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Señor D.

Superintendencia de Industria y Comercio

E.

S.

D.

Referencia: Solicitud Patente de Invención
Título: Composiciones oftalmológicas no irritantes de povidona yodada
Solicitante: Foresight Biotherapeutics, Inc.
Exp. No: 12-117.299
Asunto: Recurso de Reposición contra la Resolución No. 8179 del 27 de febrero de 2015

Yo JESÚS M. MÉNDEZ BERMÚDEZ, identificado con cédula de ciudadanía No. 13.491.525 de Cúcuta y con Tarjeta Profesional de Abogado No. 99.678 del Consejo Superior de la Judicatura, en mi calidad de apoderado de la sociedad FORESIGHT BIOTHERAPEUTICS, INC., y estando dentro del término legal, respetuosamente manifiesto a este Despacho que interpongo recurso de reposición contra la Resolución No. 8179 de 27 de febrero de 2015, por medio de la cual se deniega la patente de invención a la solicitud de la referencia.

HECHOS

- PRIMERO:** Mediante escrito radicado ante la Superintendencia de Industria y Comercio el día 12 de julio de 2012, se presentó la solicitud de patente de invención bajo el título de “**COMPOSICIONES OFTALMOLÓGICAS NO IRRITANTES DE POVIDONA YODADA**”. Dicha solicitud se radicó con el propósito de que se diera inicio a la fase nacional de la solicitud de patente internacional PCT/US2010/060489.
- SEGUNDO:** De conformidad con lo dispuesto en el Tratado de Cooperación en Materia de Patentes (PCT), el reglamento, lo previsto en el artículo 39 de la Decisión 486 de la Comisión de la Comunidad Andina, y la Circular Única de la Superintendencia de Industria y Comercio, se ordenó publicar el extracto sin que se hubieran presentado oposiciones por parte de terceros.
- TERCERO:** De conformidad con lo dispuesto en el artículo 45 de la decisión 486 se realizó el examen de patentabilidad y se requirió a la solicitante mediante el oficio 9232, notificado el 5 de agosto de 2014 para que se pronunciara sobre las objeciones efectuadas a la solicitud en cuanto a la carencia de nivel inventivo según lo establece el artículo 18 de la Decisión 486 antes mencionada.
- CUARTO:** Atendiendo dicho requerimiento y dentro del término oportuno para hacerlo, di respuesta al mencionado oficio mediante escrito radicado ante la Superintendencia de Industria y Comercio el 15 de diciembre de 2014. Se presentaron nuevas reivindicaciones 1 al 11 para dar mayor claridad y atendiendo a algunas objeciones de forma. Además se dieron las explicaciones necesarias de porqué si hay nivel inventivo en la invención.
- QUINTO:** No obstante lo anterior, la Superintendencia de Industria Comercio, mediante Resolución No. 8179 del 27 de febrero de 2015, resuelve negar la solicitud de patente a la presente solicitud con base en la falta de nivel inventivo.

SEXTO: La resolución No. 8179 del 27 de febrero de 2014, mediante la cual se niega la patente a la invención de la presente solicitud, fue notificada por edicto el día 3 de abril de 2015.

RAZONES DE INCONFORMIDAD CON EL ACTO RECURRIDO

A pesar de los argumentos aportados en mi memorial de respuesta al Oficio 9232, su Despacho considera que persiste la situación de falta de nivel inventivo. Los argumentos que, sin embargo, plantea su Despacho carecen de fundamento tanto técnico como jurídico como se demuestra enseguida.

En primer lugar, presento un nuevo capítulo de reivindicaciones en donde se hace aún más claro el objeto de la invención distinguiéndolo con claridad del estado de la técnica y por ende mostrando que la presente solicitud si tiene nivel inventivo frente al arte previo. En este nuevo capítulo de reivindicaciones se agregó una nueva reivindicación numerada 12, la cual tiene pleno soporte en la descripción.

Ahora los siguientes comentarios y argumentos deberían dejar claro la patentabilidad de la presente invención. Dichos argumentos son como sigue:

1. D2 enseña que el mentol mismo es irritante y produce incomodidad y sensaciones de quemadura, como se describe, por ejemplo, en la página 519 “el Mentol aplicado tópicamente al ojo produjo de inmediato una sensación refrescante y una irritación suave seguida en algunos casos por una sensación de incomodidad ligera algunas veces acompañada de un componente de sensación de quemadura”. Esto sin duda muestra el fracaso de D2 para obtener algo como la presente invención y en lugar de estimular, desestimula al versado en la materia. Adjuntamos una copia en inglés de D2 en caso de que su Despacho lo requiera.
2. Ahora, la combinación del mentol irritante de D2 y las propiedades irritantes bien conocidas de la PVP-I, como se describe en la presente solicitud “sorprendentemente se descubrió que las composiciones dadas aquí, comprenden povidona yodada combinada con agentes refrescantes establecidos en la presente... eliminaron el efecto irritante indeseado de la PVP-I en el ojo”, llevan juntos a menos irritación, lo que es un resultado sorprendente e inesperado de la combinación de dos productos irritantes.
3. Adjunto al presente escrito entrego una copia y su traducción de una declaración de los doctores Michael Weiser y Jason Stein inventores de la presente invención que en esta soportan el argumento presentado en el punto 2. Su Despacho ahora de notar que estos resultados sorprendentes proporcionan en piso para la patentabilidad por encima del arte previo.

Adicionalmente, conforme lo estipulado en el artículo 36 D. 486 presento ante su Despacho una solicitud divisional para que siga su respectivo trámite.

Las anteriores razones son suficientes para concluir que la presente reivindicación 1 si tiene nivel inventivo ya que una persona versada en la materia no estaría motivada para combinar las enseñanzas del arte previo para llegar a la invención mencionada en la reivindicación independiente 1. De manera respetuosa, solicito que se retire el rechazo de la reivindicación independiente 1 y sus dependientes 2 a 12.

Por las razones anteriores, quiero insistir ante su Despacho y pedirle, de manera respetuosa, reconsidere su decisión y teniendo en cuenta los argumentos presentados en mi memorial de respuesta anterior y los argumentos que se mencionan en este escrito se dé cuenta de que la presente invención si tiene nivel inventivo y merece que se le otorgue la patente.

PETICIÓN

Por lo anteriormente expuesto, respetuosamente solicito a la administración que revoque el acto administrativo por el cual se niega la patente a la solicitud de la referencia y, como consecuencia, se otorgue la patente.

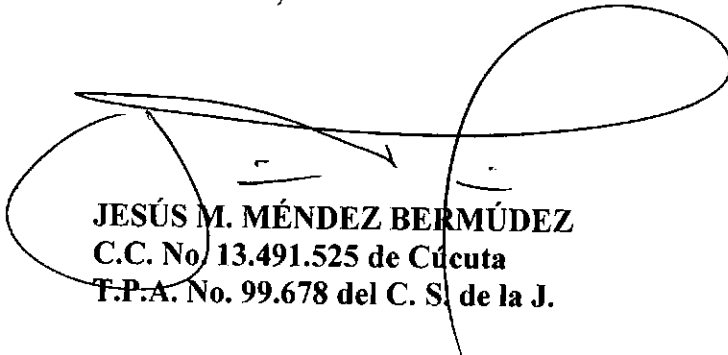
ANEXOS

1. Nuevo capítulo de reivindicaciones
2. Declaración de los inventores con su debida traducción.
3. Documento D2
4. Solicitud divisional

NOTIFICACIONES

Recibiré notificaciones en la Secretaria de este Despacho y en mi oficina, ubicada en la Calle 83ª No. 23-90 de esta ciudad.

Del señor Jefe,



JESÚS M. MÉNDEZ BERMÚDEZ
C.C. No. 13.491.525 de Cúcuta
T.P.A. No. 99.678 del C. S. de la J.

REIVINDICACIONES

1. Una preparación oftálmica comprende:
 - a. Povidona yodada en una concentración entre 0,1% a 2,5%,
 - b. un agente de enfriamiento, en una concentración que no irrite el ojo, seleccionado del grupo que consiste de mentol, camfor, y borneol.
2. La preparación oftálmica según la reivindicación 1, en donde la PVP-I está presente en una concentración seleccionada del grupo que consiste de 0,2% a 2,0%, 0,3% a 1,5%, 0,36% a 1,0%, y 0,4% a 0,75%.
3. La preparación oftálmica según la reivindicación 1, en donde la PVP-I está presente en una concentración seleccionada del grupo que consiste de 0,05%, cerca de 0,1%, cerca de 0,3%, cerca de 0,4%, cerca de 0,5%, cerca de 0,6%, cerca de 0,7%, cerca de 0,8%, cerca de 0,9% y cerca de 1,0%.
4. La preparación oftálmica según la reivindicación 1, en donde dicha preparación comprende un compuesto no esteroideal antiinflamatorio seleccionado del grupo que consiste de fumarato de cetotifeno, diclofenaco sodio, nepafenaco, bromfenaco, flurbiprofeno sodio, suprofeno, celecoxib, naproxeno, rofecoxib, y cualquier combinación de los anteriores.
5. La preparación oftálmica según la reivindicación 1, en donde dicha preparación comprende además un compuesto esteroideal antiinflamatorio se selecciona del grupo que consiste de dexametasona, dexametasona alcohol, sodio fosfato de dexametasona, acetato de flurometalona, flurometalona alcohol, etabonato de lotoprendol, medrisona, acetato de prednisolona, sodio fosfato de prednisolona, difluprednato, rimexolona, hidrocortisona, acetato de hidrocortisona, lodoxamida trometamina, y cualquier combinación de los anteriores.

