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Effects of temperature on tuning of the auditory pathway in the cicada *Tettigetta josei* (Hemiptera, Tibicinidae)

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Summary

The effects of temperature on hearing in the cicada *Tettigetta josei* were studied. The activity of the auditory nerve and the responses of auditory interneurons to stimuli of different frequencies and intensities were recorded at different temperatures ranging from 16°C to 29°C.

Firstly, in order to investigate the temperature dependence of hearing processes, we analyzed its effects on auditory tuning, sensitivity, latency and Q_{10dB}. Increasing temperature led to an upward shift of the characteristic hearing frequency, to an increase in sensitivity and to a decrease in the latency of the auditory response both in the auditory nerve recordings (periphery) and in some interneurons at the metathoracic–abdominal ganglionic complex (MAC). Characteristic frequency shifts were only observed at low frequency (3–8 kHz). No changes were seen in Q_{10dB}. Different tuning mechanisms underlying frequency selectivity may explain the results observed.

Secondly, we investigated the role of the mechanical sensory structures that participate in the transduction process. Laser vibrometry measurements revealed that the vibrations of the tympanum and tympanal apodeme are temperature independent in the biologically relevant range (18–35°C). Since the above mentioned effects of temperature are present in the auditory nerve recordings, the observed shifts in frequency tuning must be performed by mechanisms intrinsic to the receptor cells.

Finally, the role of potassium channels in the response of the auditory system was investigated using a specific inhibitor of these channels, tetraethylammonium (TEA). TEA caused shifts on tuning and sensitivity of the summed response of the receptors similar to the effects of temperature. Thus, potassium channels are implicated in the tuning of the receptor cells.

Key words: cicada, *Tettigetta josei*, hearing, temperature.

Introduction

Like many other species of insects (von Helversen and von Helversen, 1994; Stumpner and von Helversen, 2001) cicadas have developed highly specialized systems of intraspecific acoustic communication in the course of evolution. Acoustic communication is important for several behaviors, such as recognition of conspecifics, localization of the sender, mate choice and predator detection. Precise recognition of the species-specific signal is essential, especially when other sound communicating species are present, because the cicada must discern the song of its own species (Huber, 1984). Cicadas evolved a sophisticated auditory pathway capable of a fine discrimination of sound parameters, such as the spectral content and the temporal pattern of a signal (Daws et al., 1997; Fonseca and Revez, 2002a; Sueur and Aubin, 2002). Cicadas have up to 2000 receptor cells per ear, with a high frequency resolution that is maintained at the level of the central nervous system (CNS) (Fonseca et al., 2000; Fonseca and Revez, 2002a). *Tettigetta josei*, the experimental model insect species in the present study, exhibits this fine frequency resolution as revealed by the response of its auditory ascending interneurons which are sharply tuned and cover a large range of frequencies, including around the peak of the calling song – 16 kHz (Fonseca et al., 2000).

The receptor cells are organized in a bulb-like auditory organ, which is located lateroventrally in the first abdominal segment (Doolan and Young, 1981; Michel, 1975). A rod, the tympanal apodeme, provides the mechanical connection to the oscillating tympanum which acts as a pressure difference receiver (Fonseca and Popov, 1997). In *T. josei*, about 700 afferent fibers run into the auditory nerve, which is connected to first order interneurons at the metathoracic–abdominal ganglionic complex (MAC). More than a dozen auditory ascending interneurons have been found in cicadas, together with some local cells (Huber et al., 1990; Fonseca, 1994; Fonseca et al., 2000).

Cicadas are essentially ectothermic insects (Fonseca and Revez, 2002b; Sanborn et al., 1992) and therefore fluctuations in environmental temperature might influence auditory processing. *T. josei* is active during the day in June and July,

usually singing at ambient temperatures from 22°C to 35°C. Fonseca and Revez (Fonseca and Revez, 2002b) showed that, at least in three species of cicadas, some temporal parameters of their songs are deeply affected by changes in temperature. Because hearing mechanisms depend on electrochemical transduction which is likely to be affected by temperature, we might expect the auditory pathway to be temperature sensitive. In fact, Oldfield (Oldfield, 1988) discovered that, in the locust, tuning and sensitivity of single auditory receptors are temperature dependent. The characteristic frequency and sensitivity of the auditory receptors increased while latency decreased with increasing temperature. Auditory tuning properties have also been found to be affected by changes in temperature in a variety of vertebrates: amphibians (Dijk et al., 1990), reptiles (Eatock and Manley, 1981; Smolders and Klinke, 1984), hearing specialist fish (Fay and Ream, 1992) and birds (Schermuly and Klinke, 1985). By contrast, in mammals, no temperature-dependent changes in the characteristic sound frequency measured in afferent fibers were found (Gummer and Klinke, 1983). Moreover, no effects of temperature on sensitivity and latency were found in hearing generalist fish (Amoser and Ladich, 2006).

Frequency selectivity can be performed by mechanisms extrinsic or intrinsic to the receptor cells (Dallos, 1992; Fettiplace and Fuchs, 1999; Kennedy et al., 2005). Changes in temperature might affect frequency analysis mechanisms at one or both levels. Changes in the mechanical properties of the tympanic membranes and/or the auditory organ, caused by temperature variations, could not explain the shifts observed in the characteristic sound frequency of locust auditory receptors (Oldfield, 1988). Thus, these shifts might have been due to changes in the intrinsic properties of the receptors. Intrinsic mechanisms may depend on electrical properties of the individual receptors (Fettiplace, 1987; Fuchs et al., 1988; Hudspeth and Lewis, 1988).

The aim of this study was to analyze the effects of temperature on tuning, sensitivity, latency, Q_{10dB} and response strength of interneurons and auditory receptors in the species *T. josei* and to scrutinize some of the mechanisms that might be responsible for the fine frequency tuning observed in the auditory pathway of cicadas.

Materials and methods

Animals

Cicadas of the species *Tettigetta josei* Boulard 1982 were collected from the end of May until July in south eastern Portugal. The insects were transported in a cool box and kept either on a feeding shrub at ambient temperature in the shade (Lisbon University) or in a cooled environment at about 10°C (University of Southern Denmark in Odense).

Electrophysiology

Dissection and recording: Cicadas were waxed, ventral side up, to a holder 6 mm in diameter. The temperature of the holder was varied with a Peltier element (Fig. 1) and controlled through two thermocouples. One thermocouple measured the temperature of the holder itself and allowed variations in the range of 10–40°C, temperatures that the animal may face in its natural environment. The second thermocouple was inserted into the pool of insect saline where the metathoracic-abdominal ganglionic complex (MAC) was kept during the experiments (see below). This temperature sensor controlled a thermostat, the set point of which was selected from a predefined value in the range 16–30°C. Prior to each recording session the temperature of the MAC was continuously monitored and was allowed to stabilize at each of the set points selected (±0.5°C).

