

## INHIBITION OF IMMEDIATE-TYPE ALLERGIC REACTIONS BY *PRUNELLA VULGARIS* IN A MURINE MODEL

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### ABSTRACT

We studied the effect of aqueous extract of *Prunella vulgaris* (Labiatae) (PVAE) on immediate-type allergic reactions. PVAE (0.005 to 1 g/kg) dose-dependently inhibited systemic anaphylactic shock induced by compound 48/80 in rats. When PVAE was given as pretreatment, at concentrations ranging from 0.005 to 1 g/kg, the serum histamine levels induced by compound 48/80 were reduced in a dose-dependent manner. PVAE (0.001 to 1 g/kg) inhibited the passive cutaneous anaphylaxis activated by anti-dinitrophenyl (DNP) IgE antibody dose dependently. PVAE also inhibited the histamine release induced by compound 48/80 or anti-DNP IgE from the rat peritoneal mast cells (RPMC). The level of cyclic AMP in RPMC, when PVAE was added, significantly increased, compared with that of normal control. Moreover, PVAE (0.01 and 0.1 mg/ml) had a significant inhibitory effect on anti-DNP IgE-mediated tumor necrosis factor- $\alpha$  production from RPMC. These results indicate that PVAE inhibits immediate-type allergic reactions in rats.

### INTRODUCTION

The *Prunella vulgaris* var. *lilacina* Nakai (Korean name: Ha-go-cho, Labiatae) is a perennial herb which is widely distributed throughout Korea, Japan and

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China. Ha-go-cho refers to the plant as *Prunella vulgaris* itself, or its fruitspike, or inflorescence. The herba have been used as a traditional Oriental medicine for the treatment of scrofula, goitre, dermatosis and skin allergic diseases in South Korea (1–3). The primary effector cell in immediate-type allergic reactions is the mast cells. Mast cell degranulation can be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells (4). Compound 48/80 is best known as a potent inducer of degranulation and of the release, from mast cells, of histamine and other chemical mediators which are responsible for anaphylactic reactions (5). An appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylactic reaction (6,7). These cells express on their surface a receptor protein, Fcε RI, which binds with high affinity the Fc region of immunoglobulin E (IgE) (8). Aggregation of surface IgE molecules by multivalent antigen results in the activation of an array of signal transduction pathways, leading eventually to the release of inflammatory mediators from mast cell secretory granules (9,10). It has been established that the anti-IgE antibody induces passive cutaneous anaphylaxis (PCA) as a typical *in vivo* model for mast cell-dependent immediate-type allergic reaction. Rats or guinea pig skins are useful sites for studying PCA (11). Although mast cells also store small amounts of cytokines in their granules (12), these cells dramatically increase their production of tumor necrosis factor-α (TNF-α), IL-6 and other cytokines within 30 min after their surface Fcε RI receptors are cross-linked with specific antigen (13–16). Since its introduction in 1968, disodium cromoglycate (DSCG) has been widely used in the prophylactic treatment of bronchial asthma. In experimental allergic models, DSCG (a mast cell stabilizer) shows an obvious inhibitory effect on the immediate-type allergic reactions *in vivo* and *in vitro*. This effect of DSCG is thought to be based on the inhibition of the mediator release from mast cells (17,18). We evaluated the inhibitory effects of aqueous extract of *Prunella vulgaris* (PVAE) in comparison with those of DSCG.

In the present study, we showed that PVAE inhibited both compound 48/80-induced systemic anaphylactic shock and anti-dinitrophenyl (DNP) IgE antibody-induced PCA. We also investigated the influence of PVAE on compound 48/80-induced intracellular cyclic AMP (cAMP) level and anti-DNP IgE-induced TNF-α production in rat peritoneal mast cells (RPMC).

## MATERIALS AND METHODS

### Reagents

Compound 48/80, anti-DNP IgE, DSCG, ketotifen, DNP-human serum albumine (HSA), α-minimal essential medium (α-MEM), o-phthalaldehyde and metrizamide were purchased from Sigma Chemical Co.(St. Louis, MO). cAMP was purchased from Amersham Pharmacia Biotec (UK), and TNF-α was obtained from R & D Systems Inc. (USA).

