

Changes in sensory activity of ocular surface sensory nerves during allergic keratoconjunctivitis



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ABSTRACT

Peripheral neural mechanisms underlying the sensations of irritation, discomfort, and itch accompanying the eye allergic response have not been hitherto analyzed. We explored this question recording the changes in the electrical activity of corneoconjunctival sensory nerve fibers of the guinea pig after an ocular allergic challenge. Sensitization was produced by i.p. ovalbumin followed by repeated application in the eye of 10% ovalbumin on days 14 to 18. Blinking and tearing rate were measured. Spontaneous and stimulus-evoked (mechanical, thermal, chemical) impulse activity was recorded from mechanonociceptor, polymodal nociceptor and cold corneoscleral sensory afferent fibers. After a single (day 14) or repeated daily exposures to the allergen during the following 3 to 4 days, tearing and blinking rate increased significantly. Also, sensitization was observed in mechanonociceptors (transient reduction of mechanical threshold only on day 14) and in polymodal nociceptors (sustained enhancement of the impulse response to acidic stimulation). In contrast, cold thermoreceptors showed a significant decrease in basal ongoing activity and in the response to cooling. Treatment with the TRPV1 and TRPA1 blockers capsazepine and HC-030031 reversed the augmented blinking. Only capsazepine attenuated tearing rate increase and sensitization of the polymodal nociceptors response to CO₂. Capsazepine also prevented the decrease in cold thermoreceptor activity caused by the allergic challenge. We conclude that changes in nerve impulse activity accompanying the ocular allergic response, primarily mediated by activation of nociceptor's TRPV1 and to a lesser degree by activation of TRPA1 channels, explain the eye discomfort sensations accompanying allergic episodes.

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1. Introduction

Ocular allergy is an immunopathological reaction of the ocular surface triggered by contact with foreign antigens. The allergic inflammatory response affects the conjunctiva but often also the cornea and the eyelids. Clinically, ocular type I allergy is a complex disease of variable severity, ranging from the common, milder conditions of seasonal and perennial allergic conjunctivitis, to the rare and more severe forms of vernal keratoconjunctivitis and atopic keratoconjunctivitis. Allergic keratoconjunctivitis causes swelling and redness of the conjunctiva, excessive lacrimation, and mucous discharge [6,13,38,42,49]. Among the clinical symptoms of the disease, burning and itching sensations referred to the eye are particularly prominent and disturbing for the patient. However, the neural mechanisms underlying these sensations have not been established.

The cornea and bulbar conjunctiva are innervated by functionally distinct types of sensory nerve afferents, which are the source of sensations evoked by innocuous and injurious forces acting on the ocular surface [1,4,9]. Ocular surface receptors include mechanonociceptors, which are primarily activated by noxious mechanical stimulation, and polymodal nociceptors that respond to mechanical forces but also to irritant chemicals and extreme temperatures [11,12] and cold thermoreceptors, preferentially excited by moderate cooling [27]. Their specific sensitivity to the different stimulus modalities is determined by the expression of distinct transducing channels, including TRPV1 and TRPA1 for polymodal nociceptors and TRPM8 for cold thermoreceptors [61]. Additionally, the ocular sensory innervation plays a crucial role in the control of basal and reflex tearing and blinking [3,50]. The contribution of the different types of ocular surface receptors to the development of unpleasant sensations is unknown, as is the role played by the various classes of transducing channels in their activation during allergic conjunctivitis.

In this study, we used an experimental model of allergic keratoconjunctivitis in guinea pigs to analyze the changes in spontaneous

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and stimulus-evoked activity of the different functional classes of corneconjunctival sensory fibers in response to an ocular allergic challenge, as well as the contribution of TRPV1, TRPA1, and TRPM8 channels to their altered responsiveness. Preliminary results have been previously reported in abstract form [2,44].

2. Methods

2.1. Animals

Albino and pigmented guinea pigs of both sexes weighting 200 to 400 g were used. Experiments were performed in accordance with the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and the European Communities Council Directive (86/609/EEC) on the protection of animals used for experimental and scientific purposes, with the approval of the Committee for Ethics in Research of the Universidad Miguel Hernandez.

No differences in the parameters measured were observed between albino and pigmented animals. Therefore, data from both strains were analyzed together.

2.2. Induction of ocular allergy

An ovalbumin (OVA) allergic conjunctivitis was evoked following the procedure reported by Schoch [58]. For this purpose, sensitization was induced on day 0 by i.p. injection of a solution of 100 µg OVA (Sigma–Aldrich, Saint Louis, MO, USA) plus 20 mg Al(OH)₃ dissolved in 1 mL of saline solution. On days 14 to 18, 10-µL drops of 10% OVA in saline were applied daily to both eyes. Electrophysiological recordings in the excised eyes were initiated 60 min after exposure to the allergen, on day 14 for animals subjected to a single exposure, and on days 17 or 18 for animals that had been repetitively exposed to the allergen in previous days.

2.3. Measurement of blinking, eye-scratching movements, and tearing rate

Blinking as well as scratching movements of the posterior legs directed to either eye were counted by 2 different observers during a 5-min period on day 0 (before sensitization; basal values) and immediately after exposure to the allergen of both eyes of sensitized guinea pigs on days 14, 15, 16, 17, and 18. For each animal, the data from both eyes were pooled. Immediately after assessing the eye-scratching response, the tearing rate was measured in both eyes using phenol red threads (Zone-Quick, Menicon, Nagoya, Japan) placed in the nasal side of the inferior lid for 30 s. Values obtained from both eyes in each animal were pooled.

2.4. Electrophysiological recordings

Guinea pigs were humanely killed with an intraperitoneal injection of 100 mg/kg sodium pentobarbitone, and both eyes were enucleated along with a short length of the optic nerve and surrounding tissues. Excised eyes were then pinned to the bottom of a silicone-coated (Sylgard 184, Dow Corning, Midland, MI, USA) chamber filled with cold saline for further cleaning. In the preparation for ciliary nerve fiber recording (Fig. 1A), extracellular recordings were made in nerve filaments of the ciliary nerves dissected at the back of the excised eye [46]. This preparation was particularly suitable for the detection of polymodal and mechanosensory afferent nerve fibers activity. In a second type of preparation for nerve terminals recording (Fig. 1B), the activity of single corneal nerve terminals was recorded in the isolated cornea with a glass pipette applied onto the corneal epithelium surface [16]; in this preparation, cold-sensitive units were preferentially detected.

