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Effect of thielocin A1β on bee venom phospholipase A_2-induced edema in mouse paw

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Abstract

Several investigators have reported that inactivation of secretory phospholipase A_2 purified from bee venom with *p*-bromophenacyl bromide, an irreversible inhibitor, before injection resulted in attenuation of the subsequent inflammatory reaction in the mouse paw edema model. Recently, thielocin A1β, a novel secretory phospholipase A_2 inhibitor from fungi, was found to suppress histamine release from mast cells stimulated with secretory phospholipase A_2. These observations led us to examine the effect of thielocin A1β against secretory phospholipase A_2-induced paw edema. Thielocin A1β inhibited bee venom phospholipase A_2 in a dose-dependent manner ($IC_{50} = 1.4$ μM). In addition, the inhibition of bee venom phospholipase A_2 was noncompetitive ($K_i = 0.57$ μM) and reversible. Subplantar injection of bee venom phospholipase A_2 produced a rapid but transient edematous response. Coinjection of thielocin A1β (1 μg/paw) with bee venom phospholipase A_2 resulted in a $44.7 \pm 4.6\%$ reduction of edema formation. This anti-edema action was not enhanced by cyproheptadine (antihistamine/anti-serotonin). These results suggest that thielocin A1β shows edema-reducing activity via inhibition of the phospholipase A_2 activity which participates in histamine release by mast cells.

Keywords: Phospholipase A_2; Thielocin A1β; Paw edema; Anti-inflammatory drug

1. Introduction

The phospholipase A_2 enzymes are a diverse family of important enzymes which have attracted considerable attention because of their role in the production of potent inflammatory mediators such as prostaglandins, leukotrienes and platelet-activating factor (Dennis, 1983). Phospholipase A_2 enzymes exist in both extracellular and intracellular forms. The best studied extracellular phospholipase A_2, the secretory phospholipase A_2, are 14-kDa Ca^{2+}-dependent enzymes purified from venom (snake, bee, etc.) and pancreatic juice (Vadas and Pruzanski, 1986). In the last 10 years, high levels of secretory phospholipase A_2 activity have been found in association with localized sites of inflammation in both experimental animals and in humans, such as glycogen-induced ascitic fluid in rabbits (Franson et al., 1978), casein-induced peritoneal fluid in rats (Chang et al., 1987) and carrageenan-induced pleural exudate in rats (Tanaka et al., 1993). In addition, some inflammatory cytokine and lipopolysaccharides dramatically increase secretory phospholipase A_2 secretion in several tissues of rats through enhancement of gene transcription (Nakano et al., 1990; Oka and Arita, 1991). Moreover, accumulation of secretory phospholipase A_2 mRNA in tissues of endotoxin-treated rats was suppressed by dexamethasone administration (Nakano and Arita, 1990). Pruzanski and Vadas (1988) have shown the possible role of secretory phospholipase A_2 in arthritis. Approximately 25% of patients with rheumatoid arthritis were found to have high circulating secretory phospholipase A_2 activity. Serum secretory phospholipase A_2 levels in rheumatoid arthritis are significantly correlated with clinical and laboratory markers of disease activity (Pruzanski et al., 1988). Furthermore, intraarticular injection of snake venom phospholipase A_2 in rat joints caused dose-dependent inflammation which resembles the rheumatoid process in humans (Vadas et al., 1989).

Several investigators (Marshall et al., 1989; Cirino et al., 1989) have reported that inactivation of secretory

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phospholipase A_2s purified from snake venom or bee venom with *p*-bromophenacyl bromide, an irreversible inhibitor, before injection resulted in attenuation of the subsequent inflammatory reaction both in the rat and in the mouse paw edema model. These observations tend to suggest that the ability of secretory phospholipase A_2 to cause edema formation is linked to its catalytic properties. Recently, Murakami et al. (1992) reported that thielocin A1β, a novel secretory phospholipase A_2 inhibitor from fungi, suppressed degranulation in rat and mouse mast cells stimulated with various secretagogus without affecting prostaglandin D_2 synthesis, and postulated that endogenous secretory phospholipase A_2 released from activated mast cells may have a crucial role in the progression of the degranulation process. If this is the case, one might anticipate that inhibition of secretory phospholipase A_2 would attenuate the severity of inflammation via suppression of degranulation.