To expose the nervous system (auditory nerve and MAC), the legs and wings were removed and the meso- and metathoracic sterna were detached after cutting the integument laterally and sectioning the apodeme bridges, uncovered by carefully lifting the sterna. Insect saline was added to form a pool where the MAC was maintained. This preparation was alive for several hours. In order to minimize possible systematic effects, the frequency of the stimulus and the temperature of the cicada did not follow a monotonous increasing or decreasing series, and some temperatures were repeated during the experimental series. Moreover, in the TEA experiments (see below) the activity at the auditory nerve could be recovered after more than 3 h by washing the preparation with insect saline.

For intracellular recordings the MAC was stabilized with a metal spoon. The activity of the auditory nerve was recorded with a hook made from an electrolitically sharpened tungsten electrode. A silver wire indifferent electrode was placed in the insect saline pool. The intracellular electrodes consisted of 60–100 MΩ glass micropipettes (Clark GC100F-10; Reading, UK) filled with Lucifer Yellow (Sigma, St Louis, MO, USA; Cat. No. L-0259; 5% in LiCl 0.5 mol l^{-1}). After a successful recording, and when allowed by stability, the dye was injected by iontophoresis (–0.5 to –1.5 nA) in order to identify the morphology of the neuron. The microelectrodes were positioned with a Leitz micromanipulator (Germany). In order to soften the sheath of the ganglion, collagenase (Sigma C-0130) was applied to some preparations for 15–30 s, immediately followed by a thorough wash. The intracellular signal was amplified 10× (Neuro Data model IR-283, Neuro Data Instruments Corp., New York, USA), digitized (50 kHz, 12 bit resolution, low pass filtered at 5 kHz for intracellular data and 10 kHz for extracellular recordings) with a multichannel board (Digidata 1200, Axon Instruments, Foster City, USA, controlled by Axoscope 9.0) and stored for later analysis on the hard disk of a PC, along with the extracellular recording of the auditory nerve (1000× amplified with a lab made amplifier), the sound stimulus and a time marker used as trigger during analysis.

Sound stimulation: the stimuli consisted of pure tone pulses, 30 ms long with 2 ms ramps and produced at intervals of 120 ms. These stimuli, repeated five or 10 times at each of 13 sound amplitudes ranging from 30 dB to 90 dB SPL delivered in 5 dB steps (re. 20 μPa), were presented at 16 different frequencies ranging from 0.5 kHz to 24 kHz (0.5, 1, 1.5, 2, 3,

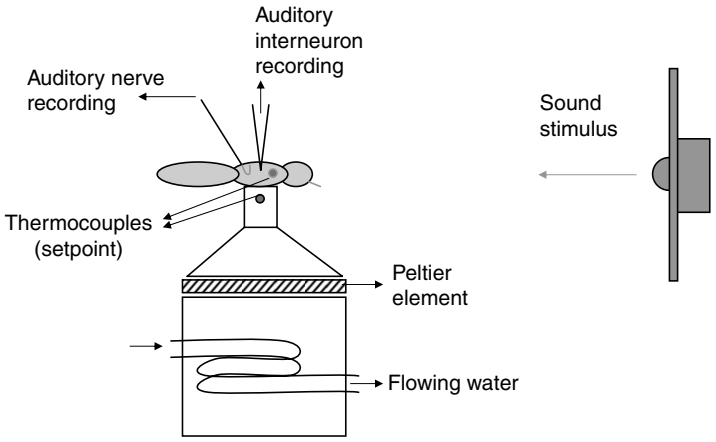

Fig. 1. Set-up used to control the body temperature of the cicada during intracellular recordings of auditory interneurons and recordings of the auditory nerve activity. The temperature of the animal holder was modified with a Peltier element and controlled *via* two thermocouples. The sensor in the holder kept its temperature within values compatible with the living tissues (10–40°C) while the second thermocouple measured and was used to control the temperature of the cicada body. The flowing water is needed to add to or remove heat from the Peltier element.

4, 5, 6, 8, 10, 12, 14, 16, 18, 20 and 24 kHz). The sound stimuli were generated by a PC computer and delivered (Sound Blaster Extigy, Creative Labs, Singapore, 96 kHz D/A conversion, 24 Bit, low pass filtered at 24 kHz) after amplification (Technics SU-V500 M2, Matsuchita Electric Industrial Co., Osaka, Japan) to a loudspeaker (Dynaudio D28/2, Skanderborg, Denmark) at 24 cm from the head of the preparation. By placing the loudspeaker in front of the cicada, the effects of directionality of the auditory periphery (Fonseca, 1993; Fonseca and Popov, 1997; Fonseca and Hennig, 2004) on the variation of the responses of interneurons that might receive input from either side of the animal were minimized. The amplitudes of the sound stimuli were measured and equalized using a Bruel & Kjær type 4135 ¼ inch microphone (Naerum, Denmark) at the position later occupied by the cicada. The microphone was previously calibrated with a pistonphone Bruel & Kjær type 4220 (Naerum, Denmark). Because the sound field in an electrophysiological set up is always disturbed by the presence of equipment close the preparation, echoes were monitored and the sound field optimized by shielding equipment and lining the Faraday cage with sound absorbing material (cotton and Illbruck 'super waffle').

Effects of TEA on the auditory responses

In order to measure the effect of TEA on the tuning, sensitivity, latency and Q_{10dB} evaluated from the summed responses of the auditory receptors, the insect saline bathing the MAC, which effectively circulated through the insect body as hemolymph, was replaced with a solution of 200 mmol l^{-1} TEA in insect saline. Recordings were made within 20–60 min after the application of the TEA solution. Then the preparation was repeatedly washed for a maximum of 2 h and 20 min with insect saline to observe the possible reestablishment of the auditory activity. The time course of the drug effect might be different from animal to animal probably caused by different timing for the drug to reach the cells within the protective sheath of the insect nervous system. The temperature in the laboratory ranged between 24–28°C.

Laser vibrometry

Laser vibrometry measurements were made at the University of Southern Denmark (Odense, Denmark).

The cicada with legs, wings and opercula removed [the effects of removing the opercula are small, see Fonseca and Hennig (Fonseca and Hennig, 2004)], was waxed to a holder (9 cm long, 2 mm across). This holder was in turn fixed to a stand that allowed great freedom of movements for positioning the cicada relative to the laser beam. In order to enhance the reflection of the laser beam from the tympanum, a few very small glass spheres, each weighing about 0.5 pg, were applied on the tympanal ridge at the position to be measured. These spheres did not affect the mechanics of the tympanum even if applied to the very thin membrane.

Because we were interested in measuring any temperature effect on the vibrations of the tympanum likely to be analyzed by the auditory organ, the laser beam was focused on the closest point externally available for measurement, which is where the tympanal ridge connects to the tympanal apodeme. Notice that in cicadas this apodeme forms a relatively stiff rod that then connects to the bulb shaped auditory organ. There were no relevant vibrations of the animal holder. Moreover, the tympanal vibrations were measured within the linear dynamic range of the tympanum which was previously evaluated.

