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Acute Stress Results in Skin Corticotropin-Releasing Hormone Secretion, Mast Cell Activation and Vascular Permeability, an Effect Mimicked by Intradermal Corticotropin-Releasing Hormone and Inhibited by Histamine-1 Receptor Antagonists

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Key Words
Azelastine · Calcium · Corticotropin-releasing hormone · Mast cells · Olopatadine · Skin · Urocortin

Abstract

Background: Mast cells play an important role in allergic inflammation by releasing vasoactive molecules, proteases and cytokines. Corticotropin-releasing hormone (CRH) and its structural analogue urocortin (Ucn) were shown to trigger skin mast cell activation and vascular permeability. We investigated the effect of acute stress on rat skin vascular permeability and CRH secretion, as well as the effect of intradermal CRH, and that of two histamine-1 receptor antagonists, azelastine and olopatadine, on vascular permeability. ***Methods:*** Rats were stressed by restraint and vascular permeability was assessed by extravasation of ^{99}Tc-gluceptate, while mast cell activation was determined by skin rat mast cell protease-1 (RMCP-1) content. Skin CRH content was evaluated by ELISA. The effect of intradermal injection of CRH and Ucn, as well as that of two histamine-1 receptor antagonists, azelastine and olopatadine, was assessed by Evan's blue extravasation. Purified rat peritoneal mast cells (RPMCs) were also pretreated with azelastine (24 μM) or olopatadine (133 μM) for 5 min before challenge with compound 48/80 (0.5 μg/ml) for 30 min. Histamine secretion was measured fluorometrically. Intracellular Ca^{2+} ions were evaluated in RPMCs loaded with calcium crimson and stimulated with compound 48/80. ***Results:*** Acute stress increased skin vascular permeability and CRH content, while it decreased RMCP-1. Intradermal injection of CRH or Ucn induced substantial Evan's blue extravasation that was inhibited by pretreatment with azelastine (24 μM) and olopatadine (133 μM). Both antihistamines also inhibited histamine release and intracellular increase of Ca^{2+} ions from RPMCs stimulated by compound 48/80. ***Conclusions:*** These results indicate that acute stress increases skin CRH that can trigger mast cell-dependent vascular permeability, effects inhibited by certain histamine-1 receptor antagonists, possibly acting to reduce intracellular Ca^{2+} ion levels.

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Introduction

Mast cells play an important role in allergic reactions through IgE and antigen-dependent release of histamine, leukotrienes, prostaglandin D_2 and proteases [1, 2]. Mast cells also secrete numerous proinflammatory cytokines that may contribute to chronic allergic diseases [2–5]. Acute stress was recently shown to activate skin mast cells [6], but its effect on vascular permeability had not been investigated. Intradermal corticotropin-releasing hormone (CRH) [7] and urocortin (Ucn) [8] were previously shown to activate rat skin mast cells and lead to mast cell-dependent vascular permeability. Increased vascular permeability due to intradermal injection of CRH was also recently confirmed in humans [9]. However, there have been no reports on whether stress could increase the skin content of CRH. As increased vascular permeability may be involved in stress-induced skin diseases [6], it appeared reasonable to test whether clinically available drugs could inhibit CRH-induced vascular permeability.

Certain histamine-1 receptor antagonists have been reported to inhibit the release of mast cell mediators [10, 11]. Azelastine is such an antihistamine present in the ophthalmic solution Optivar® and has been shown to inhibit the release of inflammatory mediators in asthma and nasal allergy [12, 13], as well as of histamine from rat peritoneal mast cells [14]. Olopatadine is another antihistamine found in the ophthalmic solution Patanol® and has been reported to inhibit human conjunctival mast cells [15, 16]. We investigated the effect of these antihistamines on CRH-induced skin vascular permeability and nonallergic activation of rat peritoneal mast cells (RPMCs), for the possibility they may be helpful in stress-induced skin disorders [17]. Here, we report that acute stress led to elevated skin CRH content in areas of increased vascular permeability; this effect was mimicked by intradermal CRH administration and was inhibited by azelastine and olopatadine.

