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Rat Paw Oedema and Mast Cell Degranulation Caused by Two Phospholipase A_2 Enzymes Isolated 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 *Trimeresurus mucrosquamatus* venom (TMV) induce rat hind-paw oedema in a dose-dependent manner. This response is suppressed by pretreatment with diphenhydramine, methysergide or compound 48/80, which reduces tissue histamine content. In isolated mast cells, $TMVPLA_2$ I and $TMVPLA_2$ II cause concentration-, time- and calcium-dependent release of histamine and β-glucuronidase. This effect is inhibited by disodium cromoglycate, mepacrine, nordihydroguaiaretic acid, piriprost and BW 755C, but not by aspirin or indomethacin. These observations indicate that the mast cell plays a predominant role in $TMVPLA_2$ I- and $TMVPLA_2$ II-induced paw oedema, and that venom PLA_2 enzyme needs an intact lipoxygenase pathway to induce mast cell degranulation.

Phospholipase A_2 (PLA_2) enzymes are widely found in snake, bee and scorpion venom. One of their richest sources is snake venom of the families *Viperidae*, *Crotalidae*, *Elapidae* and *Hydrophiidae* (Iwanaga & Suzuki 1979). These enzymes induce or accelerate several effects including neurotoxicity, myotoxicity, interference of platelet aggregation and blood coagulation, antibacterial and antiviral activities, hypotension and oedema formation (Rosenberg 1979; Kini & Evans 1989). Various snake venoms possess histamine-liberating properties, suggested as being due to enzyme action on the mast cell membrane (Uvnäs et al 1962; Uvnäs & Antonsson 1963; Ouyang & Shiau 1970), which in turn increases capillary permeability. In contrast, several groups have reported that PLA_2 from venom or pancreatic sources cause little or no degranulation of the mast cells (Rothschild 1965; Fredholm 1966; Hagen et al 1969; Hines et al 1972; Damerau et al 1975). Recently, two oedema-producing proteins possessing PLA_2 activity ($TMVPLA_2$ I and $TMVPLA_2$ II) were purified from *Trimeresurus mucrosquamatus* venom (Teng et al 1989). The aim of this study is to evaluate the role of the mast cell in $TMVPLA_2$ I- and $TMVPLA_2$ II-induced rat paw oedema, and to investigate the effect of mast cell degranulation caused by these two enzymes.

Materials and Methods

Materials

Two venom PLA_2 enzymes, $TMVPLA_2$ I and $TMVPLA_2$ II, were purified from *Trimeresurus mucrosquamatus* as described previously (Fractions XVIII and XXII, respectively, Teng et al 1989). Diphenhydramine, compound 48/80, indomethacin, aspirin, bovine serum albumin (BSA), EDTA, mepacrine, nordihydroguaiaretic acid and disodium cromoglycate were obtained from Sigma Chem. Co., USA. Methysergide was a gift from Sandoz Pharmaceutical Ltd, Switzerland. Piriprost potassium was provided by Upjohn Company, USA. BW 755C was a gift from Wellcome Res. Lab., UK. The $[^3H]LTB_4$ RIA kit was obtained from Amersham Co., UK and the $[^{125}I]PGE_2$ RIA kit was purchased from New England Nuclear, USA.

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Rat hind-paw oedema

Oedema was induced in Wistar rats (180–220 g) as previously described (Wang & Teng 1989) by subplantar injection of 0·1 mL of irritant in 0·05 M phosphate buffer saline (PBS, pH 7·4) or equal volume of PBS into the right and left hind-paw, respectively. The volumes of both hind-paws of each rat were measured by means of a plethysmometer at the beginning and at various time intervals after the injection of irritants. Hind-paw swelling was calculated as a percentage of the initial volume. The data were analysed to compare the area under the time-course curve (AUC).

Depletion of histamine and 5-HT

Rats were given compound 48/80 twice a day for 4 days as described previously (Di Rosa et al 1971). The doses used were 0·6 mg kg^{-1} for the first six injections and 1·2 mg kg^{-1} for the last two.