6. La preparación oftálmica según la reivindicación 1 en donde dicha preparación comprende adicionalmente un agente que incrementa la viscosidad.
7. La preparación oftálmica según la reivindicación 6 en donde en donde dicho agente que incrementa la viscosidad se selecciona del grupo que consiste de polivinil alcohol, polivinilpirrolidona, metil celulosa, hidroxipropilmetilcelulosa, hidroxietilcelulosa, carboximetilcelulosa, hidroxipropilcelulosa, y cualquier combinación de los anteriores.
8. La preparación oftálmica según la reivindicación 1, en donde la preparación comprende al menos un lubricante basado en lágrimas artificiales.
9. La preparación oftálmica según la reivindicación 8, en donde dicho lubricante basado en lágrimas artificiales se selecciona del grupo que consiste de propilen glicol, glicerina, polietilenglicol, dextran, polivinil alcoholes mezclados, polivinil alcohol, aceite mineral liviano, hidroxipropil metilcelulosa, hipromelosa, carbopol, carbomer 940 (ácido poliacrílico), polivinilpirrolidona, petrolato blanco, lecitina de soya, y sodio carboxil metilcelulosa y cualquier combinación de los anteriores.
10. La preparación oftálmica según la reivindicación 1, que comprende además al menos un agente bioadhesivo.
11. La preparación oftálmica según la reivindicación 10, en donde dicho agente bioadhesivo se selecciona del grupo que consiste de polivinilpirrolidona (PVP), goma de xantano, goma de algarroba, goma de acacia, hidroxipropil metilcelulosa (HPMC), alginato de sodio, pectina, gelatina, carbomer, polivinilalcohol, goma gelan, tragacanto, acacia, y sodio carboximetil celulosa, y cualquier combinación de los anteriores.
12. La preparación oftálmica de la reivindicación 1, en donde el agente de enfriamiento está presente en un rango de concentración entre 0,001% a 10%.

ELECTRONICALLY FILED

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Bo LIANG, et al.	: Group Art Unit: 1617
	:
Appln. No.: 13/515,987	: Examiner: Michael B. Pallay
	:
Filed: March 9, 2012	: Attorney Docket No. 100606-5008-US
	:
For: NON-IRRITATING OPHTHALMIC	: Confirmation No. 5853
POVIDONE-IODINE COMPOSITIONS	:

COMBINED DECLARATION UNDER 37 C.F.R § 1.132

We, Michael Weiser, M.D., Ph.D., and Jason Stein, M.D., solemnly declare and affirm the following:

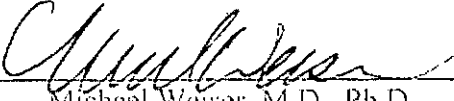
1. We are inventors of the subject matter claimed in the above-captioned patent application, U.S. Patent Application No. 13/515,987 (the "present application"). We have personal knowledge of the subject matter contained in the present application.
2. The assignee of the present application is Foresight Biotherapeutics, Inc.
3. We have extensive laboratory, clinical, and business experience in the development of ophthalmological therapies for treatment of eye disorders such as inflammation and viral, bacterial, and fungal infections.
4. The present application relates to compositions containing a cooling agent and povidone-iodine (PVP-I). PVP-I has potent antiviral and antibacterial effects and is useful as a therapy. However, the administration of PVP-I to the eye can cause undesirable effects

such as severe irritation, burning sensations, tearing, and chemosis. Cooling agents, which include compounds such as menthol, camphor, and borneol, are difficult to use in ophthalmic compositions because they also cause irritation the eye. The presently claimed invention solves these problems by the unexpected finding that a combination of two agents that cause irritation, PVP-I and a cooling agent, surprisingly provide for less irritation upon administration to the eye as a combination than when administered as the individual agents.

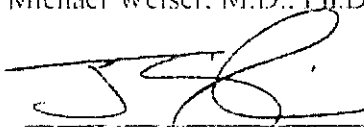
5. We have performed experiments using ophthalmic compositions containing a both PVP-I and the cooling agent menthol. In these experiments, we employed compositions that contained 0.5% and 1.0% by weight PVP-I, with each composition also containing 0.006% by weight of menthol. The use of PVP-I at these concentrations is known to be irritating. These compositions also included other ingredients that do not have irritating properties, and which do not influence the irritation experienced by the user of the composition. We then administered these compositions to the eyes of several test subjects. The administration of a combination of PVP-I and menthol together in the same ophthalmic compositions provided much less irritation than was expected from administration of these concentrations of either PVP-I alone or menthol alone.
6. We declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Application No. 13-515,987
Attorney Docket No. 100606-5008-US

Date: 4.9.15


Michael Weiser, M.D., Ph.D.

Date: 4.9.15


Jason Stein, M.D.

PRESENTADO POR MEDIOS ELECTRÓNICOS

ANTE LA OFICINA DE PATENTES Y MARCAS DE ESTADOS UNIDOS

Ref. solicitud de Bo, LIANG, et al.:	Unidad de Grupos de Técnicas: 1617
Solicitud No.: 13/515,987	Examinador: Michael B. Pallay
Presentado el: 9 de marzo de 2012:	Expediente No. 100606-5008-US
Para: COMPOSICIONES OFTÁLMICAS	Confirmación No. 5833
NO IRRITANTES DE IODOPOVIDONA:	

DECLARACIÓN CONJUNTA DE CONFORMIDAD CON EL TÍTULO 37 DEL LA
§ 1.132 DEL CÓDIGO DE NORMAS FEDERALES

Nosotros, Michael Weiser, M.D., PhD, y Jason Stein, M.D., declaramos y afirmamos solemnemente lo siguiente:

1. Que somos los inventores de la materia reivindicada en la solicitud de patente indicada anteriormente, la Solicitud de Patente de EE.UU. No. 13/515,987 (la "presente solicitud") y que conocemos personalmente la materia contenida en la presente solicitud.
2. El cesionario de la presente solicitud es Foresight Biotherapeutics, Inc.
3. Contamos con amplia experiencia de laboratorio, clínica y comercial en el desarrollo de terapias oftalmológicas para el tratamiento de trastornos oculares tales como inflamaciones e infecciones víricas, bacterianas y micóticas.
4. La presente solicitud se refiere a composiciones con un agente refrescante y iodopovidona (PVP-I). La PVP-I tiene potentes efectos antivíricos y antibacterianos y es útil como terapia. Sin embargo, la aplicación de PVP-I en el ojo puede causar efectos no deseados tales como irritación severa, sensación de ardor, lagrimeo y quemosis. Los agentes refrescantes, que incluyen compuestos tales como el mentol, el alcanfor y borneol, son difíciles de usar en composiciones oftálmicas porque también irritan el ojo. La solicitud reivindicada en el presente resuelve estos problemas con el sorpresivo hallazgo de que una combinación de dos agentes irritantes, PVP-I y un agente refrescante, sorprendentemente causan menor irritación en su aplicación en el ojo como combinación que cuando se aplican como agentes individuales.
5. Hemos realizado experimentos utilizando composiciones oftálmicas que contienen PVP-I y el agente refrescante mentol. En estos experimentos utilizamos composiciones con un 0,5% y 1,0% en peso de PVP-I, al igual que 0,006% en peso de mentol. Se sabe que el uso de PVP-I en estas concentraciones es irritante. Estas composiciones también contienen otros ingredientes sin propiedades irritantes y sin efecto sobre la irritación experimentada por el usuario de la composición. Luego aplicamos estas composiciones en los ojos de varias personas que se sometieron a la prueba. La aplicación de una combinación de PVP-I y mentol juntos en la misma composición oftálmica causó mucha menos irritación de la que se

esperaba de la aplicación de estas concentraciones de PVP-I por sí sola o de mentol por sí solo.

6. Afirmamos que todas las declaraciones realizadas en el presente son ciertas a nuestro leal saber y entender y que reflejan nuestros conocimientos. Además, estas declaraciones fueron realizadas con conocimiento de que los falsos testimonios o similares están sujetos a una multa o encarcelamiento, o ambos de conformidad con la Sección 1001 del Título 18 del Código de Estados Unidos y que dichos falsos testimonios pueden poner en riesgo la validez de la solicitud y de cualquier patente emitida con base en la misma.

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Michael Weiser, M.D., PhD

Fecha: 9.4.15

[firma]

Jason Stein, M.D.

11

Sensory experiences in humans and single-unit activity in cats evoked by polymodal stimulation of the cornea

M. Carmen Acosta, Carlos Belmonte and Juana Gallar

Instituto de Neurociencias, Universidad Miguel Hernández – Consejo Superior de Investigaciones Científicas, Aptdo. 18, 03550 San Juan de Alicante, Spain

(Resubmitted 25 January 2001; accepted after revision 13 March 2001)

1. The cornea of human subjects and of anaesthetised cats was stimulated with a jet of air of controlled flow, temperature and CO₂ concentration delivered by a gas aesthesiometer.
2. In humans, the intensity and magnitude of various components of the sensory experience (intensity of the sensation, degree of irritation, magnitude of burning and stinging pain, magnitude of the cold and warm components of the sensation) were measured using separate visual analog scales. In anaesthetised cats, the impulse response to the same stimuli was recorded from single mechanosensory, polymodal and cold-sensitive corneal fibres in the ciliary nerves.
3. Intensity–response curves for mechanical stimulation showed that all parameters of the sensation experienced by humans increased with the intensity of the stimulus. Mechanical stimuli recruited mainly phasic mechanosensory and polymodal afferents in the cat.
4. Acidic stimulation with gas mixtures of increasing CO₂ concentration evoked irritation, burning and to a lesser extent stinging pain of a magnitude roughly proportional to the intensity of the stimulus in humans. CO₂ primarily recruited polymodal afferents and weakly excited cold-sensitive fibres in the cat's cornea.
5. Heat stimuli evoked in humans a sensation profile similar to CO₂ but accompanied by a warmth component. In the cat's cornea, heat excited only polymodal fibres and silenced cold-sensitive corneal units.
6. Cold stimuli applied to the human cornea elicited a sensation of cooling that became irritant at the lowest temperatures. Corneal cold-sensitive fibres of the cat were activated in a manner proportional to the temperature drop, while polymodal nociceptor fibres were recruited only by the lowest temperatures. Topical menthol (0.2 mM) applied to humans evoked and later eliminated cold sensations produced by cold stimuli while the irritation sensation caused by low temperature stimuli still persisted.
7. Human subjects were able to identify masked mechanical, thermal and chemical stimuli applied to the cornea.
8. Irritation and cold sensations can therefore be evoked separately from the cornea by selective activation of mechanosensory, polymodal and cold corneal sensory afferents. Stimulation with different forms of energy usually leads to combined activation and/or inhibition of the different populations of sensory afferent fibres, evoking blended sensations that include irritation and thermal components in a variable degree.