Materials and Methods

Azelastine hydrochloride ophthalmic 0.05% solution (Optivar®) was provided by Asta Medica/Muro Pharmaceuticals Inc. (Tewksbury, Mass., USA), and olopatadine hydrochloride ophthalmic 0.1% solution (Patanol®) was from Alcon Laboratories, Inc. (Fort Worth, Tex., USA). The molarity of the stock concentrations of these histamine-1 receptor antagonists in the respective ophthalmic solutions were 1,195 and 2,670 μ*M*. The concentrations used for the rat skin experiments were 24 and 60 μ*M* azelastine, or 133 μ*M* olopatadine. We had determined that 24 μ*M* azelastine and 133 μ*M* olopatadine gave equivalent inhibition of mast cell activation [18]. Compound 48/80 (Sigma, St. Louis, Mo., USA) at a final concentration of 0.5 μg/ml and disodium cromoglycate (cromolyn) at a final concentration of 100 μ*M* (Sigma) were dissolved in 0.9% NaCl (normal saline).

RPMC Purification

Rats were sacrificed with carbon dioxide asphyxiation. The peritoneal cavities were lavaged with complete Locke's buffer. Cells were purified over 22.5% metrizamide in Locke's buffer to a purity of approximately 95% [19].

Histamine Release Assay

Mast cells (1×10^5 cells/100 μl sample) were preincubated with either Locke's buffer (control) or azelastine (24 μ*M*) or olopatadine (133 μ*M*) for 5 min, and challenged with compound 48/80 (0.5 μg/ml) for 30 min at 37°C in a shaking water bath. The cell suspension was centrifuged and the histamine was then measured in the supernatant fluid and pellet using a luminescence spectrometer (Perkin-Elmer, Norwalk, Conn., USA) [20].

Evan's Blue Extravasation

Male Sprague-Dawley rats, each weighing approximately 350 g (Charles River, N.Y., USA) were housed 3 per cage, provided with food and water ad libitum and maintained on a 14:10 h light:dark cycle. Each animal was handled one at a time in order to minimize stress. Rats were anesthetized with a single intraperitoneal (i.p.) injection of 0.25 ml ketamine and xylazine (100 mg/ml each). They were injected intravenously (0.6 ml for rats) via the tail vein with 1% Evan's blue 10 min after intradermal injection of the test substances; this approach reduced the background extravasation. Experimental molecules were then tested by intradermal injection in normal saline 0.9% NaCl using a tuberculin syringe. The pretreatment solutions of azelastine (24 μ*M*) or olopatadine (133 μ*M*), when appropriate, were injected first and allowed to remain in the skin for 5 min; compound 48/80 (0.5 μg/ml), CRH or Ucn (10 μ*M*) was then injected in the same spot with a different syringe; normal saline was injected as control. The animal was killed 15 min later by asphyxiation over CO_2 and decapitated; the abdominal skin was removed, turned over and photographed. Identical circular skin areas (1 cm^2) were then cut with a surgical blade around a cylindrical template. The extravasated Evan's blue was extracted by incubating the skin samples in 99% N,N-dimethyl formamide (Sigma) for 24 h at 55°C. The dye was measured fluorometrically (excitation wavelength of 620 nm and emission of 680 nm) as described previously [9].

Restraint Stress and Extravasation of ^{99}Tc-Gluceptate

Rats were stressed for 30 min by placing them into an immobilizer and skin pieces were obtained as described above. Adjacent sections were sampled for ^{99}Tc- gluceptate extravasation, as well as skin rat mast cell protease-1 (RMCP-1) and CRH content. The animals were brought into a quiet procedure room within the animal facility one at a time and were allowed to walk around on the bench, where the Plexiglas immobilizer (Harvard Apparatus, Cambridge, Mass., USA) was located, for a few minutes at the same time (9–11 a.m.) every day for 5 days to 'familiarize' them with handling outside the cages. On the day of the experiment, each rat (if not a control) was kept in the immobilizer for 30 min. The control animal was left in its cage on the bench for an equivalent amount of time, but always at least 60 min after all procedures with the experimental animal are over. At the end of the stress period (or the wait period for the con-

Table 1. Effect of acute stress on rat skin vascular permeability, CRH level and mast cell activation