Mast cell isolation

Wistar rats (300–350 g) were anaesthetized with ether and exsanguinated (McClain et al 1983; Hanahae 1984). Heparinized Tyrode solution was injected into the peritoneal cavity. The peritoneal fluid was harvested after abdominal massage, and the cell suspension obtained was layered over 38% BSA and centrifuged at 750 *g* for 25 min. The pellets were washed and suspended in Tyrode solution (composition (mM): NaCl 137, KCl 2·7, $NaHCO_3$ 12, NaH_2PO_4 0·3, $MgCl_2$ 1·0, $CaCl_2$ 1·0, dextrose 5·6 and BSA 0·1%). The mast cell count was adjusted to 1–1·5 × 10^6 cells mL^{-1}. Cell viability was assessed by trypan blue staining.

Measurement of histamine and β-glucuronidase

The mast cell suspension was prewarmed at 37°C for 3 min and the release reaction was then triggered by the addition of

either enzyme. The reaction was stopped 15 min later by the addition of ice-cold Tyrode solution and the mixture was centrifuged for 10 min at 1000 *g*. Histamine and β-glucuronidase in the supernatant were determined (Barrett 1972; Håkanson & Ronnberg 1974) and presented as a percentage of the total content. Spontaneous release was less than 5%.

Statistical evaluations

The statistical significance was determined by using Student's *t*-test. *P* values <0.05 were considered to be significant.

Results

Fig. 1 shows that $TMVPLA_2$ I and $TMVPLA_2$ II induced rat hind-paw oedema in a dose-dependent manner. Diphenhydramine (5 and 10 mg kg^{-1}), methysergide (1 and 10 mg kg^{-1}) or a combination of diphenhydramine and methysergide (each 10 mg kg^{-1}), given s.c. 1 h before $TMVPLA_2$ I or $TMVPLA_2$ II injection, reduced paw swelling by about 23, 33, 44, 57 and 69%, and 33, 35, 24, 26 and 80%, respectively (Table 1).

After several i.p. doses of compound 48/80, the histamine contents in the hind-paws of the $TMVPLA_2$ I- and $TMVPLA_2$ II-treated groups were decreased to 8·9 and 10·2%, respectively, of the control values (11·2±1·0 vs 1·0±0·1 μg (g paw)$^{-1}$ in the $TMVPLA_2$ I-treated group; 11·7±0·6 vs 1·2±0·2 μg (g paw)$^{-1}$ in the $TMVPLA_2$ II-treated group). Under these conditions, both enzyme-induced paw oedemas were reduced by more than 95% (223·7±6·7 vs 11·9±6·7 in the $TMVPLA_2$ I-treated group; 193·4±10·2 vs 7·8±2·7 in the $TMVPLA_2$ II-treated group) as calculated from the AUC.

$TMVPLA_2$ I and $TMVPLA_2$ II caused rat peritoneal mast cells to release histamine and β-glucuronidase in a concentration- and time-dependent manner (Fig. 2A, B). They were both equally potent and showed similar patterns. The concentration required to attain a maximal degranulation response was about 100–300 μg mL^{-1} and the peak was obtained 5 min after the addition of the enzymes.

To induce mast cell degranulation the enzymes required calcium (Fig. 3). Calcium-free or EDTA (1 mM) significantly

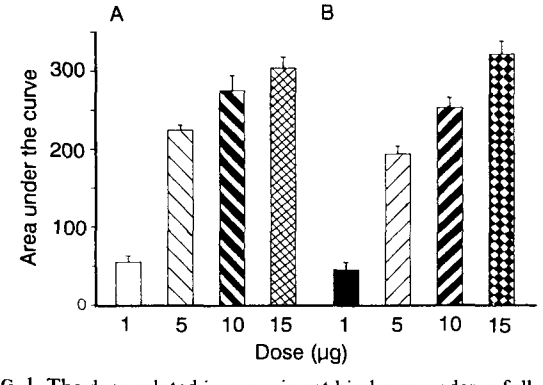

FIG. 1. The dose-related increase in rat hind-paw oedema following subplantar injection of (A) $TMVPLA_2$ I and (B) $TMVPLA_2$ II. Responses are presented as the areas under the curves measured in the 6 h period after the induction of oedematous response. Values are expressed as the means±s.e.m. of 4–6 experiments.