Peripheral nociceptive neurones are primarily characterised by their response to one or several forms of energy at intensities near or within the range of tissue injury (Sherrington, 1906; Bessou & Perl, 1969). In the skin and deep tissues of mammals including man, various functional subclasses of nociceptive neurone have been categorised, based upon the form of noxious energy (mechanical, chemical, thermal) by which they are preferentially activated (see Campbell & Meyer, 1996). Microneurography

and microstimulation in humans have provided convincing evidence that excitation by noxious stimuli of the peripheral axons of some types of primary nociceptive neurones innervating the skin is accompanied by pain sensations (Torebjörk *et al.* 1984; Ochoa & Torebjörk, 1989; Handwerker & Kobal, 1993). However, it is unclear whether a qualitatively different experience is evoked by selective excitation of each one of the various subpopulations of primary nociceptive neurones.

The contribution to pain of primary sensory neurones activated by innocuous mechanical and thermal stimulation is also still a matter of controversy. It is generally accepted that in the skin mechanical stimulation of neurones preferentially responding to low intensity mechanical forces does not elicit pain except when there is abnormal processing of peripheral information by the central nervous system (CNS) (Torebjörk *et al.* 1992). Likewise, warming of the skin becomes painful only when the population of polymodal nociceptive neurones is recruited (Van Hees & Gybels, 1981; Campbell & LaMotte, 1983; Adriaensen *et al.* 1984).

The origin of pain produced by cold is less certain. Selective activation of cutaneous cold receptors by moderate temperature reductions is the basis of thermal sensations of cooling (Hensel & Zotterman, 1951; Hensel & Roman, 1960). More intense temperature drops apparently excite a subpopulation of polymodal nociceptors located in vascular and perivascular tissues that may be responsible for the sensation of burning pain produced by intense cutaneous cooling (Kenshalo & Duclaux, 1977; LaMotte & Thalhammer, 1982; Yarnitski & Ochoa, 1990; Arndt & Klement, 1991; Campero *et al.* 1996). In contrast, it has been reported that cooling the cornea of the eye, which is innervated by cold-sensitive and polymodal sensory neurones (Belmonte *et al.* 1991; Gallar *et al.* 1993), always elicits a sensation of irritation devoid of a thermal component (Kenshalo, 1960; Beuerman & Tanelian, 1979).

The cornea is an avascular tissue of very simple structure, innervated almost exclusively by thin myelinated and unmyelinated trigeminal ganglion neurones that belong to the classes of high-threshold mechanosensory, polymodal and cold primary sensory neurones. A particular advantage is that corneal nerve terminals are easily accessible to non-noxious and noxious stimuli of different types (Belmonte & Gallar, 1996; Belmonte *et al.* 1997). Therefore the cornea is an amenable model for the analysis of the quality of sensations evoked by activation of specific subclasses of primary nociceptive neurones by their appropriate stimuli.

In the present work, we applied to the cornea of human subjects selective mechanical, thermal and chemical (acidic) stimuli and analysed the characteristics of the evoked sensation. The functional types of corneal nerve fibre recruited by these stimuli were also studied in anaesthetised cats. Preliminary results have been reported in abstract form (Acosta *et al.* 1997).

METHODS

Aesthesiometer

A gas aesthesiometer described in a previous publication (Belmonte *et al.* 1999) was used to apply selective mechanical, chemical and thermal stimulation of the cornea. Air jets of 3 s duration were applied to the corneal surface, separated by 2 min pauses. Mechanical stimulation consisted of a series of nine pulses of air at flows varying

from 0 to 264 ml min⁻¹. For chemical stimulation, the change in corneal pH produced by CO₂ applied to the corneal surface was used (Chen *et al.* 1995). Nine pulses of a mixture of air and CO₂ at different concentrations (0–80% CO₂) were employed. Thermal stimulation was performed by applying to the cornea 10 pulses of air at different temperatures in the nozzle of the probe (−10 to +90°C), which caused the corneal surface temperature to vary from −5 to +3°C around the basal value (Fig. 1A), measured by infrared thermography as previously described (Belmonte *et al.* 1999). Corneal thermography confirmed that stimulation was restricted to the cornea, with the area of detectable temperature change (> 0.5°C) ranging between 1.7 and 92.2 mm² ($n = 71$; 11–60% of the total surface of the cornea in humans; Pepose & Uebels, 1992). During selective chemical and thermal stimulation, flows 6.25 ml min⁻¹ below the mechanical threshold of the subject were used. During mechanical and chemical stimulation, the air was heated at the tip of the probe to the temperature required by each value of flow to prevent changes in corneal basal temperature (Belmonte *et al.* 1999). Room temperature and humidity ranged between 23 and 25°C and 50 and 58%, respectively.

Psychophysical experiments

Sixteen human subjects (8 male and 8 female) aged 19–27 years (mean, 22.6 ± 0.1 years) participated in the study. The research followed the tenets of the Declaration of Helsinki. The subjects gave written informed consent to a protocol approved by our Institute's ethics committee and were free to terminate the session at any time. They received financial compensation for their participation in the study. None of them had a history of corneal or ocular pathology; three wore eyeglasses (less than −2 dioptres) to correct myopia.

Stimulation procedure. The subject was seated comfortably in front of a slit-lamp table, with the head supported by a head holder. The probe was advanced towards the eye with the slit-lamp table commands, and the tip placed perpendicular to the centre of the cornea, at a distance of 5 mm from the corneal surface. The subject was asked to blink immediately before application the stimulus. He/she identified the onset of the stimulus by the click produced by the opening of the valve in the probe.

At the beginning of each experimental series, the mechanical threshold was determined using the method of levels (Yarnitski & Ochoa, 1990). Then, the series of stimuli of each modality at random intensities was applied with a 2 min interval between stimuli. Subjects ignored the modality of stimulation that was applied. In each subject, both eyes were studied in a 2 day protocol. On day 1, mechanical and heat stimuli were sequentially applied first in one eye then in the other. On day 2, CO₂ and cold stimulation were performed. Four subjects, on two different days, were additionally treated with a drop of a viscoelastic eye solution (Hylashield, 0.1% Hylan-A) or Hylashield with 0.2 mM menthol (kindly provided by Biomatrix Inc., Ridgefield, N.J, USA) applied in the conjunctival sac prior to cold stimulation. This viscoelastic solution was used to ensure a longer presence of menthol on the corneal surface.

Immediately after each stimulating pulse, the subject marked the magnitude of the various components of the sensation in separate 10 cm, horizontal visual analog scales (VAS) without marks. In preliminary experiments five different subjects were asked to define in their own words the qualities of the sensation felt after stimulation of the cornea with the gas aesthesiometer. 'Diffuse sensation of burning', 'localised, punctate stinging sensation', 'refreshing or cooling' and 'non-painful warming' were the four descriptors most often chosen by the subjects to describe the sensations experienced. Based on this information, six parameters were selected and tested on separate VAS scales: (1) intensity of the sensation; (2) degree of irritation produced by the stimulus; (3) magnitude of the burning

component of the sensation; (4) magnitude of the stinging component of the sensation; (5) magnitude of innocuous warming; (6) magnitude of innocuous cooling. In the VAS, 0 was assigned to 'no sensation' and 10 to 'maximal sensation'. Prior to the experiment, the parameters to be rated were explained to the subjects.

The intensity threshold for the chemical and thermal stimuli was taken as the lowest value that evoked a response ≥ 0.5 in the VAS.

Stimulus identification experiments. For each subject, mechanical, chemical, heat and cold stimuli of a magnitude rated in the previous experiments with the same subjective intensity value were applied on a separate day and their modality revealed to the subject. Subsequently, each stimulus was randomly applied twice to each eye and the subject was invited immediately after each application to try to identify its modality. Results were presented as a percentage of positive identifications.

Electrophysiological experiments

Experiments were performed in 16 adult cats of either sex anaesthetised with sodium pentobarbitone (Nembutal, 40 mg kg⁻¹, i.p.). Animals were maintained under anaesthesia by i.v. infusion of dilute Nembutal (2.5 mg kg⁻¹ h⁻¹) throughout the experiment. Rectal temperature, end-tidal CO₂ concentration and blood pressure were continuously monitored and maintained within physiological limits.

At the end of the experiment, animals were killed with an overdose of anaesthetic. The experiments were carried out in accordance with the guidelines laid down by the ARVO statement for the use of animals in ophthalmic research and the Universidad Miguel Hernández Ethical Committee.

