 1 h and was greatly elevated in the rat paw 3–6 h after subplantar injection of these venom PLA_2 enzymes. Methotrexate pretreatment significantly reduced not only the peripheral leucocyte count but also venom PLA_2-induced paw oedema. In rat isolated PMN leucocyte suspension, venom PLA_2 induced superoxide radical formation. Paw swelling caused by $TMVPLA_2$ I or $TMVPLA_2$ II was only slightly or not, respectively, reduced in the rats pretreated with anti-platelet plasma, which reduced peripheral blood platelet count by >96%, suggesting platelets are not involved. In isolated platelet preparation, $TMVPLA_2$ I induced platelet activation in a concentration-dependent manner, while $TMVPLA_2$ II had no effect. Pretreatment with diphenhydramine/methysergide greatly suppressed the oedematous responses caused by the two venom PLA_2 enzymes; the residual responses were significantly further depressed by aspirin. The oedematous responses caused by the enzymes were also suppressed by FPL 55712, BW 755C, dexamethasone, superoxide dismutase/catalase, isoprenaline and terbutaline. However, BN 52021 and L 652731, both platelet aggregating factor antagonists, were not effective on these responses. Thus, in addition to histamine and 5-hydroxytryptamine release by the mast cells in PLA_2-induced paw oedema (Wang & Teng 1990), the results of this study indicate minor, but significant, roles for neutrophils and inflammatory mediators including prostaglandins, leukotrienes and superoxide radicals.

Beside the systemic toxicity, the venoms of snakes belonging to the families of *Viperidae* and *Crotalidae* produce intense local irritation (Ohsaka 1979). Swelling and oedema are often the paramount early feature of snake venom poisoning at the affected part of the victims (Reid 1968). These local effects are complex in nature and are still incompletely understood. The local irritant action of cobra venom has been attributed to the cardiotoxin of the venom (Lee et al 1968; Wang & Teng 1989). The oedema-producing principles of snake venoms may be esterases (Ohtani & Takahashi 1983), kinin-releasing enzymes (Cohen et al 1970; Wang & Teng 1988) or phospholipase A_2 (PLA_2) enzymes (Rothschild 1965; Vishwanath et al 1987). Recently, two oedema-producing PLA_2 enzymes, $TMVPLA_2$ I and $TMVPLA_2$ II, were isolated from *Trimeresurus mucrosquamatus* venom (Teng et al 1989). Mast cells played a predominant role in these two venom PLA_2-induced paw oedemas in rats (Wang & Teng 1990b). The aim of this study was to investigate other inflammatory cells, beside mast cells, and possible mediators involved in these venom PLA_2-induced paw oedemas.

Materials and Methods

Materials

Two phospholipase A_2 enzymes, $TMVPLA_2$ I and $TMVPLA_2$ II, were isolated from *Trimeresurus mucrosquamatus* venom as described previously (Fractions XVIII and XXII, respectively (Teng et al 1989); the enzymatic activity of $TMVPLA_2$ I and $TMVPLA_2$ II were 18 ± 5 and 101 ± 2 μmol min^{-1} (mg protein)$^{-1}$, respectively). Diphenhydramine, methotrexate, dexamethasone, isoprenaline, superoxide dismutase, catalase, aspirin, cytochrome C, sodium arachidonate, Freund's adjuvant (complete and incomplete) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., USA. Guaiacol, cetyltrimethylammonium bromide, sodium pentobarbitone and sodium citrate were obtained from Wako Pure Chemical Ind., Japan. Terbutaline was purchased from Astra Pharmaceutical Co., Sweden. Methysergide was a gift from Sandoz Pharmaceutical Ltd, Switzerland. Captopril was generously supplied by Squibb Pharmaceutical Co., Taiwan Branch. BW 755C was provided by Wellcome Research Laboratories, UK. FPL 55712 was supplied by Fisons Pharmaceutical Division, UK. BN 52021 was a kind gift from Dr P. Braquet of Institut Henri Beaufour, France. L 652731 was kindly supplied by Merck, Sharp & Dohme Inc., USA. The structures of these four compounds have been given previously (Wang & Teng 1990a).