Table 1. Effect of diphenhydramine and methysergide on venom PLA_2-induced paw oedema.

	Oedema (AUC)[b]	
Drugs (mg kg^{-1})[a]	$TMVPLA_2$ I	$TMVPLA_2$ II
Control	223·7±6·7	193·3±10·2
Diphenhydramine (5)	163·9±14·1**[c]	130·8±7·8***
(10)	150·2±5·7***	125·8±4·1***
Methysergide (1)	126·3±10·4***	148·7±4·7**
(10)	97·1±8·7***	144·8±8·9**
Diphenhydramine (10) + methysergide (10)	69·6±5·5***	37·6±5·5***

[a] Drugs were given s.c. 1 h before subplantar injection of $TMVPLA_2$ I (5 μg) or $TMVPLA_2$ II (5 μg). [b] Areas under the curves (means±s.e.m.) measured in the 6 h period of 5–6 experiments. [c] Treatment vs control. **$P<0.01$; ***$P<0.001$.

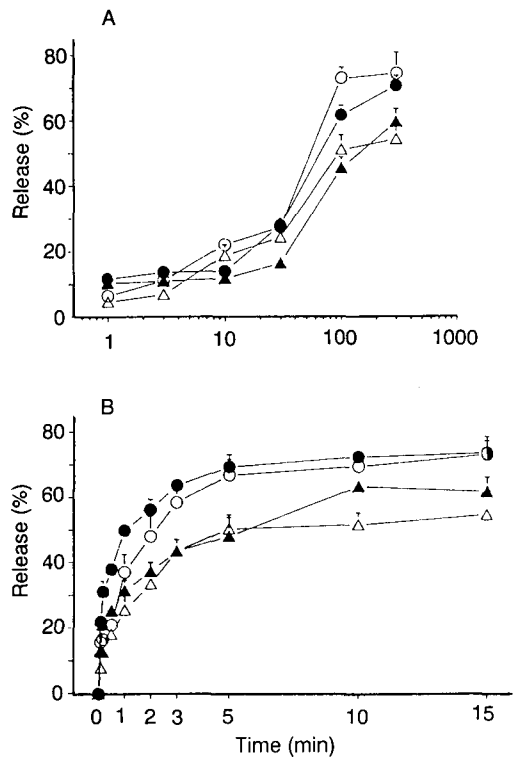

FIG. 2. Concentration (A)- and time (B)-dependent release of histamine (○,●) and β-glucuronidase (△,▲) from isolated rat peritoneal mast cells stimulated with $TMVPLA_2$ I (○,△) or $TMVPLA_2$ II (●,▲). $TMVPLA_2$ I 100 μg mL^{-1} and $TMVPLA_2$ II 200 μg mL^{-1} were used in the time-dependent experiments (B). Values are expressed as the means±s.e.m. of 5–6 experiments.

suppressed, while increase of extracellular calcium concentrations enhanced, the degranulation of mast cells. Preincubation of mast cells with disodium cromoglycate (10^{-3} M), mepacrine (3×10^{-5} M) or nordihydroguaiaretic acid (10^{-5} M) significantly inhibited the PLA_2 enzyme-induced mast cell degranulation (Table 2) while aspirin (3×10^{-4} M) and indomethacin (3×10^{-5} M) had no significant inhibition although there was a reduction in PGE_2 (Table 3). Piriprost (5×10^{-4} M) reduced LTB_4 formation to 5 and 25% of the control values of $TMVPLA_2$ I- and $TMVPLA_2$ II-treated

Table 2. Effect of disodium cromoglycate, mepacrine and nordihydroguaiaretic acid on the release of histamine and β-glucuronidase from rat peritoneal mast cells stimulated with venom PLA_2.