The sound stimulus consisted of a sine sweep burst with a frequency span of 0 to 25 kHz and 3.5 ms long. The short stimulus and the use of a rectangular force window ensured that the measurement ended before any relevant reflections were recorded. The beginning of the sampling of the vibrations was delayed relative to the generation of the sound stimulus to take into account the sound propagation from the speaker to the preparation. The stimulus was generated by a HP35665A spectrum analyzer (Hewlett Packard, Washington, USA), amplified (Xelex DD8, Stockholm, Sweden) and delivered by a loudspeaker (Dynaudio D28 AF, Skanderborg, Denmark) at 21 cm and ipsilateral to the measured tympanum. This audio chain guaranteed that the stimulus produced had enough power above 1 kHz. The analyzer computed the transfer function from the stimulus to the recorded tympanal vibrations (laser dopler vibrometer Dantec, Copenhagen, Denmark).

The temperature of the cicada was varied as described above.

Data analysis

The electrophysiological recordings were analyzed off line using dedicated home made programs and conventional spreadsheet software. The hearing thresholds were evaluated from intensity response curves using as criterion the averaged subthreshold activity plus three times the standard deviation

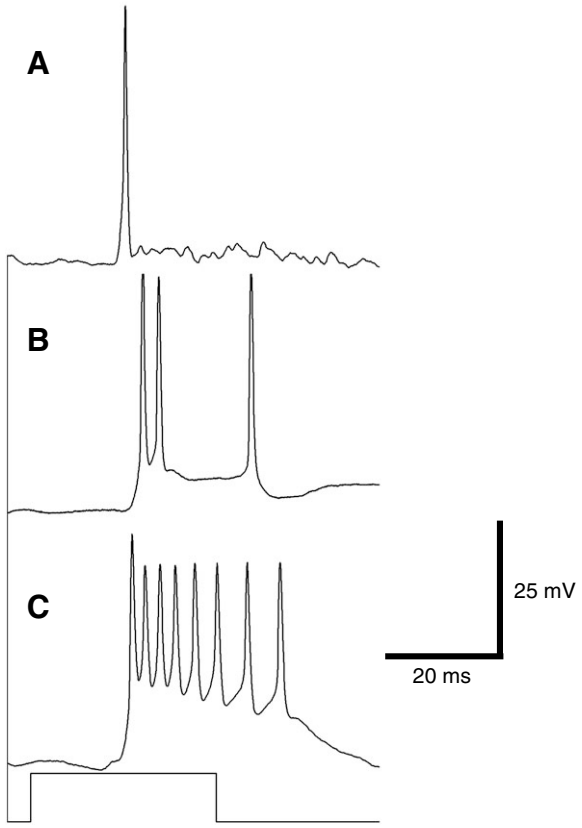

Fig. 2. Examples of electrophysiological responses of the auditory interneurons. The types varied from a phasic response (A) with a single action potential, to a phasic-tonic (B) and a more tonic response (C). The examples are from three cells recorded 20 dB above threshold at 6 kHz and at 24°C.

(Fonseca et al., 2000). For intracellular recordings the activity was evaluated as the average number of action potentials occurring in a response window defined as the stimulus duration with a latency lag, for a certain frequency and amplitude. Similarly, the activity on the auditory nerve was estimated as the peak-to-peak amplitude of the averaged recordings at each frequency and intensity which represents the summed response of the receptors. Each stimulus was repeated five times for the intracellular measurements and five or ten times for extracellular recordings. The latency of the interneurons' response was measured from the beginning of the sound stimulus to the first action potential. Although several interneurons showed spontaneous activity, the beginning of the response was usually unambiguous because it was often accompanied by an EPSP (see Fig. 2). The latencies of the auditory nerve response were measured from the beginning of the stimulus marker to the peak of the averaged recording, which was well defined because of receptor synchronization. Both latencies were measured 20 dB above threshold. The sharpness of tuning was estimated from the tuning curves dividing the characteristic frequency by the frequency band 10 dB above threshold (Q_{10dB}).

Statistic analysis

Data represented by quantitative and continuous variables was tested using parametric methods of analysis of variance in case the assumptions of linear model were verified. When this was not the case, or the data consisted of quantitative discrete variables, non parametric procedures were used. See Zar (Zar, 1999) for details on the methods. All computations were made using the packages Statistica 6.0 and R 2.1.1 or a conventional spreadsheet (Microsoft Excel).

The presence of effects caused by the change in temperature on the characteristic frequency of the interneurons and the summed response of the auditory receptors was tested using a Kruskal–Wallis (KW) non parametric method. This was followed by a Dunn's *post-hoc* pairwise test to investigate significant differences of characteristic frequency between temperatures. The same method was applied to test the effect of TEA on the characteristic frequency evaluated from the summed response of the auditory receptors.

To investigate the effects of temperature on the sensitivity of the interneurons and on the sensitivity revealed by the summed response of the auditory receptors a bi-factorial ANOVA, a one-way ANOVA or a Kruskal–Wallis test were used depending on the nature of the data. The bi-factorial ANOVA considers both the effects of the temperature and of the individual cells on the sensitivity without an interaction term. The effects of TEA on the sensitivity evaluated from extracellular recordings were tested with a one-way ANOVA followed by a Tukey's test to investigate significant differences of sensitivity between pairs of treatments.

The effects of the temperature and of the TEA on the latency of the extracellular recording were assessed using one-way ANOVA. For the influence of temperature on the latency of the interneurons a Kruskal–Wallis test was used.

Whenever the presence of significant effects of temperature on the sensitivity and latency were found, the significance of the slopes of the individual linear regression lines was tested using analysis of covariance (ANCOVA). This analysis was done using as categories auditory interneurons or auditory nerves. In case the slopes were not significantly different among cells or individuals, then a general slope was computed.

To test the significance of increasing response strength of the cells with temperature a bi-factorial ANOVA was used. Finally possible changes of the sharpness of tuning (Q_{10}) caused by temperature were tested with a bi-factorial ANOVA, used on intracellular and extracellular recordings, and with a one-way ANOVA followed by a Tukey's test, used on data from TEA experiments.

Computations were made with values in dB, and not in a linear scale, because the several observations at each condition did not differ from a normal distribution.

Results

Effects of temperature on the tuning of interneurons

We obtained 70 recordings of auditory interneurons from a total of 45 cicadas: 14 recordings from 13 insects tuned to a

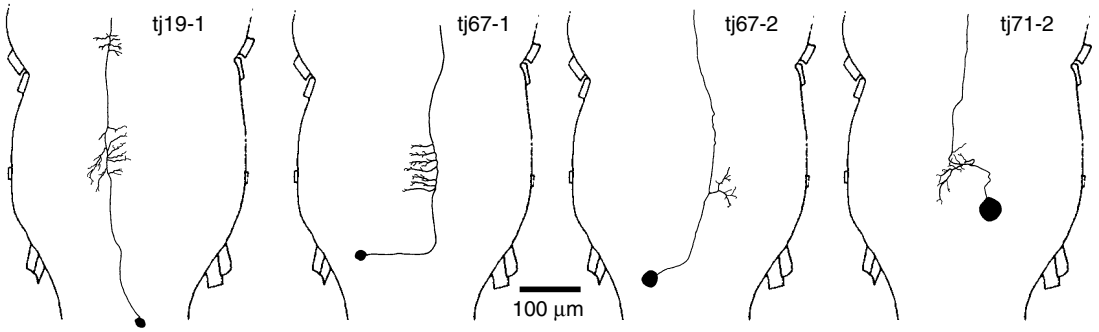

Fig. 3. Examples of four morphological types of auditory interneurons (see Table 1) (tj19-1, tj67-1, tj67-2, tj71-2) with two sensitivity maxima that revealed a shift in the characteristic frequency in the range 3–8 kHz. A fifth cell type (tj52-1) with a different morphology was only partially stained and therefore is not shown. The cells were stained with Lucifer Yellow.

low frequency band from 0.5 to 2 kHz; 3 recordings from 3 cicadas tuned to a high frequency band from 14 to 20 kHz; 3 recordings from 3 cicadas tuned to 3–6 kHz, corresponding to two physiological types (Fig. 2B,C); and 50 recordings from 36 cicadas with two sensitivity maxima, one at low (3–8 kHz) and another at high frequency (14–24 kHz). Recordings were made with temperatures ranging from 16°C to 29°C.