Rat hind-paw oedema

Wistar rats, 180–220 g, were used. Hind-paw oedema was produced by a single subplantar injection of 0·1 mL irritant in 0·05 M phosphate-buffered saline (PBS, pH 7·4) or an equal volume of PBS into the right and left hind-paw, respectively. The volumes of both hind-paws of each rat were measured with a plethysmometer at the beginning and the different periods after induction of oedema (Wang & Teng 1989). Percent hind-paw swelling was calculated as follows: hind-paw swelling (%) = ([(right paw volume − initial volume)/

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(right paw initial volume)] − [(left paw volume − initial volume)/(left paw initial volume)]) × 100. The data were also analysed to compare the area under the time-swelling curve (AUC) in the 6 h interval.

Depletion of PMN leucocytes

Rats were given methotrexate (2·5 mg kg^{-1}, i.p.) daily for 3 days as described previously (Willoughby & Giroud 1969). Three days after the last injection of methotrexate the animals were used for oedema testing. The peripheral blood cell numbers after methotrexate treatment were counted with a Coulter counter.

PMN leucocyte isolation

Blood was withdrawn and mixed with 6% dextran 70 (Markert et al 1984). The mixture was allowed to stand for 1 h and the upper layer was then decanted and centrifuged. Red blood cells in the pellet were removed by hypotonic lysis, tonicity was then restored and the pellet was washed again. The pellets were resuspended in modified Hank's balanced-salt solution of the following composition (mM): NaCl 130, KCl 5·5, Na_2HPO_4 0·6, NaH_2PO_4 0·6, $CaCl_2$ 1·4, $MgCl_2$ 0·7, Tris-HCl 25 (pH 7·4) and dextrose 10, and layered over Ficoll-Hypaque solution (Newby 1980). After centrifugation the pellets were washed, resuspended in modified Hank's balanced-salt solution and the cell count was adjusted to 10^7 cells mL^{-1}. Differential cell counting showed that more than 90% of the cells in the preparation were PMN leucocytes.

Superoxide formation

Superoxide formation was triggered by the addition of venom PLA_2 into the PMN leucocyte suspension containing cytochrome C with or without superoxide dismutase at 37°C (Markert et al 1984). The reactions were terminated 15 min later by immersing the tubes in an ice-cold bath. After centrifugation at 1500 *g* for 5 min at 4°C, the superoxide in the supernatant was determined by spectrophotometry at 550 nm, with the dismutase-free supernatant as the sample and the dismutase-containing supernatant as reference.

Measurement of recovered myeloperoxidase

Rat hind-paws were isolated and homogenized in ice-cold 0·5% cetyltrimethylammonium bromide in 0·1 M phosphate buffer pH 7·0 and freeze-thawed three times. After centrifugation at 40 000 *g* for 15 min at 4°C, enzyme activity in the supernatant was measured (Dri et al 1982). The reaction was started in the presence of guaiacol by the addition of H_2O_2. Enzyme activity was presented as guaiacol units (μmol tetraguaiacol formation min^{-1} at 37°C).

Preparation of anti-platelet plasma

Anti-platelet plasma was prepared as described by Lefort & Vargaftig (1978). Rat platelets were washed with saline and freeze-thawed five times. After centrifugation, the pellets were mixed with Freund's complete adjuvant and injected into all four foot pads of rabbit. The 2nd and 3rd immunizations were carried out at 15 day intervals. Seven days after the last immunization, blood was collected and plasma was separated. After heating at 56°C for 30 min, this plasma was lyophilized and stored at −70°C. The activity of anti-platelet plasma was estimated by measuring the platelet lysis effect in rat platelet-rich plasma.

Depletion of platelets

Wistar rats, 180–220 g, were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.p.). Anti-platelet plasma was given by i.v. (caudal vein) infusion for 30 min. One hour later the 2nd infusion was carried out which reduced peripheral blood platelet count by more than 96%, and sustained for at least 6 h. Platelet number was counted with a Coulter counter. The animals were used for hind-paw oedema experiments 30 min after the last infusion of anti-platelet plasma.