The purpose of the present investigation was to further elucidate the inhibitory mechanism of thielocin A1β against bee venom phospholipase A_2 by studying the effect of thielocin A1β on bee venom phospholipase A_2-induced edema in the mouse paw test. The combined effect of thielocin A1β and antihistamine/antiserotonin agents was also examined.

2. Materials and methods

2.1. Materials

Thielocin A1β was purified as previously reported (Yoshida et al., 1991; Matsumoto et al., 1995). L-3-Phosphatidylethanolamine, 1-palmitoyl-2-[1-^{14}C]-linoleoyl (2.18 GBq/mmol) was purchased from Amersham Corp. L-α-Phosphatidylethanolamine (from egg yolk), bee venom phospholipase A_2, *Naja mocambique mocambique* (cobra) phospholipase A_2 (p*I* 8.8), *p*-bromophenacyl bromide, cyproheptadine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phospholipase A_2s were used as supplied. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis of these commercial enzymes was more than 85–95% pure. All other reagents were of analytical grade or better.

2.2. Assay of phospholipase A_2

Phospholipase A_2 activity was measured by a method described previously (Tanaka et al., 1992). The substrate was prepared by diluting 1-palmitoyl-2-[1-^{14}C]linoleoyl phosphatidylethanolamine with L-α-phosphatidylethanolamine to a specific activity of 2000 dpm/nmol. The reaction was started by addition of the enzyme. The amount of phospholipase A_2 was adjusted to optimize the linear kinetics for quantitation, i.e., hydrolysis of the substrate was less than 20% in all experiments. Thielocin A1β and *p*-bromophenacyl bromide were added to the assay tubes in dimethyl sulfoxide (DMSO) solution (2% of the final volume), using a DMSO-enzyme control. Control experiments showed that DMSO at this concentration had no effect on enzymatic activities. Inhibition is expressed as the percent of enzyme control.

2.3. Bee venom phospholipase A_2-induced paw edema in mice

The edema produced by bee venom phospholipase A_2 was assayed according to the method of Vishwanath et al. (1988) Male 6- to 8-week-old JCL-ICR mice (weighing 25–35 g) were injected subplantarly into the left paw with the indicated amounts of bee venom phospholipase A_2 in a total volume of 25 μl of sterile phosphate buffer saline (PBS) solution. At the time indicated (0–60 min), the mice were killed under ether anesthesia, and both hind limbs were removed at the ankle joint and weighed individually. The increase in weight due to edema was calculated by subtracting the weight of each non-treated right hind limb. Thielocin A1β, up to 20 mg/ml, was solubilized in DMSO (not more than 0.2% final concentration in sterile PBS solution). DMSO at this concentration had no effect on the volume of the hind limb when compared to the PBS vehicle control alone, and also did not alter phospholipase A_2-induced edema formation. For oral administration, cyproheptadine was suspended in sterile water containing 5% gum arabic and administered in a volume of 0.25 ml 1 h before phospholipase A_2 injection. The significance of the difference between the mean of the control and that of the drug-treated group was evaluated using Student's *t*-test.

3. Results

3.1. Inhibition of bee venom phospholipase A_2 activity by thielocin A1β

Thielocin A1β showed strong inhibitory activity against bee venom phospholipase A_2 in a dose-dependent manner with an IC_{50} value of 1.4 μM. In contrast, *p*-bromophenacyl bromide, an irreversible phospholipase A_2 inhibitor, showed weak inhibitory activity (IC_{50} = 80 μM, Fig. 1) against bee venom phospholipase A_2. Even at 1000 μM, phospholipase A_2 activity remained 35.0 $\pm$ 4.8% of the control. The reversible characteristics of thielocin A1β were confirmed using the dilution method according to Lister et al. (1989) (Table 1). Bee venom phospholipase A_2 was preincubated with thielocin A1β (37° C, 20 min) at 30 μM, a