2.4.1. Recording of ciliary nerve fibers in whole eyes

Connective tissue and extraocular muscles at the back of the eye were carefully removed to expose and isolate the ciliary nerves around the optic nerve. The excised eye was then placed in a chamber divided in 2 compartments by a Sylgard-coated plastic wall. The front of the eye was introduced into a round perforation made in the center of the dividing wall to which the bulbar conjunctiva was pinned, thereby isolating the front from the back of the eye (Fig. 1A). This prevented direct exposure of the ciliary nerves located in the back compartment to the chemical substances applied onto the corneal surface. The anterior compartment was continuously bathed with warmed (34°C) physiological saline solution of the following composition (in mM): NaCl, 133.4; KCl, 4.7; CaCl₂, 2; MgCl₂, 1.2; NaHCO₃, 16.3; NaH₂PO₄, 1.3; glucose, 7.8, and gassed with 95%O₂–5% CO₂ to maintain a pH of 7.4. The rear compartment was filled with warm mineral oil. Thin nerve filaments were teased apart from the ciliary nerve trunks and placed on an Ag–AgCl electrode for monopolar recording of single unit impulse activity, using conventional electrophysiological equipment (DAM50 amplifier, WPI, Sarasota, FL, USA). Electrical signals were fed into a PC through an acquisition system (CED Micro1401, Cambridge Electronic Design, Cambridge, UK) and analyzed with Spike 2 software (v6.0, Cambridge Electronic Design, Cambridge, UK). Receptive fields of afferent fibers innervating the corneoscleral surface were located using mechanical stimulation with a fine paint brush and mapped thereafter using a von Frey hair (5.88 mN). Spontaneous activity was measured during 1 min from recordings obtained before any stimulation was applied. Mechanical threshold was determined assessing the first impulse response evoked by calibrated von Frey hairs of increasing diameter (range 0.25–4 mN, Bioseb, Vitrolles, France). For chemical stimulation, a gas jet of 98.5% CO₂ was applied on the corneal receptive field during 30 s [1,25]. Thermal stimulation was performed by heating (up to 45°C) or cooling (down to 20°C) the perfusion solution with a custom-made Peltier device.

2.4.2. Recording of nerve terminals in isolated corneas

Corneas were excised with a circular cut around the limbus and pinned to the bottom of a recording chamber superfused continuously with physiological saline solution and maintained at 34°C with a feedback-controlled Peltier device. Nerve terminal impulse (NTI) activity was recorded as described elsewhere [16,17,22,23,46], using a glass micropipette applied to the surface of the cornea with a micromanipulator, fixed by slight suction. Pipettes had a tip diameter of around 50 µm and were filled with the physiological saline solution. Signals were recorded with respect to an Ag/AgCl pellet in the bath. Electrical activity was amplified (AC preamplifier NL 103, Digitimer, Welwyn, UK), filtered (high pass 150 Hz, low pass 5 kHz; Filter Module NL 125, Digitimer, Welwyn, UK), transferred to a PC with a CED micro1401 acquisition system, and analyzed with the indicated software (Fig. 1B). The corneal surface was explored with the tip of the micropipette until a site in which spontaneous activity of a single nerve terminal was detected. Nerve impulses originating at single cold-sensitive nerve endings were identified by their rhythmic, regular ongoing discharge that increased upon cooling and decreased upon warming [16,17,27,46]. For thermal stimulation, the temperature of the physiological solution bathing the cornea was changed with the Peltier cell, from the control temperature of 34°C down to 20°C or up to 50°C (cooling rate approximately –0.5°C/s; heating rate approximately +0.4°C/s). Spontaneous NTI activity at basal temperature was recorded for at least 1 min before the application of a cooling ramp to 20°C, followed by rewarming to the basal temperature. After recovering a stable baseline activity, the solution bathing the cornea was heated to about 50°C and immediately cooled again to baseline temperature (Fig. 2).

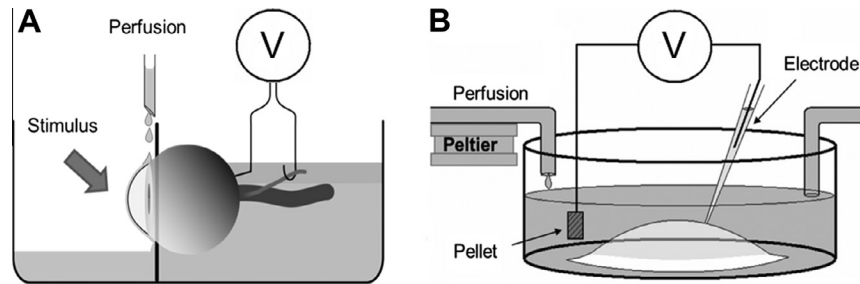


Fig. 1. Schematic diagram of the experimental setup used for the recording of ciliary nerve fibers in the excised eye (A) and single nerve terminals in the isolated cornea (B).

Different parameters (Fig. 2) were calculated to quantify the cooling response: (1) mean frequency of the ongoing NTI activity at the basal temperature of 34°C; (2) cooling threshold (expressed as the decrement in temperature required to increase by 25% the previous basal frequency of discharge); (3) peak frequency (maximal firing frequency reached during the cooling ramp); (4) temperature change to reach peak frequency (expressed as the temperature change in °C required to reach maximal frequency per second during the cooling pulse); (5) silencing threshold (temperature required to silence the terminal during the heating pulse); (6) threshold to resume NTI activity when decreasing temperature from 50°C to 34°C (expressed as the temperature decrement required to recover NTI firing after silencing); (7) peak frequency of discharge induced by decreasing temperature from 50°C to 34°C; (8) heating threshold for paradoxical response (when present) (expressed as the value of temperature change, in °C required to evoke firing with a heating pulse); and (9) peak frequency of the paradoxical response (maximal firing frequency in imp/s obtained during the firing response to a heating pulse).

2.5. Drugs

In a separate group of experiments, the effects of the TRPV1 blocker capsaizipine (Tocris Bioscience, Bristol, UK) or of the TRPA1 blocker HC-030031 (Sigma–Aldrich, Saint Louis, MO, USA) were tested; capsaizipine was dissolved in phosphate-buffered saline from a 25 mM stock in absolute ethanol to a final concentration of 5 mM; HC-030031 was dissolved in saline solution from a

100 mM stock in DMSO to a final concentration of 100 µM. The direct effect of capsaizipine and HC-030031 on spontaneous and stimulus-evoked corneal nerve activity, in control eyes and in eyes that had been repetitively exposed to the allergen was explored. For this purpose, in the whole eye preparation a 6-mm piece of paper soaked in the corresponding drug was placed on the receptive field of the recorded mechano- or polymodal nociceptor fiber during 1 min; mechanical and chemical stimulation were applied before and immediately after application of the drug, and 15 min afterward. To study the direct effects of the drugs on cold nerve terminals, they were added to the physiological saline solution and perfused during 8 min; responses to thermal stimulation were tested before and during exposure to the drug.