All 14 recordings tuned to low frequency (0.5–2 kHz) and the three tuned to 3–6 kHz, a total of 24%, maintained their characteristic frequency with changing temperature. Similarly, the three recordings (4%) tuned only to high frequencies (14–20 kHz) did not exhibit any clear tuning shifts with temperature. From the remaining 50 recordings with two sensitivity maxima, 29 recordings (41%) from 26 cicadas, corresponding to at least three physiological (Fig. 2) and five morphological types (Fig. 3), revealed changes in the characteristic frequency (KW, $H_{4,91}$=43.91, P<0.01). This characteristic frequency shifted up to 3 kHz with changing temperature (Table 1) at the low frequency (3–8 kHz) sensitivity maximum, but not at the highest frequency range (Fig. 4A,B). The sharpness of tuning at the characteristic frequency evaluated by the Q_{10dB} was not affected by temperature (bi-factorial ANOVA, $F_{13,55}$=1.28, P>0.1). From the remaining 21 (30%) cell recordings from 18 cicadas, 18 maintained their tuning and another three increased their characteristic frequency up to 24–26°C but at the highest temperature tested they showed a lower than expected frequency tuning (data not shown).

Cells repeatedly recorded at the same temperature in the course of an experiment maintained their tuning despite some changes in sensitivity occasionally observed. These might be due to some deterioration of the cell response caused by very long recordings or to other mechanisms allowing variable gain (see Discussion).

Effects of temperature on the sensitivity of interneurons

At the low frequency band (3–8 kHz), 20 of the recordings referred to in Table 1, exhibited a decreasing threshold, that is, they became more sensitive with increasing temperature (bi-factorial ANOVA, $F_{9,29}$=15.87, P<0.01) (Fig. 4C). Their

Table 1. *Characteristic frequency of 29 responses of interneurons from 26 cicadas exhibiting changes in their low frequency tuning in the range 3–8 kHz with changes in body temperature*

	Frequency				
Recording*	16–17°C	18–19°C	20–21°C	22–24°C	25–29°C
1-1 (C)	4			6 and 8	
3-1 (C)	3		4	6	
10-3 (A)				3	4
14-3 (C)	4		4		5
19-1 (C)†	5	5	5	6	6
20-2 (A)		4		5/6	
23-2 (C)	3		3	4	
27-1 (B)	4		5	6	
27-2 (B)				5	6
30-3 (B)	4		5		6
31-1 (C)	5	5	5	6	6
33-1 (B)	5		5	6	
37-1 (B)		5	5	6	6
45-1 (C)		3		5	
50-5 (B)			5	5	8
51-1 (B)	4	5		6	6
52-1 (B)†	5	5		6	6
54-1 (C)		5		6	
54-2 (B)	3			5/6	
55-1 (B)	4			6	
56-1 (C)		3		5	5
61-1 (C)			5	6	
64-1 (B)			5	6	
64-2 (B)	4/5	6	6	6	6
67-1 (C)	3	3	6	6	6
67-2 (B)†	3		3	5	5
71-2 (C)†	4	4	4	6	6
73-2 (B)			5	6	
75-3 (B)	3	4	4	5	6

Frequency values are in kHz.

*A, B and C refer to the physiological response types of the interneurons, as indicated in Fig. 2.

†Cells that were stained at the end of the experiment and all revealed different morphologies as shown in Fig. 3.

