Original article

Inhibitory effect of 2,5-bis(4-hydroxy-3-methoxybenzylidene) cyclopentanone on mast cell histamine mediated-rat paw edema

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Abstract:

2,5-Bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone, known as pentagamavunon-0 (PGV-0), is a benzylidene cyclopentanone analog of curcumin. This compound posses stronger pharmacological activities such as anti-inflammatory, antioxidative and antibacterial effects in comparison to curcumin. PGV-0 was reported to also have antiallergic effect in rat basophilic leukemia cell line and rat peritoneal mast cell *in vitro.* This study will demonstrate the inhibitory effect of PGV-0 on the histamine release and edema formation stimulated by compound 48/80 in animal model. The study was conducted using an *in vivo* microdialysis technique implanted into rat hind paw, and histamine content in dialysate was quantified using HPLC-fluorometric method. In addition to the compound 48/80-induced edema formation, the skin histamine content was also evaluated. Intragastric administration of PGV-0 at 1 h prior to compound 48/80 subplantar injection inhibited the histamine release from the rat hind paw in a dose-dependent manner. PGV-0 at dose of 40 mg/kg body weight decreased the rat paw edema and inhibited the decrease of the histamine content and the degranulation of mast cell in skin tissue. These data demonstrated that the inhibition on the histamine release by PGV-0 resulted in the suppression of edema formation. These effects were suggested mainly due to inhibition of the histamine content and mast cell degranulation in rat skin tissue.

Keywords: Benzylidene cyclopentanone; Curcumin; Histamine; Mast cells; Paw edema

Introduction

Attempts to design new molecules possessing better properties than curcumin has been reported. One of the trigger is the stability of curcumin which is strongly affected by pH and light [1]. Based on kinetic studied reported by Tonnesen and Karlsen, curcumin in aqueous solution at pH less than 7 is quite stable, but this compound is decomposed at increasing pH more than 7 [2]. The main products of this decomposition are ferulic acid and 4-phenyl-3-butene-2-one, which rapidly undergo the retro-aldol condensation to vanillin and acetone. The instability of curcumin at alkaline pH is associated with the active methylene moiety. In addition to pH, the stability of curcumin is also affected by light which generates degradation products such as ferulic aldehyde, ferulic acid, dihydroxynaphthalene, vinylguaiacol, vanilin and vanilinic acid and is also influenced by the active methylene moiety [1,2]. Converting this active methylene moiety and one carbonyl moiety becomes cyclopentanone resulting in more stable compounds known as 2,5-bis(4-hydroxy- 3-methoxybenzylidene) cyclopentanone or pentagamavunon-0 (PGV-0) according to United States Patent (Figure 1) [1-4]. The modification, however, is still able to maintain the hydroxy moiety (active moiety) of the compound which is responsible for antioxidant effect [3,4].

2,5-Bis(4-hydroxy-3-methoxybenzylidene) cyclopentanone has been synthesized for a number of purposes such the one made by Kodak in 1961 for developing film-forming photosensitive polymers [5]. This compound was also reported to have various pharmacological properties including antibacterial and antifungal effects as well as anti-inflammatory effect through inhibition of prostaglandin biosynthesis in cyclooxygenase pathway [3]. In addition to these effects, PGV-0 also inhibited lipid peroxidation and function as free hydroxy radical scavenger [3,4]. The antioxidative, and anticyclooxygenase activities of this compound at dose of 20 mg/kg, po was 2-and 7-times stronger than those imposed by curcumin while its anti-inflammatory activity was reported to be 5-times higher [3,4].

In addition to the pharmacological activities mentioned, curcumin and its analogs was reported to possess inhibitory effect of histamine release which was closely related to its antioxidative property [6,7]. In our previous study, PGV-0 showed the inhibitory effect on the histamine release in RBL-2H3 cells and rat peritoneal mast cells (RPMCs) in response to several histamine releasing agents such as thapsigargin, ionophore agent, compound 48/80 and phorbol myristate acetate (PMA). The inhibitory effects of PGV-0 were stronger than that imposed by curcumin and was co-founding with its antioxidative activity. The rigidity and stability of the chemical structure of this compound contributed to the better effects imposed by PGV-0 although both compounds possess the same moieties at the aromatic ring. The mechanism underlying the inhibitory effect on the histamine release was through inhibition of the Ca^{2+} uptake into mast cells since the Ca^{2+} is critical substance in histamine-releasing process [8].