To test whether the blocking drugs prevented the various components of the allergic inflammatory reaction, a group of animals was treated before the allergic challenge with either capsaizipine or HC-030031, applying a 10-µL drop of the drug in each eye, 20 to 60 min before the application of OVA on days 14 to 18. Blinking and tearing rates measured in drug-treated animals were compared with those from nontreated allergic guinea pigs. The effects of this pretreatment on the spontaneous and stimulus-evoked impulse activity of the various functional types of corneoconjunctival nerve fibers were also assessed.

2.6. Statistical analysis

Data were collected and processed for statistical analysis using SigmaStat software (SigmaStat v3.5; Systat Software Inc, Point

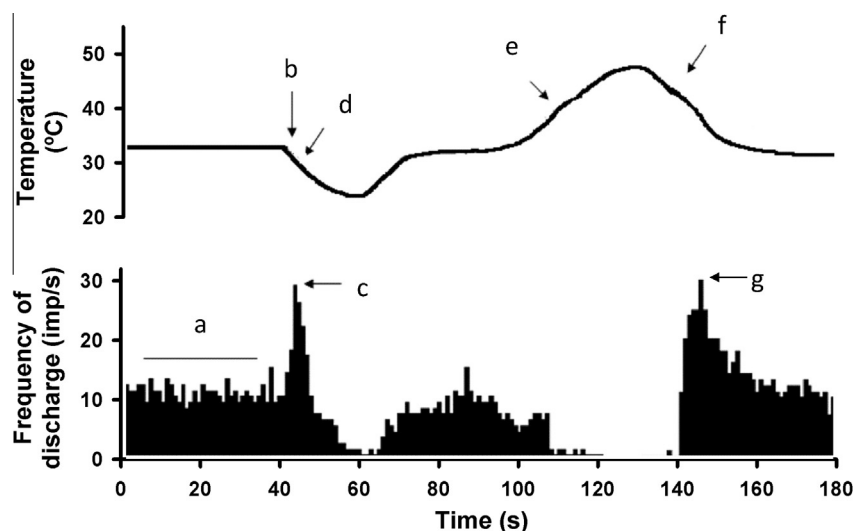


Fig. 2. Example of the change in NTI activity in a cold thermoreceptor terminal evoked by thermal stimulation. Upper trace: Temperature change. Lower trace: histogram of the firing frequency in impulses/s. The parameters used to quantify the cooling response were: a, mean frequency of the ongoing NTI activity at the basal temperature of 34°C; b, cooling threshold; c, peak firing frequency; d, temperature required to reach peak frequency during the cooling pulse; e, temperature required to silence the terminal during heating (silencing threshold); f, threshold to resume NTI activity when cooling from 50°C; g, peak frequency induced by cooling from 50°C to 34°C.

Richmond, CA, USA). Data were expressed as mean $\pm$ SEM, with n being the number of explored nerve fibers or terminals. Differences in mechanical threshold, spontaneous activity, and firing frequency in response to CO₂ pulses or temperature changes (cooling or heating) between control and allergic eyes as well as between allergic eyes pretreated or not with TRP channel blockers were compared using the independent t test or its nonparametric equivalent Mann–Whitney rank sum test. Differences between responses in allergic eyes before and after treatment with TRP ion channel blockers were analyzed with the paired t test or the Wilcoxon signed rank test. One-way ANOVA or Kruskal–Wallis ANOVA on ranks were used to compare 2 or more experimental groups, as indicated. Z test or Fisher's exact test were used to compare proportions. A P value of .05 or below was considered significant.

3. Results

3.1. Responses to the allergic challenge

Immediately after ocular instillation of the OVA solution, immunized animals developed conjunctival hyperemia and edema.

3.1.1. Changes in tear rate, blinking frequency, and eye scratching

Tearing rate was significantly higher in allergic animals at all studied time points. Also, a significant increase in blinking frequency was measured after the allergic challenge (Table 1). Only 8 of 27 animals during all poststimulus measurements displayed scratching movements (3.2 ± 0.9 scratching movements per 5 min) directed to one or both eyes, mostly within the first min of the 5-min measurement period after ocular application of OVA.

3.1.2. Changes in nerve impulse activity

Data on nerve impulse activity were obtained on day 14 (single exposure) or on days 17 and 18 after daily repeated OVA challenges (repeated exposure). Measurements made on these 2 last days produced very similar values; hence, data from both days were pooled to obtain a single value of the various parameters of nerve impulse activity corresponding to the repeated exposure to the allergen.

3.1.2.1. Mechanonociceptors and polymodal nociceptors. Spontaneous activity. Spontaneous activity was measured in 118 mechanonociceptor fibers (control, $n = 55$; after single exposure to the allergen, $n = 47$; after repeated exposures, $n = 16$) and in 89 polymodal nociceptor fibers (control, $n = 34$; after single exposure, $n = 30$; after repeated exposures, $n = 25$). The percentage of mechanonociceptors exhibiting ongoing activity after single or repeated exposures to the allergen (10.6% and 6.3%, respectively) was not different from that of control animals (7.2%, $P = .799$ and $.665$, respectively). This was also the case for the value of mean firing frequency in the fibers displaying spontaneous activity (mean 0.3 ± 0.2 imp/s in control eyes vs 0.5 ± 0.1 imp/s after a single exposure to the allergen, $P = .415$, and 0.7 imp/s in the sole fiber exhibiting spontaneous activity after repeated exposures). Likewise, in polymodal nociceptor fibers the incidence of ongoing activity (16.7% after single

exposure; 12.0% after repeated exposures) was slightly higher than in control animals (8.8%), but the differences were not significant ($P = .484$ and $.983$, respectively). Altogether, mean ongoing firing frequency of active polymodal units was not statistically modified after allergen exposure (1.5 ± 0.9 imp/s in controls; 1.1 ± 0.5 imp/s after single exposure; 0.6 ± 0.2 imp/s after repeated exposures; differences from control $P = .723$ and 0.385 , respectively).

Response to mechanical stimulation. Mechanical threshold was measured in 115 mechanonociceptors (control, $n = 55$; after single exposure, $n = 46$; after repeated exposures, $n = 14$) and in 87 polymodal nociceptors (control, $n = 34$, after single exposure, $n = 28$; after repetitive exposure, $n = 25$). In recordings from control corneas, mechanonociceptors responded phasically, firing 1 to 3 impulses under sustained (3 s) suprathreshold mechanical stimulation, while polymodal nociceptors gave a more prolonged, irregular discharge. After a single allergic challenge, mechanical threshold of mechanonociceptor fibers was significantly reduced (from 0.89 ± 0.12 mN to 0.38 ± 0.06 mN, $P < .05$; Fig. 3A) and remained low after repeated exposures (0.55 ± 0.12 mN; Fig. 3A). In contrast, the mechanical threshold of polymodal nociceptors was unaltered after single or repeated exposure to the allergen (Fig. 3B). No differences between intact and allergic eyes were noticed in the duration or firing pattern of the response to mechanical stimuli of mechanonociceptor and polymodal nociceptor fibers (data not shown).