Platelet isolation

Blood was withdrawn from Wistar rats, mixed with sodium citrate (3·8% (1 : 14 to blood)) and centrifuged for 10 min at 120 *g* and room temperature (21°C). The supernatant was obtained as platelet-rich plasma, mixed with EDTA to a final concentration of 6 mM and centrifuged at 500 *g* for 10 min. The pellets were washed twice with Tyrode solution (calcium-free) with EDTA 2 mM, and with BSA 3·5 mg mL^{-1}, and suspended in Tyrode solution of the following composition (mM): NaCl 136·8, KCl 2·8, $NaHCO_3$ 11·9, $MgCl_2$ 1·1, $CaCl_2$ 1·0, NaH_2PO_4 0·33, dextrose 11·2 and BSA 3·5 mg mL^{-1}.

Platelet aggregation and ATP release reaction

Aggregation was measured by a turbidimetric method (O'Brien 1962; Born & Cross 1963). ATP released from the platelets was detected by bioluminescence (DeLuca & McElory 1978). Both the aggregation and the release reaction were simultaneously and continuously measured by Lumi-aggregometer. Lactate dehydrogenase was measured by Wroblewski & La Due (1955) as the indicator of cell lysis.

Statistical evaluations

The results are expressed as the means ± s.e.m. of the indicated number of experiments and Student's *t*-test was used for the statistical evaluation. *P* values $<0·05$ were considered to be significant.

Results

The role of PMN leucocytes

Myeloperoxidase activity was significantly increased within 1 h (6·3 ± 1·3 units/paw in the $TMVPLA_2$ I group, 6·9 ± 2·2 units/paw in the $TMVPLA_2$ II group vs 1·0 ± 0·2 units/paw in the control group) and remained elevated at 3 and 6 h (15·5 ± 3·9 and 24·4 ± 3·9 units/paw, respectively, in the $TMVPLA_2$ I group, 15·9 ± 4·8 and 29·4 ± 6·6 units/paw, respectively, in the $TMVPLA_2$ II group) in the rat hind-paw after subplantar injection of venom PLA_2 (5 μg/paw). Pretreatment of rats with methotrexate (2·5 mg kg^{-1}, i.p.) for 3 days decreased the total leucocyte and PMN leucocyte counts in peripheral blood to 36 and 8%, respectively, in the $TMVPLA_2$ I-treated group, and to 34 and 20%, respectively, in the $TMVPLA_2$ II-treated group (Table 1). The platelet counts and haematocrit values were not significantly changed. Under these conditions, $TMVPLA_2$ I- and $TMVPLA_2$ II-induced paw oedema were reduced to 36 and

Table 1. Effect of methotrexate pretreatment on peripheral blood cell counts.

	Cells μL^{-1}			
	Total leucocytes	PMN leucocytes	Platelets	Haematocrit (%)
Control	10,180 ± 1,092	1,856 ± 201	395,354 ± 12,833	40·0 ± 0·7
Methotrexate pretreatment				
$TMVPLA_2$ I	3,673 ± 788***	146 ± 12***	365,790 ± 35,554	42·6 ± 1·4
$TMVPLA_2$ II	3,521 ± 506***	388 ± 93***	398,064 ± 37,077	41·6 ± 2·6

Values are expressed as mean ± s.e.m. of at least five separate determinations. ***$P < 0.001$.

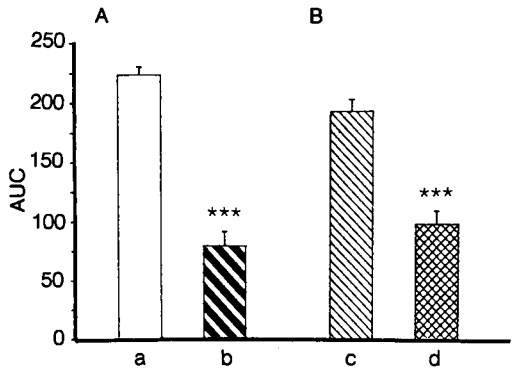

FIG. 1. Effect of pretreatment with methotrexate on hind-paw oedema induced by $TMVPLA_2$. Responses are presented as the areas under the curves (AUC) measured in the 6-h period after subplantar injection of $TMVPLA_2$ I (A) and $TMVPLA_2$ II (B). The oedematous response was induced by the injection of $TMVPLA_2$ I, 5 μg (a), or $TMVPLA_2$ II, 5 μg (c), in the normal rats, and injection of $TMVPLA_2$ I (b) or $TMVPLA_2$ II (d) in the methotrexate-pretreated rats. Values are expressed as means ± s.e.m. of 6 experiments. Statistically significant differences from the corresponding control values are noted as ***$P < 0.001$.