Experimental conditions	n	Vascular permeability ^{99}Tc, cpm/100 mg	Skin CRH ng/ml	Mast cell activation residual RMCP-1 ng/ml	Serum corticosterone ng/ml
Control	4	67,728 ± 14,669	0.007	94.7 ± 28.3	19.9 ± 8.1
Restraint stress (30 min)	4	176,413 ± 29,613*	0.103*	16.7 ± 2.4*	328.6 ± 45.8*
Compound 48/80	3	123,814 ± 17,980$^+$	–	42.4 ± 3.0$^+$	N/A
Stress vs. control*		p = 0.002	p = 0.001	p = 0.001	p = 0.001
Compound 48/80 vs. control$^+$	4	p = 0.002	–	p = 0.024	N/A

*/$^+$ Using the Mann-Whitney U test.

trol), the animal was killed with asphyxiation over CO_2, and decapitated. Skin samples were then removed carefully using a circular template to preserve consistency and fixed in 4% paraformaldehyde overnight at 4°C, or handled as required for quantitation of extravasated ^{99}Tc.

For ^{99}Tc extravasation, the morning of the experiment (9–12 a.m.), animals were injected with 0.5 ml of 500 μCi of ^{99}Tc. Binding of technetium to gluceptate prevents ^{99}Tc from escaping the circulation and constitutes a good marker from extravasation [21]. To be stressed, rats were placed in Plexiglas immobilizers immediately following ^{99}Tc injection.

To assess skin permeability, the animals were anesthetized immediately after stress with one intraperitoneal injection (0.1 ml) of a mixture of ketamine and xylazine (1.0 and 0.02 ml, respectively, of 100 mg/ml each). Rats were perfused intracardially with 100 ml normal saline to remove any intravascular tracer; skin samples were removed as described. Skin permeability was assessed by the amount of ^{99}Tc extravasated in the skin using a Gamma Well Counter, as reported previously [21]. The samples were then weighed and the amount of radioactivity was expressed as counts/100 mg of tissue.

Serum Corticosterone Measurements

Serum corticosterone was measured as an index of HPA axis activation. Blood was collected from the neck vessels after the rat was decapitated. Blood samples were allowed to clot overnight at 2–8°C before centrifuging for 20 min at 2,000 *g*. The serum was collected and subjected to corticosterone radioimmunoassay using a corticosterone ^{125}I-RIA kit (Phoenix Pharmaceuticals, Belmont, Calif., USA).

Skin RMCP-I Content

Mast cell activation was quantitated indirectly by measuring the residual skin content of RMCP-1. Same size skin samples were removed as described above and RMCP-1 content was evaluated, before and after stress by extracting it after freeze-thaw and homogenization in PBS using a Polytron at 4°C (Brinkmann Instruments, Westbury, N.Y., USA). The supernatant fluid was then assayed for RMCP-1 by ELISA (Moredun, Scotland).

Skin CRH Content

CRH was measured in the fluid removed from a skin blister made on the abdominal area (after shaving) as follows: a plastic intravenous catheter (24 G, Sandy, Utah, USA) was inserted subcutaneously while the animal was under anesthesia as described before and 50 μl of normal saline were introduced to create the 'blister'. The animal was then allowed to wake up and was restrained for 30 min. At the end of the stress period, 50 μl more of 0.9% NaCl was introduced in the 'blister' through the same catheter and the total fluid of about 70–80 μl was removed. Control animals were treated similarly except that they remained anesthetized. CRH was then assayed by ELISA (Phoenix Pharmaceuticals).

Intracellular Ca^{2+} Ion Measurement

RPMCs were incubated with 50 μ*M* calcium crimson probe (Molecular Probes, Eugene, Oreg., USA) for 8 min at 37°C, washed free of dye and then treated with compound 48/80 (0.5 μg/ml). The fluorescence intensity was evaluated at 10, 20, 30, 40, 690, 700, 710 and 720 s during mast cell activation using confocal microscopy and notably increased over time [22]. RPMCs were also pretreated with azelastine (24 μ*M*) or olopatadine (133 μ*M*) for 5 min prior to stimulation with compound 48/80 (0.5 μg/ml) and the fluorescence intensity was again evaluated for the same duration.