In the present study, we investigated the inhibitory effect of PGV-0 on the histamine release *in vivo*. We measured endogenous histamine levels in the subplantar space of the rat hind paw using a microdialysis technique coupled with high performance liquid chromatography (HPLC)-fluorometry. We determined the effect of PGV-0 pretreatment on the histamine release and edema formation stimulated by compound 48/80. The result of these studies may provide useful information for further discovering pharmacological synthetic compound for treatment some disease related to histamine or mast cells.
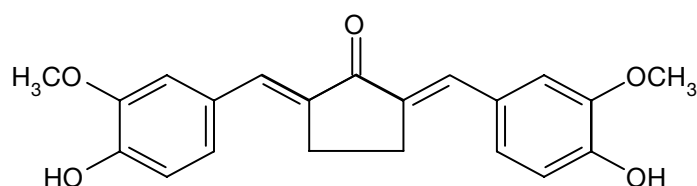
Figure 1 The structure of 2,5-bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone or PGV-0

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Materials and Methods

Material

2,5-Bis(4-hydroxy-3-methoxybenzylidene) cyclopentanone (PGV-0) was synthesized by and obtained from Dr. Sardjiman [4]. The histamine release inducer used in the study is compound 48/80 (Sigma-Aldrich, St. Louis USA).

Animals

The animal experiments performed in the present study were conducted according to the guidelines of the Animal Care Committee of Ehime University School of Medicine, and the experimental protocol were approved by the committee. Wistar rats weighing 180-230 g were used. They were housed at a constant temperature (22 ± 2°C) with a constant relative humidity (55 ± 10%) on the an automatically controlled 12:12 h light-dark cycle (light on at 7:00 a.m.) and had free access to food and water.

Microdialysis of rat hind paw

The experiment was conducted according to Guo *et al.* [9]. The rats (n = 4-6 rats in each group) were anaesthetized with diethyl ether followed by intraperitoneally injection of 50 mg/kg nembutal, and placed on their backs. The body temperature was kept at 37°C by exposing to a warming light. Each hind paw was fixed tightly on a platform, and a dummy needle covered with the guide tubing for the microdyalisis probe was inserted into the subcutaneous space of the plantar area. The dummy needle was removed from the tubing, and then the microdialysis probe (CMA/20, membrane length 4 mm; Carnegie Medicin, Stockholm, Sweden) was inserted through the tubing into hind paw tissue. Subsequently, the tubing was apartly torn to expose the membrane surface to the subcutaneous tissue. The area was perfused with normal saline at a flow rate of 2 μl/min. Recovery of histamine from surrounding fluid in the dialysate (relative recovery) was 13.62 ± 2.42% (mean ± SEM, n = 7) as estimated by an *in vitro* perfusion test, in which the probe was placed in a test tube containing a 100 pmol/ml standard solution of histamine and was perfused at 37°C. Animal was pretreated with intragastric administration of PGV-0 at 10, 20 or 40 mg/kg body weight (BW) or normal saline solution (as a control group) one hour prior to subplantar injection of 50 μg compound 48/80 dissolved in 50 μl normal saline solution. The needle was injected subcutaneously in the area near the dialysis site, approximately 2 mm next to the dialysis membrane. The dialysate was collected every 20 min for 6 h, and was kept at -20°C for histamine release quantification. Response was expressed as percentage of increase of histamine release from baseline (basal output or basal histamine release), then plotted as an observation time-response curve. The area under the curve for each period of time was calculated for further evaluation.