### Animals

The original stocks of male Sprague-Dawley rats (200–300 g) were purchased from Dae Han Experimental Animal Center (Taejon, South Korea) and the animals were maintained in the College of Pharmacy, Woosuk University. The rats were housed five per cage in a laminar air flow room maintained under the temperature of  $22 \pm 2^\circ\text{C}$  and relative humidity of  $55 \pm 5\%$  throughout the study.

### Preparation of PVAE

The plants of *Prunella vulgaris* were collected in Wanju, South Korea, on the 19th of July, 1999. The plant was identified by D.K.Kim (College of Pharmacy, Woosuk University) and the voucher specimen (WSP-99-36) was deposited at the Herbarium of the College of Pharmacy, Woosuk University. The plant sample was extracted with distilled water at  $70^\circ\text{C}$  for 5 h (two times). The extract was filtered through a  $0.45 \mu\text{m}$  filter, lyophilized and kept at  $4^\circ\text{C}$ . The yield of dried extract from starting crude materials was about 10.1%. The dried extract was dissolved in saline or Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 5.6 mM glucose, 0.1% bovine serum albumin) before use.

### Compound 48/80-Induced Systemic Anaphylactic Reaction

Compound 48/80-induced systemic anaphylactic reaction was examined as previously described (19). Rats were given an intraperitoneal injection of 8 mg/kg of the mast cell degranulator compound 48/80 ( $n = 10/\text{group}$ ). PVAE was administered intraperitoneally from 0.001 to 1 g/kg, 1 h before the injection of compound 48/80. Mortality was monitored for 1 h after induction of anaphylactic shock. After the mortality test, blood was obtained from each rat's heart. DSCG was used as a drug of positive control.

### PCA Reaction

An IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE, followed 48 h later with an injection of DNP-HSA into the rat's tail vein. The DNP-HSA was diluted in PBS. The rats were injected intradermally with  $0.5 \mu\text{g}$  ( $50 \mu\text{l}$ ) of anti-DNP IgE into each of four dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. After 48 h, each rat received an injection of  $100 \mu\text{g}$  ( $100 \mu\text{l}$ ) of DNP-HSA in saline containing 4% Evans blue (1:4) via the

tail vein. The rats were sacrificed 30 min after the challenge and the dorsal skin was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 1 ml of 1M KOH and 9 ml of a mixture of acetone and phosphoric acid (5:13) based on the method of Katayama *et al.* (20). The absorbant intensity of the extraction was measured at 620 nm in a spectrophotometer (Shimadzu, UV-1201, Japan).

### Preparation of Serum and Histamine Determination

The blood was centrifuged at  $400 \times g$  for 10 min. The serum was withdrawn and the histamine content was measured by the o-phthaldialdehyde spectrofluorometric procedure of Shore *et al.* (21). The fluorescent intensity was measured at 438 nm (excitation at 353 nm) in a spectrofluorometer (Shimadzu, RF-5301 PC, Japan).

### Preparation of RPMC

RPMC were isolated as previously described (22). In brief, rats were anesthetized by ether and injected with 20 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO<sub>3</sub>, 2.7 mM KCl, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub> and 0.1% gelatin), into the peritoneal cavity and the abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened and the fluid containing peritoneal cells was aspirated by a Pasteur pipette. Thereafter, the peritoneal cells were sedimented at  $150 \times g$  for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells, i.e. macrophages and small lymphocytes, according to the method described by Yurt *et al.* (23). In brief, peritoneal cells, suspended in 1 ml Tyrode buffer B, were layered on 2 ml of metrizamide (22.5 W/V%) and centrifuged at room temperature for 15 min at  $400 \times g$ . The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml Tyrode buffer A containing calcium. Mast cell preparations were about 95% pure, as assessed by Toluidine blue staining. More than 97% of the cells were viable, as judged by Trypan blue uptake.