Response to CO₂. The response evoked by 98.5% CO₂ pulses was tested in 66 polymodal fibers (control, $n = 18$; after a single exposure $n = 27$; after repeated exposures, $n = 21$). After a single allergic challenge, the firing response evoked by a CO₂ pulse in polymodal nociceptors was not significantly changed compared with controls (Fig. 4A). However, in eyes repeatedly exposed to the allergen, the frequency of impulses in response to CO₂ was significantly higher (control: 1.4 ± 0.2 imp/s; repetitive exposures: 3.6 ± 0.7 imp/s, $P < .01$, Fig. 4A) and the latency of the impulse response was significantly shorter (control: 18.3 ± 1.7 ms; repetitive exposures: 9.1 ± 1.6 ms, $P < .001$ Fig. 4B). Also, the values of latency and magnitude of the response to CO₂ were different from those measured after a single exposure to the allergen ($P < .03$ and $P < .075$, respectively).

Response to heat. In control eyes, 38% of polymodal nociceptor fibers (13 of 34) were activated by a heating ramp to 45°C. After a single exposure to the allergic challenge the incidence of heat responsiveness was slightly lower (6 of 30, 20%; $P = .194$, Z test), but returned to control levels after repetitive exposures (9 of 25, 36%). When present, the irregular firing pattern evoked by heating ramps looked very similar in control and allergic eyes, although responses were not quantified.

3.1.2.2. Cold thermoreceptors. At 34°C, corneal cold thermosensitive nerve terminals discharged spontaneously NTIs at variable frequency (range: 0.9 to 17.5 imp/s, $n = 66$). NTI firing increased with cooling pulses. In response to a heating ramp, cold thermoreceptor endings usually gradually reduced their impulse firing until they silenced at around 37°C to 38°C (silencing threshold; Fig. 2). However, a fraction of them resumed the impulse firing when

Table 1

Values of tearing (wet length of the phenol red thread) and blinking rate measured before and after the allergic challenge.

Characteristic	Before	After allergic challenge (topical treatment with ovalbumin)				
	Day 0	Day 14	Day 15	Day 16	Day 17	Day 18
Tearing rate (mm)	8.7 ± 1.0	$30.8 \pm 2.2^{**}$	$24.6 \pm 0.9^{**}$	$25.3 \pm 1.0^{**}$	$28 \pm 1.0^{**}$	$26.5 \pm 2.0^{**}$
Blinking (no. per 5 min)	3.6 ± 1.5	$14.8 \pm 1.8^{**}$	$23.8 \pm 2.5^{**}$	$22.4 \pm 1.4^{**}$	$29.6 \pm 4.2^{**}$	$42.5 \pm 4.1^{**}$
No. of animals	14	20	5	5	5	2

^{**} $P < .001$ (1-way ANOVA); data from all groups are different from control values (day 0).

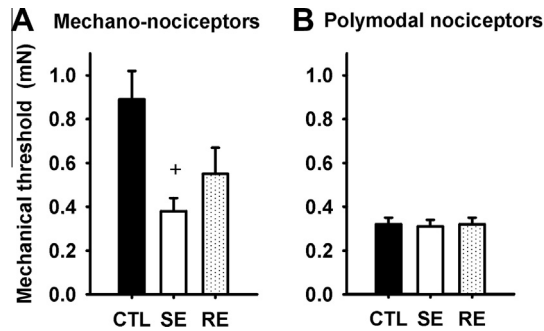


Fig. 3. Mechanical threshold of mechanonociceptors (A) and polymodal nociceptors (B) in control animals (CTL), and in allergic animals after single (SE) and repeated (RE) exposures to the allergen. $^*P < .05$, Mann–Whitney rank sum test.

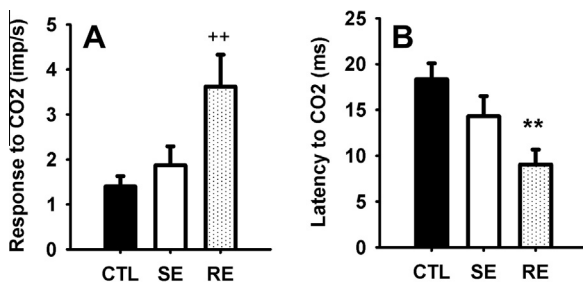


Fig. 4. Response of polymodal nociceptors to chemical stimulation with a 30-s CO₂ pulse in control eyes (CTL) and after single (SE) and repeated exposures (RE) to the allergen. (A) Mean discharge rate of the impulse response to CO₂. (B) Latency of the onset of firing response to CO₂. $^{**}P < .001$, $^{**}P < .01$, t test or Mann–Whitney rank sum test.

the temperature reached values over 40°C to 42°C (paradoxical response). Table 2 summarizes the values of different parameters of the impulse response to temperature variations in corneal cold-sensitive terminals. After repeated allergic challenges, the ongoing activity of cold thermoreceptors at the basal temperature of 34°C decreased significantly (Table 2, Fig. 5A). Moreover, in challenged eyes, the impulse discharge evoked by thermal stimuli was altered after single and repeated exposures to the allergen. This was evidenced in the reduction of the maximal firing response attained with cold pulses from 34°C to 20°C (Fig. 5B) or when cooling was performed at the end of a 50°C heating pulse (Table 2). Also, the maximal response to cold was achieved in both instances with comparatively smaller reductions of temperature (Table 2). Notably, in allergic eyes no significant changes were observed in cooling threshold measured with cooling pulses from 34°C to 20°C (Table 2, Fig. 5C).

After the allergic challenge, the mean temperature value required to silence cold nerve endings by heating (silencing threshold) was significantly lower than in control eyes (Table 2, Fig. 5D). The percentage of cold thermoreceptors exhibiting paradoxical response increased slightly ($P = .078$) after repeated allergen exposure (Table 2).

3.2. Effects of TRPV1 and TRPA1 blockers

3.2.1. Tear secretion and blinking rate

The enhanced blinking evoked by the first ocular OVA application on day 14 was not modified by pretreatment with capsazepine or HC-030031. However, both drugs significantly attenuated the increased blinking evoked by repeated exposure to the allergen (Fig. 6A).

