population size was taken to be 10^4, on the basis of estimates for other loci[29]. We tried three different values for the selection coefficient: $s = 5\%$, 1% and 0.5%. For these parameters, an s of 1% resulted in the highest likelihoods, so we reported the results for $s = 1\%$. If we use the chi-squared approximation with one degree of freedom for the log-likelihood ratio statistic $2\ln(\mathrm{Lik}(\hat{T})/\mathrm{Lik}(T))$, we obtain an approximate 95% confidence interval for T of [0, 4,000 generations]. However, this approximation may not be appropriate in this context. Thus, we also ran 100 simulations to examine the distribution of $\hat{T}$ when the true T is equal to our maximum likelihood estimate of $T = 0$ (here, $n = 5 \times 10^5$ and $\varepsilon = 0.2$). These simulations suggested an approximate 95% confidence interval of [0, 6,000 generations]. We assumed a generation time of 20 years for converting T into years.

Received 11 November 2001; accepted 29 July 2002; doi:10.1038/nature01025.
Published online 14 August 2002.

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Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com/nature).

Acknowledgements

We thank F. Heissig for help with the cDNA sequencing; A. von Haeseler, G. Weiss and S. Zöllner for help with the data analysis on an earlier version of the manuscript; J. Wickings at the Centre International de Recherches Medicales for DNA samples of central chimpanzees; and the Bundesminsterium für Bildung und Forschung, the Max Planck Society and the Wellcome Trust for financial support. M.P. was supported by a National Science Foundation postdoctoral research fellowship in bioinformatics. S.E.F. is a Royal Society Research Fellow and A.P.M. is a Wellcome Trust Principal Research Fellow.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to S.P. (e-mail: paabo@eva.mpg.de). *FOXP2* cDNA sequences of the mouse, rhesus macaque, orang-utan, gorilla, chimpanzee and human have GenBank accession numbers AY079003, AF512950, AF512949, AF512948, AF512947 and AF337817, respectively. Accession numbers for genomic sequences for the twenty humans, two chimpanzees and one orang-utan are AF515031–AF515050, AF515051–AF515052 and AF515053, respectively.

A corollary discharge maintains auditory sensitivity during sound production

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Speaking and singing present the auditory system of the caller with two fundamental problems: discriminating between self-generated and external auditory signals and preventing desensitization. In humans[1] and many other vertebrates[2–7], auditory neurons in the brain are inhibited during vocalization but little is known about the nature of the inhibition. Here we show, using intracellular recordings of auditory neurons in the singing cricket, that presynaptic inhibition of auditory afferents and postsynaptic inhibition of an identified auditory interneuron occur in phase with the song pattern. Presynaptic and postsynaptic inhibition persist in a fictively singing, isolated cricket central nervous system and are therefore the result of a corollary discharge from the singing motor network. Mimicking inhibition in the interneuron by injecting hyperpolarizing current suppresses its spiking response to a 100-dB sound pressure level (SPL) acoustic stimulus and maintains its response to subsequent, quieter stimuli. Inhibition by the corollary discharge reduces the neural response to self-generated sound and protects the cricket's auditory pathway from self-induced desensitization.

We have examined auditory information processing in the singing cricket, *Gryllus bimaculatus*. Males attract females or warn off rival males with songs that are generated by rubbing their forewings together. Calling song can be produced for many hours on end with a sound intensity greater than 100 dB SPL measured 50 mm from the ear[8]. It consists of a 250-ms series of chirps that is separated by a 300-ms chirp interval (Fig. 1a). A chirp itself comprises four syllables of 21-ms duration, which are generated by the closing movements of the forewings. Crickets' ears are located on their forelegs and are therefore exposed fully to the self-generated sounds. About 60 auditory afferent neurons project from the ear along the fifth prothoracic nerve and terminate in the auditory neuropile of the prothoracic ganglion[9]. Two local, mutually inhibitory omega 1 neurons (ON1s) are located in the prothoracic ganglion[10]. This identified pair of bilaterally symmetrical interneurons receives auditory information from the ear ipsilateral to their soma. These interneurons are most sensitive to the carrier frequency (4.5 kHz) of the male calling song.