Statistics

Results are presented as the mean ± standard deviation. Significance was evaluated by ANOVA for histamine release and by the non-parametric Mann-Whitney U test for skin ^{99}Tc, RMCP-1 and CRH content, and was set at $p < 0.05$.

Results

Effect of Acute Restraint Stress on Rat Skin Mast Cell Activation and Vascular Permeability

In this study, ^{99}Tc levels were significantly increased in tissues of rats (n = 4) restrained for 30 min (176,413 ± 29,613 cpm/100 mg tissue) when compared with control (n = 4) rats (67,728 ± 14,669 cpm/100 mg tissue) as shown in table 1 (p = 0.002). Compound 48/80 (0.5 μg/ml i.v.) used as positive control by stimulating mast cells (n = 3) increased ^{99}Tc extravasation to 123,814 ± 17,980 cpm/100 ng (table 1).

Effect of Acute Stress on Skin CRH Content

Serum corticosterone level significantly increased ($p = 0.001$) from 19.9 ± 8.1 ng/ml (control group) to 328.6 ± 45.8 ng/ml in restraint-stressed rats (table 1), indicating the rats were stressed adequately. Acute stress for 30 min significantly increased ($p = 0.001$) CRH content from 0.007 to 0.103 ng/ml after stress in skin areas adjacent to those sampled for ^{99}Tc extravasation (table 1).

Effect of Acute Stress on Skin Mast Cell Activation

Skin RMCP-1 level decreased ($p = 0.024$) in rats following restraint stress for 30 min from 94.7 ± 28.3 to 16.7 ± 2.4 ng/ml in control rats (table 1). This result indicates that skin mast cells were activated and secreted RMCP-1.

Effect of Azelastine and Olopatadine on Rat Skin Evan's Blue Extravasation

Injection of compound 48/80 (0.5 μg/ml), used as positive control, as well as of CRH or Ucn (10 μ*M*), induced substantial Evan's blue extravasation (left) as compared to normal saline (right) (fig. 1a) indicating mast cell activation. The extent of dye extravasation due to CRH or Ucn was equivalent to that of compound 48/80. However, when the site was pretreated with azelastine (24 or 60 μ*M*) or olopatadine (133 μ*M*) for 5 min before injection of compound 48/80 (fig. 1a), CRH (fig. 1b) or Ucn (fig. 1c) the extravasation was completely inhibited (left). Injection of the drugs alone (right) had no effect. Notably, cromolyn (100 μ*M*) did not prevent extravasation (fig. 1a). These subjective results were confirmed with objective assessment of Evan's blue extracted in formamide and measured fluorometrically. The inhibition exerted by 24 μ*M* azelastine was equivalent to that seen with 133 μ*M* olopatadine (fig. 2).

We then studied the effect of compound 48/80, CRH and Ucn on activation of PRMCs; we also investigated the effect of azelastine and olopatadine on this process. This approach was undertaken because acute stress, compound 48/80, CRH and Ucn were shown previously [6, 7] and here to activate skin mast cells.

Effect of Azelastine and Olopatadine on Histamine Release from RPMCs

RPMCs stimulated with compound 48/80 (0.5 μg/ml) for 30 min at 37°C, secreted 27.6 ± 1.6% histamine ($p < 0.05$, fig. 3). Neither CRH nor Ucn (10 m*M*) could stimulate histamine release (results not shown). Azelastine (24 μ*M*) or olopatadine (133 μ*M*) significantly ($p < 0.05$)

