

Effect of Menthol on Cold Receptor Activity

Analysis of Receptor Processes

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ABSTRACT The effect of menthol on the discharge pattern of feline nasal and lingual cold receptors was analyzed in order to elucidate the underlying sensory transducer mechanism. A repetitive beating activity and burst (grouped) discharges were observed in both cold receptor populations at constant temperatures and after rapid cooling. An analysis of the impulse activity revealed a cyclic pattern of impulse generation, which suggested the existence of an underlying receptor potential oscillation that initiates impulses in the afferent nerve when it exceeds a threshold value. The frequency and amplitude of the periodic impulse-inducing receptor processes were characterized by the burst frequency, which increased with warming, and by the average number of impulses generated during each cycle, which increased with cooling. Menthol at micromolar concentrations induced an acceleration of the burst frequency at higher temperatures, but reduced the burst frequency in the midtemperature range. At temperatures above 25°C, menthol increased the number of impulses elicited during each cycle and induced bursting in previously repetitively discharging fibers. At low temperatures, menthol suppressed bursting and finally inhibited all cold receptor activity. The impulse pattern at constant temperatures and during the dynamic response to rapid cooling was comparably affected by menthol. Calcium application completely abolished the stimulating menthol effect. Since, in equal concentrations, menthol specifically impairs neuronal calcium currents, the results are consistent with the conjecture that in cold receptors, menthol reduces the activation of a calcium-stimulated outward current by an impeding effect on a calcium conductance, thereby inducing depolarization and a modification of bursting behavior. The data confirm the hypothesis of a calcium-controlled outward conductance being involved in the generation of cyclic afferent activity in cold receptors.

INTRODUCTION

Menthol (2-isopropyl-5-methyl-cyclohexanol) induces cold sensations when applied to human skin and mucous membranes, the underlying mechanism being

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a stimulating action on peripheral cold receptors (Hensel and Zotterman, 1951*b*). The effect is not restricted to menthol, but it seems to be rather specific. In a survey of more than 1,200 compounds possessing this quality, several molecular requirements were identified as prerequisites for intrinsic activity (Watson et al., 1978), which suggests a specific drug-receptor interaction at the level of the sensory structure.

Studies on various neuronal membrane channels similarly indicate a specific action of menthol. In molluscan neurons and vertebrate dorsal root ganglion cells, calcium currents were reversibly reduced by external menthol application (Swandulla et al., 1985, 1986). The mode of action of menthol appeared to be a specific interaction with calcium channels rather than a nonspecific alcohol effect.

An analysis of the grouped (burst) discharge apparent in various cold receptor populations indicates that afferent activity is generated by cyclic membrane events, which might be identical to the mechanisms controlling pacemaker activity in bursting molluscan neurons (Braun et al., 1980). A calcium-stimulated outward current, acting as a negative feedback system, is generally viewed as an essential link in the cycle of events controlling periodic neuronal activity (Eckert and Lux, 1976; Lux and Heyer, 1979; Gorman et al., 1982). Studies of the effect of calcium on thermoreceptor discharge have made the participation of a calcium-activated outward conductance in eliciting the bursting pattern most likely (Schäfer et al., 1982).

Cold receptors are free nerve endings (Hensel, 1982), and their small size has so far prevented the direct study of their transducer processes. However, we consider menthol a promising tool to elucidate the underlying sensory mechanisms, since these various observations suggest that menthol might interfere with calcium channels of cold receptors. We therefore analyzed the changes of impulse pattern induced by menthol with respect to the underlying receptor processes. The purpose of the present study was to verify the proposals concerning thermal transduction in cold receptors (Braun et al., 1980, 1984; Schäfer et al., 1982, 1984) and to gain additional insight into the participating membrane processes. We present evidence that cold receptor transducer processes in fact include a system able to induce and maintain cyclic membrane potential changes, and that these oscillations are controlled by calcium currents and a calcium-activated outward conductance. A preliminary account of a part of our results has appeared elsewhere (Isenberg et al., 1984).

METHODS

The results were drawn from 23 adult cats anesthetized with intravenous sodium pentobarbital (35 mg/kg). Additional anesthetic was given during the experiment to maintain an areflexic condition. Afferent neuronal activity of cold receptors was recorded from the intact animal in 17 cats and from an isolated organ preparation in 6 cats.

Preparation and Recording

Intact animals. The method of recording afferent impulses from nasal and lingual feline cold fibers has been described elsewhere (Hensel and Zotterman, 1951*a*; Duclaux et al., 1980). The method was modified in that the dissection of nerve and recording of activity was always performed under mineral oil. Receptive fields of nasal cold fibers were identified using a cold brass cylinder of 0.8 mm² cross-sectional area.

Isolated organ preparations. After resection of the mandibula, both lingual arteries were cannulated and the tongue was perfused by a modified Krebs' solution. The tongue was then excised and placed upside down in a Perspex chamber, the apical part located on a nickel-plated brass thermode. The recording chamber was filled with mineral oil, and dissection of the lingual nerve and recording of afferent activity were performed using the techniques described above.

Receptive fields of identified cold fibers were stimulated by water-circulated thermodes connected with seven thermostats set either at 40, 35, 30, 25, 20, 15, and 10°C or at 50, 45, 40, 35, 30, 25, and 20°C. With a multiway tap, successive cooling steps of 5°C were applied within the range of temperatures available, the constant phase between each step being 3–5 min. The slope of the temperature change against time was nonlinear, but 80% of the final temperature was reached within 1 s. The diameter of the thermode used to stimulate the nasal area was 10 mm, and the dimensions of the lingual thermodes were 20 × 30 mm (intact animal) and 35 × 35 mm (isolated tongue). In the lingual experiments, the tongue was permanently attached to the thermode during preparation and recording. A thermocouple was secured in a groove in the stimulating surface of the thermodes.

Menthol Application

After the control values of cold fiber activity were recorded at constant temperatures and after cooling steps (i.e., static and dynamic responses), a 0.9% NaCl solution containing 0.4 g/liter menthol was infused into the femoral vein. The concentration used corresponds to a saturated aqueous menthol solution. The rate of infusion that caused distinct changes of discharge rate was evaluated in preliminary experiments. We did not measure the blood or plasma menthol concentration in the intact animal, but we compared the menthol-induced changes of activity in these experiments with the quantitative data of the isolated tongue.

The rate of infusion in the experiments on intact animals ranged from 0.23 to 0.67 mg min⁻¹ kg⁻¹ with a mean of 0.57 ± 0.11 (SD) mg min⁻¹ kg⁻¹ menthol. Within an average infusion period of 60 min, this value corresponds to a supply of 34.2 mg/kg menthol, which is well below toxic doses (LD₅₀ = 700 mg/kg) and in the range of the effective dose (ED₅₀ = 35 mg/kg) in another mammal, the rat (Wei, 1983). In some experiments, after termination of the menthol infusion, a solution of calcium gluconate containing 9.23 mg/ml calcium was infused at a rate of 4 ml/min into the femoral vein.