Measurement of paw edema

The experiment was conducted based on the development of edema in the rat hind paw by weight displacement method [10]. Rats (n = 4-6 rats in each group) were ether anesthetized under light for the purpose of injection and edema measurement. Prior to treatment, baseline paw weights were measured for each hind paw by dipping them up to a fixed level in a beaker containing water. The beaker, sited on a top pan balance (AE 240, Mettler-Toledo, Switzerland), was calibrated before each reading to zero weight. Animals were treated with PGV-0 at 40 mg/kg BW as per microdialysis experiment and the increased volume (Δ ml) of paw caused by the swelling at a particular time was calculated by subtraction of paw weight from the baseline paw weight. Measurement of paw edema was conducted every hour for 5 h, following by skin tissue collections. At the end of each experiment, the skin was quickly removed for the purpose of histamine content and mast cells determination. The area under the curve was calculated to evaluate the anti-inflammatory effect. Response was expressed as an increased volume of paw edema, then plotted as an observation time-response curve. The area under the curve each period of time was calculated to evaluate the response of each group.

Determination of histamine content

The histamine release in the dialysate (first

experiment) and the histamine content in the tissue homogenate (second experiment) were measured by HPLC-fluorometry [11]. Forty microliters of dialysate were diluted 5 times with 5 mM Na_2EDTA, and 50 μl of the sample was injected directly into column packed with cation exchanger TSKgel SP2SW (150 × 6 mm i.d.; Tosoh, Tokyo, Japan). Histamine was eluted with 0.25 M potassium phosphate at a flow rate of 0.6 ml/min, and post-labeled with *o*-phthalaldehyde in alkaline condition, and detected by fluorometric detector (Hitachi, Tokyo) using excitation and emission wavelengths of 360 and 450 nm, respectively. For examining histamine content in tissue sample, paw skins were washed with cold normal saline, blotted with filter paper, weighed, and promptly homogenized in 1 ml of 3% perchloric acid with a polytron homogenizer operated at maximal speed in an ice bath. The homogenate was centrifuge at 10,000 × g for 10 min at 4°C, and the supernatant was collected. Thirty microliters of 2 M $KOH/1$ M KH_2PO_4 was added for each 300 μl supernatant, mixed and then centrifuged at 10,000 × g for 15 min at 4°C. Fifty microliters of the supernatant were injected directly onto a column packed with TSKgel SP-2SW cation exchanger for histamine determination.

Mast cell determination

The skin tissue was quickly removed and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4°C. After washing with 0.1 M phosphate buffer, specimens were immersed in 20% sucrose/0.1 M phosphate buffer overnight at 4°C. Fixed skin tissue were embedded in optimal cutting temperature (OCT) compound and stored at -80°C. The frozen skin tissues in OCT compound were cut into 4-5 μm sections with a cryotome. For mast cell identification, the alcian blue/safranin staining procedure was used [12]. Briefly, sections were incubated for 2 h with alcian blue staining solution and then stained with safranin solution for 1 min, and finally mounted on slides with permount. Mast cells were detected and scored in each section for quantitative analysis. The detection was performed with a X 10 objective, a X 10 eyepiece, and the area observation was randomly photographed with a Olympus

Digital Camera. The mast cells density per tissue area (mm) was counted in each photograph.

Statistical analysis

All experiment were designed as completely randomized multifactorial with 4-6 rats for each group. The results were expressed as the mean ± SEM. Data were statistically analyzed using repeated measures analysis of variance (ANOVA) followed by LSD test. P values < 0.05 indicated significant differences.

Results

PGV-0 inhibited histamine release in dialysate

Histamine was used in this study as the main inflammatory mediator involved in allergic edema formation. Compound 48/80, a potent inducer of mast degranulation and histamine release from connective tissue-type mast cell was used to develop the allergic edema formation. Subplantar injection of compound 48/80 induced rapidly a 120-fold increase in histamine release compared with the basal level soon after the injection in Wistar rats. Then, the histamine release gradually declined monoexponentially into the basal level within 3 h. The histamine release peak was reached during first 20-40 min after compound 48/80 subplantar injection. The injection of normal saline solution alone induced only 6-fold increase in the histamine release, and return to basal level in an hour. The basal level in the present experiment was about 1026.96 ± 464.75 pmol/ml (n = 5).