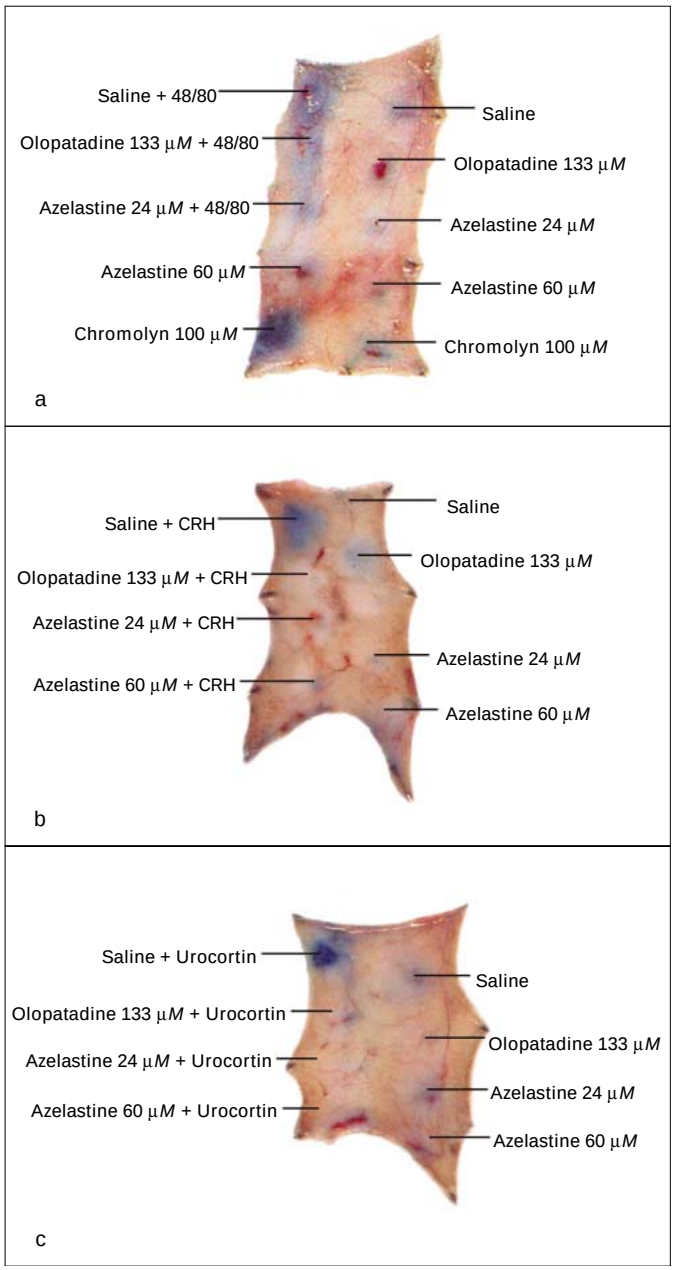

Fig. 1. Increased rat skin vascular permeability by compound 48/80, CRH and Ucn and its inhibition by azelastine or olopatadine. Photographs showing the effect of 0.5 μg/ml C48/80 (a; left), CRH (b; left) or Ucn (c; left) alone for 10 min or after pretreatment with azelastine or olopatadine. Normal saline was injected as control. Administration of azelastine or olopatadine alone is shown on the corresponding right panels.