Data Analysis

Data were stored on magnetic tape and analyzed with the aid of a PDP 12 laboratory computer (Digital Equipment Corp., Maynard, MA), using a program developed for identification and analysis of neuronal burst discharges (Braun and Hensel, 1977). Commonly, the term "burst discharge" is used to describe a regular impulse activity, consisting of groups of impulses separated by pauses, which is induced by membrane potential oscillations (Eckert and Lux, 1976; Lux and Heyer, 1979; Gorman et al., 1982). Thus, in this study, the term "burst frequency" represents the frequency of the cyclic receptor membrane events, inducing the bursting pattern. It has to be considered, however, that a varying number of impulses will be initiated by each cycle, and therefore a burst may consist of either several impulses, or only one impulse, or even no impulses, if the underlying process fails to initiate afferent impulses at every cycle.

The burst frequency and number of impulses per burst were calculated using the mean interval duration and the number of intervals representing different distribution maxima of the interval histogram (Braun and Hensel, 1977):

$$BF = \frac{1}{BI}$$

$$BI = \frac{n_1 \cdot t_1 + n_2 \cdot t_2 + n_3 \cdot t_3 + n_4 \cdot t_4 \dots}{n_2 + 2n_3 + 3n_4 \dots}, \quad (1)$$

where BF is the burst frequency and BI is the burst interval;

$$SB = \frac{n_1 + n_2 + n_3 + n_4 \dots}{n_2 + 2n_3 + 3n_4 \dots}, \quad (2)$$

where SB is the number of impulses per burst, n is the number of intervals representing one distinct distribution maximum of the interval histogram (n_1 = number of intraburst intervals; the term is deleted when grouping is absent), and t is the mean of the interval durations within the distribution defined above (t_1 = intraburst interval duration; the term is deleted when grouping is absent).

It has to be noted, however, that the aim of the program is not to detect a periodic pattern in neuronal activity, but to render a quantitative analysis of discharges with an apparent cyclic pattern (e.g., regular bursting activity).

As an example, the burst frequency of one cold receptor at two different temperatures will be calculated (Table I). The data are from Fig. 4 (control, at 25 and 40°C).

TABLE I
Calculation of the Burst Frequency

25°C, Bursting activity		40°C, Beating activity	
$n_1 = 306$	$t_1 = 34$	$n_1 = \text{—}^*$	$t_1 = \text{—}$
$n_2 = 199$	$t_2 = 194$	$n_2 = 292$	$t_2 = 105$
$n_3 = \text{—}$	$t_3 = \text{—}$	$n_3 = 78$	$t_3 = 220$
$n_4 = \text{—}$	$t_4 = \text{—}$	$n_4 = 56$	$t_4 = 350$
BI = 246 ms		BI = 109 ms	
BF = 4.0 s ⁻¹		BF = 9.1 s ⁻¹	

* No intraburst intervals were apparent. Time (t) is in milliseconds.

RESULTS

17 single afferent fibers showing the characteristics of specific cold fibers (Hensel, 1982) were identified during experiments on intact animals. Since the intravenous administration of menthol caused long-lasting changes of cold receptor activity, only one receptor per cat was studied in these experiments. All fibers were tested throughout the whole range of static and dynamic thermal stimuli under control and infusion conditions. Afferent activity was recorded from 4 lingual nerve fibers (temperature range, 10–40°C), and from 13 infraorbital nerve fibers (temperature range, 10–40°C, $n = 8$; 20–50°C, $n = 5$). Nasal cold receptors had spotlike receptive fields in the glabrous skin of the nose. Because of the position of the lingual thermode, the dimensions of the lingual receptive fields could not be evaluated, but they were all located on the apical upper surface of the tongue.

16 cold receptors were identified during experiments on the isolated tongue preparation, and 4 of them were tested under control and menthol conditions with a complete stimulation series in the range of 10–40°C. Unless otherwise indicated, the data that follow were drawn from the intact animal.

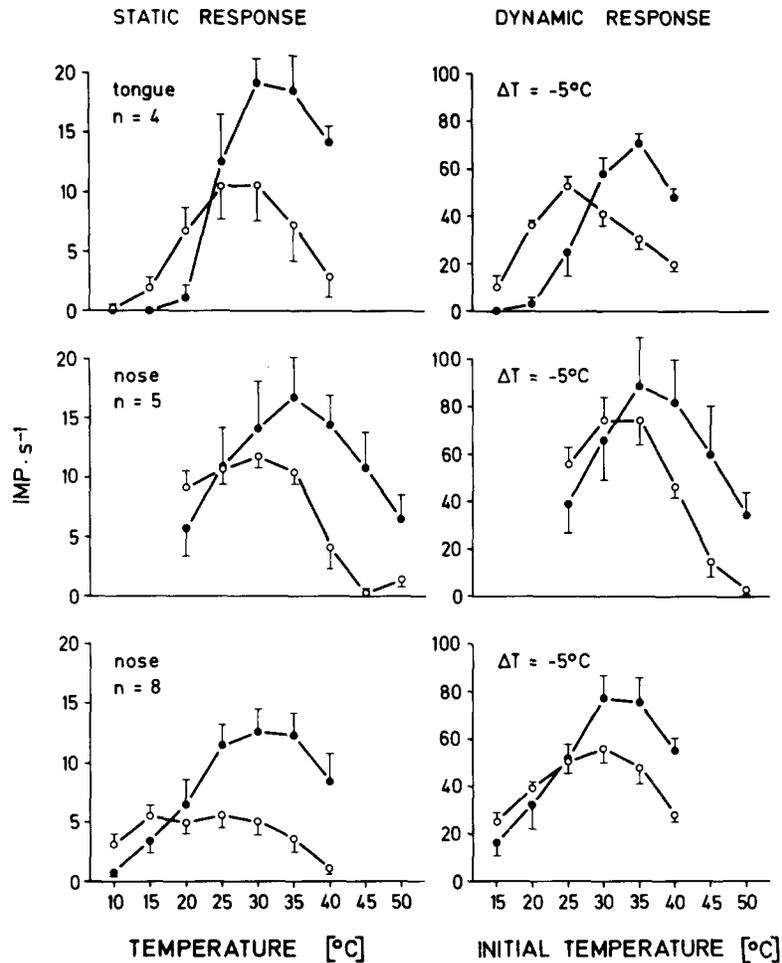


FIGURE 1. Effect of menthol on cold receptor activity at constant temperatures (left) and after rapid cooling steps (right). Open circles: control values; solid circles: menthol application. Static responses are impulse rate per second, averaged over a period of 1 min before a temperature change; dynamic responses are characterized by the peak impulse frequency obtained during cooling. A paradoxical discharge is visible at 50°C under control conditions. Lingual and nasal cold receptor populations are indicated. An average dose of $0.57 \pm 0.11 \text{ mg min}^{-1} \text{ kg}^{-1}$ menthol was infused. Bars indicate SE.