### Inhibition of Histamine Release

Purified RPMC were resuspended in Tyrode buffer A for the treatment of compound 48/80. RPMC ( $2 \times 10^5$  cells/ml) were preincubated for 10 min at 37°C before the addition of compound 48/80. The cells were preincubated with PVAE for 10 min at 37°C and washed with Tyrode buffer A, and then incubated (10 min) with compound 48/80. RPMC ( $2 \times 10^5$  cells/ml) were also sensitized with 10 µg/ml anti-DNP IgE for 16 h and preincubated with PVAE at 37°C for 10

min prior to challenge DNP-HSA (1 µg/ml). The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at  $400 \times g$  for 5 min at 4°C. Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at  $400 \times g$  for 5 min at 4°C. PVAE did not absorb the compound 48/80 and anti-DNP IgE.

### Assay of Histamine Release

The inhibition percentage of histamine release was calculated using the following equation:

$$\frac{\text{Histamine release without PVAE} - \text{Histamine release with PVAE}}{\text{Histamine release without PVAE}} \times 100$$

### Assay of TNF- $\alpha$ Production

TNF- $\alpha$  production was measured with the quantitative sandwich enzyme immunoassay technique, using a commercial kit (R & D Systems, U.S.A). RPMC ( $3 \times 10^5$  cells/ml) were sensitized with anti-DNP IgE (1 µg/ml) and incubated for 18 h in the absence or presence of PVAE (0.001 to 0.1 mg/ml) before the challenge DNP-HAS (0.1 µg/ml). TNF- $\alpha$  production was measured by ELISA. The ELISA was performed by coating 4-well plates with murine polyclonal antibody with specificity for murine TNF- $\alpha$  Standard, controls, and samples are pipetted into the wells and any mouse TNF- $\alpha$  present is bound by the immunobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse TNF- $\alpha$  is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution (100 µl) is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop solution (100 µl) is added. The intensity of the color measured is in proportion to the amount of mouse TNF- $\alpha$  bound in the initial step. Optical density readings were made on a Titertek Multiscan (Flow Laboratories) with a 405 nm filter. The sample values are then read off the standard curves.

### Measurement of cAMP Level

The cAMP level was measured according to the method of Peachell *et al.* (24). In brief, purified mast cells were resuspended in prewarmed (37°C) Tyrode buffer A. Typically, an aliquot of cells ( $2 \times 10^5$  cells) were added to an equivalent volume (50 µl) of prewarmed buffer containing the drug in an Eppendorf tube. The reaction was allowed to proceed for discrete time intervals, terminated by the addition of ice-cold acidified ethanol (0.9 ml of 86% ethanol/1 M HCl, 99:1) with brief vigorous vortexing and then snap frozen in liquid nitrogen. The

sample was later thawed and vortexed, then the debris was sedimented in a centrifuge ( $400 \times g$  at  $4^{\circ}\text{C}$ , for 5 min), and an aliquot (0.9 ml) of the supernatant was removed and evaporated to dryness under reduced pressure. The dried sample was reconstituted in assay buffer (150–200  $\mu\text{l}$ ) and stored frozen. The cAMP level was determined by enzyme immunoassay, using a commercial kit (Amersham Pharmacia Biotech).

### Statistical Analysis

The results obtained were expressed as mean  $\pm$  S.E. The Student's t-test was used to make a statistical comparison between the groups. Results with  $p < 0.05$  were considered statistically significant.

## RESULT

### Effect of PVAE on Systemic Anaphylactic Reaction

Initial experiments examined the effects of PVAE on compound 48/80-induced systemic anaphylactic reaction. When PVAE was pretreated at concentrations ranging from 0.001 to 1 g/kg for 1 h, the mortality with compound 48/80 was dose-dependently reduced. Especially, PVAE completely inhibited anaphylactic reaction with the doses of 0.5 and 1 g/kg. In this same *in vivo* experiment, DSCG (0.5 and 1 g/kg) also had an inhibitory effect on compound 48/80-induced systemic anaphylactic reaction 100%. Treatment with drugs (1 g/kg) detected no physiological differences by appearance (Table 1).