Crickets must maintain auditory sensitivity during bouts of singing because they respond behaviourally to auditory stimulation

during chirp intervals[11]. They do not, unlike some other animals[12–15], modulate the responsiveness of their peripheral auditory system during sound production[16]. To understand how auditory sensitivity is maintained despite the intense self-stimulation, we examined the activity of the auditory afferents and ON1 to acoustic stimulation during sonorous, silent and fictive singing.

During pharmacologically elicited sonorous singing, we made intracellular recordings from the dendritic region of ON1. The neuron responded with bursts of spikes in phase with the syllables (Fig. 1a). The maximum spike frequency of ON1 to self-generated syllables was on average 176 ± 28 Hz (mean ± s.e.m; $n = 10$ crickets). As this was much lower than the response of ON1 to 100 dB SPL sound pulses at rest (376 ± 50 Hz, $n = 5$ crickets), it indicated that an inhibitory input influenced auditory processing during singing.

If singing was elicited but sound production was prevented by removing one forewing to cause 'silent singing' then ON1 did not spike, which indicated that its response during sonorous singing was caused by the crickets' song. Instead, ON1 received inhibitory postsynaptic potentials (IPSPs) during the syllables (Fig. 1b). In five silently singing crickets, the average duration of a wing movement cycle underlying sound production was 38.1 ± 1.5 ms. IPSPs started 5.0 ± 0.2 ms after the start of wing closing and reached a maximum at 23.3 ± 1.4 ms, just after the transition between wing closing and opening at 19.0 ± 0.7 ms. If a sequence of acoustic stimuli was presented during silent singing, then ON1 spiked continuously during the chirp intervals but was inhibited during the chirps (Fig. 1c). Only excitatory postsynaptic potentials (EPSPs), and occasionally a spike, were elicited by the stimuli during silent chirps. Thus, the activity of ON1 during sonorous stridulation is due to excitation from reafferent sound stimulation, which is caused by the crickets' own song and a synchronous inhibitory input.

To determine whether this inhibition was elicited by sensory feedback or by the network of neurons that generate the motor pattern, we induced fictive singing in crickets with either their thoracic ($n = 3$) or thoracic and abdominal ganglia ($n = 2$) isolated from muscles and sense organs, except for the fifth prothoracic nerve. IPSPs were also present in the fictively singing cricket. The IPSPs were sufficient to suppress the spiking response to acoustic stimuli (Fig. 1d), as in silent singing. The IPSPs even persisted when the ears were removed ($n = 4$; Fig. 1e).

To analyse whether auditory afferents are also affected by inhibition, we made intracellular recordings close to their terminals. During sonorous singing, the afferents responded in phase with sound production (Fig. 2a). During quieter chirps, the presence of primary afferent depolarizations (PADs) before the spikes was indicated by a gradual depolarization (Fig. 2a, arrow). The PADs might be masked by the spike activity, we therefore recorded auditory afferents in six silently singing crickets. Spikes were only occasionally produced during silent chirps, which confirmed that the afferents responded to the self-generated sound. PADs were now obvious during the syllables (Fig. 2b).

In four silently singing crickets, the average duration of a wing movement cycle was 36.0 ± 1.8 ms. The PADs started 4.2 ± 0.4 ms after the start of wing closing and reached a maximum at 19.3 ± 1.4 ms, just after the transition between wing closing and opening at 17. 8 ± 1.0 ms. Thus, the PADs have a similar timing to