Effects of Menthol on Mean Impulse Activity

After the onset of menthol application at constant temperatures of 30, 35, or 40°C, the discharge rate increased with a latency of ~ 30 s to achieve a fairly constant level of activity after an average period of 2 min. Fig. 1 shows the effect of menthol on the static and dynamic responses of cold fibers after their activity had stabilized to an approximately constant level. The mean impulse activity at

constant temperatures was depressed at lower and enhanced at higher temperatures; the range of activity was therefore shifted into the upper temperature range. The dynamic response, which was characterized by the peak frequency obtained during cooling steps from different adapting temperatures, was similarly affected.

Successive series of stimulation during menthol administration progressively shifted the activity range of cold receptors into the upper temperature range (Fig. 2). This effect was a consequence of the advancing suppression of activity at lower temperatures and the enhancement of activity at higher temperatures. Such a depression of activity at low temperatures can also be observed when

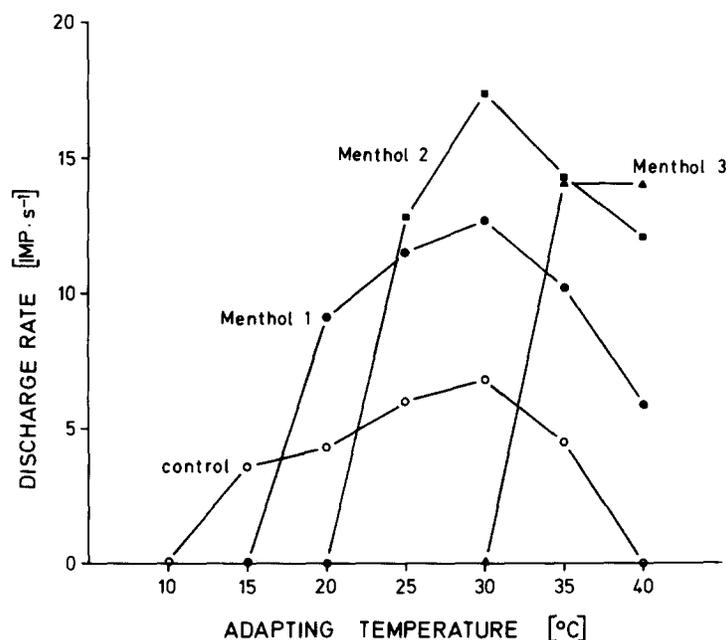


FIGURE 2. Modification of menthol effect on cold receptor activity with continued application. The time interval between the successive stimulation series (i.e., 1, 2, 3) amounts to 30 min (constant phase at each temperature, 5 min). Infused dose of menthol: $0.50 \text{ mg kg}^{-1} \text{ min}^{-1}$. Lingual cold receptor.

external calcium is reduced (Schäfer et al., 1982; Schäfer, K., manuscript in preparation).

These results are in agreement with previously reported effects of menthol (Hensel and Zotterman, 1951*b*; Dodt et al., 1953; Pierau, 1967). The earlier observations by these authors concerning a narrowing of the range of cold fiber activity can neither be confirmed nor refuted by our data, because 50°C as the uppermost stimulation temperature is still too low to evaluate the upper threshold of cold receptor activity during menthol administration. A relative increase of activity, however, was maximal at temperatures of 40°C and above. A so-called paradoxical discharge (Dodt and Zotterman, 1952; Long, 1977) was present in

three out of five fibers tested at temperatures above 40°C, and was obliterated by the shift of cold receptor activity after menthol administration (Fig. 1). After applying the 50°C stimulus, a second control series was added, because a modification of cold fiber activity after stimulation with noxious temperatures has been reported (Dubner et al., 1975; Long, 1977). In our experiments, no alterations of activity by noxious heat were observed.

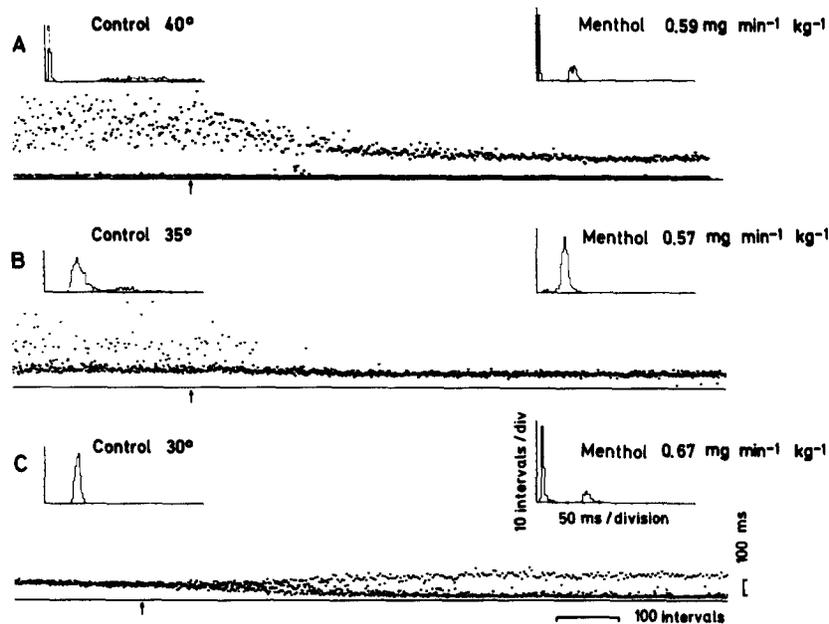


FIGURE 3. Changes of impulse pattern at the onset of menthol infusion at various constant temperatures. Each dot represents one interval, with dimensions as indicated. (A) Burst discharge at control conditions; intervals shorter than 50 ms represent intraburst intervals. (B) Discharge with irregularly appearing silent periods at control conditions. (C) Repetitive (beating) impulse activity, which changes into a burst discharge after the onset of menthol supply. (Insets) Interval distribution at the beginning and the end of the displayed section. The arrows indicate the onset of menthol application. Intervals shorter than 50 ms are intraburst intervals. Dimensions are given in C. The transition from control to menthol values was completed within 1.6 min in these experiments. A: lingual; B and C: nasal cold receptor.