The elevation of tear secretion observed since the onset of the allergic challenge at day 14 was reduced significantly by pretreat-

Table 2

Background activity and response to thermal stimulation (cooling and heating) in cold thermosensitive terminals of control eyes and of eyes challenged with the allergen.^a

Parameter	Control eyes	Allergic eyes	
		Single	Repeated
Ongoing activity (imp/s)	9.0 ± 0.5	8.0 ± 0.7	6.0 ± 0.8*
<i>Cooling from 34°C</i>			
Cooling threshold (change in temperature from 34°C, in °C)	−2.6 ± 0.2	−3.3 ± 0.5	−1.8 ± 0.2
Peak frequency (imp/s)	29.9 ± 1.3	20.3 ± 1.8**	16.6 ± 1.6**
Temperature change to reach peak frequency (change in °C)	−6.1 ± 0.4	−4.8 ± 0.5 [‡]	−3.5 ± 0.4**
<i>Cooling from 50°C</i>			
Cooling threshold (change in temperature from 50°C, in °C)	−14.6 ± 0.7	−12.6 ± 0.7	−10.5 ± 0.6**
Peak frequency (imp/s)	20.2 ± 1.1	12.8 ± 1.0**	14.1 ± 1.5*
Temperature change to reach peak frequency (change in °C)	−17.2 ± 0.3	−16.3 ± 0.4	−11.9 ± 0.4**
<i>Heating</i>			
Silencing threshold (change in temperature from 34°C, in °C)	+4.6 ± 0.5	+2.8 ± 0.6**	+1.6 ± 0.2**
Incidence of paradoxical response (% of units)	20%	17%	28%
Heating threshold for paradoxical response (change in temperature from 34°C, in °C)	+11.4 ± 1.6	+15.3 ± 1.3	+12.8 ± 0.8
Peak frequency of paradoxical response (imp/s)	15.3 ± 4.6	24.3 ± 5.8	14.8 ± 3.9
No. of terminals	66	34	25

$^{**}P < .001$, $^*P < .005$, $^‡P < .05$, t test difference from control.

^a Data are presented as mean ± standard error of the mean.

ment with capsazepine (Fig. 6B). In contrast, HC-030031 was ineffective in attenuating the increased tear flow induced by the allergic reaction (Fig. 6B).

3.2.2. Nerve impulse activity

3.2.2.1. Mechanonociceptors. Direct application of capsazepine or HC-030031 onto the receptive field of mechanosensory fibers in allergic corneas did not evoke an impulse discharge or change the mechanical threshold ($0.31 ± 0.04$ and $0.31 ± 0.04$ mN before and after capsazepine, respectively, $n = 5$; $0.30 ± 0.03$ vs $0.30 ± 0.03$ mN before and after HC-030031, $n = 8$). A lack of effect was also seen in control corneas with capsazepine, tested in 5 fibers, and HC-030031, tested in 7 fibers (data not shown).

Pretreatment of the animal with capsazepine before exposure to the allergen did not alter the low incidence of spontaneous activity seen in mechanonociceptors of eyes subjected to repetitive allergic challenges (1 of 9 fibers, 11.1%) or the value of mean firing frequency (0.58 imp/s) in this spontaneously active fiber. The reduction in the mechanical threshold of mechanonociceptors caused by the allergic challenge was slightly affected by capsazepine pretreatment ($0.43 ± 0.16$ vs $0.55 ± 0.12$ mN, $P = .051$, $n = 8$ and 14, capsazepine-treated and nontreated allergic corneas, respectively). Likewise, pretreatment with HC-030031 in eyes subjected to repeated allergic challenge did not modify significantly the incidence of spontaneous activity (4 of 42 recorded fibers, 9.5%) or the mean firing frequency of the spontaneously active fibers ($0.3 ± 0.1$ imp/s). Notably, the tendency of the allergic challenge to decrease mechanical threshold of mechanonociceptors became more prominent after pretreatment with HC-030031 ($0.40 ± 0.05$ vs $0.55 ± 0.12$ mN, $P = .001$, $n = 40$ and 14, HC-030031 and nontreated allergic corneas, respectively).

3.2.2.2. Polymodal nociceptors. Capsazepine directly applied on the receptive field of 18 polymodal nociceptor fibers of allergic eyes did not modify significantly their mechanical threshold

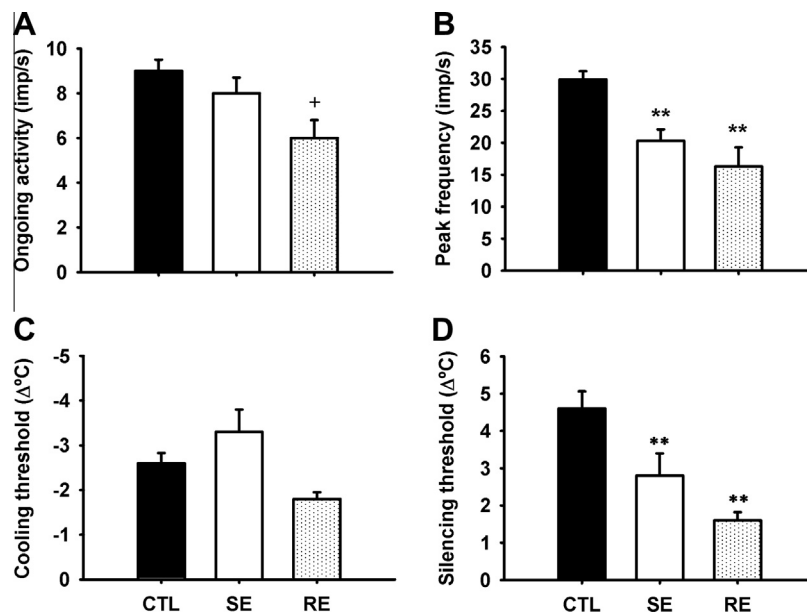


Fig. 5. Values of the different parameters of the response of cold thermoreceptors to cooling in control (CTL) and in challenged eyes after single (SE) and repeated (RE) exposures to the allergen. (A) Ongoing activity at basal temperature (34°C). (B) Peak frequency of discharge in response to cooling ramps from 34°C to 20°C . (C) Decrement in temperature required to increase by 25% the basal firing frequency of discharge (cooling threshold). (D) Increase of temperature needed to silence NTI firing (silencing threshold) during heating ramp from 34°C to 50°C . $^{**}P < .001$, $^+P < .05$, t test or Mann–Whitney rank sum test.