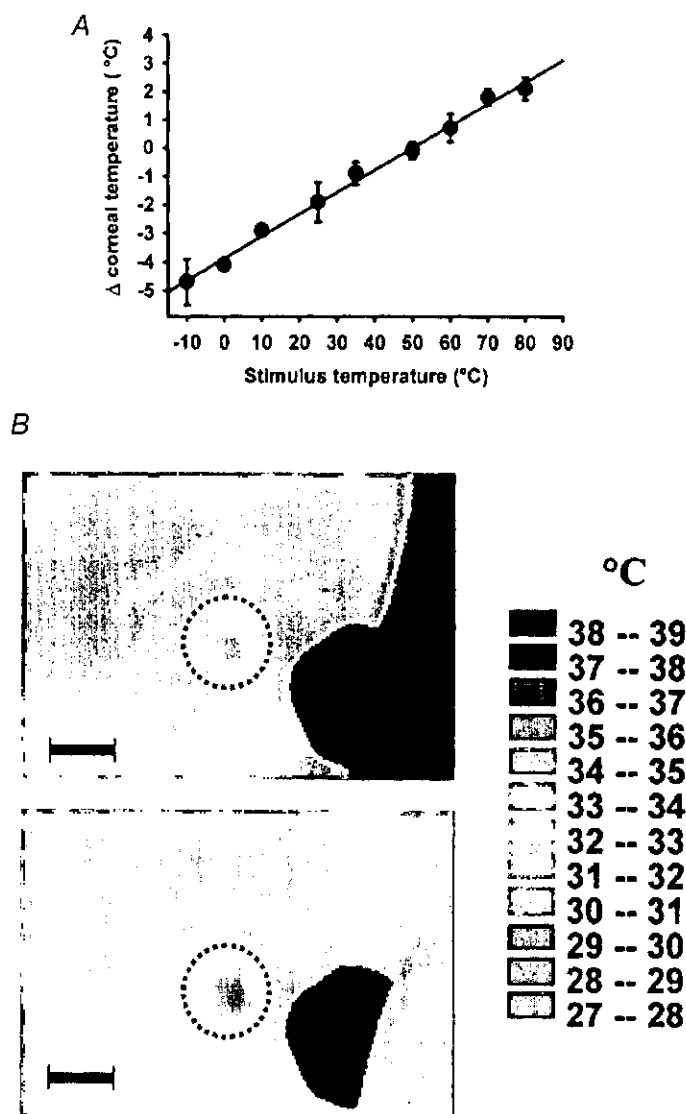
Single-unit corneal afferent activity was recorded in thin nerve filaments, dissected from the ciliary nerves at the back of the eye, using Ag-AgCl electrodes and conventional electrophysiological equipment, as described elsewhere (Belmonte *et al.* 1991).

Mechanical threshold was measured using a calibrated von Frey-type aesthesiometer (Cochet-Bonnet aesthesiometer) provided with a 0.12 mm diameter nylon filament of adjustable length (force, 0.11–1.96 mN). The receptive field was subsequently mapped using a suprathreshold force value (0.16–0.25 mN above threshold). Responsiveness to cold was explored applying a drop of saline at 4 °C onto the corneal surface. The receptive fields of cold-sensitive units were then located and mapped with the tip of an ice-cooled metal bar (0.5 mm diameter). The chemical sensitivity of the mechanically identified units was assayed by applying a 30 s pulse of gas containing 98.5% CO₂ (at 80 ml min⁻¹ flow) to the receptive field. The conduction velocity of each unit was determined from the latency of the nerve impulse evoked by a 0.1–0.5 ms, 0.5–3 mA, suprathreshold electric shock, applied to the receptive area with a

Figure 1. Temperature change at the corneal surface during thermal stimulation

A, corneal surface temperature changes during stimulation with 3 s air jets of different temperatures (–10 to +80 °C), applied at flows below 150 ml min⁻¹, $n = 6$ –7. The 50 °C point is the average of 32 determinations performed at variable flows (0–267 ml min⁻¹). The regression curve ($y = 0.08x - 3.9$; $r^2 = 0.994$) was used to estimate the corneal temperature change evoked by the stimulus in the experiments in which no simultaneous thermography was performed.

B, example of thermography temperature profiles in the ocular surface at the end of a stimulation with an air jet of 3 s duration, at a subthreshold flow (39 ml min⁻¹). Stimulus temperature at the tip of the probe was –10 °C in the upper and +80 °C in the lower picture. Scale bars, 10 mm. Dotted lines show the limits of the cornea.



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pair of silver electrodes, and the conduction distance, measured with an 8.0 gauge thread placed along the trajectory of the nerve. When the characterisation of the unit was complete, the probe of the gas aesthesiometer was placed 5 mm away from the receptive field on the cat's cornea. The same stimulation protocol used in the experiments with humans was applied. The impulse response of the unit was recorded continuously until completion of the experimental protocol. Fibre threshold to the stimulus was calculated as the minimum stimulus intensity required to evoke consistently at least one nerve impulse.

A total of 105 corneal units (85 with latencies corresponding to the A δ range and 20 C-units) were studied. Thirty-five responded to touching of the corneal surface with the Cochet-Bonnet aesthesiometer and were insensitive to a 30 s CO₂ pulse and to cold saline, and so were classified as mechanosensory units. Sixty-two fibres were classified as polymodal units because in addition to displaying mechanical sensitivity they also produced a train of impulses during the application of CO₂. These fibres were also activated by heat.

Eight fibres were initially identified as cold units based on their prominent ongoing activity in the absence of intended stimulation that increased markedly after application of a drop of cold saline to the cornea. In a separate group of four cold-sensitive and seven polymodal units, the responses to cold and chemical stimulation were tested before and after treatment with a 50 μ l drop of 0.2–0.6 mM menthol applied to the unit's receptive field.

Neural discharges and traces of the stimulating pulses were recorded on FM tape for later off-line computer analysis (CED 1401 plus and Spike 2 program, Cambridge Electronic Design Ltd, Cambridge, UK). The frequency of spontaneous discharges and changes in mean firing frequency (impulses s⁻¹ (imp s⁻¹)) evoked by application of the various stimuli were measured.

Analysis of data

Data are expressed as means $\pm$ S.E.M. Pearson's correlation was used to determine association between variables. Linear regression and the r^2 coefficient were applied to see how well the regression model

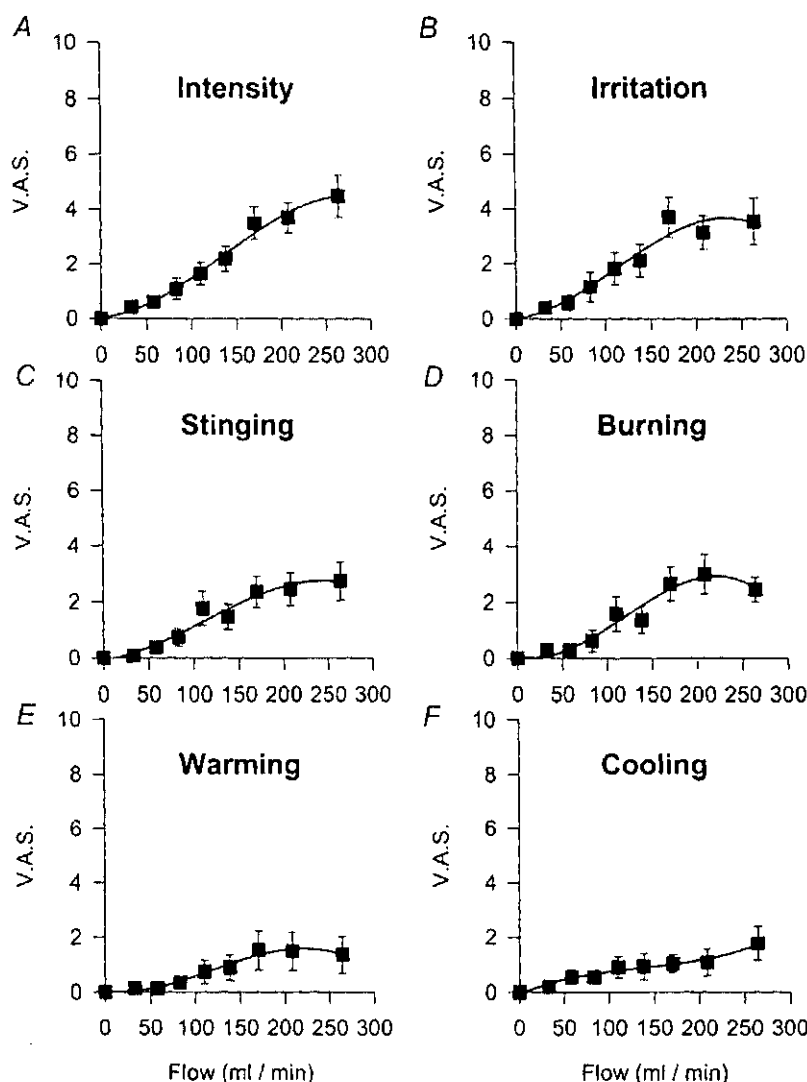


Figure 2. Relationship between the amplitude of mechanical stimulation and VAS values in human subjects

A, intensity. B, irritation. C, stinging. D, burning. E, warming. F, cooling. Data are means $\pm$ S.E.M., $n = 16$ subjects.

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described the data. Differences between groups were compared with parametric or non-parametric tests for paired or unpaired data, two-way repeated measures (RM) ANOVA and Tukey's test. Significance was set at $P < 0.05$.

RESULTS

General

Stimulation with pulses of air at a nozzle temperature of 50°C did not modify the basal corneal temperature ($34.4 \pm 0.1^{\circ}\text{C}$, $n = 94$ measurements performed in both eyes of 3 subjects), except when air flows over 264 ml min^{-1} were attained. Air at temperatures below or above this neutral value (from -10 to $+80^{\circ}\text{C}$ at the nozzle of the probe), applied to the centre of the cornea, produced a central point of highest temperature change (range, -7.5 to $+3.4^{\circ}\text{C}$) surrounded by almost concentric

areas of progressively smaller temperature variation that were restricted to the cornea (Fig. 1*B*).

Mechanical stimulation

The minimum flow of air at neutral temperature required to evoke a sensation was $79 \pm 5 \text{ ml min}^{-1}$ (median, 83 ml min^{-1} ; range, $33\text{--}208 \text{ ml min}^{-1}$; $n = 32$, both eyes of 16 subjects). Threshold flow varied greatly between individuals, but for the same individual similar values were measured in the two eyes and on different days (data not shown). The sensation evoked by threshold flow was always defined as slightly unpleasant.