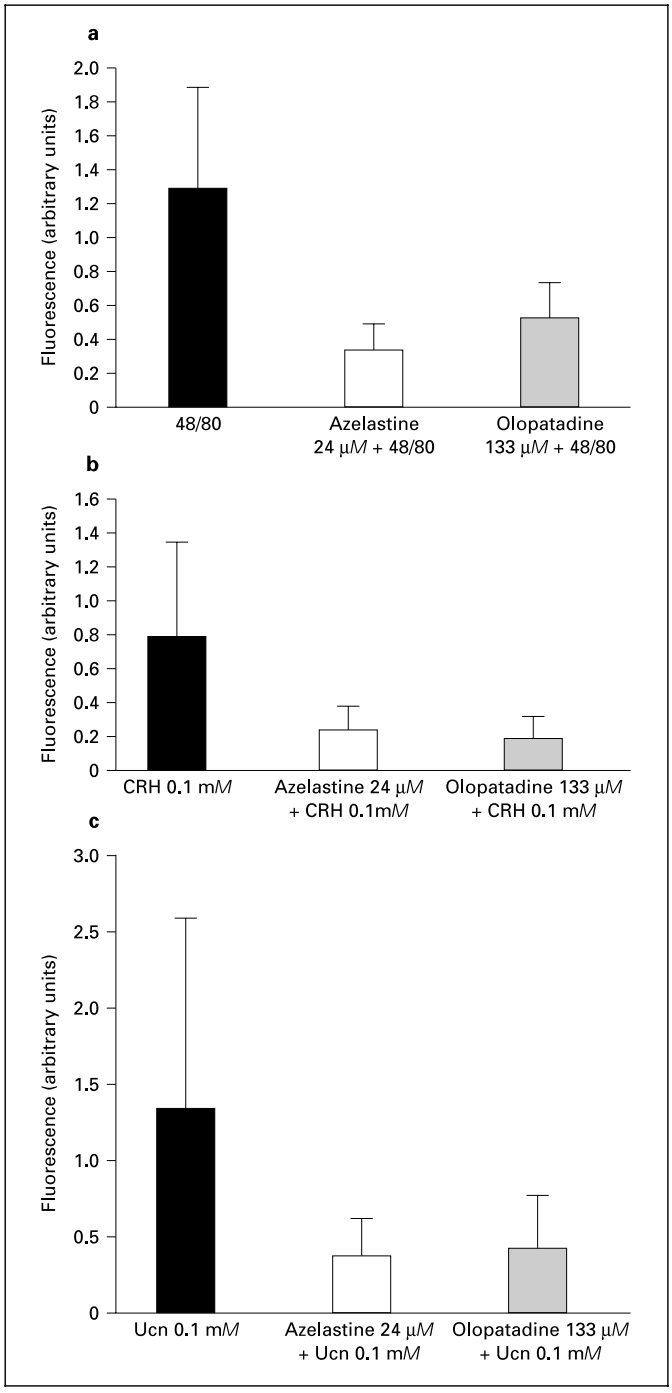

Fig. 2. Effect of azelastine and olopatadine on compound 48/80 (a), CRH (b) and Ucn (c)-induced rat skin Evan's blue extravasation (n = 8). The skin was pretreated intradermally with azelastine (24 μ*M*) or olopatadine (133 μ*M*) for 5 min before injection of Evan's blue (1%) in the tail vein, followed by compound 48/80 (0.5 μg/ml), CRH (10 μ*M*) or Ucn (10 μ*M*) intradermally for 10 min. Skin was removed and the extravasated Evan's blue-associated fluorescence was measured after extraction in formamide. Compound 48/80, CRH or Ucn alone induced significant increases in fluorescence ($p < 0.05$).

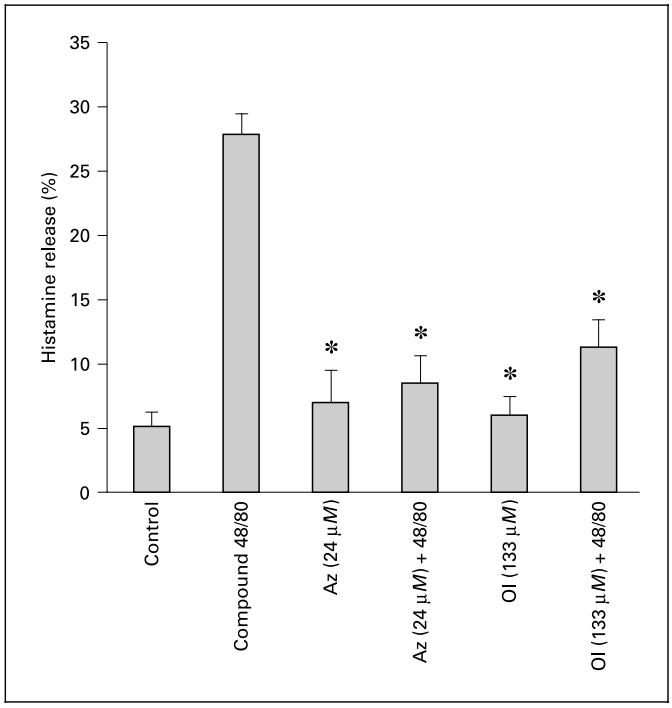

Fig. 3. Effect of azelastine and olopatadine on histamine release from RPMCs (n = 3). RPMCs were preincubated with azelastine (Az, 24 μ*M*) or olopatadine (Ol, 133 μ*M*) for 5 min before stimulation with compound 48/80 (0.5 μg/ml) for 30 min and histamine release was measured fluorometrically in both the supernatant fluid and the pellet (* $p < 0.05$).

inhibited compound 48/80-induced histamine release to about the same extent (fig. 3).