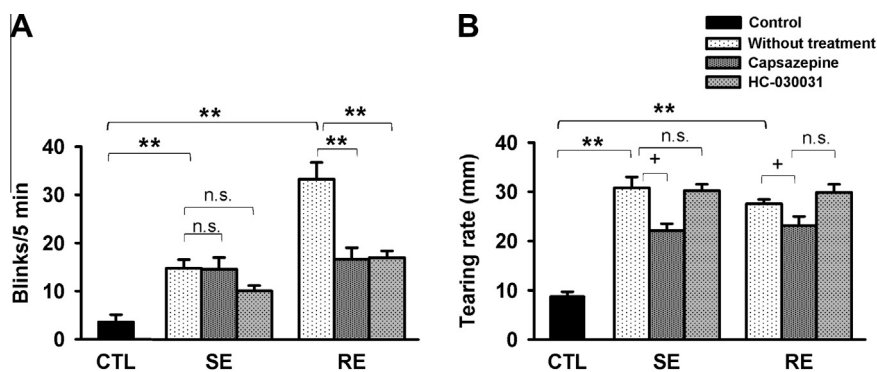


Fig. 6. Blinking (A) and tearing rate (B) in intact eyes (CTL, black columns) and after single (SE) or repetitive (RE) exposure to the allergen, without (white columns) and after treatment with capsazepine (dark gray columns) or HC-030031 (light gray columns). $^{**}P < .001$, $^+P < .05$, NS, not significant; t test or Mann–Whitney rank sum test.

(0.25 ± 0.01 vs 0.26 ± 0.01 mN, before and after, respectively) or the spontaneous firing rate, when present (0.1 ± 0.1 vs 0.3 ± 0.1 imp/s, $P = .432$). However, capsazepine greatly reduced the firing response to CO_2 (4.3 ± 1.2 vs 0.8 ± 0.2 imp/s, $P < .001$). This effect was seen immediately after application of the drug and was reversed with 15-min washing. A similar blockade of the CO_2 response was seen in polymodal nociceptors recorded from control corneas ($n = 11$, data not shown).

In allergic eyes treated with capsazepine before the OVA challenge, no polymodal nociceptor fibers exhibiting spontaneous activity or responding to heat pulses (heating to 45°C) were found (0 of 12), in contrast with untreated allergic challenged corneas. Mechanical threshold of polymodal fibers of capsazepine-pretreated eyes (0.29 ± 0.04 mN, $n = 10$) was not different from that of untreated allergic animals. However, capsazepine reversed the enhancement in firing response to CO_2 induced by the allergic challenge (cf Figs. 4A and 7A), whereas the shortening of the discharge latency was not affected by the drug (Fig. 7B).

Direct application of HC-030031 to the receptive field of polymodal nociceptor fibers from allergic eyes ($n = 20$) did not modify their mechanical threshold (0.30 ± 0.03 vs 0.30 ± 0.03 mN before

and after treatment, respectively), spontaneous activity (0.30 ± 0.01 vs 0.30 ± 0.01 imp/s) or amplitude of the response to CO_2 (5.0 ± 1.4 vs 5.0 ± 1.4 imp/s). A similar absence of effect on polymodal nociceptor responsiveness to these stimuli was found when the drug was applied into intact corneas ($n = 9$, data not shown).

In allergic eyes pretreated with HC-030031 before the repetitive exposure to the allergen, the proportion of polymodal nociceptor fibers exhibiting spontaneous activity and the mean ongoing firing frequency values were similar to those seen in untreated animals (4 of 15, 27% and 0.4 ± 0.1 imp/s, respectively). This was also the case for the percentage of polymodal nociceptors responding to heat (36%, 9 of 25). However, HC-030031 reduced mechanical threshold (0.25 ± 0.01 mN, $n = 13$, $P < .05$) and tend to attenuate the enhanced responsiveness to CO_2 and the shorter latency of the impulse response to CO_2 stimulation normally observed after the allergic challenge although differences did not reach the significance level (Fig. 7).

3.2.2.3. Cold thermoreceptors. In recordings performed in repeatedly challenged eyes, capsazepine added to the perfusing solution

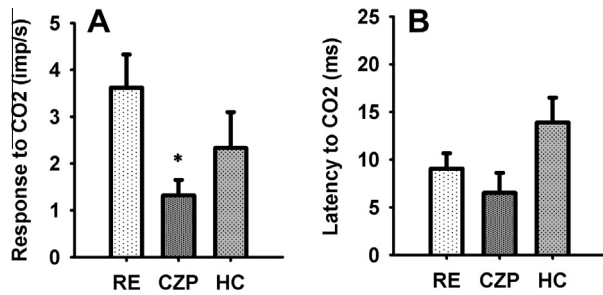


Fig. 7. Response of polymodal nociceptors to chemical stimulation with a 30-s CO₂ pulse, in allergic animals repeatedly exposed to the allergen without (RE) or after pretreatment with capsazepine (CZP) or HC-030031 (HC). (A) Mean discharge rate of the impulse response to CO₂. (B) Latency for the onset of the firing response to CO₂. **P* < .005, *t* test or Mann–Whitney rank sum test.

at a final concentration of 50 μ M did not modify the ongoing or cold-evoked NTI activity (ongoing activity: 6.5 ± 0.7 vs 5.9 ± 0.7 imp/s; cooling threshold: -1.7 ± 0.3 vs -2.0 ± 0.4 °C; peak response to cold: 22.4 ± 1.3 vs 19.9 ± 0.9 imp/s; before and after capsazepine, respectively, *n* = 7). Similar results were found in control corneas (*n* = 5, data not shown).

Pretreatment with capsazepine before the allergic challenge eliminated some of the effects of the allergic reaction on cold thermoreceptor activity, such as the reduction of mean ongoing firing frequency, the lower firing frequency peak evoked by a cooling ramp, and the lower temperature increase required to silence cold thermoreceptors seen after OVA application (Fig. 8A–D). However, the lower temperature value required to attain the peak response to a cooling ramp in challenged eyes was unaffected by capsazepine (data not shown). Six of 17 cold-sensitive endings of eyes pretreated with capsazepine before repeated OVA application presented a paradoxical response to heat, the same proportion as in untreated eyes (*P* = .887). The mean threshold of the paradoxical response was significantly lower than in challenged eyes without capsazepine treatment ($+8.6 \pm 1.2$ °C, *n* = 6 vs $+12.8 \pm 0.8$, *n* = 25; *P* < .05).

HC-030031 directly applied in the perfusion solution did not have any effect on the spontaneous and thermal evoked activity of cold nerve terminals (spontaneous activity: 6.0 ± 0.5 vs 5.7 ± 0.4 imp/s; cooling threshold: -1.7 ± 0.5 vs -2.3 ± 0.7 °C; peak response to cold: 24.1 ± 1.5 vs 23.3 ± 1.4 imp/s; before and after, *n* = 8). Similar results were found in control corneas (*n* = 6, data not shown).

Moreover, HC-030031 did not reverse the lower spontaneous activity at basal temperature or the decreased responsiveness to a cooling ramp exhibited by cold receptors under the allergic challenge (Fig. 8A–C). Likewise, in eyes pretreated with HC-030031, 16 of 27 cold-sensitive terminals still presented paradoxical response, with a mean threshold of $+11.8 \pm 0.7$ °C and a mean peak frequency of discharge of 15.7 ± 2.04 imp/s. These values are not different from those of untreated eyes ($+12.8 \pm 0.8$ °C and 14.8 ± 3.9 imp/s, respectively). Notably, the significant reduction in allergic eyes of the temperature value required to silence ongoing activity of cold thermoreceptors by warming disappeared after pretreatment with HC-030031 (Fig. 8D).