As shown in Fig. 2*A*, subjects reported an increasing intensity of the sensation with increases in the strength of the stimulus, the correlation between the two parameters being significant (Pearson's correlation

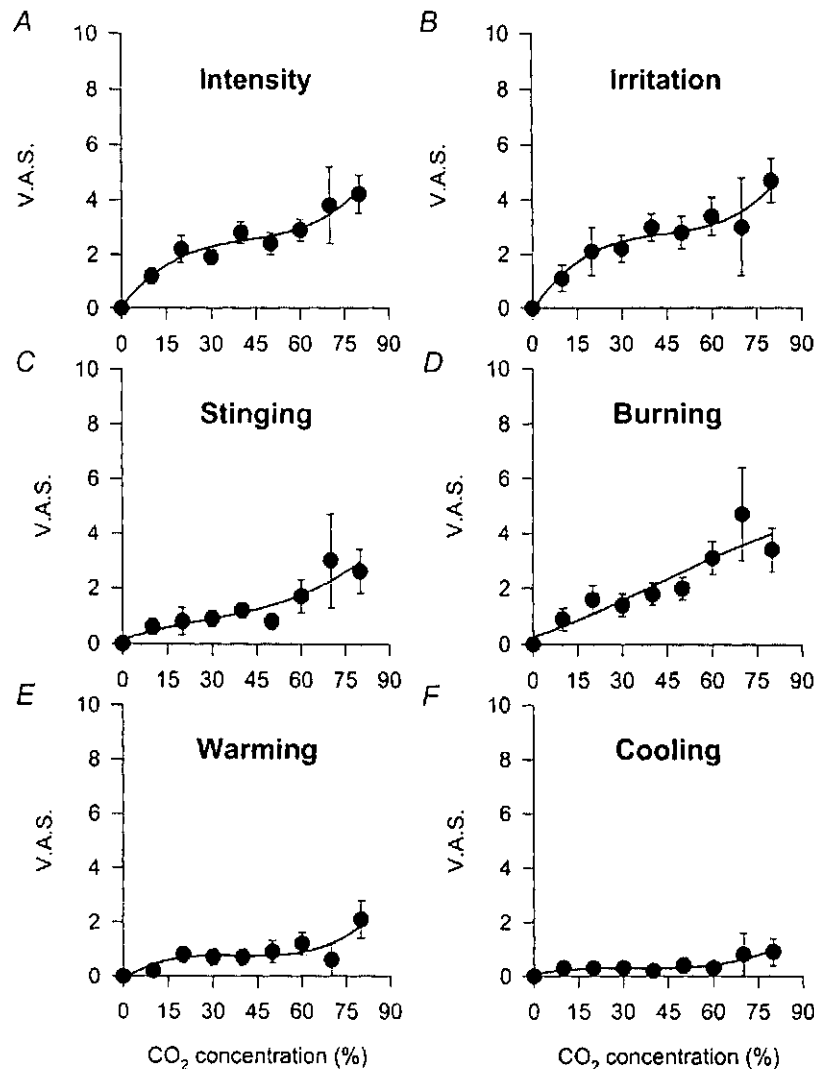


Figure 3. Relationship between the magnitude of chemical stimulation and VAS values in human subjects

A, intensity. B, irritation. C, stinging. D, burning. E, warming. F, cooling. Data are means $\pm$ S.E.M., $n = 16$ subjects.

coefficient, 0.987; $P < 0.001$). The same was also true for the parameters 'irritation', 'stinging' and 'burning', which increased in proportion to the magnitude of the stimulus and reached similar maximal values (Fig. 2B–D).

Subjects assigned low VAS values to the thermal components of the sensation evoked by mechanical stimuli (Fig. 2E–F). Moreover, after a given stimulus, some individuals assigned the same VAS value to warmth and cold, suggesting that temperature sensations associated with mechanical stimulation were not clearly defined.

When mechanical stimuli were applied to the cornea of the cat, they evoked in 35 out of 58 polymodal units a sustained, irregular discharge of impulses that usually outlasted the duration of the stimulating pulse and

occurred at a mean threshold of $79 \pm 9 \text{ ml min}^{-1}$, $n = 35$. Eight out of 35 mechanosensory units responded with one to three impulses with no relation to the intensity of the stimulus. In seven cold-sensitive fibres a modest increase in the ongoing frequency of discharge was elicited with air pulses over $62 \pm 8 \text{ ml min}^{-1}$ that was not related to flow values.

Stimulation with CO_2

The mean CO_2 concentration necessary to evoke a sensation in 16 subjects was $21 \pm 3\%$ (median, 10%; range, 10–50%; $n = 32$). The threshold sensation evoked by CO_2 was defined by subjects as irritating. The intensity of the sensation experienced increased with the magnitude of the stimulus (Pearson's correlation coefficient, 0.940; $P < 0.001$; Fig. 3A). The same was true

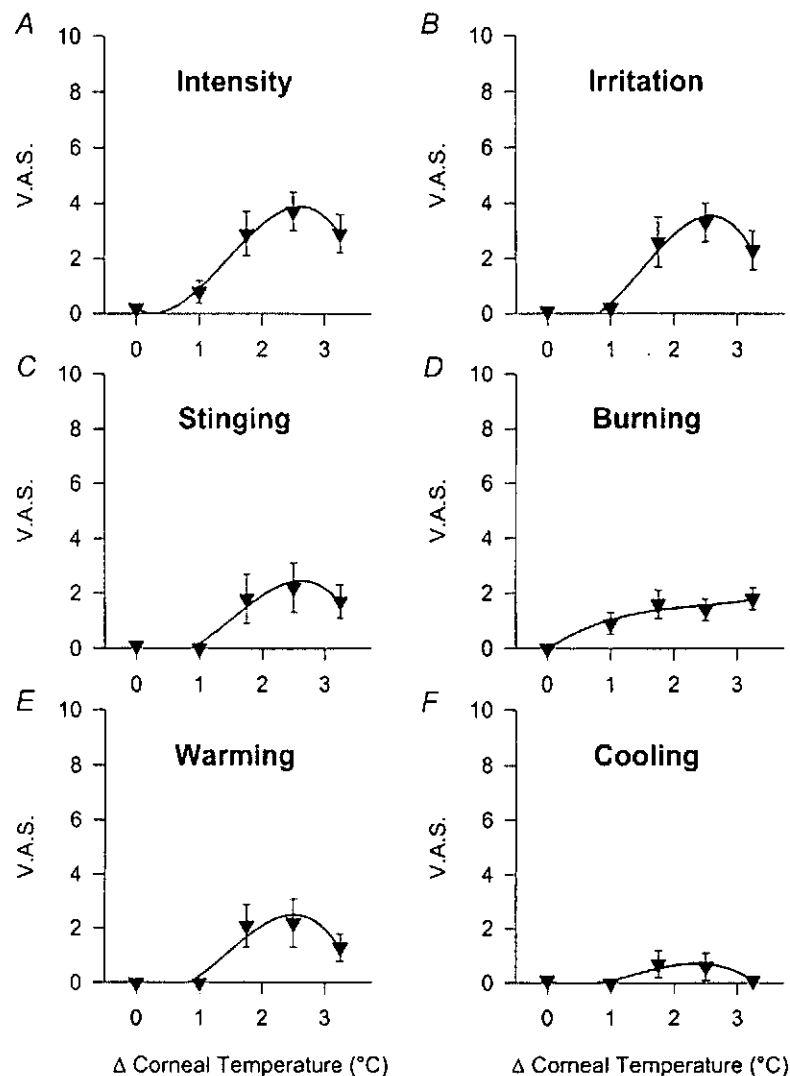


Figure 4. Relationship between the magnitude of heat stimulation and VAS values in human subjects

A, intensity. B, irritation. C, stinging. D, burning. E, warming. F, cooling. VAS data (means $\pm$ S.E.M., $n = 16$ subjects) are plotted against the change in corneal temperature.

for the curve of irritation (Fig. 3*B*). The sensation of burning pain was more prominent than that of stinging pain (Fig. 3*C* and *D*), while the warming component attributed to the sensation was slightly greater than the cooling component, although both were of low magnitude (Fig. 3*E* and *F*).

In the cat cornea, about half of the polymodal units (28 out of 54) produced within the first second an impulse discharge in response to CO₂ pulses of increasing CO₂ concentration. The discharge usually outlasted the duration of the pulse. Mean CO₂ threshold concentration was $34 \pm 3\%$ CO₂ with a maximal mean firing frequency obtained at $52 \pm 3\%$ CO₂. Mechanosensory fibres fired only occasionally during the application of CO₂ pulses, without any correspondence with CO₂ concentration. Moderate increases of ongoing firing frequency were

obtained in four out of five cold units during the application of CO₂ pulses, with a threshold CO₂ concentration of $21 \pm 4\%$.

Stimulation with hot air

Heat threshold had a mean value of $+1.2 \pm 0.2^\circ\text{C}$ above the basal corneal temperature (range, $+1$ to $+2.5^\circ\text{C}$; $n = 18$). The threshold sensation evoked by heat was defined as irritating. The magnitude of irritation was paralleled by the subjective assessment of intensity, reaching similar maximal values (Fig. 4*A* and *B*). Stimulation with heat also evoked a sensation of burning and, to a lesser degree, stinging pain as was the case for chemical stimulation (Fig. 4*C* and *D*). Likewise, the thermal attribute of the sensation was predominantly warmth (Fig. 4*E* and *F*).