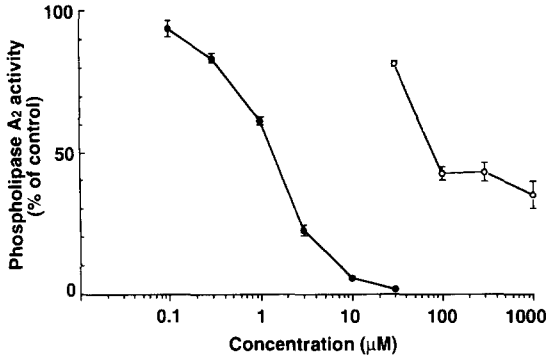

Fig. 1. Inhibition of bee venom phospholipase A_2 by thielocin A1β (•) and *p*-bromophenacyl bromide (○). Inhibition is expressed as the percent of enzyme control. Each value represents the mean ± S.E.M. of three independent experiments, each performed in duplicate and corrected for no enzymatic hydrolysis (0.5% or less in all experiments). *p*-Bromophenacyl bromide was preincubated with bee venom phospholipase A_2 for 20 min at 37° C without Ca^{2+}.

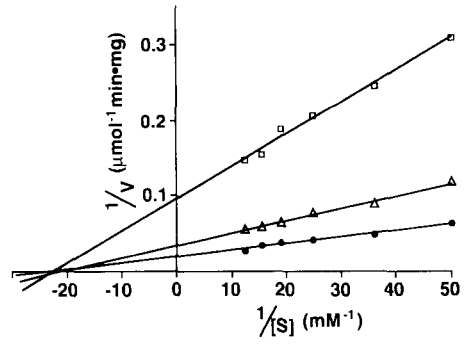

Fig. 2. Noncompetitive inhibition of bee venom phospholipase A_2 by thielocin A1β. Double reciprocal plot of bee venom phospholipase A_2 activity toward phosphatidylethanolamine in the presence (0.5 μM, △ and 2 μM, □) or absence (•) of thielocin A1β. Standard assay conditions were used and the lines were drawn on the basis of regression analysis.

concentration high enough to sufficiently reduce the enzymatic activity (see Fig. 1). After the preincubation, an aliquot was removed and diluted 30-fold to 1 μM in the assay mixture. Slight inhibition was observed, indicating reversible inhibition. A similar result was observed when the thielocin A1β concentration in the preincubation period was set at 90 μM and then diluted to 3 μM during the assay. However, *p*-bromophenacyl bromide showed similar inhibitory activity before (at 300 μM, 43 ± 3%; at 1000 μM, 35 ± 5%) and after dilution (at 10 μM, 45 ± 5%; at 33 μM, 38 ± 4%), indicating irreversible inhibition. Furthermore, the double reciprocal plot showed that thielocin A1β behaves kinetically as a noncompetitive inhibitor for bee venom phospholipase A_2 with a K_i value of 0.57 μM (Fig. 2).

3.2. Effect of thielocin A1β on bee venom phospholipase A_2-induced paw edema in mice

Injection of bee venom phospholipase A_2 (1 μg) into mouse hind footpad resulted in significant formation of paw edema even 5 min after injection. Maximum edema was achieved within 30 min after the injection (Fig. 3A). The dose-response relationship for edema formation (0.01–3.0 μg/paw) was also determined (Fig. 3B). Because in vitro bee venom phospholipase A_2 was strongly inhibited by thielocin A1β (IC_{50} = 1.4 μM), we examined the effect of thielocin A1β on the edema-inducing activity of bee venom phospholipase A_2. Thielocin A1β did not cause edema formation when compared with the PBS vehicle control alone. Coinjection of thielocin A1β (1 μg/paw) with phospholipase A_2 resulted in significant reduction of edema formation (42.4 ± 8.3% or 63.8 ± 6.5% respectively, Table 2). However, coinjection of thielocin A1β at doses of 0.1 and 0.3 μg/paw did not result in a significant reduction in paw edema. Subplanter injection of the venom phospholipase A_2 from *Naja mocambique mocambique* also produced an edematous response. Nevertheless, coinjection of thielocin A1β (1 μg/paw) with *Naja mocambique mocambique* venom phospholipase A_2 (1 μg/paw) did not result in a significant reduction in paw edema (enzyme alone, 39.8 ± 4.9 mg; enzyme with thielocin A1β, 46.0 ± 4.8 mg).