Modification of Discharge Pattern by Menthol at Different Constant Temperatures

The menthol-induced changes of mean afferent activity result from modifications of the temporal pattern of the cold receptor discharge. Our data indicate that cyclic receptor membrane processes are involved in the increase of the discharge rate induced by menthol administration. Three different modifications of the impulse pattern can be observed, depending on the adaptation temperature (Fig. 3): a burst discharge apparent under control conditions is accelerated by a

progressive shortening of the intervals between bursts of action potentials (Fig. 3A), the regular beating activity is transformed into bursting activity by the generation of shorter (intra-burst) intervals (Fig. 3C), and activity in fibers showing longer intervals that appear irregularly is enhanced by progressively substituting shorter intervals for the longer ones. This corresponds to the filling of silent periods with additional impulses (Fig. 3B). The few intervals shorter than 50 ms appearing at the end of the diagram (Fig. 3B), which are visible in the interval histogram as a small distribution maximum, indicate that the pattern is finally changing from beating to bursting. Changes of the discharge pattern similar to those obtained with menthol can be induced by a reduction of plasma calcium by the administration of the chelating agent EDTA (Schäfer et al., 1982; Braun et al., 1984).

In 9 out of 13 nasal cold fibers and in all lingual cold fibers, a regular grouping of impulses was apparent at various static temperatures under control conditions. In general, bursting was more pronounced in lingual cold fibers, relative to both the number of impulses per burst and the number of temperatures inducing this pattern. This observation accords with previous investigations (for references, see Hensel, 1982). During menthol administration, however, all fibers studied showed static bursting. Discharge under control and menthol conditions can be characterized by bursting at low temperatures and beating at high temperatures (Fig. 4, diagram *a*). With increasing thermode temperatures, the number of impulses per burst decreases, whereas the longer intervals, representing the pauses between the bursts, are shifted to shorter values (Fig. 4, diagrams *b* and *c*), which indicates an increased burst frequency. Interval histogram data of different constant temperatures clearly indicate that the distribution maxima representing repetitive impulse activity at higher temperatures (Fig. 4, control, 35°C, and menthol, 50°C) emerge from the distribution maxima that represent the pauses between the bursts at lower temperatures (Fig. 4, control, 30°C, and menthol, 45°C).

The regular interval distribution of discharges consisting of longer intervals that appear irregularly (Fig. 4, control, 40°C) suggests the existence of a periodic process. The intervals are clustered at values corresponding to simple multiples of the basic value, which represents the burst period. This pattern has been observed by others as well (Braun et al., 1980, 1984; Schäfer et al., 1984) and is attributed to cyclic impulse-inducing processes, which occasionally fail to initiate the appropriate impulse.

Menthol either causes complete inhibition of activity at low temperatures or at least increases the duration of both intra-burst intervals and the pauses between the bursts. At higher temperatures, burst discharges are induced or, if present at control conditions, enhanced by menthol. Beating activity at high temperatures is accelerated (Fig. 4).

The data reported by us and others (Braun et al., 1980, 1984; Schäfer et al., 1984) indicate that the regular grouping of impulses at lower temperatures, as well as the repetitive impulse activity at higher temperatures, represents a manifestation of an underlying oscillating receptor potential. Using interval histogram data, underlying hypothetical oscillations were calculated for seven bursting cold fibers (Fig. 5). Additionally, the average number of impulses per

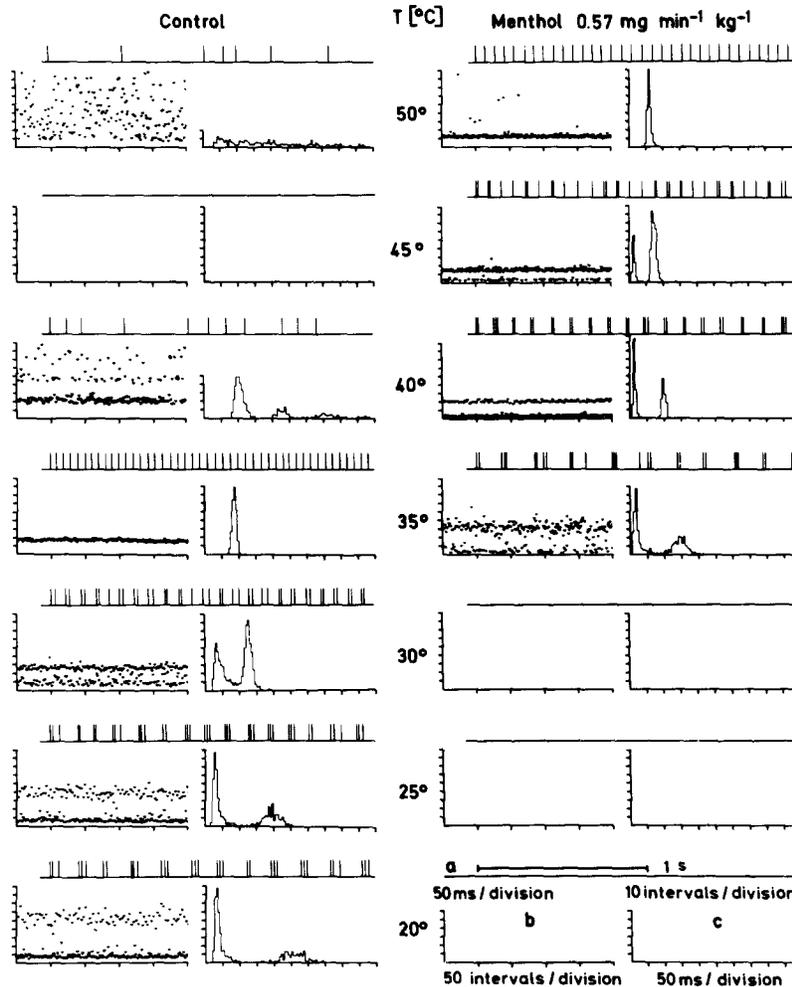


FIGURE 4. Effect of menthol on impulse pattern of a nasal cold receptor. The diagram shows the impulse discharge (*a*), the duration of successive intervals (*b*), and the interval distribution (*c*) at different constant temperatures for control and menthol conditions. Intervals shorter than 100 ms at 20, 25, and 30°C during control, and intervals shorter than 50 ms during menthol application, are intraburst intervals. The dimensions of the graphs are given at 30°C, menthol application. Menthol inhibited impulse activity at temperatures below 35°C. The main interval at 40°C at either the control or menthol condition is 100–110 ms, but whereas at control conditions intervals appear that represent multiples of the basic interval (225 ms, 350 ms), during menthol application, grouping is induced. Activity at 50°C (control) corresponds to paradoxical discharge.

burst was calculated (see Methods). Menthol administration particularly increases the number of impulses per burst, at least until its inhibiting action prevails. Under both control and menthol infusion conditions, the burst frequency increases and the number of impulses per burst decreases with higher static

temperatures. Under control conditions, however, the impulse activity at temperatures above 40°C was too low to reliably calculate either the burst frequency or the number of impulses per burst, which in this case may have been considerably less than 1.