4. Discussion

Most of the previous experimental studies on allergic ocular diseases [18,21,29] were aimed at deciphering the molecular and cellular events underlying the allergic reaction and to find possible treatments for the disease [48,63]. Our work demonstrates that during an experimental ocular allergic reaction, increased tearing and blinking are accompanied by disturbances of threshold, firing

pattern, and responsiveness to natural stimuli of mechano-, polymodal, and cold afferent fibers innervating the eye surface. Such changes are particular for each receptor type, vary along the time course of the allergic reaction, and are presumably the peripheral neurophysiological substrate of the discomfort sensations accompanying allergic conjunctivitis. Our study further shows that pharmacological blockade of TRPV1 and in a minor extent of TRPA1 channels before the allergic reaction, differentially attenuated the enhanced blinking and tearing. In polymodal nociceptors, acute blockade of TRPV1 but not of TRPA1 channels reduced chemical responsiveness, while in cold thermoreceptors activity was not affected by the blockade of these channels. These results, taken together, suggest that part of the behavioral and neurophysiological signs of irritation developed during ocular surface allergy are mediated by activation of TRPV1 and TRPA1 channels, involving, at least in part, those expressed at the peripheral endings of corneal polymodal sensory afferents, whose responsiveness to inflammatory chemicals was expectedly reduced by capsazepine treatment before the allergic challenge.

Guinea pigs have been frequently used as an experimental model for allergic conjunctivitis because of the analogies of the vascular, cellular, and molecular characteristics of the ocular allergic response between this species and humans [19–21]. An additional advantage is that morphology and functional properties of sensory nerve fibers innervating the ocular surface of guinea pigs are known in detail [15–17,22,23,34,46]. Therefore, experimental allergic conjunctivitis induced by OVA in the guinea pig eye [18,21,29,58] appears to be an appropriate model to correlate the molecular and cellular changes induced by an allergic challenge, with the sensory activity of peripheral sensory receptors of the cornea and conjunctiva during the allergic reaction.

The early phase of an ocular allergic response (which clinically lasts for 20 to 30 min) is characterized by an immediate, IgE-mediated mast cell degranulation with minimal presence of migratory inflammatory cells. Mast cells degranulation leads to local release of histamine, tryptase, prostaglandins, and leukotrienes, enhancing their tissue and tear levels [42,49], and also activating vascular endothelial cells and expression of chemokines and adhesion molecules. These molecules initiate the recruitment of inflammatory cells in the conjunctival mucosa [42,49], which leads to mucosal infiltration of eosinophils, neutrophils, basophils, and T lymphocytes and produces the persistent clinical inflammation that determines the ocular signs and symptoms observed in perennial and chronic allergic diseases [42,49]. Many of these inflammatory substances are known to activate and evoke sensitization of polymodal nociceptor fibers in various tissues, including those of the ocular surface [10], thereby causing development of ongoing activity, lowered threshold, and enhanced firing responses to natural stimuli [12]. Thus, the augmented responsiveness of polymodal nociceptors of the cornea and bulbar conjunctiva after OVA application are likely attributable to a combined action of the cascade of cytokines released by the allergic challenge.

The overall changes in nerve activity observed in our experimental conditions after the first, acute exposure to the allergen were less prominent than those obtained after repeated exposure, in spite of the fact that guinea pigs displayed some behavioral signs of irritation (blinking and tearing) at that stage. As mentioned above, the buildup of inflammatory mediators occurs gradually and is expected to be lower at the initial steps of the allergic reaction. Besides, in the continuously perfused excised eye, it is possible that part of the inflammatory substances locally released after the allergen challenge are washed out when the eye is placed in vitro, thus reducing or even preventing the development of some of the signs of sensitization. In later stages of the allergic reaction, the recruitment of inflammatory cells by neuropeptides liberated by active sensory nerves and cytokines, such as various

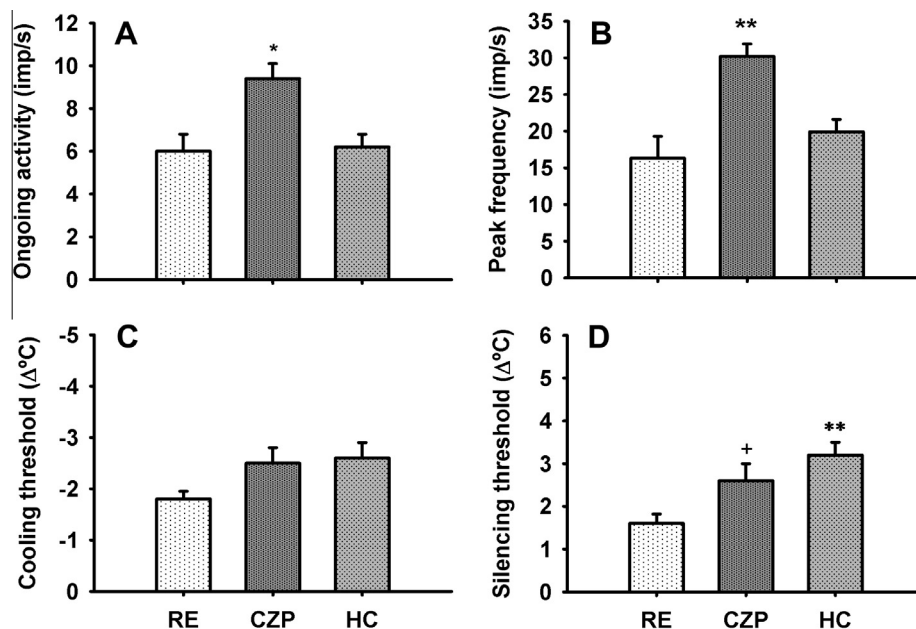


Fig. 8. Values of the different parameters of the response of cold thermoreceptors to cooling, in challenged eyes after repeated exposures to the allergen without (RE) or with pretreatment with capsaizipine (CZP) or HC-030031 (HC). (A) Ongoing activity at basal temperature (34°C). (B) Peak frequency of discharge in response to cooling ramps from 34°C to 20°C . (C) Decrement in temperature required to increase by 25% the basal firing frequency of discharge (cooling threshold). (D) Increase of temperature needed to silence NTI firing (silencing threshold) during heating ramp from 34°C to 50°C . * $P < .05$, ** $P < .001$, + $P < .005$, t test or Mann–Whitney rank sum test.

interleukins, $\text{TNF-}\alpha$, and $\text{TGF-}\beta$ released by activated mast cells, further contribute to accumulation of inflammatory agents. These act on their cognate receptors at the nociceptor terminal and rapidly activate intracellular kinases, thereby producing posttranslational changes in transducer and voltage gated ion channels, ultimately leading to increased insertion of the channels into the membrane, altered thresholds, and modified membrane kinetic properties [33]. Such longer-lasting changes could explain the more prominent sensitizing effects seen in our experiments after repeated allergic challenges.