Marshall et al. (1989) have reported that oral administration of cyproheptadine (antihistamine/antiserotonin) results in a dose-dependent reduction of bee

Table 1
Distinction between reversible and irreversible inhibition by thielocin A1β and *p*-bromophenacyl bromide of bee venom phospholipase A_2

Compound	Concentration (μM)		Phospholipase A_2 activity (% of control) [c]
	Preincubation [a]	Assay [b]	
Thielocin A1β	30	1.0	93 ± 11
	90	3.0	86 ± 4
p-Bromophenacyl bromide [d]	1000	33	38 ± 4
	300	10	45 ± 5

[a] Bee venom phospholipase A_2 was preincubated with inhibitor at the designated concentration for 20 min at 37° C. [b] Inhibitor concentration after dilution for assay. [c] Results are means ± S.E.M. of triplicate determinations, each performed in triplicate. [d] *p*-Bromophenacyl bromide was preincubated without Ca^{2+}.

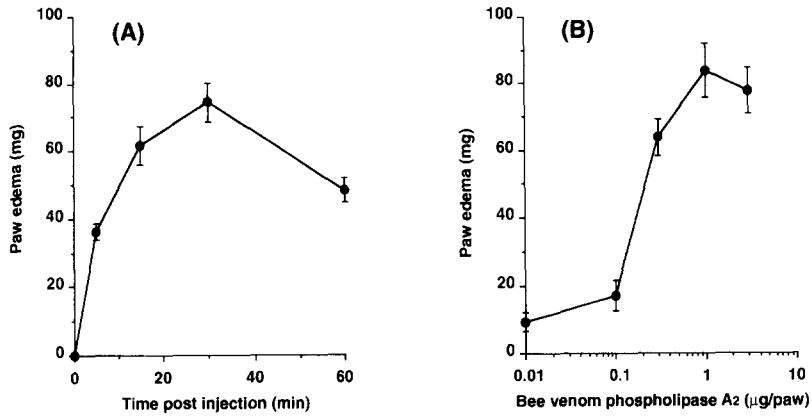

Fig. 3. Bee venom phospholipase A_2-induced mouse paw edema. (A) Time course of edema induced by bee venom phospholipase A_2. Phospholipase A_2, 1 μg, was injected into mouse paw and edema was measured at the indicated time. Each value represents the mean ± S.E.M. obtained from 5 animals. (B) Effect of protein concentration in edema-inducing activity of bee venom phospholipase A_2. Phospholipase A_2, 0.01–3.0 μg, was injected into mouse paw and edema was measured at 30 min. Each value represents the mean ± S.E.M. obtained from 5 animals.

venom phospholipase A_2-induced paw edema (0.3 and 3.0 mg/kg). However, we found that cyproheptadine (0.3 mg/kg p.o.) did not significantly enhance the anti-edema action of thielocin A1β (1.0 μg/paw) (Fig. 4).

4. Discussion

Secretory phospholipase A_2 enzymes can be classified into two types, group I and group II, based on their primary structures. Mammalian group I phospholipase A_2 abundantly occurs in the pancreas and has long been thought to act as a digestive enzyme (Nevalainen, 1980). The other type, mammalian group II phospholipase A_2, is considered to be involved in the pathogenesis of both experimental and clinical inflammatory states (Pruzanski and Vadas, 1991; Vadas et al., 1993). This led many investigators to expend enormous efforts to find secretory phospholipase A_2 inhibitors to better understand the role of this enzyme in the inflammatory process and to enable its clinical use in the treatment of inflammation and related disorders. Contrary to popular belief, many secretory phospholipase A_2 inhibitors might be amphipathic with a low critical micelle concentration (Wilkerson, 1990; Tanaka and Arita, 1995).