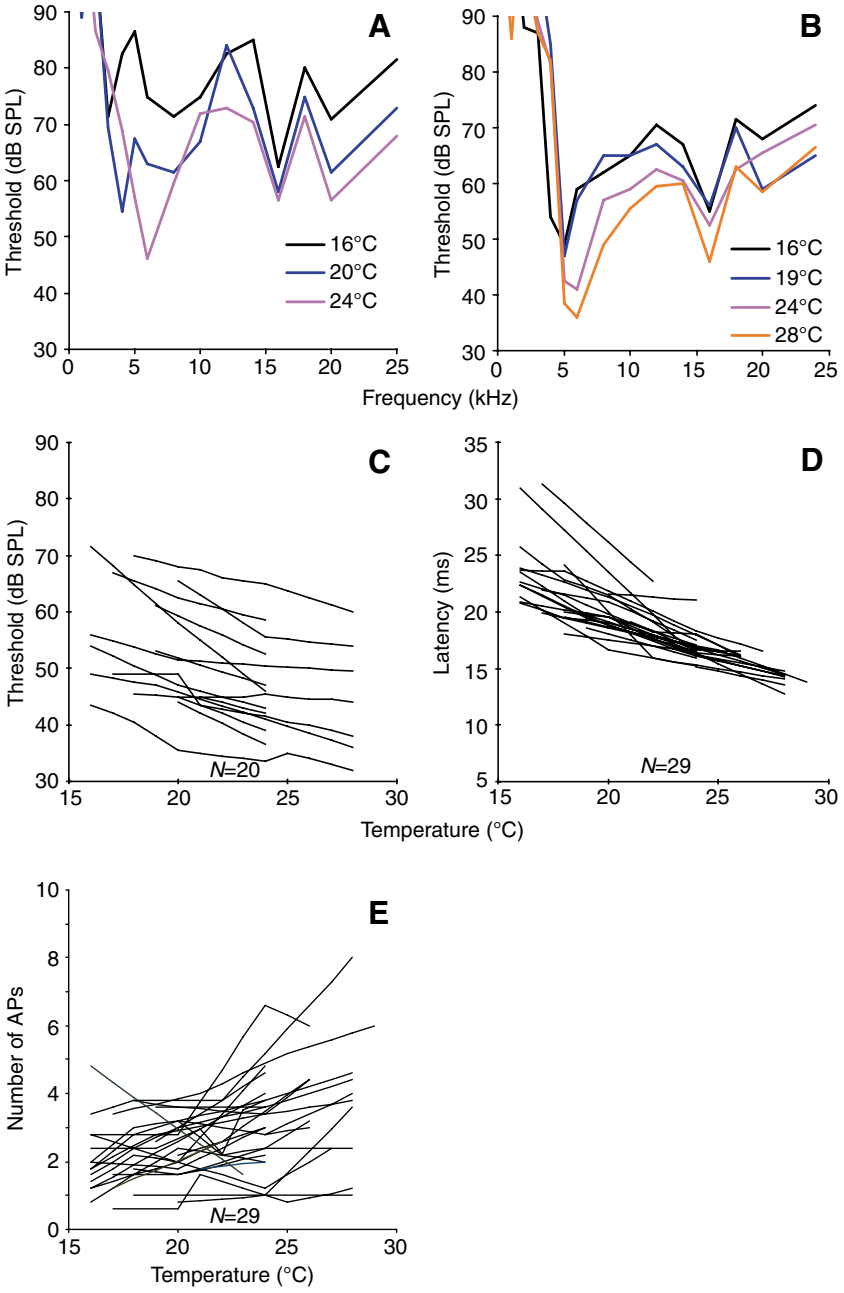

Fig. 4. Effects of body temperature on tuning, sensitivity, latency and response strength of auditory interneurons of the cicada *T. josei*. (A,B) Tuning curves of two interneurons exhibiting shifts in their tuning and sensitivity with body temperatures ranging from 16°C to 28°C. Maximum effects are observed at temperatures from 16–18°C to 24°C in the frequency range 3 to 8 kHz. At higher frequencies the characteristic frequency remains constant, but some effect on sensitivity is still present. (C) Sensitivity at the characteristic frequency of 20 recordings, of the 29 cells listed in Table 1, which exhibited an increased sensitivity with temperature. The lines connect the sensitivities of each interneuron. (D) Dependence of latency on temperature, obtained from 29 recordings of interneurons. Latencies were measured 20 dB above threshold at the characteristic frequency and decreased with increasing temperature. (E) Dependence of the number of action potentials on the temperature in 29 recordings. At each temperature the number of action potentials is an average of five stimulus presentations at the characteristic frequency and 20 dB above threshold.

increase in sensitivity averaged about 2.2 dB °C^{-1} (ANCOVA, $F_{17,15}$=0.52, P>0.05, computed common slope: 2.2). In the remaining recordings sensitivity did not seem to have any direct relationship with temperature.

At the high frequency band (14–24 kHz), the threshold values were not significantly different at different temperatures (one-way ANOVA, $F_{12,70}$=0.66, P>0.05). A similar result was obtained for the cells that were tuned just to a low frequency band (0.5–2kHz), where sensitivity did not change significantly with temperature (KW, $H_{8,35}$=5.17, P>0.05).

Fig. 4A,B are examples of tuning curves of two auditory interneurons whose characteristic frequency and sensitivity changed with temperature at the low frequency minimum. At high frequency a smaller shift in sensitivity can be seen.

Effects of temperature on the latency of interneurons

Each cell responds to the stimuli with a given delay, which depends on stimulus intensity and on temperature. Fig. 5 shows an example of the effect of temperature on the response of one auditory interneuron. The latency decreased with temperature while the strength of the response increased, as revealed by an increase in the number of spikes. Indeed, all the recordings represented in Table 1 showed a clear similar decrease in latency with increasing temperature (Fig. 4D; KW, $H_{13,82}$=64.30, P<0.05). Latencies decreased about 0.83 ms °C^{-1} (ANCOVA, $F_{24,32}$=0.37, P>0.05, computed common slope: 0.83).

Effects of temperature on response strength of interneurons

The response strength of the 29 cells in Table 1, evaluated as the average number of action potentials per stimulus 20 dB above threshold, increased with temperature in most of the interneurons (Fig. 4E). This effect was observed both at the characteristic frequency (bi-factorial ANOVA, $F_{13,55}$=6.19, P<0.01) and at the second higher frequency sensitivity maximum (bi-factorial ANOVA, $F_{13,46}$=12.73, P<0.01). In three of those 29 cells, the general increasing tendency at the characteristic frequency was accompanied by an oscillation of the number of action potentials. In the other three interneurons the increase in temperature was not followed by a change in response strength. Only one notable exception of a reduction with increase in temperature was seen.

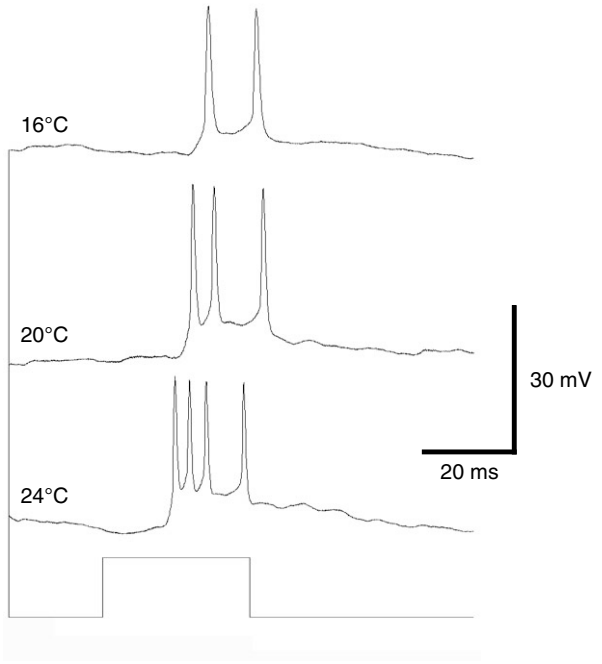

Fig. 5. Intracellular recording of an auditory interneuron showing the variation in latency and strength of the response with temperature. The sound stimulus at 6 kHz was delivered 20 dB above threshold at each temperature.

Table 2. *Characteristic frequency measured from the summed response of the auditory receptors in 19 cicadas*

	Frequency				
Recording*	16–17°C	18–19°C	20–21°C	22–24°C	25–29°C
14-3	3	4		4	6
19-1	3/4	4	5		5
31-1	3	3	6	6	6
39-4	3	5	5	5	
42-2	3		6		6
48-2	3	3	6		6
49-2	3	3	3		6
50-4	3	3	6		4–6
51-1	3		3		6
52-1	3/4		6		6
54-2	3		4		
56-1	3		5		5
58-1	3		4	5	4/5
63-5	4	4	6		6
64-2	3	4	5	6	6
65-3	3	6	6		6
67-1	3	5	6	6	6
71-2	3/4	4	4–6	4–6	6
75-3	3	3/4	4–6	6	6

Frequency values are in kHz.

Effects of temperature on the tuning measured from the summed response of the auditory receptors

The effect of temperature on tuning observed at the level of the auditory interneurons was already present at the periphery. Nineteen out of the 20 cicadas in which auditory nerve recordings were analyzed (Table 2) showed a significant change in the characteristic frequency up to 3 kHz with changing temperature (KW, $H_{4,75}$=48.18; P<0.01). The characteristic frequency increased from 3–4 kHz at low temperature to 5–6 kHz at high temperature. In contrast, and along with the results obtained with intracellular recordings, the effects at high frequency were negligible (Fig. 6A,B). The sharpness of tuning at the characteristic frequency evaluated by the Q_{10dB} was not affected by temperature (one-way ANOVA, $F_{13,67}$=0.74, P>0.1).