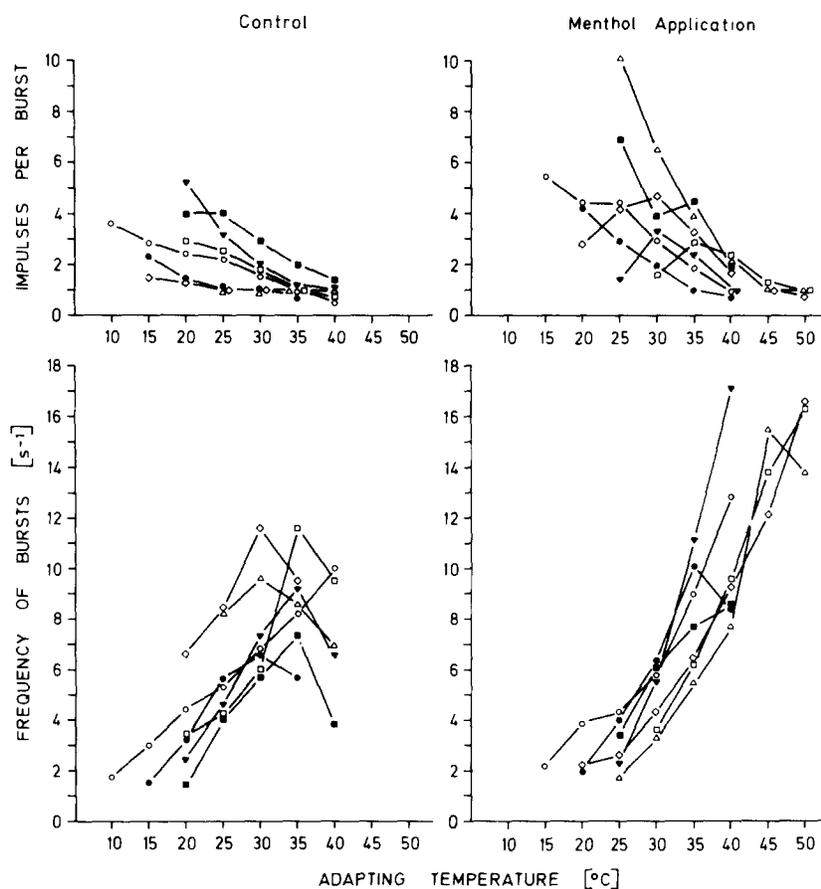


FIGURE 5. Modification of burst discharges by menthol. The number of impulses per burst and the burst frequency are calculated from interval distribution data (see Methods). A “burst” may consist of several, one, or less than one impulse (see Methods). An example of a burst discharge of less than one impulse per burst is the diagram representing 40°C, control, of Fig. 4. The low number of intervals obtained during stimulation at either 45 or 50°C did not permit calculation of either the number of impulses per burst or the burst frequency at control conditions. Infused doses ranged from 0.47 to 0.67 mg min⁻¹ kg⁻¹ menthol. Open symbols: nasal; solid symbols: lingual cold receptors.

Both the mean discharge rate and burst frequency are affected by menthol, and for comparison, the changes of the burst frequency are related to the alterations of the mean impulse activity in Fig. 6. While the burst frequency corresponds to the frequency of the underlying impulse-eliciting cyclic receptor

process, the mean impulse frequency represents the degree of a suprathreshold condition for impulse generation, and is determined by both the oscillation frequency and the number of impulses initiated during each cycle (Braun et al., 1980). Menthol increases the burst frequency at higher temperatures, but decreases it in the temperature range of 15–35°C. The reduction of the slope of

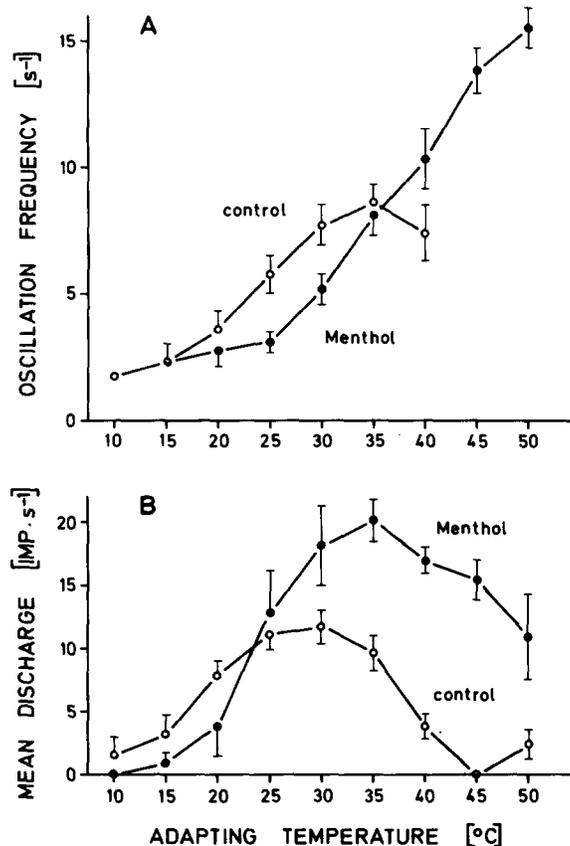


FIGURE 6. Modification of cold receptor activity by menthol. Data are from seven cold fibers exhibiting burst discharges at constant temperatures. (A) Burst frequency (same data as Fig. 5). (B) Mean impulse rate, averaged over a period of 1 min before a temperature change. Bars indicate SE. At 25, 30, and 35°C, the burst frequency (which represents the underlying receptor membrane oscillation) was reduced by menthol, whereas mean impulse activity was increased.

the burst-frequency-temperature relations seen under control conditions at temperatures above 35°C does not appear during menthol administration; in the temperature range studied, the burst frequency increases monotonically with higher temperatures (Fig. 6A). The mean discharge rate is also reduced at lower temperatures and enhanced at higher temperatures by menthol administration; the changes plotted against control are shown in Fig. 6B. The burst frequency

and mean discharge rate, however, are not evenly modified by menthol: although the burst frequency is considerably reduced by menthol in the temperature range of 25–30°C, the mean impulse activity is elevated at these temperatures.