Groups of rats ( $n = 10/\text{group}$ ) were intraperitoneally pretreated with 200  $\mu\text{l}$  saline or drugs were given at various doses, 1 h before the compound 48/80

**Table 1.** Effect of PVAE on Compound 48/80-Induced Systemic Anaphylactic Reaction in the Rats

Treatment	Dose (g/kg)	Compound 48/80 (8 mg/kg)	Mortality (%)
None (saline)	—	+	100
PVAE	0.001	+	100
	0.005	+	80
	0.01	+	80
	0.05	+	20
	0.1	+	10
	0.5	+	0
	1	+	0
DSCG	1	—	0
	0.5	+	0
	1	+	0

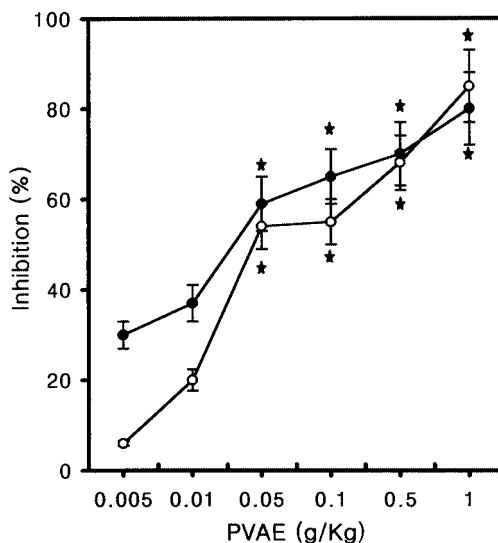
injection. Mortality (%) within 1 h following the compound 48/80 injection was represented as number of dead rats  $\times$  100/number of total experimental rats.

### Effect of PVAE on Serum Histamine Release

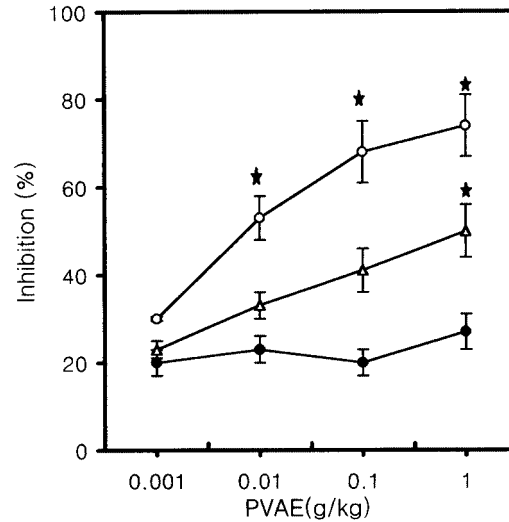
To confirm that the effect of PVAE or DSCG on systemic anaphylactic reaction was due to an inhibited serum histamine release, we analyzed the histamine content after injection of rats with compound 48/80. PVAE was given from 0.005 to 1 g/kg, 1 h before the compound 48/80 injection. The correlative results with those of the mortality test were shown when their sera histamine contents were measured (Fig. 1). The inhibition rate of histamine by PVAE was significant at the doses of 0.05 to 1 g/kg.

### Effect of PVAE on PCA Reaction

Another way to test allergic reactions is to induce PCA (25). When drugs (0.001 to 1 g/kg) was given orally to rats, PVAE inhibited PCA dose-dependently. However, it did not show inhibitory activity when DSCG was given orally to rats. Another reference drug, ketotifen inhibited PCA dose-dependently (Fig. 2).



**Figure 1** Effect of PVAE on compound 48/80-induced serum histamine release in the rat. Groups of rats were intraperitoneally pretreated with PVAE (○) or DSCG (●). Each drug was given with various doses 1 h before ( $n = 10$ /group) the compound 48/80 injection. The compound 48/80 solution was intraperitoneally given to the groups of rats. Each datum represent the mean  $\pm$  S.E. of 3 independent experiments. \* $p < 0.05$ ; significantly different from the saline value.



**Figure 2.** Effect of PVAE on PCA in the rat. PVAE (○) or DSCG (●) or ketotifen (△) was administered orally 1 h prior to the challenge with antigen (DNP-HSA). Each datum represents the mean  $\pm$ S.E. of 3 independent experiment. \* $p < 0.05$ ; significantly different from the saline value.