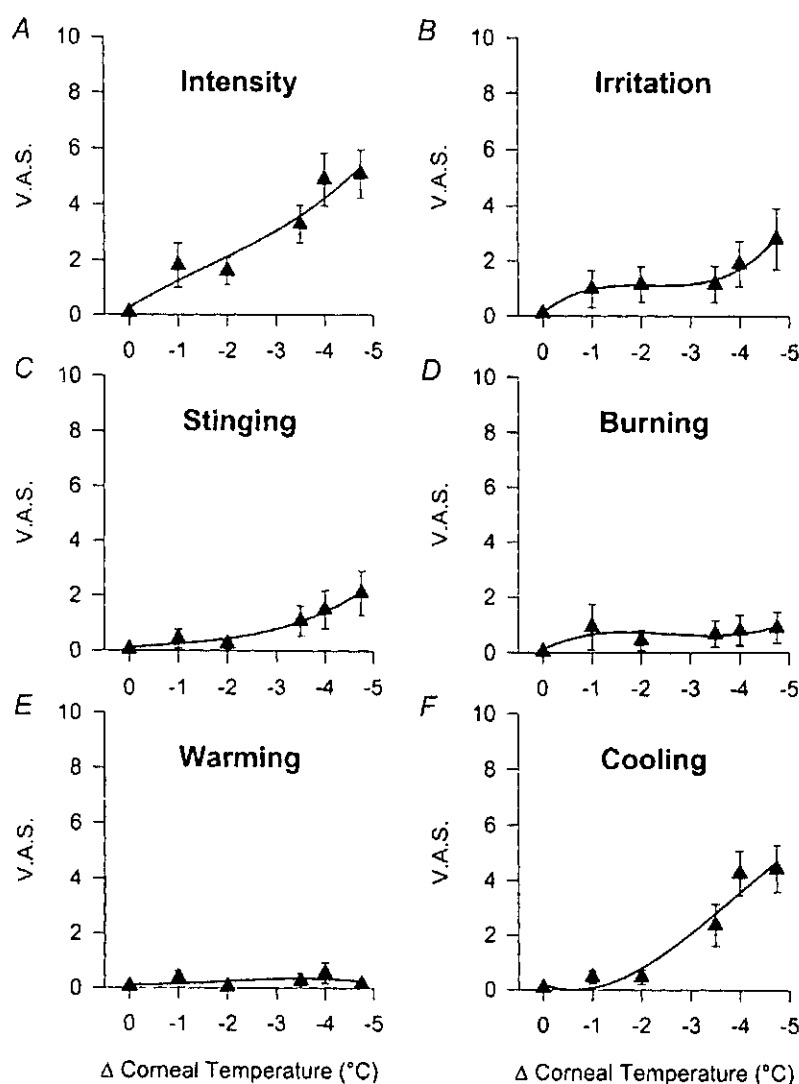


Figure 5. Relationship between the magnitude of cold stimulation and VAS values in human subjects

A, intensity. B, irritation. C, stinging. D, burning. E, warming. F, cooling. Data are means $\pm$ S.E.M., $n = 16$ subjects.

Heat pulses applied to the cornea of the cat evoked a tonic impulse discharge in one-third of polymodal units (12 out of 35). No consistent impulse response to heat was observed in mechanosensory fibres. In three cold-sensitive fibres the ongoing activity decreased or stopped with heat stimulation.

Stimulation with cold air

Cooling threshold was established at $-2.4 \pm 0.4^\circ\text{C}$ below the corneal surface temperature (range, -4.75 to -1°C ; $n = 12$). The threshold sensation evoked by cold was defined as cooling and in some cases as slightly unpleasant. With pulses of air of decreasing temperature, a dissociation between the curves for subjective intensity and irritation was observed (Fig. 5*A* and *B*). Only the

lowest tested temperature evoked a sensation of irritation in all the studied subjects. At the same levels, a moderate stinging pain sensation was also reported with almost no burning component (Fig. 5*C* and *D*). With cold stimulation, a warming sensation was never signalled (Fig. 5*E*), whereas the cooling sensation was prominent, being the dominant quality evoked by cold stimulation, with a magnitude proportional to the intensity of the stimulus (Fig. 5*F*).

A quarter of corneal polymodal units in the cat (6 out of 26) produced a low frequency discharge during the application of the lowest temperature cold stimuli (corresponding to 29°C in the human cornea). In contrast, cold did not evoke a consistent response in mechanosensory units.

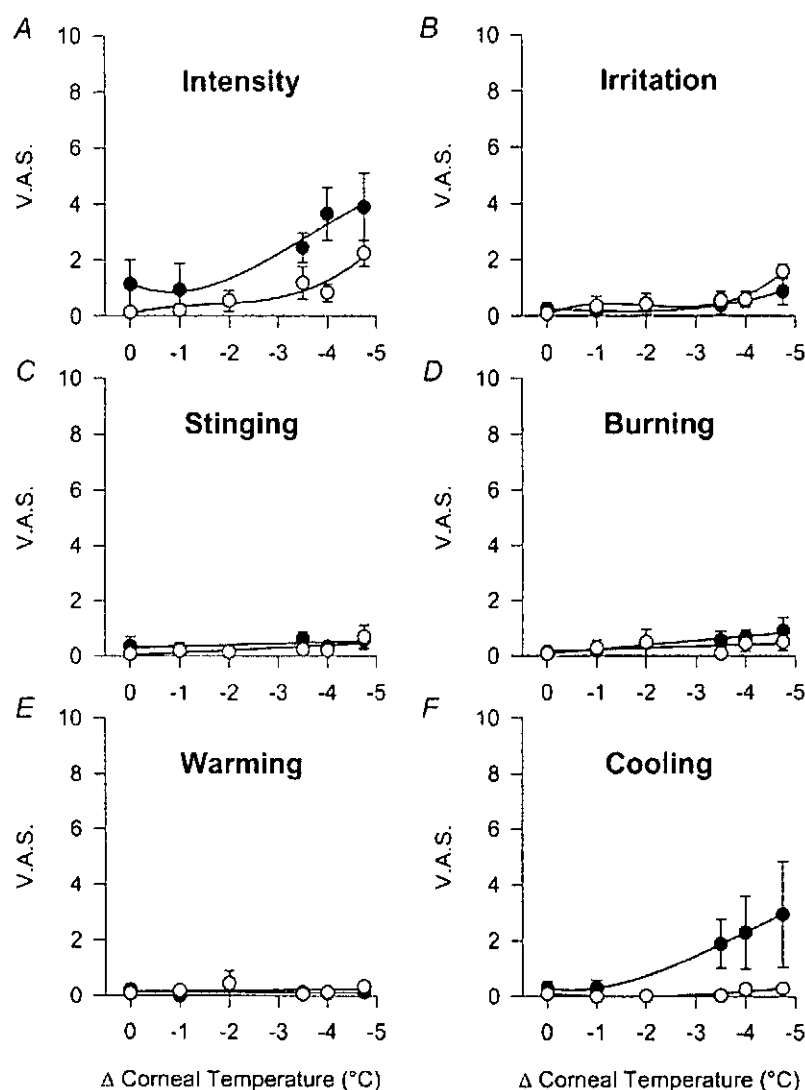


Figure 6. Effect of 0.2 mM menthol on the VAS response to cold stimulation

A, intensity. *B*, irritation. *C*, stinging. *D*, burning. *E*, warming. *F*, cooling. The subject's cornea was treated with Hylashield alone (●) and Hylashield plus 0.2 mM menthol (○) prior to stimulation. Data are means $\pm$ s.e.m., $n = 4$ subjects. In menthol-treated subjects, the value of irritation at -4.75°C below the basal corneal temperature was significantly different from the value at this basal corneal temperature (34.4°C ; $P < 0.05$, paired *t* test).

All cold-sensitive units responded to a decrease of the corneal surface temperature of -1°C , with the maximal response at a mean temperature change of -4.75°C . The response to cold pulses was composed of a brief, high frequency impulse discharge followed by sustained firing at lower frequency for the duration of the pulse and a post-stimulus silence lasting several seconds.

Effect of menthol

Menthol applied topically to the eye produced an immediate sensation of cooling and mild irritation followed in some cases by a feeling of light discomfort sometimes with a burning component. Cold stimulation performed 15–30 min after menthol application showed that in comparison with the curves obtained in the same eyes treated with the control viscoelastic solution, the intensity curve flattened and the extreme cold stimuli also produced a mild but significant sensation of irritation (Fig. 6*A* and *B*). Warm sensations were not reported, but the most striking observation was that no cold sensations were elicited by cooling of the cornea in the subjects pretreated with topical menthol (Fig. 6*E* and *F*). The control viscoelastic solution itself, which reduces corneal evaporation (Acosta *et al.* 1999), also had a mild attenuating effect on the response to cold stimuli (compare Figs 5 and 6).

In single-fibre recordings in the cat, four cold units increased their firing frequency immediately after application of menthol, with a discharge that lasted for 20–75 s. Five minutes after application of menthol, the response to cooling was reduced to $29 \pm 18\%$ of the control value ($P = 0.033$, paired *t* test, data not shown). No changes in the thermal or chemical responsiveness of polymodal units ($n = 7$) were observed after menthol.

Profiles of the sensations evoked by the different stimuli

Figure 7 represents the VAS values for irritation, stinging and burning pain, warming and cooling components evoked by the different stimulus modalities. The data selected for each stimulus modality are those corresponding to a mechanical stimulus of air flow at 208 ml min^{-1} , a chemical stimulus of $80\% \text{ CO}_2$, a heat stimulus of $+2.5^{\circ}\text{C}$ and a cold stimulus of -4°C . These stimuli were selected because they evoked a mean VAS value of subjective intensity of 4 VAS units in the previously described experiments in which stimulus–response curves were obtained.

Mechanical, chemical and heat stimuli had a low cooling component and high irritation and burning pain components. In contrast, intense cold stimuli had a prominent cooling component significantly greater than the sensation components associated with irritation and pain. Moreover, cross-comparison between data obtained with the different stimulus modalities showed that with cold stimulation, values for cooling were significantly higher and for the burning pain component significantly

lower than in the case of the other modalities ($P < 0.05$, Tukey's test).