Modification of Impulse Pattern by Menthol During Rapid Cooling Steps

Cold fibers respond to rapid cooling with a transient increase of impulse activity, which decays to a new static level after the dynamic phase of the stimulus has

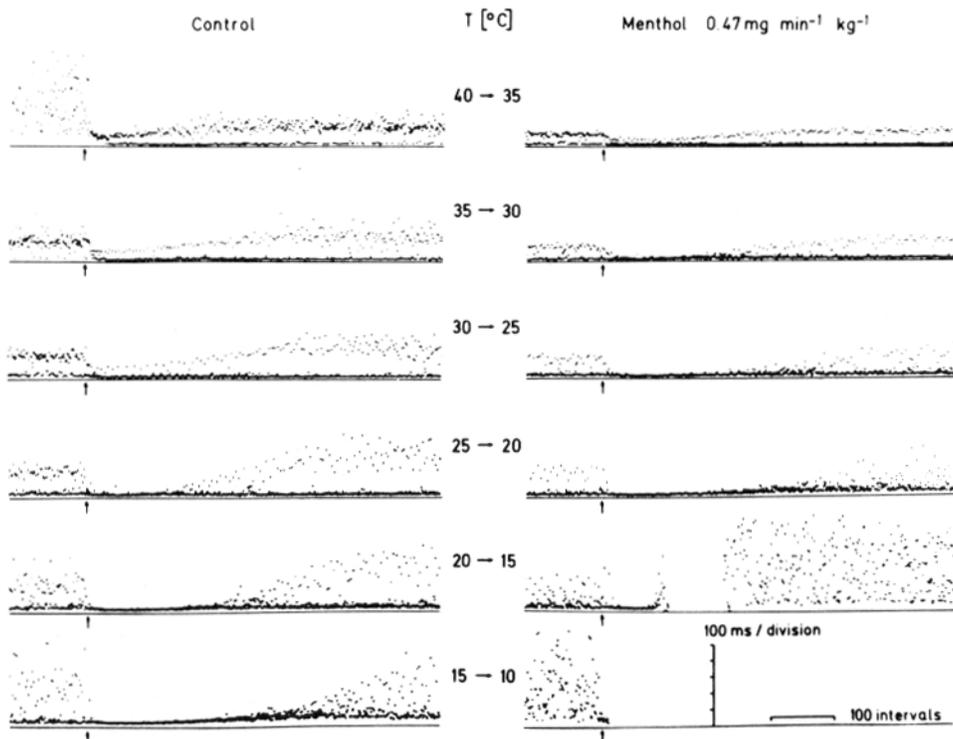


FIGURE 7. Modification by menthol of impulse pattern during cooling steps. Nasal cold fiber. The duration of successive intervals is represented; each dot corresponds to one interval. 80% of the temperature change during the various cooling steps was completed within 1 s; the dynamic increase of impulse activity (dynamic response) lasted ~10 s. The arrows indicate the onset of the 5°C cooling step. Burst discharges can be identified by a successive order of shorter and longer intervals, the shorter intervals representing intraburst intervals. Activity was suppressed transiently after cooling from 20 to 15°C, and was totally inhibited after cooling from 15 to 10°C (menthol application).

been completed (Hensel, 1982). We observed the following response patterns to cooling: bursting fibers respond either throughout the dynamic response with bursting, or bursting is transiently replaced by repetitive activity. Nonbursting fibers usually respond with a transient acceleration of repetitive activity, followed by a transient burst discharge. These patterns, however, are temperature dependent, and can be observed occasionally in the same fiber during cooling from different adapting temperatures (Fig. 7, control). With respect to this pattern,

the differences between lingual and nasal cold receptors are only quantitative in nature.

Menthol enhances the dynamic response, particularly at higher temperatures, by accentuating the dynamic burst discharge, whereas at low temperatures, the inhibiting action of menthol is already effective and suppresses the dynamic response by blocking impulse activity completely before a burst discharge can develop (Fig. 7). An analysis of successive intervals proves that there is a considerable reduction of the interval duration during the dynamic response to cooling. If a burst discharge is apparent at the adapting temperature, intervals representing burst pauses merge with the intraburst intervals (Fig. 7), thereby causing the repetitive discharges already mentioned above. During adaptation to the new static temperature, the shortened intervals become progressively

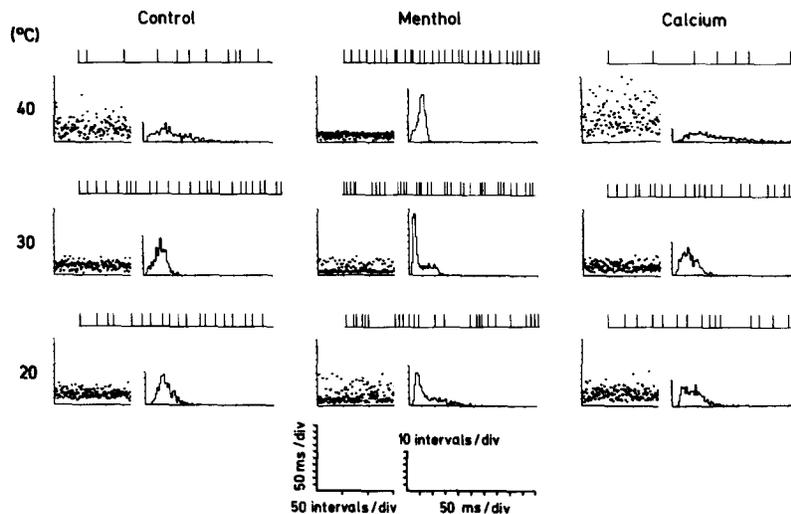


FIGURE 8. Abolition by calcium of the menthol effect on cold receptors. For three constant temperatures, the impulse activity (top), the duration of successive intervals (lower left), and the interval distribution (lower right) are shown. Dimensions are given in the inset; impulse activity is represented for a period of 1.5 s. Control: before menthol administration; menthol: after 30 min of application; calcium: after infusion of a single dose of 7 mg/kg body weight calcium. Nasal cold receptor.

longer, eventually separating into intervals representing burst pauses and intraburst intervals (Fig. 7), which correspond to a bursting pattern.

At a given adapting temperature, menthol has a negligible effect or no effect at all on the duration of intraburst intervals, but raises the probability of their appearance. This applies to both the dynamic and the static response. The data indicate that menthol mainly modulates the duration of either the intervals representing the pauses between the bursts, or the intervals of discharges, where grouping is absent. These changes are consistent with the assumption that menthol mainly affects the period of the cyclic impulse-inducing receptor membrane process.

In various cold fiber populations, a transient suppression of impulse activity has been monitored to follow the dynamic increase of the discharge rate during

cooling (Hensel, 1953; Poulos and Lende, 1970; Kenshalo and Duclaux, 1977; Duclaux et al., 1980). Several of the cold fibers studied by us showed this behavior as well, and the effect was accentuated by menthol at lower temperatures, varying from impulse inhibition for several seconds after the dynamic response (Fig. 7, 20–15°C) to complete suppression of the dynamic response (Fig. 7, 15–10°C).