With the use of secretory phospholipase A_2 purified from rat platelets, we successfully isolated a novel secretory phospholipase A_2 inhibitor from fungi and designated it thielocin A1β (Yoshida et al., 1991). Thielocin A1β inhibited various secretory phospholipase A_2s in a dose-dependent manner. Among them, rat group II phospholipase A_2 was the most sensitive to thielocin A1β (IC_{50} = 0.0033 μM). However, it showed weak inhibitory activity against phospholipase A_2 purified from rat pancreas, which belongs to group I phospholipase A_2, with an IC_{50} of 21 μM. Thus, thielocin A1β inhibition of rat group II phospholipase

Table 2
Effect of thielocin A1β on bee venom phospholipase A_2-induced paw edema in mice

Treatment (1 μg/paw)		Paw edema (mg)	
Phospholipase A_2	Thielocin A1β	Exp. 1	Exp. 2
(−)	(−)	7.7±5.0	3.8±1.1
(−)	(+)	10.2±2.2	7.8±1.6
(+)	(−)	80.5±4.7	64.0±4.8
(+)	(+)	46.3±6.7 [b]	23.2±4.1 [c]
		(57.6±8.3)	(36.2±6.5)

Each value represents the mean ± S.E.M. obtained from five animals. The numbers in parentheses express the percentage of the phospholipase A_2-treated group. [b] $P < 0.005$, [c] $P < 0.001$ vs. phospholipase A_2-treated group.

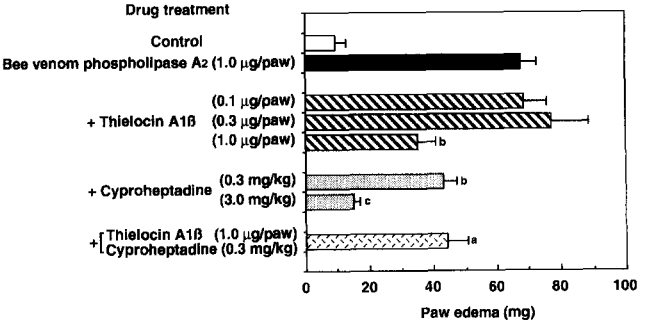

Fig. 4. Effect of thielocin A1β and cyproheptadine on bee venom phospholipase A_2-induced mouse paw edema. The indicated amount of thielocin A1β was mixed with 1 μg of bee venom phospholipase A_2 and injected into the mouse paw. Cyproheptadine was suspended in sterile water containing 5% gum arabic and administered orally in a volume of 0.25 ml 1 h before phospholipase A_2 injection. After 30 min, edema was measured. Each value represents the mean ± S.E.M. obtained from 5 animals. [a] $P < 0.05$, [b] $P < 0.005$, [c] $P < 0.001$ vs. phospholipase A_2-treated group.

A_2 was 6.4×10^3 times greater than its inhibition of rat group I phospholipase A_2. In addition, its inhibition was independent of both Ca^{2+} and substrate concentration and was also not affected by the substrate form (*Escherichia coli* membranes, phospholipids presented as surfactant mixed micelles or sonicated liposomes) or the species of phospholipid used. These observations indicated that inhibition of secretory phospholipase A_2 by thielocin A1β results from direct interaction with the enzyme (Tanaka et al., 1992). Recently, we demonstrated the antiinflammatory effect of thielocin A1β and the involvement of secretory phospholipase A_2 in the pathogenesis of rat carrageenan pleurisy. At 5 h after coinjection of thielocin A1β with carrageenan in the pleural cavity, both the number of leukocytes and the amount of protein in the exudate significantly decreased, and the exudate volume in the pleural cavity decreased dose dependently ($ED_{50} = 0.54$ mg/kg). Furthermore, secretory phospholipase A_2 activity in the pleural exudate was also decreased ($IC_{50} = 0.060$ mg/kg) by coadministration of thielocin A1β. The decrease in secretory phospholipase A_2 activity after various doses of thielocin A1β correlated well with the reduction in the exudate volume ($r = 0.85$; $P < 0.01$) (Tanaka et al., 1993).