Effects of temperature on the sensitivity evaluated from the summed response of the auditory receptors

Changes in threshold at the auditory nerve level were observed at the characteristic frequency. The sensitivity of the recordings in Table 2 increased with temperature (one-way ANOVA, $F_{13,68}$=3.68, P<0.01). However, only 10 out of these 19 recordings showed a clear enhanced sensitivity with increasing temperature (Fig. 6C), exhibiting a shift of 2.4 dB °C^{-1} (ANCOVA, $F_{9,20}$=0.39, P>0.05, computed common slope: 2.4).

At high frequency (14–24 kHz), the sensitivity did not seem to vary significantly with temperature (one-way ANOVA, $F_{13,63}$=0.60, P>0.05; cf. Fig. 6A,B).

As shown in the previous tables and figures, the effect of temperature was already present at the auditory periphery and was maintained at the level of the first order interneurons.

Effects of temperature on the latency measured from the summed response of the auditory receptors

The auditory response of all cicadas listed in Table 2, measured from the auditory nerve recordings, became faster as the temperature increased from 16°C to 28°C (Fig. 6D). The latency decreased significantly (one-way ANOVA, $F_{13,66}$=12.40, P<0.01) about 0.48 ms °C^{-1} (ANCOVA, $F_{18,42}$=0.25, P>0.05, computed common slope: 0.48).

Effects of temperature on mechanosensory structures

The tympanum and the tympanal apodeme are the structures that are driven into oscillation by the sound, and they transmit the oscillation to the receptor cells at the auditory organ, where transduction occurs.

Measurements by Doppler laser vibrometry in three cicadas demonstrated that the vibrations at the tympanum and the tympanal apodeme were not significantly affected by temperature, in the biologically relevant range (18–35°C). The vibration velocity and the phase angles, measured at different points on these structures, namely where the tympanal apodeme attaches to the tympanal ridge (see an example in Fig. 7), did not show any considerable change up to 15 kHz. Although some changes were observed at higher frequencies, they were not observed in all cicadas nor in the same cicada in other measurements on the ridge. Consistently, no effect was seen in the frequency range where the strong

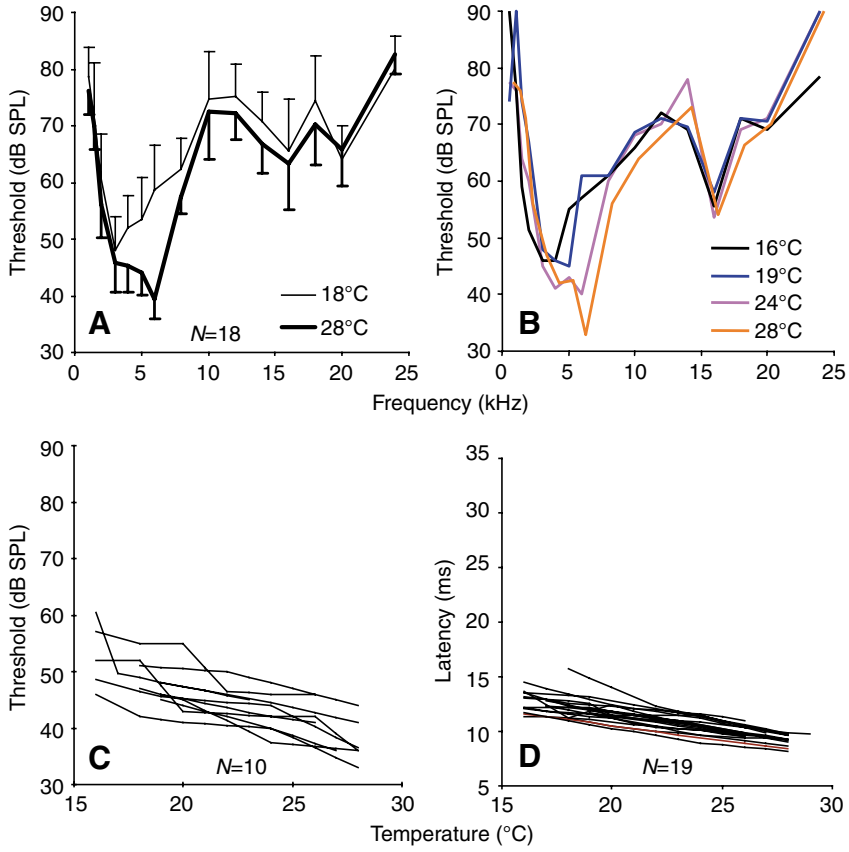

Fig. 6. Effects of body temperature on tuning, sensitivity and latency evaluated from recordings of the auditory nerve of the cicada *T. josei*. (A) Averaged and (B) example tuning curves measured at different temperatures ranging from 16°C to 28°C. There is a strong effect in the characteristic frequency and sensitivity in the range 3–8 kHz, but not at higher frequencies. Error bars indicate the standard deviation. (C) Sensitivity at the characteristic frequency measured in the 10 cicadas, from 19 recordings (see Table 2), which exhibited increased sensitivity with temperature. The lines connect the sensitivities evaluated from each auditory nerve recording. (D) Dependence of latency on temperature measured in 19 cicadas. Latencies were measured 20 dB above threshold and decreased with increasing temperature.

effects of temperature were detected in the nervous system (3–8 kHz).

Effects of tetraethylammonium (TEA) on tuning, sensitivity and latency evaluated from the summed response of the auditory receptors

TEA is an inhibitor that blocks nonspecifically a variety of potassium channels. An application of TEA (200 mmol l^{-1}) diluted in insect saline and applied to the body cavity entered the nervous system but did not block all the potassium channels, since auditory processing was affected but not abolished. Moreover, the effects of this drug could be removed by repeatedly washing the preparation with insect saline.

The tuning evaluated from the auditory nerve recordings on 10 cicadas kept at room temperature (24–28°C; Table 3) were modified by TEA application (KW, $H_{2,30}$=18.19; P<0.01). The characteristic frequency usually decreased in the presence of the drug, with shifts of up to 3 kHz and, when TEA was washed out, the auditory nerve responses recovered (Dunn's *post-hoc* test) and frequency tuning was re-established (Fig. 8A,B). The Dunn's pairwise *post-hoc* test did not find differences in the characteristic frequency between the initial condition and the washed preparation. The sharpness of tuning at the characteristic frequency evaluated by the Q_{10dB} was not affected by TEA (one-way ANOVA, $F_{2,27}$=1.68, P>0.1).

Regarding sensitivity (Fig. 8), the threshold increased with the presence of TEA and decreased after the TEA has been washed out (one-way ANOVA, $F_{2,27}$=12.80, P<0.01). Again a Tukey's *post-hoc* test revealed no differences in the sensitivity before TEA application and after washing the preparation. The effects on tuning and sensitivity paralleled the effects of reducing temperature (compare Fig. 8A with Fig. 6A). By contrast, latency did not appear to change significantly with the different treatments (one-way ANOVA, $F_{2,27}$=1.57, P>0.1).