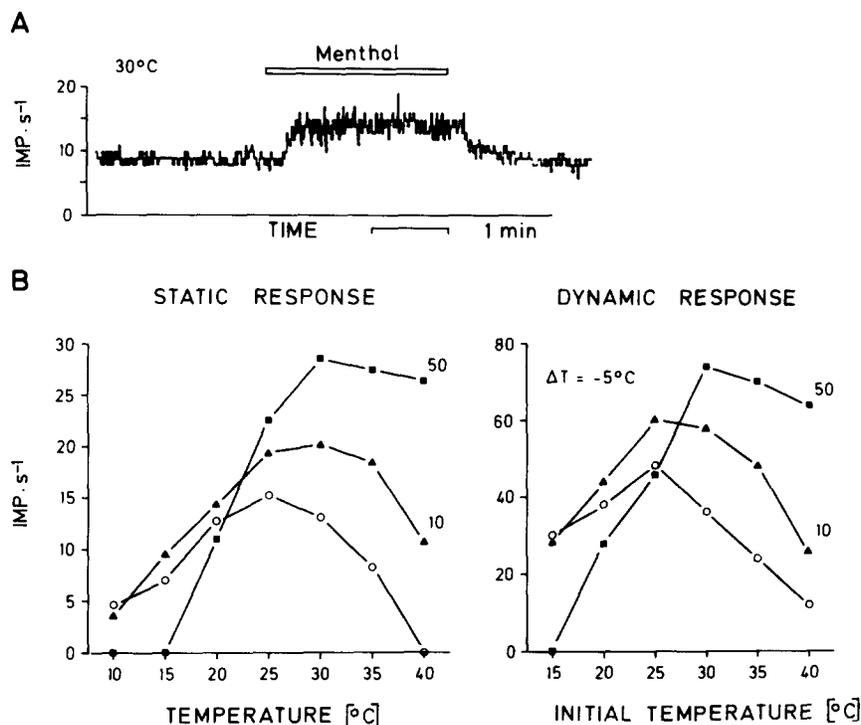


FIGURE 9. Effect of menthol on cold receptor activity of an isolated perfused tongue preparation. (A) Mean impulse activity in impulses per second, averaged for 10 s. The bar indicates perfusion of isolated tongue with a Krebs' solution containing 10 $\mu\text{mol/liter}$ menthol. (B) Static and dynamic responses of a second cold receptor during control conditions (open symbols) and during perfusion with a solution containing 10 (triangles) or 50 (squares) $\mu\text{mol/liter}$ menthol. The effect of menthol was fully reversible; the effect of various concentrations was reproducible. This receptor allowed recording of afferent activity for 4 h. The changes in the discharge pattern induced by menthol were comparable to those observed in intact animals.

Abolition of Menthol Effect by Calcium

Since menthol interferes with the conductance of mammalian neuronal calcium channels (Swandulla et al., 1985), we analyzed the effect of calcium administration on cold fiber activity, which had been already modulated by menthol. A single dose of 7 mg/kg calcium was sufficient to reduce cold fiber activity to control values (Fig. 8). The discharge pattern modulated by menthol was equally well modified by calcium to become identical to the control conditions (Fig. 8). Similar changes were observed with respect to the dynamic responses.

Effect of Menthol on Cold Receptors of an Isolated Preparation

To study the effect of menthol quantitatively, afferent activity of cold receptors was recorded from the lingual nerve of an isolated cat tongue preparation. The tongue was perfused either with a Krebs' solution or with a Krebs' solution containing various concentrations of menthol. 16 cold receptors were identified; 4 were studied at adapting temperatures between 40 and 10°C during control and menthol conditions. Recording from an isolated perfused preparation did not alter the response characteristics of cold receptors. Perfusing the tongue with a solution containing 10 $\mu\text{mol/liter}$ menthol reversibly increased cold fiber activity (Fig. 9A). The effect developed within seconds. Repeated perfusion of solutions containing various concentrations of menthol resulted in identical responses to thermal stimulation, when equal concentrations were present. The temporal sequence of the application had no effect. By comparison of Figs. 2 and 9B, it can be concluded that the effective concentration during the experiments on intact animals varies in the range of values around 50 $\mu\text{mol/liter}$. These values accord with the concentrations, which are already effective in modulating calcium channel conductance in a variety of neurons (Swandulla, D., K. Schäfer, and H. D. Lux, unpublished data), and they are in the range reported as being effective on cold receptor activity, when applied to the mucous membranes of the tongue (32–640 $\mu\text{mol/liter}$, calculated from data of Hensel and Zotterman, 1951*b*). The menthol-induced changes of discharge pattern in cold receptors of the isolated preparation were comparable to those observed in intact animals.

DISCUSSION

The small size of cold receptors has so far prevented any direct study of their sensory transducer mechanisms. However, the application of menthol has provided some insight into the underlying receptor membrane processes. Our results suggest that cold receptors possess calcium channels and calcium-controlled outward conductances, that these membrane processes are involved in the generation of cyclic afferent activity, and that a calcium-dependent process participates in the impulse frequency adaptation after dynamic responses. Additionally, our data indicate that sensory transduction includes cyclic membrane processes in all cold receptors.

Mode of Action of Menthol

Of the various sensory structures present in the skin, only cold receptors seem to be affected by menthol in low concentrations (Hensel and Zotterman, 1951*b*). Several findings suggest that the effect is the result of a highly specific interaction of menthol with the cold receptor membrane. In inducing cold sensations, *d*-menthol is 45 times less active than its natural enantiomer *l*-menthol, and several molecular requirements (for instance, stereochemical configuration and a functional group capable of hydrogen bonding) need to be satisfied for a compound to exert a menthol-like effect (Watson et al., 1978). Observations in a variety of molluscan neurons and in dorsal root ganglion cells suggest a specific impeding action of menthol on calcium currents. The effects of menthol develop in seconds, they are fully reversible and stereochemically selective, and they cannot be evoked by intracellular application (Swandulla et al., 1985; 1986; Swandulla, D.,

K. Schäfer, and H. D. Lux, unpublished data). Related compounds, such as cyclohexanol, fail to produce any comparable effect, even in considerably higher concentrations, which suggests a specific interaction of menthol with calcium channels rather than a nonspecific alcohol effect. Menthol reduces current through two types of calcium channels present in sensory neurons (Carbone and Lux, 1984) by different mechanisms. The high-voltage-activated, long-lasting conductance (HVA type) is impaired by an accelerated inactivation, whereas the low-voltage-activated transient current (LVA type) is reduced without its inactivation time course being affected (Swandulla et al., 1985). Menthol has practically no effect on a third type of calcium current recently identified by Nowycky et al. (1985) in sensory neurons (Swandulla, D., E. Carbone, K. Schäfer, and H. D. Lux, manuscript submitted for publication). Taken together, these observations strongly support the view that in cold receptors, the modulating effect on afferent activity depends on a specific action of menthol that interferes with calcium channels of the sensory membrane. The similar range of menthol concentrations needed to affect either cold receptor activity or neuronal calcium channels further corroborates this proposal.