As shown in Figure 2a, intragastric administration of PGV-0 1 h prior to subplantar injection of compound 48/80 inhibited histamine release from the rat hind paw in a dose-dependent manner. At doses of 10 and 20 mg/kg BW, this compound decreased significantly the histamine release only during the first 20 min, and then there were no significant differences found at next time points. However, the compound at the dose of 40 mg/kg BW showed significantly inhibitory effects until the first 80 min. Figure 2b shows area under the curve of each treatment group for a purpose to evaluate the inhibitory effect of histamine release or anti-allergy effect. PGV-0 at the dose of 10, 20 and 40 kg/kg BW

inhibited the histamine release in paw edema by 8, 30 and 50%, respectively.

PGV-0 inhibited paw edema formation

Figure 3a shows the time-course of the development of paw edema stimulated by subplantar injections with or without pretreatment of PGV-0. Subplantar injection of compound 48/80 could elicit rapidly the paw edema formation and reached a maximum during 1-2 h (1.25-1.29 ml) after the injection and gradually decreased. These edema formation caused by compound 48/80 during 5 h were significantly different compared to these of normal saline injection at each time point. Besides, intragastric administration of PGV-0 (40 mg/kg BW) could decrease the edema formation at each time point for 5 h significantly. Figure. 3b shows area under the curve of each group for a purpose to evaluate the inhibitory effect of edema formation or anti-inflammatory effect. PGV-0 at the dose of 40 mg/kg BW inhibited the paw edema formation by 25%.

PGV-0 restored the histamine content in rat paw skin

Table 1 shows the histamine content in paw skins at 5 h after subplantar injection of compound 48/80 with or without pretreatment of PGV-0 (40 mg/kg BW). The histamine content in paw skin at 5 h, which was injected previously by normal saline was 143.57 ± 23.91 nmol per gram. However, after subplantar injection of

50 µL of 1 mg/ml compound 48/80 solution, the histamine content decreased 8 times becoming 18.45 ± 2.50 nmol per gram. Intragrastric administration of PGV-0 1 h prior to compound 48/80 injection restored the histamine content into the value of 48.51 ± 17.81 nmol per gram.

PGV-0 prevented mast cell degranulation in rat paw skin

After subplantar injection of compound 48/80, the number of stained mast cell decreased indicating that degranulation of mast cell occurred (Figure. 4). By semi-quantitative analysis, the density of mast cell with subplantar injection of normal saline (saline group) and compound 48/80 (control group) were 80.33 ± 10.27 and 12.32 ± 3.38 cells and per area unit (mm), respectively. Intragrastric administration of PGV-0 (40 mg/kg BW) 1 h prior to compound 48/80 injection prevented mast cell degranulation into the value of 30.71 ± 3.62 cells and per area unit (mm).

Discussion

Benzylidenecyclopentanone analogues of curcumin could be synthesized referring to curcumin as a lead compound. Sardjiman *et al.* had synthesized a series benzylidene cyclopentanone analogue of curcumin in order to develop curcumin stability [4]. These analogues of curcumin were supposed to be more stable than their lead compound, curcumin. In our previous study,

Table 1 The histamine contents of compound 48/80-induced rat paw following 5-h administration of PGV-0 (n = 4-6). Saline group was the rats only received a saline subplantar injection in stead of compound 48/80 injection. *, $P < 0.05$ compared to control group; #, $P < 0.05$ compared to saline group

Chemicals	Histamine content (nmol/gram)	% Decrease compared to saline
Saline 50 µL	143.57 ± 23.91	-
Compound 48/80 50 µL/50 mL saline (control)	18.45 ± 2.50#	87.15 ± 1.74
Saline 50 µL, PGV-0 40 mg/kg BW	149.62 ± 20.65	-4.21 ± 14.38
Compound 48/80, PGV-0 40 mg/kg BW	48.51 ± 17.81 *,#	66.21 ± 12.40

The percentage of decrease in histamine content was calculated according to the following equation:

% decrease = (histamine content of control (saline) rat - histamine content of treatment rat)/(histamine content of control (saline) rat) × 100.

among fourteen benzylidenecyclopentanone analogues of curcumin, compound 2,5-bis(4-hydroxy-3- methoxy-benzylidene) cyclopentanone or PGV-0 showed the inhibitory effects of histamine release from RBL-2H3 cells line and rat peritoneal mast cells better than these of curcumin [8].