### Effect of PVAE on Histamine Release from RPMC

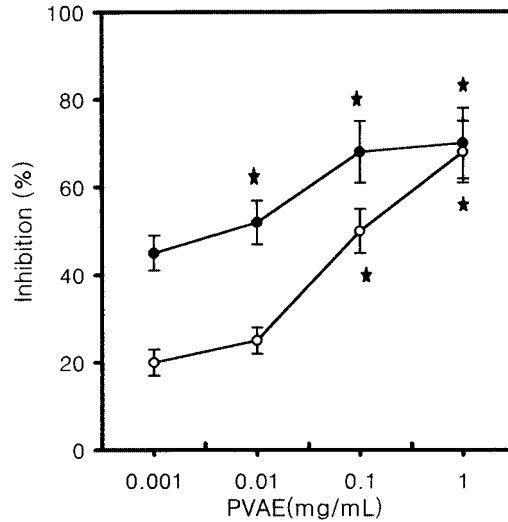
The inhibitory effects of PVAE or DSCG on compound 48/80-induced or IgE-mediated histamine release from RPMC are shown in Fig. 3 and Fig. 4. PVAE significantly inhibited compound 48/80-induced or anti-DNP IgE-mediated histamine release at concentrations 0.1 and 1 mg/ml. DSCG significantly inhibited compound 48/80-induced or IgE-mediated histamine release at concentrations from 0.01 to 1 mg/ml.

### Effect of PVAE on TNF- $\alpha$ Production from RPMC

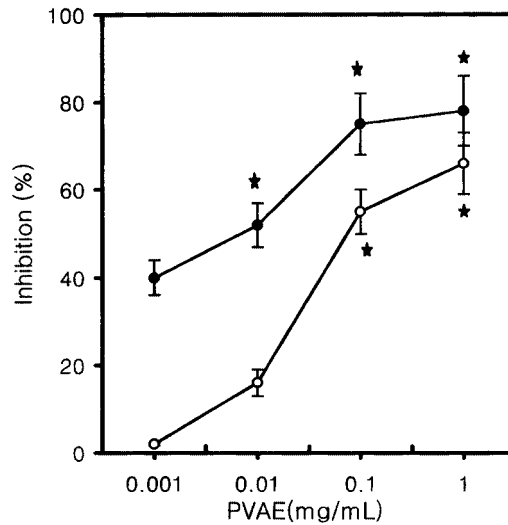
The inhibitory effect of PVAE on immediate-type TNF- $\alpha$  production from RPMC are shown in Table 2. PVAE (0.01 and 0.1 mg/ml) significantly inhibited IgE-mediated TNF- $\alpha$  production. No significant cytotoxicity of PVAE on the culture was observed in the concentrations used in the experiments, as assessed by Trypan blue uptake.

### Effect of PVAE on cAMP Level of RPMC

Finally, We investigated the cAMP content to clarify the mechanism by which PVAE inhibits histamine release from RPMC. RPMC were preincubated with or without PVAE at 37°C for 10 min prior to incubation with or without



**Figure 3.** Effect of PVAE on compound 48/80-induced histamine release from RPMC. RPMC ( $2 \times 10^5$  cells/ml) were preincubated with drug at 37°C for 10 min prior to incubation with compound 48/80 (5  $\mu$ l/ml) for 10 min. (○), PVAE + compound 48/80; (●), DSCG + compound 48/80. Each datum represents the mean  $\pm$  S.E. of 3 independent experiments. \* $p < 0.05$ ; significantly different from the saline value.



**Figure 4.** Effect of PVAE on anti-DNP IgE-mediated histamine release from RPMC. RPMC ( $2 \times 10^5$  cells/ml) were preincubated with drug at 37°C for 10 min prior to challenge with antigen (1  $\mu$ l/ml) for 10 min. (○), PVAE + anti-DNP IgE; (●), DSCG + anti-DNP IgE. Each datum represents the mean  $\pm$  S.E. of 3 independent experiments. \* $p < 0.05$ ; significantly different from the saline value.