Menthol Modulates Cyclic Activity of Cold Receptors

Grouped discharges have been observed in cold fiber populations of various species, including man (for references, see Hensel, 1982). The bursting pattern is viewed as being generated by a receptor potential oscillation that initiates impulses when it exceeds a threshold value (Iggo and Young, 1975; Braun et al., 1980). A qualitative model can be formulated that incorporates the membrane processes that are needed to induce the observed cyclic activity pattern in cold receptors. This model closely follows that proposed for the control of pacemaker activity in molluscan neurons (Eckert and Lux, 1976; Lux and Heyer, 1979; Gorman et al., 1982). The model includes (a) an imbalance of membrane inward and outward currents, which constitutes a continuous depolarizing drive; (b) a voltage-dependent calcium current, activated during depolarization; and (c) a burst-terminating, hyperpolarizing outward current, activated by the increasing intracellular calcium during the positive-going phase of the cycle. Warming augments the depolarizing drive, because the inward conductances are more strongly temperature dependent than the outward conductances (Gorman and Marmor, 1970; Marchiafava, 1970; Marmor, 1971; Willis et al., 1974), and therefore causes an acceleration of the oscillation frequency (Murray, 1966; Junge and Stevens, 1973; Barker and Gainer, 1975). This behavior can be observed in cold receptors (Figs. 5 and 6).

In some molluscan neurons, the calcium inward current activating the burst-terminating outward current is present at all potentials obtained during normal activity (Lux and Heyer, 1979). It is therefore possible that the calcium-sensitive outward current is permanently activated to a certain degree. This seems to be the case in cold receptors, since a reduction of external calcium increases the impulse discharge, which indicates a depolarization induced by a reduction of the calcium-activated outward current (Schäfer et al., 1982). If the calcium-activated outward current contributes to the imbalance underlying oscillating

behavior, then any increase of outward current activation will reduce the depolarizing drive (imbalance), which will in turn decrease the oscillation frequency. Such an increase of conductance has been reported to occur with warming for both the calcium and the calcium-activated outward channel (Johnston, 1980; Gorman et al., 1982; Lux and Brown, 1984), and it seems reasonable to assume that this mechanism underlies the reduction of burst frequency in cold receptors at temperatures above 35°C, which has also been observed by others (Braun et al., 1980; Schäfer et al., 1982).

The changes of burst frequency induced by menthol administration indicate that menthol interferes with the membrane processes controlling cyclic afferent activity. As in molluscan neurons (Swandulla, D., K. Schäfer, and H. D. Lux, unpublished data), any reduction of the persistent calcium inward current by menthol will result in an impaired activation of the calcium-stimulated outward current. This constitutes an augmented depolarizing imbalance that causes the membrane potential oscillation to accelerate, which can be seen at temperatures above 35°C. For the menthol-induced slowing of the burst-triggering oscillations in cold receptors at temperatures below 35°C, the following mechanism is proposed. Both the amplitude and the activation kinetics of the inward calcium current are strongly temperature dependent and may be rather low at low temperatures (Donaldson and Beam, 1983; Lux and Brown, 1984), so that the amount of calcium entering the receptive structure during each cycle may not be sufficient during menthol application to adequately activate the hyperpolarizing outward current. The increase in the mean impulse discharge, which can be observed concomitantly with the reduced burst frequency in the temperature range of 25–35°C, indicates a prolonged suprathreshold phase of the underlying oscillation and therefore further corroborates the proposed concept.

Furthermore, the analysis of the menthol effect on impulse pattern allows us to speculate on the type of calcium channel involved in sensory transduction. Since the afferent impulses released by the receptive structure do not modulate cyclic cold receptor activity (Braun et al., 1980; this report), any action potential-activated calcium conductance, such as the HVA type, seems to be absent in peripheral cold receptors. The data indicate that a calcium channel with characteristics of the recently discovered LVA type is involved, which accords with the assumption of this type of channel contributing to rhythmic neuronal activity (Llinas and Yarom, 1981). Additionally, several lines of evidence suggest the existence of a biochemical receptor specific for compounds with chemical characteristics like menthol. This receptor, located on the cold receptor membrane, may be close to or identical with a calcium channel.

Modification of Dynamic Responses by Menthol

In most of the mammalian cold fibers studied so far, the adaptation of impulse frequency to the new static level after a dynamic increase of activity is accompanied by the transient appearance of a grouped discharge (Dubner et al., 1975; Braun et al., 1980; Sumino and Dubner, 1981; Schäfer et al., 1982). Our results indicate that dynamic bursting is usually induced only within a midtemperature range of cold receptor activity, whereas at lower and higher temperatures, the

dynamic response mainly consists of a repetitive discharge of high frequency. The data obtained so far (Braun et al., 1980; Schäfer et al., 1982; this report) indicate that the burst-inducing receptor potential oscillation accelerates during rapid cooling. At higher temperatures, the stimulus-induced transient depolarizing shift of the oscillation may not be sufficient to release more than one impulse per cycle. At low temperatures, however, the depolarizing drive during cooling may be transiently stronger than the oscillating process can follow. Then the limiting factor preventing impulse grouping is the maximal oscillation frequency attainable during the dynamic response, which is determined by the imbalance of the ionic currents representing the depolarizing drive, and by the time constants of activation and inactivation of the underlying currents. Both factors are strongly temperature dependent (Gorman and Marmor, 1970; Marchiafava, 1970; Marmor, 1971; Eckert and Lux, 1976; Johnston, 1980; Gorman et al., 1982; Beam and Donaldson, 1983; Donaldson and Beam, 1983; Carpenter and Gregg, 1984; Lux and Brown, 1984). The conductances of the currents controlling the oscillation frequency are low at low temperatures, and the low time constants of these currents at low temperatures result in a defective temporal differentiation of the cyclic events underlying the oscillation. In addition, the low calcium conductance present at low temperatures accounts for a reduced activation of the calcium-stimulated hyperpolarizing outward current. Oscillating behavior is therefore suppressed and repetitive firing of high frequency can be observed throughout the dynamic response.

Our data indicate that, in addition to interfering with the membrane events inducing cyclic discharges, menthol affects the process of impulse frequency adaptation to new static levels after rapid cooling steps. Several lines of evidence indicate that a slow outward current underlies impulse frequency adaptation after dynamic responses (Partridge and Stevens, 1976; Swerup, 1983). This slow outward current may be partially activated by calcium entering the receptor during stimulus-induced depolarization (Swerup, 1983). The enhancement of the dynamic response by menthol therefore may also result from the suppressive action of menthol on a calcium conductance.

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