Effect of Azelastine on Intracellular Ca^{2+} Ion Level in RPMCs

RPMCs loaded with calcium crimson probe for 8 min, washed and then treated with compound 48/80 (0.5 μg/ml) increased their intracellular Ca^{2+} level as was demonstrated by increased fluorescence intensity (fig. 4a). Neither CRH or Ucn (10 μ*M*) induced any fluorescence increase (results not shown). In contrast, RPMCs pretreated with azelastine (24 μ*M*) for 5 min and then stimulated with compound 48/80 showed almost no increase in fluorescence intensity (fig. 4b). Similar results were seen with 5 times higher concentrations of olopatadine (results not shown). These results indicate that azelastine inhibited compound 48/80-induced mast cell activation possibly by preventing intracellular Ca^{2+} ion increase.

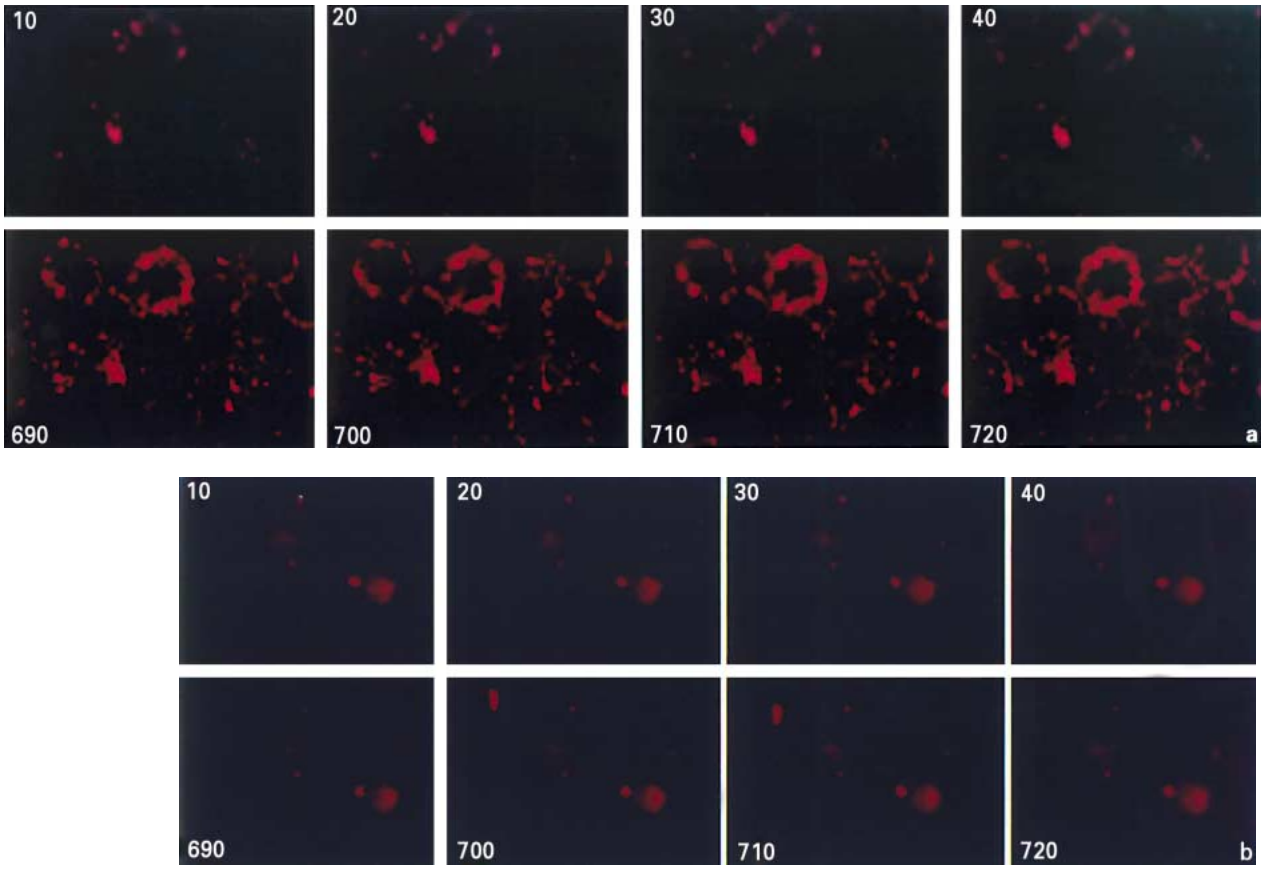

Fig. 4. Effect of azelastine on compound 48/80-induced intracellular Ca^{2+} level (n = 3). RPMCs loaded with calcium crimson probe and treated with compound 48/80 contained increased intracellular Ca^{2+} as shown by increased fluorescence intensity (a) measured at 10, 20, 30, 40, 690, 700, 710 and 720 s. RPMCs pretreated with azelastine (24 μM) for 5 min and then treated with compound 48/80 did not show increased fluorescence (b).

Discussion

Here we report for the first time that acute restraint stress increased skin vascular permeability, as well as skin CRH levels, an index of local stress. The increase in skin CRH under stress indicates that, in addition to release of hypothalamic CRH, local release of CRH occurs and may have proinflammatory effects, as suggested previously [6]. Increased serum corticosterone levels, an index of HPA axis activation, indicated that the rats were stressed as the HPA axis was activated sufficiently, as shown previously [21]. Had the extravasation results been negative in the absence of any serum corticosterone elevation, one may have concluded that the skin response was negative because the rats had not been stressed enough. The RMCP-1 increase in skin areas adjacent to those showing CRH elevation indicates that skin mast cells had degranulated and released their RMCP-1, leading to decreased content. This finding is supported by our present and previously reported [6, 8] findings that intradermal administration of CRH, or its structural analogue Ucn, induced skin vascular permeability. A similar effect was recently reported in humans [9].