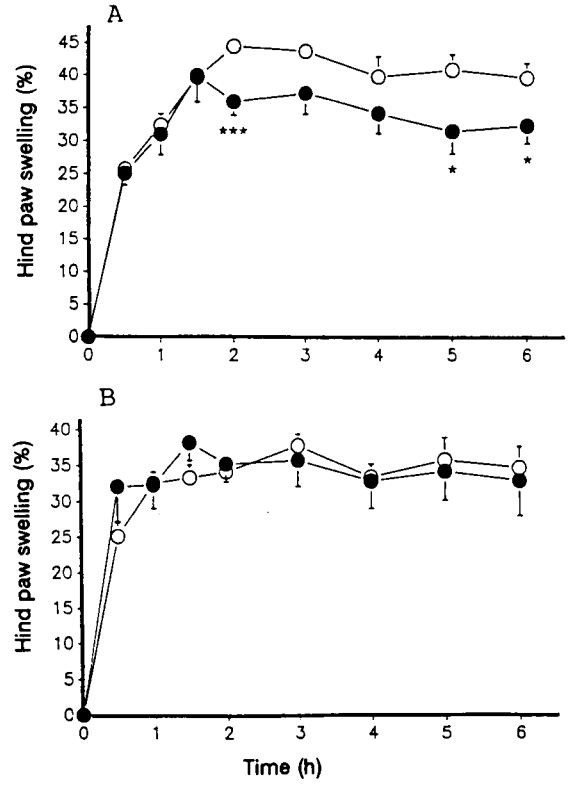

FIG. 2. Effect of pretreatment with anti-platelet plasma on hind-paw swelling caused by $TMVPLA_2$. Paw swelling was induced by subplantar injection of $TMVPLA_2$ I, 5 μg (A), and $TMVPLA_2$ II, 5 μg (B), in normal (○) or anti-platelet plasma pretreated (●) rats. Values are expressed as means ± s.e.m. of 4–6 experiments. Statistically significant differences from the corresponding control values are noted as *$P < 0.05$, ***$P < 0.001$.

51% of the control values, respectively (Fig. 1). In isolated PMN leucocyte suspension, superoxide formation was increased (1·72 ± 0·13 nmol/2·5 × 10^6 cells in the $TMVPLA_2$ I group, 1·80 ± 0·20 nmol/2·5 × 10^6 cells in the $TMVPLA_2$ II group vs 0·03 ± 0·01/nmol 2·5 × 10^6 cells in the control group) when stimulated with $TMVPLA_2$ I (100 μg mL^{-1}) or $TMVPLA_2$ II (100 μg mL^{-1}).

The role of platelets

The platelet count in peripheral blood was decreased to less than 4% of the control value after i.v. infusion of anti-platelet plasma. $TMVPLA_2$ I-induced paw oedema was only slightly reduced, while $TMVPLA_2$ II-induced paw oedema was unaffected (Fig. 2). In rat isolated platelet suspension, arachidonate (100 μM) induced platelet aggregation and release reaction in the platelet-rich plasma and the washed platelet suspension (Fig. 3). In these platelet preparations, $TMVPLA_2$ I was able to induce platelet activation in a concentration-dependent manner, while $TMVPLA_2$ II was not effective at the highest concentration tested (50 μg mL^{-1}). Less than 10% lactic dehydrogenase was released at 200 μg mL^{-1} $TMVPLA_2$ I.