	Release (%)[a]	
Drugs[b]	Histamine	β-Glucuronidase
$TMVPLA_2$ I (200 μg mL^{-1})	73·1 ± 4·1	50·8 ± 2·8
+ disodium cromoglycate (10^{-3} M)	46·0 ± 7·0**[c]	33·6 ± 3·8***
+ mepacrine (3×10^{-5} M)	44·9 ± 4·2***	34·4 ± 2·6**
+ nordihydroguaiaretic acid (10^{-5} M)	59·8 ± 2·2***	32·7 ± 3·5**
$TMVPLA_2$ II (100 μg mL^{-1})	64·8 ± 3·5	45·9 ± 0·9
+ disodium cromoglycate (10^{-3} M)	19·2 ± 2·6***	16·1 ± 2·4***
+ mepacrine (3×10^{-5} M)	47·8 ± 3·3**	30·6 ± 1·3**
+ nordihydroguaiaretic acid (10^{-5} M)	50·8 ± 2·4*	37·8 ± 2·8*

[a] Values are expressed as the means ± s.e.m. of at least four experiments. [b] Mast cell suspension was preincubated with disodium cromoglycate (15 min), mepacrine (5 min) or nordihydroguaiaretic acid (5 min) before stimulation with venom PLA_2. [c] Treatment vs control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 3. Effect of piriprost, aspirin and indomethacin on the release of histamine and β-glucuronidase, and formation of PGE_2 and LTB_4 from rat peritoneal mast cells stimulated with venom PLA_2.

	Percent release[a] (%)		Arachidonate metabolites[b] (pg/10^5 cells)	
Drugs[c]	Histamine	β-Glucuronidase	PGE_2	LTB_4
$TMVPLA_2$ I (200 μg mL^{-1})	74·0 ± 2·3	54·5 ± 1·6	32·5 ± 8·4	147·6 ± 17·3
+ piriprost (5×10^{-4} M)	55·4 ± 4·9**[d]	41·2 ± 2·3***	93·2 ± 16·6***	7·9 ± 0·7***
+ aspirin (10^{-3} M)	74·1 ± 6·6	54·2 ± 3·5	0·6 ± 0·1***	615·0 ± 209·4*
+ indomethacin (3×10^{-5} M)	70·9 ± 3·3	56·0 ± 3·4	0·6 ± 0·2***	264·0 ± 56·8*
+ BW 755C (10^{-3} M)	36·8 ± 1·8***	29·7 ± 2·9***	0·4 ± 0·1***	4·1 ± 0·4***
$TMVPLA_2$ II (100 μg mL^{-1})	60·9 ± 3·5	44·7 ± 5·5	61·6 ± 9·8	35·5 ± 11·3
+ piriprost (5×10^{-4} M)	45·0 ± 6·2*	33·4 ± 5·7	91·2 ± 14·3	8·8 ± 0·2*
+ aspirin (10^{-3} M)	63·7 ± 7·0	44·5 ± 3·4	1·0 ± 0·3***	54·5 ± 3·9*
+ indomethacin (3×10^{-5} M)	65·7 ± 3·5	45·2 ± 2·7	1·8 ± 0·6***	88·2 ± 28·0*
+ BW 755C (10^{-3} M)	34·8 ± 3·6***	30·3 ± 2·2**	0·4 ± 0·2***	7·6 ± 0·6**

[a,b] Values are expressed as the means ± s.e.m. of at least four experiments. [c] Mast cell suspension was preincubated with drugs for 5 min before stimulation with venom PLA_2. [d] Treatment vs control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

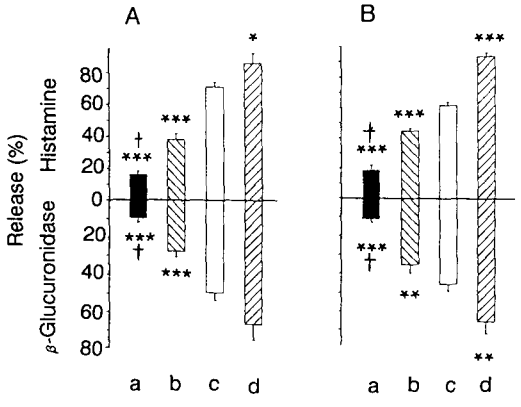

FIG. 3. Effects of various extracellular calcium concentrations on mast cell degranulation caused by $TMVPLA_2$ I 100 μg mL^{-1} (A) or $TMVPLA_2$ II 100 μg mL^{-1} (B). EDTA 1 mM (a); saline (b); Ca^{2+} 1 mM (c) or 2 mM (d) was added into Ca^{2+}-free reaction mixture prewarmed at 37°C for 3 min before $TMVPLA_2$ challenge. Values are expressed as the mean ± s.e.m. of 5–6 experiments. Statistically significant differences from the corresponding control values (c) are noted as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and from the corresponding values in (b) groups are noted as † $P < 0.001$.

groups, respectively, and also suppressed their mast cell degranulation response to about 75%.