In the present study, this compound was evaluated for the inhibitory effects of histamine release from mast cell histamine mediated-rat paw edema. In this system, the histamine was released from the connective tissue-type mast cell in rat skin tissue. Stimulation with compound 48/80, a potent inducer of mast degranulation and of histamine release from connective tissue-type mast cell, will cause an effective increase on the histamine release from rat skin tissue.

Compound 48/80 is a substance which can directly activate mast cell secretory processes by increasing the rate of guanosine-5'-triphosphate (GTP) binding to G-proteins (Go/Gi mixture) [13-16]. In turn, the activation of G-proteins could emerge some intracellular signaling events such as phospolipase C, protein kinase C, Ca^{2+} signaling event which can stimulate the exocytosis process of mediators-containing granules into membrane surface of mast cell, cause mast cell degranulation, and then finally release the histamine and other mediators such as serotonin, proteoglicans (heparin, chondroitins), eicosanoids (PGD_2, LTC_4, LTB_4), cytokines and some proteins and peptides from these cells [17-19]. This substance could release the histamine from rat peritoneal mast cells both in the presence and absence of extracellular calcium [12]. In the previous study, the histamine release from rat peritoneal mast cells (a kind of connective tissue-type mast cell) stimulated by compound 48/80 could be suppressed by PGV-0 until more than 40% [8]. *In vivo* study (rat paw edema experiment), subplantar injection of compound 48/80 acted rapidly to stimulate histamine release from +/+ rat skin tissue and mainly on mast cells present in the subcutaneous tissue around the injection site, and did not influence the blood histamine concentration [9]. In our study, the quick release of histamine from Wistar rat skin tissue was soon followed by a more gradual increase of edema formation. The histamine release

reached its maximal volume 1-2 h after the injection, when the histamine output was already decreased into the basal level. Guo *et al.* reported that the histamine promoted immediately the vascular permeability, and soon caused the swelling or edema formation [9]. In the present study, PGV-0 especially at the dose of 40 mg/kg BW could inhibit the histamine release from rat skin tissue and the edema formation stimulated by compound 48/80. It suggests that the inhibitory effect on mast cell degranulation in the rat skin tissue, and subsequently inhibition on the decrease of histamine content in the tissue underlie the effect of PGV-0 on histamine-mediated rat paw edema.

PGV-0 especially at the dose of 40 mg/kg BW seems more effective to inhibit the histamine releases than inhibit the edema formation in the study. The fact indicates that eventhough histamine is a main mediator release in compound 48/80-stimulated edema formation, the full development of edema is complex and may require other mediators such as serotonin, a more potent inflammatory agent than histamine, or membrane-derived lipid mediators [20], such as prostaglandins and leukotrines synthesized in minutes, and a number of cytokines including tumor necrosis factor (TNF-α) and interleukins produced for several hours [21]. So, even PGV-0 succeeded to suppress the histamine release from skin tissue mast cells induced by compound 48/80, and was also reported possessing anti-inflammatory effect, there were still some mediators working to develop the edema formation. These facts cause the inhibitory effect on the edema formation induced by compound 48/80 is a bit less than this on the histamine release.