The present data also show that the effects of the allergic reaction on the various functional classes of ocular surface sensory receptors were quite different for each receptor type. In mechanonociceptors, only threshold was transiently affected, while no spontaneous activity developed. Similar changes have been described in the inflamed skin, where mechanical threshold decreased in postinjury inflammation in only a fraction of A δ mechanonociceptors without development of ongoing activity [30,54]. This lowering of mechanical threshold was not prevented by application of TRPV1 or TRPA1 channel blockers, thus suggesting that other mechanosensory channels are modulated by inflammatory mediators at corneal mechanonociceptor afferents; TRPV4, TRPC5, 2-pore domain K^+ channels, or Piezo channels reportedly mediating sensory mechanotransduction in other cells [5,26,28,51] are possible candidates. In contrast, chemosensitivity of polymodal nociceptors exhibited a sustained sensitization after repeated allergic challenges, reflected in the decreased latency and larger responses to CO_2 stimulation. A similar change in responsiveness to acid has been observed in more than a half of the cutaneous polymodal nociceptors after skin inflammation resulting from surgical incision [35].

The enhanced sensitivity of polymodal nociceptive afferents is possibly the principal cause of unpleasant sensations and behavioral responses induced by the allergic inflammation. TRPV1 and TRPA1 are the main ion channels conferring to polymodal nociceptors their characteristic sensitivity to a large variety of exogenous chemicals and endogenous inflammatory mediators [7,8,36,39,45,47,59,60].

These channels are variably expressed by the different functional subclasses of nociceptive neurons, in some of which they coexist and interact [14,55]. Transducing channel activity sets the initial level of nerve impulse firing in each class of nociceptive sensory afferent, while the variable recruitment of these fibers determines their contribution to the final quality of the evoked sensation [1,4].

Selective blockade of TRPV1 or TRPA1 affected differentially the characteristics of the response of polymodal nociceptors to chemical stimulation. TRPV1 appears to play a central role in the acute response of polymodal nociceptor terminals to the pH drop evoked by CO_2 exposure, in agreement with the well-established sensitivity of this channel to protons [24]. Moreover, the absence of spontaneously active fibers and the attenuated response to CO_2 seen after capsaizipine treatment confirms that TRPV1 channels are also important for chemical responsiveness and sensitization of polymodal nociceptors [37,52]. This is further supported by the attenuation of the enhanced blinking and tearing caused by the repeated allergic challenge with capsaizipine pretreatment. TRPA1 blockade by HC-030031 also decreased augmented blinking but not tearing, and had limited effects on chemical responsiveness of corneal polymodal nociceptors, suggesting that the contribution of TRPA1 to the enhancement of excitability in polymodal nociceptors is more modest at least for nociceptors innervating the cornea.

In contrast, both the background and stimulus-evoked impulse activity in corneal cold thermoreceptors was depressed after the allergic challenge. Analogous reductions of cold receptor activity have been reported in corneal cold thermoreceptor endings of mice after application of bradykinin, histamine or a mixture of inflammatory mediators (so-called inflammatory soup), an effect that is conveyed through direct inhibition by a $\text{G}\alpha_q$ protein of the cold-transducing channel TRPM8 [64]. Interestingly, under the allergic challenge less warming was required to silence activity during heating pulses (silencing threshold), confirming that the channels activated by heat in cold endings were sensitized by mediators released during the allergic challenge [64]. A decrease of cold thermoreceptor sensory inflow to the central nervous system may enhance discomfort sensations evoked by mechano- and

polymodal nociceptor sensitization, whose activity is inhibited in part at higher levels of the pain sensory pathways by the input of peripheral cold thermoreceptors [53].

The depression of cold receptor activity elicited by inflammation was reversed under pretreatment with capsazepine. This is likely an indirect effect, the consequence of the blockade by the drug of polymodal nociceptor sensitization, which in turn reduces their release of neuropeptides and a cascade of events leading to massive release of inflammatory mediators accompanying neurogenic inflammation [32].

In humans, experimental stimulation of ocular polymodal nociceptors with CO₂ or heat causes irritation and pain [1,4]. Thus, augmented blinking in allergic challenged guinea pigs could in principle be attributed to discomfort caused by the higher sensory input from polymodal nociceptors expressing TRPV1 and TRPA1 channels, while any tonic effect on blinking of cold thermoreceptors would be reduced. Itching is a prominent symptom of allergic conjunctivitis, particularly in the initial stages of the disease [49]. In our experiments, guinea pigs did not display scratching movements directed to the eye in response to the allergic challenge, a behavioral response seen in cutaneous itch [40]. However, the possibility that blinking represents a behavioral reaction to ocular itch cannot be excluded. It has been proposed that itch is caused by activation of a specific subset of unmyelinated polymodal nociceptor neurons whose peripheral nerve endings are activated by serotonin and histamine, acting, respectively, on 5HT-3 and H1 receptors and ultimately on TRPV1 channels [31,56,57]. Additionally, a part of such neurons possess a histamine-independent itch mechanism, activated by distinct pruritogenic mediators released by mast cells [41], which interact with Mas-related G-protein receptors (Mrgpr) [43] opening downstream TRPA1 channels [62]. Hence, it can be speculated that in our experiments, a fraction of the corneoconjunctival polymodal nociceptor fibers sensitized by the allergen belong to the subpopulation of itch-evoking nociceptive neurons coexpressing TRPV1 and TRPA1 channels [62] that would be responsible of itching sensations accompanying allergic conjunctivitis.

In humans, basal tear secretion appears to be partly maintained by the tonic activity of ocular cold thermoreceptors [50], while reflex tearing is evoked by polymodal nociceptor activation by noxious stimuli [3,50]. Augmented tearing caused by the allergic reaction in guinea pigs was partly reversed by TRPV1 blockade, suggesting that it is partially caused by reflex excitation of polymodal nociceptors. Plasma extravasation and perhaps direct stimulation of accessory lacrimal glands by locally released inflammatory mediators may also contribute to the higher tear flow.

In conclusion, our experimental data in guinea pigs suggest that inflammatory mediators released during allergic keratoconjunctivitis evoke direct chemical activation and sensitization of polymodal nociceptors, discrete sensitization of mechanonociceptors, and reduction of cold thermoreceptor activity. These changes in sensory inflow, taken together, could explain the different discomfort sensations and reflex responses found in patients during the evolution of the ocular allergic reaction.

Conflict of interest statement

The authors report no conflict of interest.

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