Discussion

A dose-dependent oedematous response of rat paw was induced by $TMVPLA_2$ I and $TMVPLA_2$ II. More than 70% of the intensity of these paw oedemas was suppressed by diphenhydramine and methysergide. In addition, pretreatment of the rats with compound 48/80 significantly reduced the paw histamine and 5-HT content (Di Rosa et al 1971), and also suppressed the enzyme-induced paw oedema. Evidence from experiments with rat isolated peritoneal mast cells showed that $TMVPLA_2$ I and $TMVPLA_2$ II released histamine and β-glucuronidase in a concentration- and time-dependent manner. This in-vitro effect and the in-vivo oedematous response were produced over a comparable dose-range. These observations indicate that mast cells play a predominant role in the enzyme-induced paw oedema.

The enzymatic activity of $TMVPLA_2$ I and $TMVPLA_2$ II was 18 ± 5 and 101 ± 2 μmol min^{-1} (mg protein)$^{-1}$, respectively (Teng et al 1989), but the enzymes showed similar

potency in inducing oedema formation and mast cell degranulation. The dissociation between phospholipid hydrolytic activity and pharmacological properties of PLA_2 have been demonstrated (Condrea et al 1981, 1982). In the case of pharmacological effects independent of catalytic activity, absolute affinity is the primary factor which determines pharmacological potency (Kini & Evans 1989). The presence of separate sites for catalytic and pharmacological properties has also been suggested (Rosenberg et al 1983; Rosenberg 1986), which could explain the lack of correlation between the enzymatic and pharmacological activities of the two enzymes used in this study. However, the substrate(s) for these enzymes in the cell membrane is so far unknown.

Both enzymes induced mast cell degranulation that was influenced by the extracellular calcium concentration, with more than a 70% depression in the presence of EDTA, and an enhanced reaction with an increase in concentration.

Disodium cromoglycate acts against the triggering action of PLA_2 on mast cells (Orr & Cox 1969; Rosenberg 1979). It also inhibits the mast cell degranulation caused by concanavalin A, dextran, antigen and compound 48/80 (Garland & Mongar 1974; Foreman et al 1975; Ennis et al 1980; Hanahae 1984). This effect could be due to a block of calcium influx (Ennis et al 1981; Pearce et al 1984) or to the promotion of phosphorylation of a 78 000 daltons protein from mast cell (Sieghart et al 1981). In general, mast cell activation is associated with activation of membrane phospholipid metabolism (Kennerly et al 1979; Martin & Lagunoff 1979). Mepacrine, a PLA_2 inhibitor (Vargaftig & Dao Hai 1972), nordihydroguaiaretic acid and piriprost, both lipoxygenase inhibitors (Hamberg 1976; Bach et al 1982), reduced $TMVPLA_2$ I- and $TMVPLA_2$ II-induced mast cell degranulation. In contrast, the cyclo-oxygenase inhibitors, aspirin and indomethacin, at a concentration that greatly reduced PGE_2 formation by mast cells, had no effect on these venom PLA_2-induced mast cell degranulation. Similar findings were also demonstrated in the degranulation of mast cells pretreated with cyclo-oxygenase inhibitor then triggered by other secretagogues (Lewis et al 1979; Sullivan & Parker 1979; Chi et al 1982). BW 755C, a dual inhibitor of cyclo-oxygenase/lipoxygenase (Higgs et al 1979), greatly suppressed the PGE_2 and LTB_4 formation and also suppressed the release of histamine and β-glucuronidase from mast cells. These results suggest that intact lipoxygenase pathways could be necessary to venom PLA_2-induced mast cell degranulation.

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