Related to inflammatory process, PGV-0 was reported possessing several biological activities such as free radical-related anti-oxidative and anti-cyclooxygenase activities [3,4]. Free radical derived from metabolites of unsaturated fatty acid participated in inducing the histamine release [16-18]. Another previous study also reported that the inhibitory effect of histamine release of curcumin and its analog was closely related to its antioxidative property [7]. Besides, its anti-inflammatory activity in carrageenan-induced edema experiment was 5-times higher than those of curcumin at dose of 20 mg/kg,
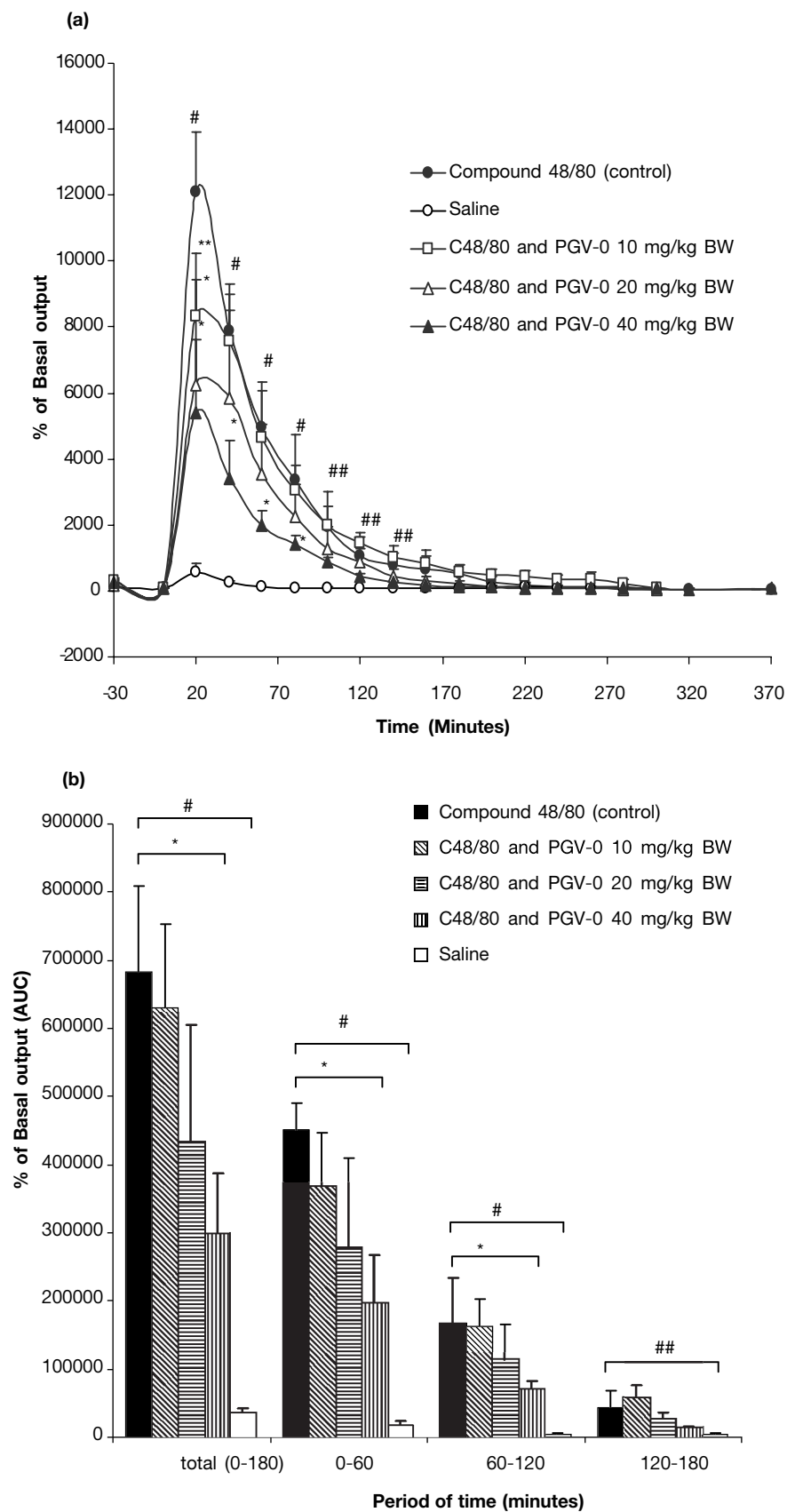
Figure 2 The effects of PGV-0 on the histamine output from the paw of rats following subplantar injection of compound 48/80 (50 µg dissolved in 50 µl of saline). PGV-0 was intragastrically administered 1 h prior to compound 48/80. The data