The effect of TEA on tuning was only observed at low frequencies (3–8 kHz). Sensitivity exhibited stronger changes at low frequencies, but some effect seemed also to be present at high frequencies (Fig. 8A). The shift on the tuning curves caused by the presence of TEA was similar to that caused by low temperatures. Hence, both the application of TEA and changes in temperature affected the frequency selectivity measured at the auditory nerve in a similar manner and at the same frequency range (3–8 kHz).

Discussion

Our results demonstrate that about 50% of the recordings of auditory interneurons with two sensitivity maxima showed temperature dependence (Fig. 4), in contrast to interneurons tuned to very low (0.5–2 kHz) or to high frequency (14–20 kHz). Moreover, in the cells with two sensitivity maxima the effect on tuning was only observed at the low frequency range (3–8 kHz) and not at the second higher frequency sensitivity maximum (14–20 kHz). The characteristic frequency in the 3–8 kHz range increased up to 3 kHz with increasing temperature, and this temperature-dependent shift in tuning was already present in the auditory nerve recordings (Fig. 6). This suggests that the temperature-induced modifications are effected through frequency selectivity mechanisms at the level of the receptor cells or on the biophysics of the ear. The same shifts caused by temperature on auditory tuning were observed in lower vertebrates (Dijk et al., 1990; Eatock and Manley, 1981; Fay and Ream, 1992; Schermuly and Klinke, 1985; Smolders and

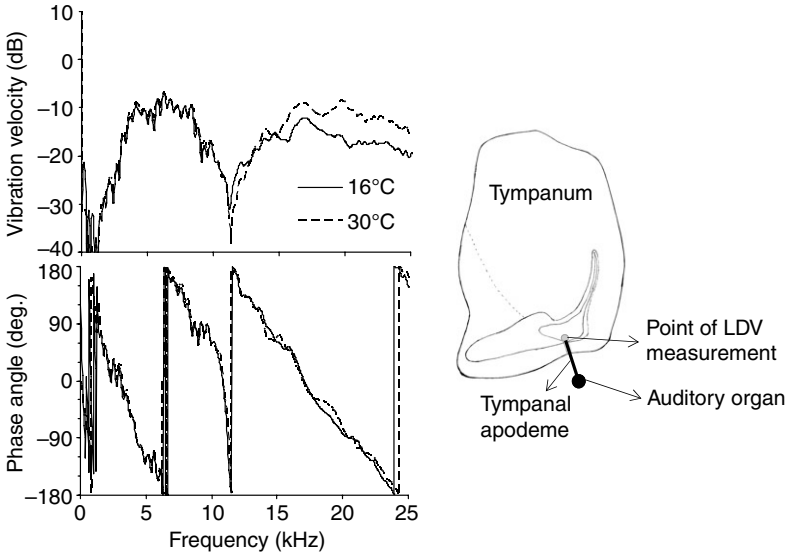

Fig. 7. The typical effect of body temperature, in one of three males measured, on the vibrations of the tympanal apodeme measured by laser Doppler vibrometry (LDV). There is no clear effect on the vibration velocity (presented in arbitrary units) and the phase angle, especially in the frequency range 3–8 kHz, where a strong effect of body temperature on auditory tuning and sensitivity was measured in the nervous system. The diagram on the right is of a the tympanum, tympanal apodeme and auditory organ, indicating the point where the laser beam was focused.

Klinke, 1984) and in the locust that, to our knowledge, is the only report in insects (Oldfield, 1988).

Frequency analysis may be performed by extrinsic mechanisms, prior to the receptor cell transduction. This is currently accepted in insects, where the mechanics of the tympanal structures is considered to be responsible for frequency analysis [e.g. crickets (Ball and Hill, 1978; Kleindienst et al., 1983), locusts (Michelsen, 1971) (but see Windmill et al., 2005)], and is recognized as the primary mechanism in mammals, where the place of maximal vibration at the basilar membrane varies systematically with sound frequency, although here complemented by cellular amplification (Dallos, 1992; Kennedy et al., 2005). In other systems frequency analysis can be performed by intrinsic receptor mechanisms [e.g. electrical tuning (Fettiplace, 1987; Fuchs et al., 1988; Hudspeth and Lewis, 1988)].

Mechanical properties of the tympanum (tympanal ridge and apodeme) are likely important components of the frequency selectivity of the cicada auditory receptors. However, measurements on those structures by laser vibrometry showed no significant vibration dependence on temperature in the low frequency range up to 15 kHz (Fig. 7). Hence, it seems that the mechanical properties of the peripheral structures do not explain the effects of temperature on the frequency selectivity of the receptors in *T. josei*, observed in the range 3–8 kHz.

Studies performed in the turtle, where measurements of basilar membrane motion using laser interferometry revealed no position-dependent mechanical tuning (O'Neill and Bearden, 1995), indicated electrical tuning of the receptors (hair cells) as the prime mechanism for frequency selectivity. By contrast, in the mammalian cochlea, filtering is primarily performed by mechanical properties of the basilar membrane, enhanced by electromechanical amplification mechanisms of the outer hair cells (Dallos, 1992) and possibly the stereocillia bundles (Kennedy et al., 2005). In the electrical tuning of receptor cells, first described for the turtle auditory papilla, the filter is totally intrinsic to the hair cell, where the receptor potential is modulated by voltage-dependent ionic currents that generate a series of damped oscillations of the membrane potential. If the sound frequency of the stimulus matches the resonant oscillations of the membrane potential an amplified response will arise (Fettiplace, 1987). This electrical tuning, observed in hair cells of a hearing specialist fish (Sugihara and Furukawa, 1989), amphibians (Hudspeth and Lewis, 1988), reptiles (Fettiplace, 1987) and birds (Fuchs et al., 1988), might also be present in insect receptor cells (Oldfield, 1984; Oldfield, 1988).

Because higher temperatures increase molecular motion and speed of chemical reactions, we would expect higher temperatures to increase the neurons' excitable state. Indeed, kinetics of ion channels has for a long time been known to be highly temperature sensitive (Hodgkin et al., 1952). Moreover, characteristics of BK channels were recognized as the rate-limiting step for determining the frequency of electrical tuning. BK channels, which belong to the potassium channel family, are large conductance voltage and Ca^{2+}-activated potassium channels whose activity is regulated by membrane voltage and/or intracellular Ca^{2+}. Higher frequency tuning was accompanied by an increase in the number and speed of the BK channel kinetics (Fettiplace and Fuchs, 1999). Therefore, if higher temperatures enhance the ion channel kinetics, increased

Table 3. *Characteristic frequency evaluated from auditory nerve recordings of ten cicadas kept at room temperature (24–28°C) treated with 200 mmol l^{-1} tetraethylammonium*

	Frequency		
Animal	Before TEA	TEA (200 mmol l^{-1})	Washed
tj3	6	3	6
tj5	5	3/4	5
tj10	6	4	6
tj11	5	4	6
tj12	6	4	4/6
tj13	4	3	6
tj14	6	3	4
tj15	6	4	6
tj18	4	4	6
tj20	6	4	6

Frequency values are in kHz.
TEA, tetraethylammonium.