Identification of stimulus modality

Table 1 gives the percentage of correct identifications made by nine subjects to whom mechanical, chemical, heat and cold stimuli were randomly presented twice for each eye. Chemical and cold stimuli were accurately

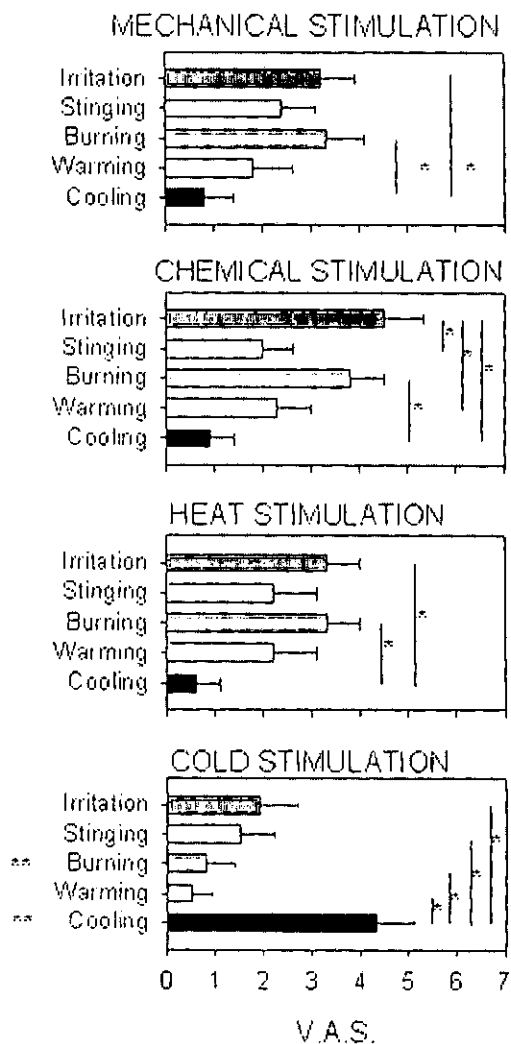


Figure 7. Comparison between components of the sensation evoked by different modalities of stimulation

Data are means $\pm$ S.E.M., $n = 9$ subjects. Values of the different components of sensation evoked by selective mechanical, chemical, heat and cold stimuli with the same mean subjective intensity (4 VAS units, see text) were compared. * $P < 0.05$, significant difference between the magnitudes of the sensation components signalled by the end of the vertical lines inside each modality of stimulation. ** $P < 0.05$ significant difference between the magnitudes of the sensation component comparing all the modalities of stimulation ($P < 0.05$, Tukey's test after two-way RM ANOVA).

Table 1. Percentage of correct identification of the stimulus modality applied to the human cornea

Stimulus applied	Stimulus reported				
	Mechanical	Chemical	Heat	Cold	No stimulus or undefined
Mechanical	47 ± 7%	17 ± 7%	17 ± 8%	19 ± 9%	0%
Chemical	8 ± 4%	75 ± 7%	17 ± 7%	0%	0%
Heat	3 ± 3%	31 ± 9%	64 ± 9%	0%	3 ± 3%
Cold	11 ± 7%	17 ± 7%	0%	72 ± 10%	0%
Sham	0%	6 ± 6%	6 ± 6%	0%	88 ± 7%

Data are means ± S.E.M. of the percentage of correct identifications determined in 9 subjects.

Table 2. Sensation threshold in human and firing threshold of corneal units in the cat

Stimulus applied	Human	Cat		
	Sensation threshold	Mechanosensory	Polymodal units	Cold-sensitive units
Mechanical (ml min ⁻¹)	79 ± 5	109 ± 24 (23%)	79 ± 9 (60%)	62 ± 8 (25%)
Chemical (% CO ₂)	21 ± 3	(3%)†	34 ± 3 (54%)	21 ± 4 (80%)
Heat (°C)	+1.2 ± 0.2	(4%)†	+2 ± 0 (36%)	(-60%)*
Cold (°C)	-2.4 ± 0.4	(4%)†	-2.7 ± 0.8 (9%)	-1 ± 0 (100%)

Data are means ± S.E.M. Percentage of corneal sensory units recruited by each type of stimulation is also shown in parentheses. Data correspond to units that changed their basal firing frequency by more than 30% in response to at least one-third of the pulses of each modality. * Percentage of units that reduced their firing discharge during application of heat stimuli. † For values below 5% thresholds were not calculated.

identified in more than 70% of the cases. The incidence of correct identification was slightly lower for heat stimuli. Mechanical stimuli were confounded with other types of stimuli in about half of the trials.

Correlation between sensation parameters and single-fibre activity

Table 2 compares the stimulus intensities necessary to excite primary corneal sensory units in the cat (firing

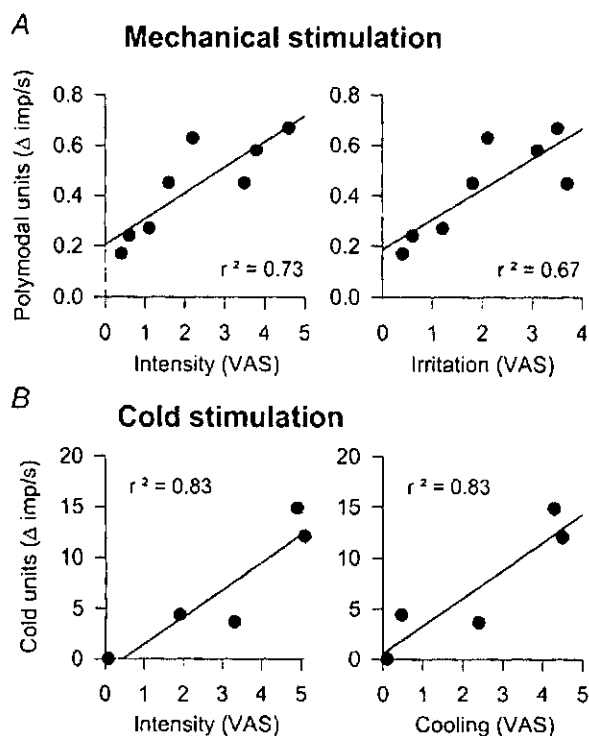


Figure 8. Correlation between VAS ratings in humans and impulse response of corneal units in cats

A, mechanical stimulation. B, cold stimulation. Data were obtained using an identical stimulation protocol for the psychophysical and the electrophysiological experiments. Pearson's correlation and linear regression (continuous lines) were statistically significant.

threshold) with those required for stimulus detection in humans and the proportion of each type of corneal sensory afferent that was recruited by the different stimulus modalities applied with the gas aesthesiometer. The sensation threshold for mechanical stimulation in humans was similar to that required to activate polymodal units in the cat, whereas the threshold value of corneal mechanosensory fibres was higher. In the case of chemical stimulation, the sensation threshold in humans was below the CO_2 concentration necessary to recruit polymodal nociceptive units and near that required to activate cold units with CO_2 in the animal experiments. For heat stimulation, the sensation threshold in humans was close to the heat threshold of polymodal afferents in the cat. The sensation threshold for cold stimulation in humans had an intermediate value between that of cold-sensitive and polymodal fibres. Irritation evoked by cold stimulation appeared at temperature values required to excite polymodal fibres with cold.

VAS values for the intensity and irritation components of the sensation evoked by increasing mechanical stimulation correlated well with the discharge of polymodal nociceptors ($P = 0.00715$ and 0.0178 , respectively; Pearson's correlation; Fig. 8A) but were not correlated with the impulse activity of mechanosensory or cold-sensory units (data not shown). Although the firing frequency of polymodal nociceptive fibres in the cat was augmented with pulses of CO_2 of increasing concentration, no significant correlation was found between the impulse response in polymodal fibres and VAS scores evoked in humans by similar stimuli. The same was also true for the firing frequency evoked by CO_2 and heat in mechanosensory and cold-sensory units and the human psychophysical responses evoked by these stimuli. In contrast, for cold stimuli the correlation between intensity and cooling VAS values with cold-sensory fibre impulse activity was significant ($P = 0.049$ and 0.045 , respectively; Fig. 8B). A positive correlation was also found between the VAS values of the irritation component in humans and the gross discharge of all types of corneal afferent fibre evoked by cold stimulation ($P = 0.002$).

DISCUSSION

This study shows that two main sensations can be evoked by mechanical, thermal or chemical stimulation of the cornea: one of 'irritation' that was obtained by application of mechanical force, acid, heat and extreme cold, and one of 'cooling' that was elicited by application of moderate cold. Thus, the data suggest that in the human cornea, as occurs in the skin (Wolf & Hardy, 1941; Chery-Croze, 1983; Chen *et al.* 1996), separate peripheral and central afferent channels exist for the perception of pain and innocuous cold.

Characteristics of the stimulus and sensation ratings

The gas aesthesiometer used in this study (Belmonte *et al.* 1999) was suitable for restricting stimulation to only one

modality and to the limits of the corneal tissue. Only mechanical stimulation with high flows of air produced additional cooling of the corneal surface. Thermography also showed that stimuli were restricted to the cornea. Therefore, sensations evoked by the various stimulus modalities in humans and neural discharges in corneal fibres of cats can be safely ascribed to the independent effects of mechanical, chemical and thermal stimuli on corneal nerve endings.

The subjective parameters selected to determine the magnitude, affective quality and thermal attributes of the evoked sensation in humans were based on the descriptors most often chosen by the subjects in previous corneal stimulation experiments (Lele & Weddell, 1956; Kenshalo, 1960; Beuerman *et al.* 1977; Belmonte *et al.* 1999). Information about intensity and irritation was collected separately in an effort to discriminate between the capacity to detect the magnitude of the stimulus and the degree of unpleasantness elicited by that stimulation (Price, 1988). It is difficult to determine to what extent the subjects confused intensity with irritation, or the sensation of warmth with that of burning pain, but the fact that often these parameters were seen changing independently of each other suggests that they reflect separate sensory experiences.