are expressed as the percentage of the basal release vs. observation time (Fig. 2a), and the percentage of the basal output (AUC) vs. period of time (Fig. 2b), and as mean ± SEM (n = 4-6). *, P < 0.05; **, P < 0.01 compared to control group; [#], P < 0.05; [##], P < 0.01 compared to saline group
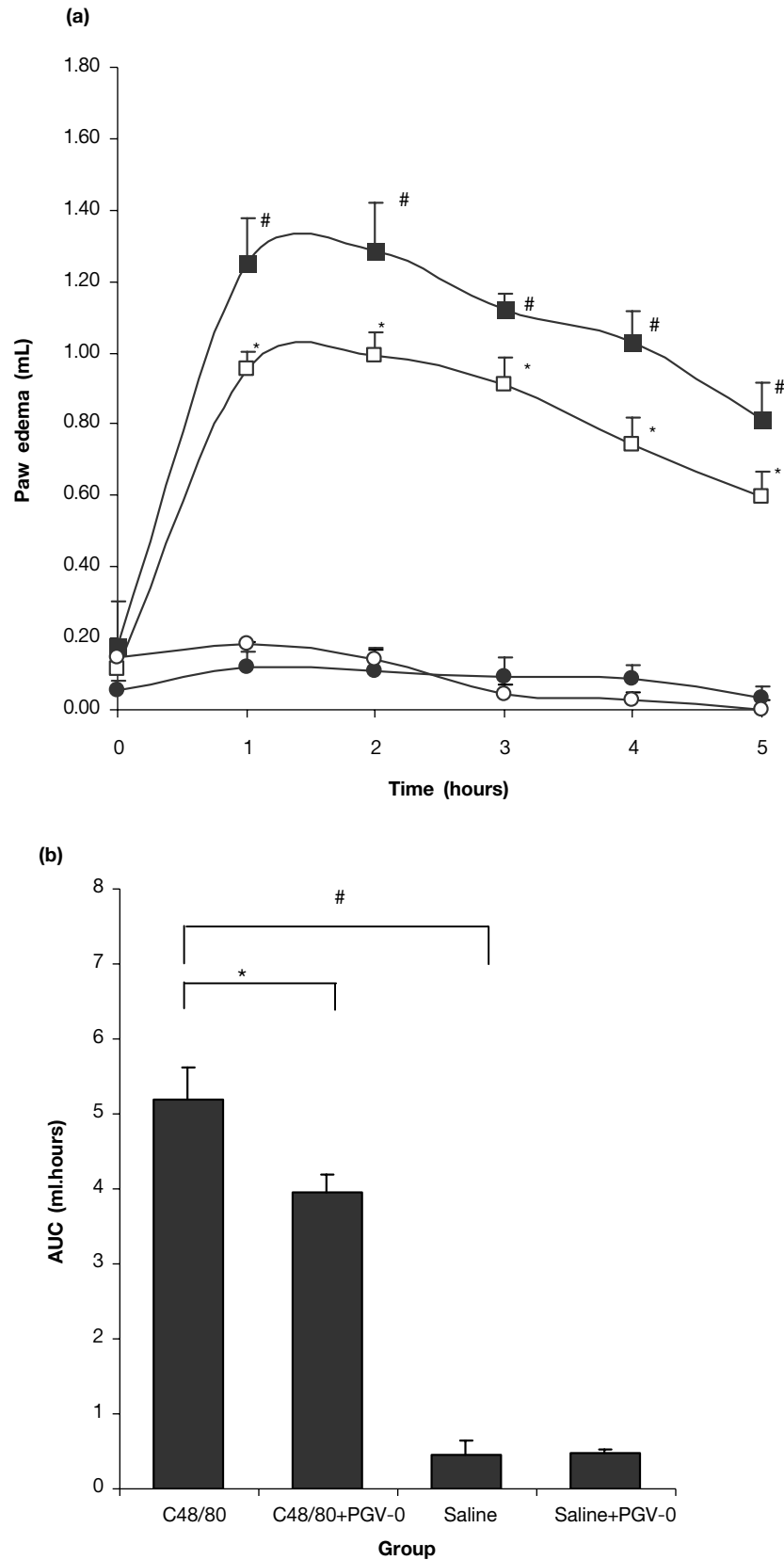
Figure 3 The effects of 40 mg/kg BW PGV-0 on the development of paw edema elicited by subplantar injection of compound 48/80. The data are expressed as the increased paw edema volume (Δ ml) vs. time observation (Fig. 3a), and the increased paw edema volume (AUC) during observation times (Fig. 3b), and as mean ± SEM (n = 4-6). Saline group (control group) was the rats only received a saline subplantar injection in stead of compound 48/80 injection. *, P < 0.05 compared to control group; #, P < 0.05 compared to saline group. ■ = compound 48/80 (control group); □ = compound 48/80 and PGV-0 group; ● = saline group; and ○ = saline and PGV-0 group