Neither CRH nor Ucn could stimulate histamine release or increase intracellular Ca^{2+} ion levels in RPMCs. A possible explanation could be that skin mast cells express CRH receptors, while peritoneal mast cells do not. This possibility is supported by recent reports that skin [23] and articular [24] mast cells express CRHR-1. Mast cells may express CRH only in tissues that are innervated especially by CRH- or Ucn-containing nerves, an unlikely possibility in the peritoneal cavity. Another explanation is that CRHR are stripped from RPMCs during the purification process, especially if they are few in numbers.

Here, we also report that pretreatment with azelastine or olopatadine inhibited vascular permeability induced by intradermal CRH or Ucn. Similar inhibition was noted for histamine release from RPMCs challenged with compound 48/80. Azelastine was about five times more potent than olopatadine in blocking rat skin vascular permeability and histamine release. Furthermore, azelastine and olopatadine inhibited intracellular Ca^{2+} ion levels, typically induced by compound 48/80. Such inhibition had been previously reported for the inhibitory effect of chondroitin sulfate [22]. Azelastine has been reported to inhibit the allergic response [25, 26], as well as the release of inflammatory mediators [27–30] and histamine from rat mast cells following immunologic and nonimmunologic stimuli [14]. Azelastine also inhibited tryptase and IL-6 release from immunologically challenged normal human mast cells [18], while olopatadine prevented mediator release from human conjunctival mast cells [15, 16, 30].

Cromolyn had little effect on skin vascular permeability in our studies. Even though cromolyn is known to inhibit RPMC secretion [31], it does not inhibit human lung mast cells [32] or cultured human mast cells, except at much higher concentrations [25].

Certain histamine-1 receptor antagonists may, therefore be of help in stress-induced skin disorders.

Our results support previous studies indicating that acute stress worsens skin disorders [32] and upregulates CRH-2β receptor expression in areas of stress-induced alopecia [33]. The skin has been proposed to have a local HPA axis [34] and skin mast cells have been hypothesized to act as 'universal sensors of environmental and emotional stress' [35]. In fact mast cells are now considered as a major regulator of neuroimmunoendocrine disorders [36].

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