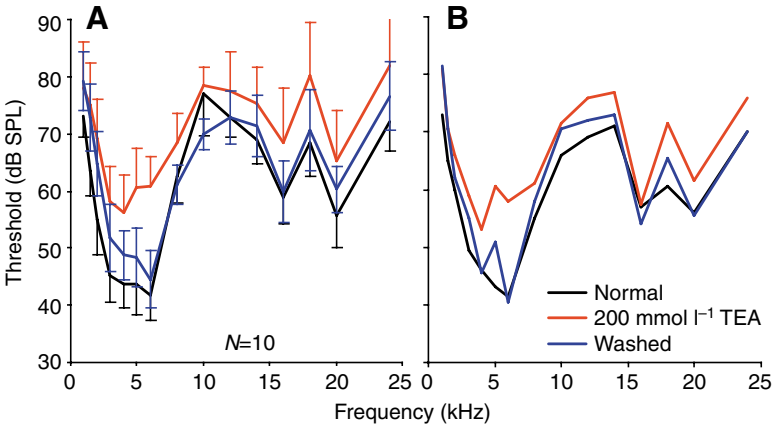

Fig. 8. Effects of 200 mmol l^{-1} tetraethylammonium (TEA) on tuning and sensitivity evaluated from recordings of the auditory nerve of the cicada *T. josei*. (A) Averaged and (B) example tuning curves measured before and after drug application, and the effect of repeatedly washing with insect saline. TEA resulted in a downward shift of the characteristic frequency and a reduced sensitivity. Sensitivity and tuning were reestablished after repeatedly washing the preparation with insect saline for up to 2 h 30 min. Error bars in A indicate the standard deviation. Recordings were made at ambient temperature of 24–28°C.

frequency at the electrical oscillation of the membrane potential will arise and thus will result on an upward shift of the tuning of the cell. Furthermore, the electrical resonance of the hair cells was shown to be highly temperature sensitive in the leopard frog saccular hair cells (Smotherman and Narins, 1998) and in the chick cochlea (Fuchs and Evans, 1990). If a similar mechanism is present in *T. josei*, it might explain the temperature effects observed at low frequency (3–8 kHz), despite the fact that electrical tuning has been restricted to even lower frequencies. At high frequency (14–24 kHz) this mechanism is unlikely since it would require too high frequency oscillations of the membrane potential.

In order to assess the effects of potassium ion channels on hearing of *T. josei*, we used 200 mmol l^{-1} tetraethylammonium (TEA) to block potassium channels. This drug caused a shift in tuning similar to that caused by temperature variations (Fig. 8, compare with Fig. 6A,B; see also Fig. 4A,B). Thus, TEA and temperature are likely to have affected tuning mechanisms in the same way. Low temperature and TEA might have affected the kinetics or number of active potassium channels available. Hence, it is likely that those channels play an important role in frequency selectivity in the auditory receptors of *T. josei*.

Because higher temperatures make the neuron more excitable we would expect that sensitivity would increase with temperature. Accordingly, the interneurons' auditory thresholds decreased with increasing temperature at low frequency (3–8 kHz) but kept constant at very low (0.5–2 kHz) and high frequencies (14–24 kHz). Again, this effect was already present at the level of the receptors. Unexpectedly, in three cells a decrease in sensitivity occurred at the highest temperature tested. This might be explained by a hyperpolarizing mechanism, similar to the one present in crayfish motoneurons, caused by an excess of sodium extrusion at high temperatures [Aréchiga and Cerbón (Aréchiga and Cerbón, 1981) in Smolders and Klinke (Smolders and Klinke, 1984)]. In the locust auditory receptors most of the cells recorded maintained their sensitivity relatively constant, in contrast to 20% of them that increased their sensitivity (Oldfield, 1988). Our recordings on *T. josei* revealed that the very low (0.5–2 kHz) and high (14–24 kHz) frequency ranges seemed to be less affected by temperature. If receptor cells are tuned in to those frequency ranges, they should remain relatively temperature insensitive as well. If so, it would indicate different tuning mechanisms among the receptor cells. Unfortunately it has not yet been possible to confirm this hypothesis in cicadas.

TEA affected sensitivity in the same way as a reduction in temperature, causing an increase in the threshold of the auditory nerve responses. This suggests that blocking of potassium channels interferes with the transduction mechanisms involved in the generation of the receptor potential.

In *T. josei* some recordings of a single preparation showed differences in sensitivity at the same temperature (up to 30 dB). Thus, some oscillations of the auditory threshold were temperature independent. Variations in sensitivity might arise from several causes. Hennig et al. (Hennig et al., 1994) discovered that the folding of the tympanum that occurred during singing caused an increase in auditory thresholds by about 20 dB. Cicadas are thus able to adjust their hearing threshold within this range. De-tension accompanied by folding of the tympanum occurs when the cicadas prepare to sing and is probably a mechanism to protect the tympanum and the auditory receptors from damages that might be caused by the high pressures created in the abdomen during singing. This state might affect measurements but is normally short, unless the cicada starts singing (P.J.F., unpublished observations). This condition could be easily detected in our recording of the auditory nerve that includes the axon of the tymbal motoneuron. In addition, the sensitivity of a sensory system can usually be raised or lowered by efferent neuronal connections, which may intervene at various sites in the sensory system (Reichert, 1992) and might also be present in cicadas. Furthermore, insects' tympanal membrane vibrations may be affected by ventilation and abdominal movements in such a way that sensitivity to external sounds can be reduced (Meyer and Elsner, 1995). These effects, however, are rhythmic and usually do not last long enough to interfere markedly with a stimulation series used in electrophysiological experiments.

In contrast to the weak effect of TEA on latency, temperature caused a decrease in the response delay of interneurons and auditory receptors. Notice that temperature strongly affected the conduction velocity of the neurons, an important component of the time lag measured. This could be seen in the distinct decrease of the latency in the response observed at the

auditory nerve and especially at the interneurons, which are located further away in the auditory pathway, while the application of TEA did not produce any relevant effect on the time lag from sound stimulus to the nerve activity. A similar result was observed in the neostriatal neurons of the rat brain (Bargas et al., 1989). However, TEA affected sensitivity, causing an increase in the threshold of the auditory nerve responses. This might be due to a blocking of potassium channels involved in the generation of the receptor potential, while the effect on the speed of axonal conduction, mostly dependent on sodium channel excitability, was probably largely unaffected by this chemical but strongly modified by temperature.

Finally, in *T. josei* the tuning shift caused by temperature might generate a mismatch between relevant behavioral stimuli and the characteristic frequency in the 3–8 kHz range. Nevertheless, this should not cause any real constraint on this communication system because, (1) insects typically communicate at signal-to-noise levels well above thresholds, (2) in this cicada the calling song peak is at a much higher frequency (16 kHz) and (3) singing usually occurs at temperatures above 22°C.

Much work has already been performed, in lower vertebrates and insects, in order to identify the mechanisms responsible for tuning of auditory receptors. From our experiments we can conclude not only that the tuning of the auditory receptors is temperature dependent but also that the potassium channels are likely implicated in the tuning of receptor cells in this cicada, at least in the frequency range 3–6 kHz. However, further studies on the transduction mechanisms and on the characterization of the channels at the auditory receptors are needed to clarify their role in this system.

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