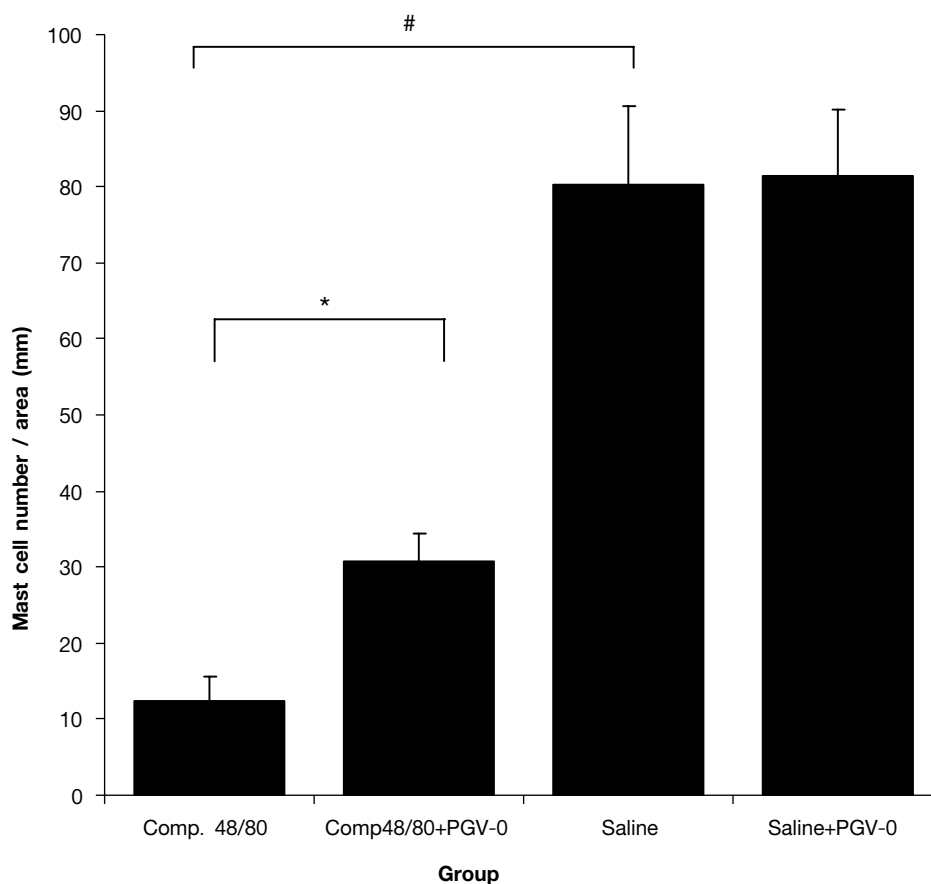
Figure 4 The mast cell density in compound 48/80-induced paw following 5-h administration of PGV-0 at the dose of 40 mg/kg BW (n = 4-5). The mast cell density is expressed as mast cell number per area unit (mm). Saline group (control group) was the rats only received a saline subplantar injection in stead of compound 48/80 injection. *, P < 0.05 compared to control group; #,P < 0.05 compared to saline group

po [3,4].

In conclusion, 2,5-bis(4-hydroxy-3-methoxybenzylidene) cyclopentanone or PGV-0 inhibited the rat paw edema related to histamine release or mast cell by preventing the mast cell degranulation and decreasing of histamine content in the rat skin tissue.

Acknowledgements

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