Mechanical stimulation

The magnitude of mechanical stimulation with increasing gas flows was accurately reflected in the subjective stimulus-intensity curve. There was a complete overlap between intensity and irritation curves for low and moderate flow values. This suggests that no innocuous sensations can be evoked by mechanical stimulation of the cornea even with near-threshold forces, excluding the possibility that touch is a separate modality from pain in the human cornea as previously suggested (Lele & Weddell, 1956; Draeger, 1984). Pure mechanosensory units responded to increasing gas flows with only one or two spikes irrespective of the magnitude of the stimulus, suggesting that they mainly signal the onset of high intensity stimulation. Therefore, encoding of mechanical stimulus intensity was presumably produced by activation of polymodal units, which exhibited a somewhat higher capability of translating the stimulus force into firing frequency changes. Nevertheless, the limited encoding by polymodal fibres of the magnitude of the stimulus observed in our electrophysiological experiments suggests that progressive recruitment of units is an important mechanism for intensity perception in the cornea as occurs in other tissues (Torebjörk & Hallin, 1974, 1976; Van Hees & Gybels, 1981; see Price, 1988). This is not surprising considering the high density of nerve endings and the partial overlapping of receptive fields of polymodal neurones in the cornea (Belmonte & Giraldez, 1981; Beuerman & Kupke, 1982; Brock *et al.* 1998; De Felipe *et al.* 1999). The ongoing discharge of cold-sensory fibres was slightly altered by all levels of mechanical

stimuli with the gas jet, but particularly when high flows were applied. This may explain the separation of intensity and irritation curves for the highest flow values, which was coincidental with the occurrence of a distinct sensation of cooling, additionally suggesting that the firing of cold-sensory fibres modified corneal pain sensation. Changes in the quality of sensations evoked by interactions between mechanical and thermal sensory inputs have been reported in other somatosensory territories (Bini *et al.* 1984; Craig *et al.* 1996). Co-activation of cold fibres by air pulses could also be the reason for the confusion between mechanical and cold stimuli sometimes observed in the stimulus identification experiments.

Chemical stimulation

Our data in the cat confirm that CO₂ application is an appropriate chemical stimulus for polymodal nociceptors, possibly through the production of a local pH drop (Belmonte *et al.* 1991; Steen *et al.* 1992; Gallar *et al.* 1993; Chen *et al.* 1995). Increasing concentrations of CO₂ augmented the subjective magnitude of the sensation and the degree of irritation experienced by the subject, with a complete overlap between the curves of intensity and irritation, suggesting that irritation is the sole sensation evoked by activation of corneal polymodal nociceptors. Correlation between the firing frequency of polymodal fibres in the cat and subjective values of the various parameters of the sensation in humans was poor. This may indicate again that progressive recruitment of polymodal fibres by higher CO₂ concentrations was more important for the encoding of the intensity of the stimulus than increasing the firing of individual units. Nevertheless, it is also possible that short CO₂ pulses that stimulate corneal fibres in the human cornea were insufficient to evoke a similar response in the cat, which has a mechanical threshold, measured with the Cochet-Bonnet aesthesiometer, higher than that in humans (Chan-Ling, 1989). Also, the possibility cannot be excluded that mechano-insensitive nociceptors (Schaible & Schmidt, 1985; Handwerker *et al.* 1991; Meyer *et al.* 1991; Brock *et al.* 1998; Weidner *et al.* 1999), which were not detected with our fibre sampling procedure, contributed to the encoding of CO₂ stimuli.

Subjects described the sensation evoked by CO₂ in terms of both stinging and burning pain. Activation of A δ and C polymodal nociceptors by electrical or natural stimuli applied to the skin of the hand during microneurographic studies in humans (Ochoa & Torebjörk, 1989) produced pricking and burning pain, respectively. The cornea possesses both A δ and C polymodal nociceptors and it could be speculated that like in the skin, A δ mechanosensory and polymodal fibres mediate sensations of stinging pain while C fibres evoke predominantly burning pain sensations. Chemical stimulation weakly excited cold-sensory fibres (Gallar *et al.* 1993). Accordingly, a weak

conscious cold sensation was elicited at the highest CO₂ concentration value.

Thermal stimulation

Stimulation with hot air at flows below mechanical threshold produced a distinct sensation of irritation that overlapped fully with the intensity curve and had stinging and burning pain components. These noxious sensations are attributable to the excitation of polymodal nociceptors (Bessou & Perl, 1969; Croze *et al.* 1976), which in the cornea belong to the A δ and C fibre type and are activated by temperatures over 37–39°C (Belmonte & Giraldez, 1981; Gallar *et al.* 1993) as well as by stimulation with CO₂. The main difference in terms of fibre recruitment between CO₂ and heat stimuli is that heating completely silenced cold-sensory fibres. This may explain why a feeling of warmth accompanied this stimulus modality, and was interpreted as being different from chemical stimulation in the experiments of stimulus identification.

Sensations evoked by cold stimuli had a well-defined profile and were clearly distinguishable from those elicited by other stimulus modalities. While changes in stimulus intensity were well detected and correlated closely with the magnitude of the cooling component attributed to the stimulus, irritation was reported only with the lowest temperatures and was of moderate value. Lele & Weddell (1956) described in human subjects defined thermal sensations evoked by stimulation of the cornea with cold air. These results were challenged by Kenshalo (1960), who compared the sensation evoked by the cooled bulb of a thermometer applied to the human cornea, conjunctiva, forehead skin and lip and concluded that in the cornea, in contrast to the other tissues, cold evoked a specific quality of irritation, without a defined thermal component. Likewise, Beuerman and colleagues (Beuerman *et al.* 1977; Beuerman & Tanelian, 1979) presented data showing that only irritation was evoked by a jet of saline directed at the human cornea at temperatures between 28 and 30°C. This led to a general acceptance that irritation is the sole sensation that can be evoked from the cornea with any form of stimulating energy.

In our study, in which the sensation evoked by cooling was divided into various subcomponents, it became evident that small corneal temperature decreases were detected and interpreted mainly as a thermal experience. Cold stimuli selectively activated cold-sensory fibres of the cat cornea, in a manner proportional to the temperature reduction. Sensory fibres responding to cold in the cornea present some differences to those of the episclera, such as larger receptive fields, more prominent dynamic responses to cooling and sensitivity to capsaicin (Gallar *et al.* 1993). Based on the psychophysical data of Kenshalo (1960) indicating the absence of temperature

sensations arising from the cornea, it was tentatively concluded (Gallar *et al.* 1993; Belmonte & Gallar, 1996; Belmonte *et al.* 1997) that corneal cold-sensitive fibres are 'cold nociceptors' similar to those described in the tooth (Jiväsjärvi & Kniffki, 1987). The present results indicate that this is not the case and that corneal fibres responding to cold appear to behave as innocuous thermal receptors sensitive to temperature reductions, giving rise to thermal sensations of cooling in the human cornea.

At low temperature values (around 29°C at the corneal surface), cold stimuli also recruited a reduced proportion (about 25%) of corneal polymodal nociceptors. The possibility that noxious low temperatures of the skin are detected by a subpopulation of nociceptors has been proposed repeatedly (Torebjörk, 1974; Georgopoulos, 1976, 1977; LaMotte & Thalhammer, 1982; Saunet *et al.* 1985; Arndt & Klement, 1991; Klement & Arndt, 1992; Campero *et al.* 1996). The present data in the cornea favour this interpretation. The action of menthol on corneal sensitivity further supports the idea that low temperatures evoke a blended sensation resulting from the simultaneous activation of specific cold thermoreceptors and a fraction of polymodal nociceptors. Menthol induces a cold sensation in the skin and mucosae due to a rather selective initial activation of cold receptor fibres (Hensel & Zotterman, 1951) followed by an inhibition of the impulse discharge (Schäfer *et al.* 1986). Likewise, in the cat cornea, menthol produced a transient excitation of cold-sensory fibres followed by a prolonged reduction in their responsiveness to cooling. In agreement with these observations, topical application of menthol to human eyes evoked a transient sensation of 'freshness' often followed by a feeling of warmth and the disappearance of any thermal sensation upon stimulation of the cornea with cold air. These effects would explain the absence after menthol of thermal sensations evoked by cold air in the human cornea but the persistence of a mild irritation with the lowest temperature stimuli, which are expected to maximally recruit the fraction of polymodal fibres sensitive to cold. Furthermore, the frequent reporting of warm sensations in the eye following ocular instillation of menthol supports the hypothesis that an inhibition of cold-sensory fibres rather than an activation of 'warm receptors' (never identified in the cornea), is responsible for warm sensations arising from this tissue, as was suggested by the experiments involving stimulation with hot air.

Sensations evoked by combined activation of corneal fibres

The present experiments showed that stimuli that produced a selective activation of polymodal nociceptive fibres of the cornea, such as CO₂ or heat, evoked a distinct sensation of irritation and pain. When mechanical force was applied pure mechanosensory fibres were also recruited and the quality of the unpleasant sensation changed,

suggesting that the sensory input provided by this class of mechanosensory Aδ fibres does not simply add to the inflow supplied by polymodal Aδ fibres but elicits a pain quality that is distinct from that produced by polymodal fibres of either conduction velocity. Pure mechanosensory fibres may contribute to the evocation in the cornea of a sharp pain sensation as occurs in the skin following application of acute noxious stimuli (Head, 1920; Lewis & Pochin, 1937; Bishop & Landau, 1958; Price *et al.* 1977; Meyer & Campbell, 1981). Activation or inhibition of thermosensitive (cold) fibres concomitant with the discharge of nociceptive fibres changes the quality of the irritating sensation by the addition of a thermal component that can be distinguished as independent by the subject if appropriately explored. Finally, the demonstration that selective stimulation of corneal cold-sensitive fibres evokes a pure thermal sensation, resolves in favour of Weddell and co-workers (Weddell, 1955; Lele & Weddell, 1956) the controversy about the presence or not of sensory modalities other than pain in the human cornea (Kenshalo, 1970).

This study provides direct evidence that the cornea is equipped with thermal and nociceptive sensory nerve endings functionally similar to those of the skin and external mucosae. Their separate activation evoked qualities of sensation interpreted either as innocuous thermal sensations or irritant sensations. The advantageous characteristics of this tissue for the application of separate stimulus modalities further allowed us to establish that selective stimulation of Aδ and C polymodal nociceptors produces a pain sensation that changes in quality when mechanosensory and/or thermal fibres are activated concomitantly. However, the ability to distinguish the thermal component of the stimulus was maintained, thus supporting the hypothesis that separate sensory channels exist for the transmission to the CNS of the modality and the spatial-temporal characteristics of peripheral non-noxious and noxious stimuli.

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