Kinins, arachidonate metabolites, platelet activating factor (PAF) and superoxide radical in venom PLA_2-induced paw oedema

Captopril (1 mg kg^{-1}, s.c.) significantly potentiated the paw oedema induced by low doses of these two venom PLA_2 enzymes (Fig. 4). Paw swelling caused by $TMVPLA_2$ II was significantly suppressed by pretreating the rats with aspirin (180 mg kg^{-1}, s.c.) alone, while $TMVPLA_2$ I-induced paw swelling was unaffected (Fig. 5). However, the residual responses caused by $TMVPLA_2$ I and $TMVPLA_2$ II in rats pretreated with diphenhydramine and methysergide (each

Table 2. Effect of anti-inflammatory drugs on $TMVPLA_2$-induced paw oedema.

Drugs	Oedema (AUC)	
	$TMVPLA_2$ I	$TMVPLA_2$ II
Control	229·4 ± 8·3	200·5 ± 11·8
Removal of superoxide radical		
Superoxide dismutase/catalase (each 500 units)	161·5 ± 12·0***	129·8 ± 14·5**
Drugs affecting the eicosanoid pathway		
Aspirin (180 mg kg^{-1}, s.c.)	201 ± 12·3	127·3 ± 10·8***
FPL 55712 (100 μM)	144·3 ± 14·6***	121·3 ± 13·8***
BW 755C (50 mg kg^{-1}, i.p.)	96·3 ± 9·9 ***	90·9 ± 10·6***
Dexamethasone (1 mg kg^{-1}, s.c.)	106·4 ± 10·4***	52·9 ± 7·9 ***
PAF antagonists		
BN 52021 (50 μg)	214·6 ± 8·9	212·9 ± 15·9
L 652731 (50 μg)	226·2 ± 14·2	209·8 ± 23·5
β-Adrenoceptor agonists		
Isoprenaline (1·5 mg kg^{-1}, s.c.)	88·1 ± 13·5***	74·7 ± 18·1***
Terbutaline (0·5 mg kg^{-1}, i.p.)	140·6 ± 21·7*	109·9 ± 19·6***

$TMVPLA_2$ I and $TMVPLA_2$ II (each 5 μg/0·1 mL/paw). Aspirin, indomethacin or diphenhydramine/methysergide (1 h); isoprenaline and terbutaline (30 min); dexamethasone (2 consecutive days) before venom PLA_2 subplantar injection. FPL 55712, superoxide dismutase/catalase, BN 52021 or L 652731 was co-injected with venom PLA_2. Values are expressed as means ± s.e.m. of 5–9 experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

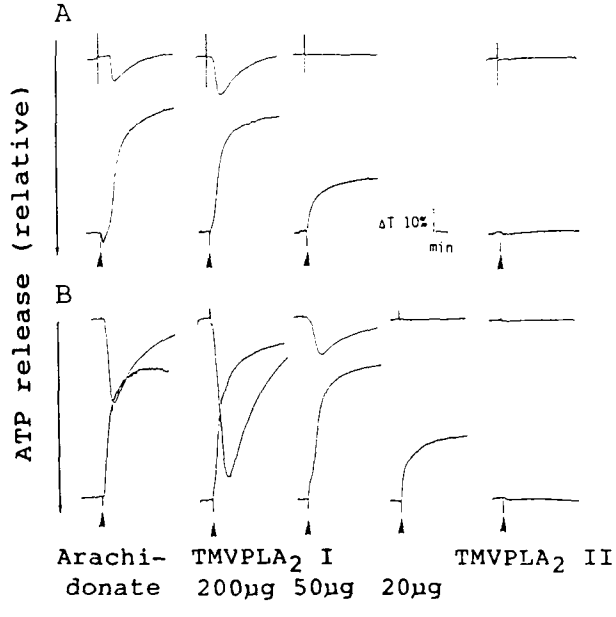

FIG. 3. Platelet aggregation and release reaction. Platelet aggregation (upward tracings) and ATP release (downward tracings) were induced by arachidonate (100 μM), $TMVPLA_2$ I (20, 50 and 200 μg mL^{-1}) or $TMVPLA_2$ II (50 μg mL^{-1}) in rat platelet-rich plasma (A) and washed platelet suspension (B). Similar results were obtained in three other experiments.