**Table 2.** Effect of PVAE on IgE-Mediated TNF- $\alpha$  Production in RPMC

PVAE Treatment (mg/ml)	Anti-DNP IgE + DNP-HSA	TNF- $\alpha$ Production (pg/ml)
None (saline)	—	69 $\pm$ 5.8
None (saline)	+	209 $\pm$ 18.7
0.001	+	175 $\pm$ 16.3
0.01	+	108 $\pm$ 9.6*
0.1	+	84 $\pm$ 9.0*

RPMC ( $3 \times 10^5$  cells/ml) were sensitized with anti-DNP IgE (1  $\mu$ g/ml) and incubated for 18 h in the absence or presence of PVAE before the challenge with DNP-HAS (0.1  $\mu$ g/ml). Each datum represents the mean  $\pm$  S.E. of 3 independent experiments.

\*  $p < 0.05$ ; significantly different from the saline value.

compound 48/80. When RPMC were incubated with PVAE, the cAMP content increased, compared with that of control cells (Table 3). The increase in cAMP content by PVAE was little affected by the addition of compound 48/80.

## DISCUSSION

We have demonstrated that PVAE pretreatment profoundly affected compound 48/80-induced systemic anaphylactic reaction and anti-DNP IgE-induced PCA reaction. PVAE inhibited the compound 48/80 and anti-DNP IgE-induced histamine release from RPMC. When drugs were given orally to rats, PVAE potentially inhibited PCA reaction in rats but DSCG did not show inhibitory activity. The inhibitory effects of DSCG on anaphylactic reactions in rats are reported to derive from inhibition of mediator release from the mast cells (16,26,27). In the

**Table 3.** Effect of PVAE on Compound 48/80-induced cAMP Content in RPMC

PVAE Treatment (mg/ml)	Compound 48/80 (5 $\mu$ g/ml)	cAMP (p mol)
None (saline)	—	0.39 $\pm$ 0.03
None (saline)	+	0.21 $\pm$ 0.03
0.1	—	1.04 $\pm$ 0.21*
1	—	1.21 $\pm$ 0.18*
1	+	0.73 $\pm$ 0.08*

RPMC ( $2 \times 10^5$  cells/ml) were pretreated with PVAE at 37°C. Each datum represents the mean  $\pm$  S.E. of 3 independent experiments.

\*  $p < 0.05$ ; significantly different from the saline value.

present study, PVAE and DSCG potently inhibited histamine release activated by compound 48/80 or anti-DNP IgE antibody. The results demonstrate that PVAE inhibits the mast cell-dependent anaphylactic reaction in rats and suggest that the activity may be due to the inhibition of histamine release from the mast cells. Some recent studies have shown that compound 48/80 is able, apparently directly, to activate G-proteins (28,29). The evidence indicates that the protein is Gi-like and that the activation is inhibited by benzalkonium chloride (30). Tasaka *et al.* (31) reported that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane. This result indicates that the membrane permeability increase may be an essential trigger for the release of the mediator from mast cells. PVAE might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80. This is supported by a previous report that benzalkonium chloride and other selective antagonists inhibit the histamine release induced by compound 48/80 (32). Rats administered PVAE are protected from IgE-mediated cutaneous allergic reaction. The possible mechanism of these effects appears to be related to the activation of adenylate cyclase and a subsequent increase in intracellular cAMP (33). The intracellular cAMP content of the mast cells, when incubated with PVAE, increased about 3-fold in comparison with that of basal cells. The mode of action of PVAE is likely related to the prevention of calcium release from the calcium store of mast cells due to elevation of the intracellular cAMP level by inhibition of the cAMP phosphodiesterase. Our data showed that PVAE inhibited anti-DNP IgE-induced TNF- $\alpha$  production. The effect of PVAE on mast cell cytokine production *in vivo* and the relative importance of mast cells as a source of TNF- $\alpha$  during inflammatory and immune responses are important areas for future studies. In conclusion, the results obtained in the present study provide evidence that PVAE inhibited the immediate-type allergic reaction *in vivo* and *in vitro* in a murine model. Further work should address the possibility that PVAE may also be active in the inhibition of human mast cell degranulation and, therefore, in the treatment of human allergic disorders.

#### ACKNOWLEDGMENTS

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