Marshall et al. (1989) reported that injection of snake venom phospholipase A_2 into the mouse hind footpad produced a significant 3- to 4-fold rise in paw edema, compared to the saline control. Edema formation depended on the enzyme concentration and appeared to be specific for secretory phospholipase A_2 because it did not occur on enzyme pretreatment with *p*-bromophenacyl bromide, a nonspecific irreversible phospholipase A_2 inhibitor (Kyger and Franson, 1984). This led us to examine the effect of thielocin A1β against secretory phospholipase A_2-induced mouse paw edema. Although thielocin A1β possesses strong inhibitory activity against rat group II phospholipase A_2, this enzyme did not cause a stable significant rise in paw edema like that reported by Murakami et al. (1990). In support of this, Mounier et al. (1994) proposed that, in contrast to venom phospholipase A_2s, mammalian phospholipase A_2s may not hydrolyze the phospholipids contained in the outer leaflet of the cell membrane by the 'penetrating' capacity. More recently, Cirino et al. (1994) also reported that human recombinant group II phospholipase A_2, injected in the rat paw, did not cause edema formation. However, subplantar injection of human group II phospholipase A_2 was able to induce edema formation by co-injection of a cationic substance (poly-L-arginine). These observations maybe suggest that mammalian phospholipase A_2 needs a physiological counterpart (such as cationic substance, cytokine, growth factor and so on which makes the cell surface susceptible to the enzyme) to trigger the inflammatory responses. Other than rat group II phospholipase A_2, bee venom phospholipase A_2 was most sensitive to thielocin A1β in vitro (Tanaka et al., 1992). Interestingly, bee venom phospholipase A_2 has the characteristics of mammalian group II phospholipase A_2 except that it lacks the two half-cystines that form the Cys69–100 disulfide bridge (Davidson and Dennis, 1990). Therefore, we chose bee venom phospholipase A_2 as a proinflammatory enzyme to study secretory phospholipase A_2-induced edema in mouse paw.

The present experiments showed that inhibition of bee venom phospholipase A_2 by thielocin A1β was noncompetitive and reversible (Table 1, Fig. 2). These results distinguish thielocin A1β from agents such as manoalide and *p*-bromophenacyl bromide, which inhibit secretory phospholipase A_2 by covalent modifications of lysine and histidine residues, respectively (Lombardo and Dennis, 1985; Kyger and Franson, 1984). Thielocin A1β inhibited bee venom phospholipase A_2 completely at 30 μM (Fig. 1). Coinjection of thielocin A1β at 1 μg/paw (equivalent to 40 μM which can completely inhibit bee venom phospholipase A_2 activity in vitro) with the same dose of bee venom phospholipase A_2 resulted in a significant reduction in edema formation (Table 2). Coinjection of thielocin A1β with the same amount of *Naja mocambique mocambique* phospholipase A_2 did not show significant reduction. This might be because of the less potent inhibitory activity of thielocin A1β against *Naja mocambique mocambique* phospholipase A_2 ($IC_{50} = 9.3$ μM) than against bee venom phospholipase A_2 ($IC_{50} = 2.0$ μM). These results also demonstrate that thielocin A1β shows anti-edema activity due to inhibition of phospholipase A_2 activity. Cirino et al. (1989) reported that pretreatment of bee venom phospholipase A_2 with 1 mM of *p*-bromophenacyl bromide resulted in a weak (39%) reduction in bee venom phospholipase A_2-induced paw edema. In our experiment, 1 mM of *p*-bromophenacyl bromide could not inhibit bee venom phospholipase A_2 activity completely, but showed partial inhibition in vitro (Fig. 1). These observations indicate that complete inhibition of bee venom phospholipase A_2 is needed for a significant reduction of bee venom phospholipase A_2-induced paw edema.

Several investigators (Brain et al., 1977; Morita et al., 1983) reported that secretory phospholipase A_2, including bee venom phospholipase A_2, provoked the release of mast cell histamine in vitro. Thielocin A1β can suppress histamine release from both rat and mouse mast cells (Murakami et al., 1992). Also, cyproheptadine (antihistamine/antiserotonin) did not enhance the anti-edema action of thielocin A1β (Fig. 4). These results indicated that thielocin A1β shows edema-reducing activity by inhibiting phospholipase A_2 activity, which participates in histamine release by mast cells or basophils.

All the findings described thus far suggest that a secretory phospholipase A_2 inhibitor might be useful as a novel therapeutic and/or prophylactic drug for allergic diseases. We are now conducting further studies using thielocin A1β to clarify the role of secretory phospholipase A_2 in allergic diseases.

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