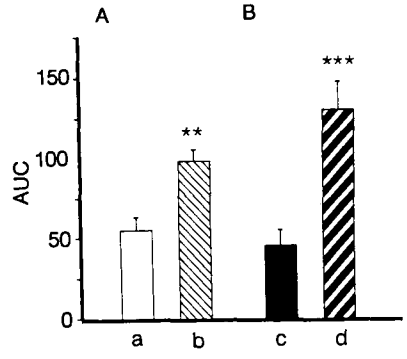

FIG. 4. Effect of captopril on the hind-paw oedema induced by $TMVPLA_2$. Responses are presented as the areas under the curves (AUC) measured in the 6-h period after the subplantar injection of $TMVPLA_2$ I (A) or $TMVPLA_2$ II (B). The oedematous response was induced by subplantar injection of $TMVPLA_2$ I, 1 μg (a), or $TMVPLA_2$ II, 1 μg (c), with no pretreatment with other drugs as control. Captopril (1 mg kg^{-1}, s.c.) was given 30 min before injection of $TMVPLA_2$ I (b) or $TMVPLA_2$ II (d) into the paw. Values are expressed as means ± s.e.m. of 5 experiments. Statistically significant differences from the corresponding control values are noted as $**P < 0.01$, $***P < 0.001$.

10 mg kg^{-1}, s.c.) was further suppressed by aspirin to 55 and 32% of the control values, respectively. After pretreatment with dexamethasone (1 mg kg^{-1}) for 2 consecutive days, the paw oedema caused by venom PLA_2 was reduced (Table 2). Pretreatment with BW 755C, or subplantar co-injection with FPL 55712, also suppressed venom PLA_2-induced paw oedema. $TMVPLA_2$ I- and $TMVPLA_2$ II-induced paw oedema was reduced by subplantar co-injection with superoxide dismutase/catalase, but not with BN 52021 or L 652731. However, pretreatment with terbutaline or isoprenaline greatly reduced the paw swelling caused by these two venom PLA_2 enzymes.

Discussion

PMN leucocyte infiltration in the rat hind-paw during inflammation was estimated by measuring the recovered myeloperoxidase activity in the paw tissue. The peripheral eosinophil count in rat was low, about 1–4% of the total leucocyte count (Ringler & Dabich 1979). The cellular infiltrates in inflammatory rat skin, neutrophils appeared

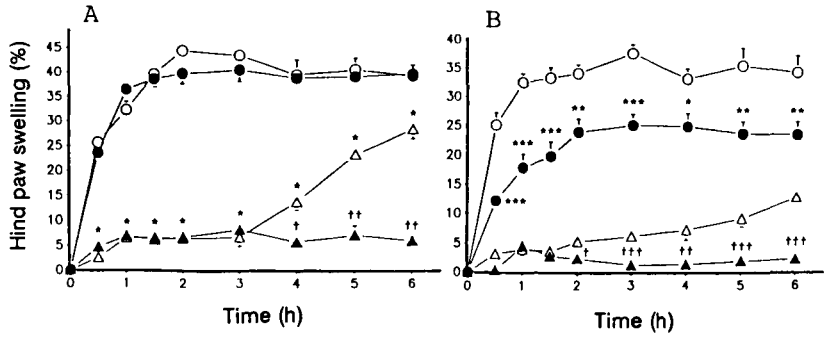

FIG. 5. Effect of aspirin on the hind-paw swelling caused by TMVPLA$_2$. TMVPLA$_2$ I, 5 μg (A), or TMVPLA$_2$ II, 5 μg (B), was given subplantarly without any anti-inflammatory agents (○). Aspirin (180 mg kg^{-1}, s.c. ●), diphenhydramine/methysergide (each 10 mg kg^{-1}, s.c., △), diphenhydramine/methysergide in combination with aspirin (▲) were given 1 h before subplantar injection of irritant. Values are expressed as means ± s.e.m. of 5–9 experiments. For A the statistically significant differences from the corresponding control values (○) are noted as $*P<0.001$ and from the corresponding values without aspirin are noted as $\dagger P<0.01$ and $\dagger\dagger P<0.01$. For B the statistically significant differences from the corresponding control values (○) are noted as $*P<0.05$, $**P<0.01$ and $***P<0.001$ and from the corresponding values without aspirin are noted as $\dagger P<0.05$, $\dagger\dagger P<0.01$ and $\dagger\dagger\dagger P<0.001$.

within 1–2 h and peaked by 8 h, and eosinophils were absent in rat skin (Tannenbaum et al 1980). Thus, the myeloperoxidase activity in rat paw, measured within a 6 h period after induction of the inflammatory response, was mainly from neutrophil. This enzyme activity was significantly increased within 1 h and greatly elevated at 3 and 6 h after subplantar injection of venom PLA$_2$. Superoxide radical can be produced through a respiratory burst of PMN leucocytes under proper stimulation (Green et al 1979) and released superoxide radical contributes to the increase of vasopermeation (Del Maestro et al 1981).

In isolated PMN leucocyte suspension, venom PLA$_2$ increased superoxide formation. Moreover, this venom PLA$_2$-induced paw oedema was significantly suppressed by superoxide dismutase/catalase. These results indicate that PMN leucocytes involved in the TMVPLA$_2$ I- and TMVPLA$_2$ II-induced paw oedema and superoxide radical released from PMN leucocyte participated in these responses.

Platelets, as inflammatory cells (Henson & Ginsberg 1981), contribute to the process by increasing vascular permeability, attracting leucocytes and promoting blood coagulation. In isolated platelet suspensions, TMVPLA$_2$ I concentration-dependently induced platelet activation, while TMVPLA$_2$ II was not effective in this respect. Paw swelling caused by TMVPLA$_2$ I or TMVPLA$_2$ II was only slightly or not, respectively, reduced in the rats pretreated with anti-platelet plasma, which greatly reduced peripheral blood platelet count. Thus, unlike mast cells and PMN leucocytes, platelets do not play a significant role in TMVPLA$_2$ I- and TMVPLA$_2$ II-induced paw oedema.

PLA$_2$ induces the release of arachidonic acid from membrane phospholipids; the arachidonate is transformed into prostaglandins and leukotrienes, which lead to increased vascular permeability and cause oedema formation (Damerau et al 1975; Samuelsson 1983). In this study, aspirin alone suppressed TMVPLA$_2$ II-induced paw oedema, but did not affect the hind-paw oedema caused by TMVPLA$_2$ I. However, in rats pretreated with diphenhydramine/methysergide to antagonize H_1 and 5-HT_1 receptors the residual responses caused by these two venom PLA$_2$ enzymes were further reduced by aspirin. In addition, BW 755C, a dual inhibitor of cyclo-oxygenase/lipoxygenase (Higgs et al 1979), and FPL 55712, an SRS-A antagonist (Samhoun & Piper 1986), and dexamethasone significantly reduced these venom PLA$_2$-induced paw oedemas. These results indicate that prostaglandins and leukotrienes participated in these venom PLA$_2$-induced paw oedemas, and that the role of prostaglandins is more important in paw oedema caused by TMVPLA$_2$ II than that of TMVPLA$_2$ I.

Captopril, a kininase II inhibitor, increased kinin concentration and potentiated the vasopermeation effect of histamine and prostaglandins (Williams 1979; Boura & Svolmanis 1984). Since captopril potentiated venom PLA$_2$-induced paw oedema, kinins could be involved in this response. Neither TMVPLA$_2$ I nor TMVPLA$_2$ II possess kinin activity, nor did they not release kinins from plasma. Therefore, these kinins may be generated from the reaction of exudate and kinin-releasing enzymes released from mast cells or neutrophils (Greenbaum et al 1969; Rothschild et al 1974).

BN 52021 and L 652731, both PAF antagonists (Braquet & Godfroid 1986), did not affect these venom PLA$_2$-induced paw oedemas, while they significantly suppressed PAF-induced oedema. Thus, PAF played a minor role in these oedematous responses. The inhibitory effects of β-adrenoceptor agonists, terbutaline and isoprenaline, could partly be due to direct actions on endothelial cells (Dobbins et al 1982) and partly through inhibition of mast cell degranulation (Undam et al 1985).

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