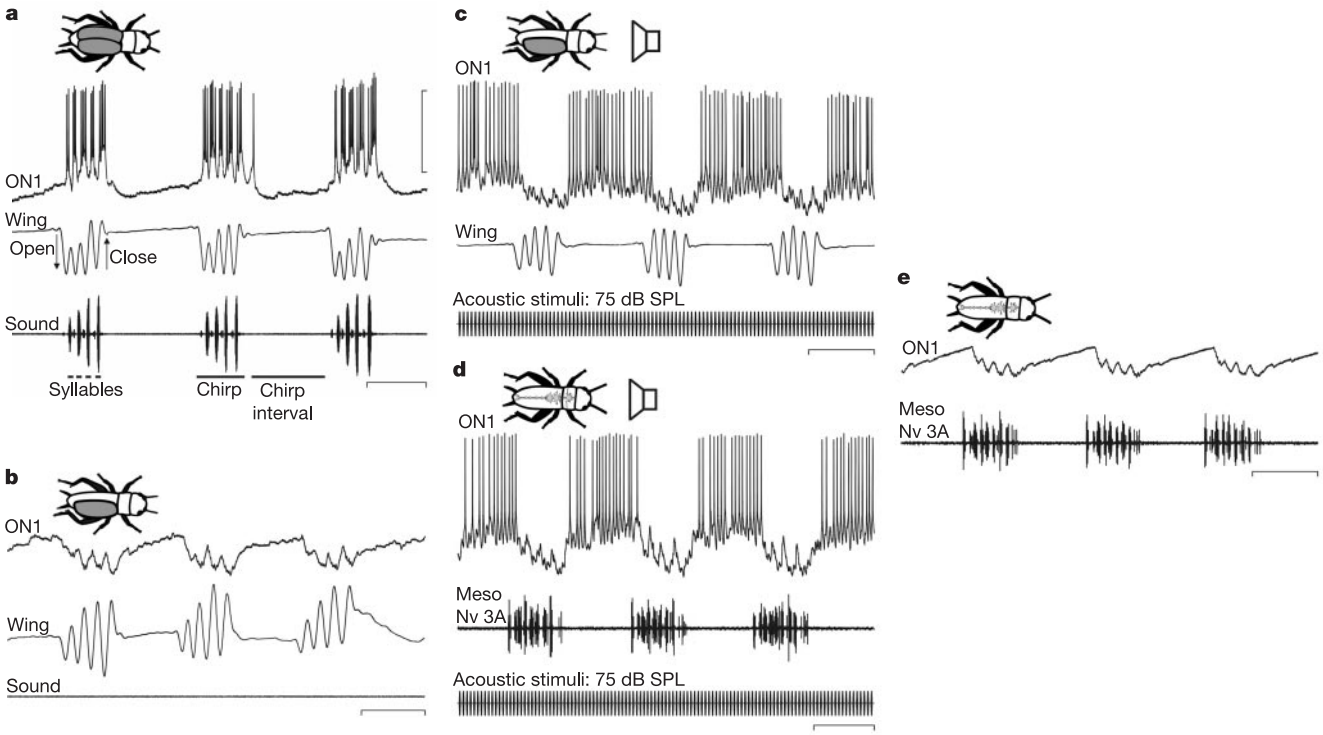

Figure 1 Responses of ON1 during singing. **a**, During sonorous singing ON1 produces bursts of spikes in phase with each syllable. A syllable, a whole chirp and a chirp interval are marked below the sound recording. **b**, During silent singing, IPSPs are present in ON1 in phase with the silent wing movements. They increase in amplitude from −2.3 ± 0.3 mV at the first closing wing movement to −5.3 ± 0.5 mV at the last ($n = 8$ crickets). **c**, **d**, When stimulated with a sequence of acoustic pulses, ON1 responds during the chirp intervals, but is inhibited during both silent (**c**) and fictive (**d**) chirps. **e**, During fictive singing, IPSPs occur in phase with the chirps even when the animal's ears are removed. Mean amplitude of the IPSPs increases from −2.4 ± 0.6 mV for the first syllable of the fictive chirp to −5.1 ± 0.7 mV at the last ($n = 5$ crickets). Symbols above the figures represent singing with two wings, singing with one wing, fictively singing and fictively singing with ears removed. ON1, intracellular ON1 recording; wing, wing movements; sound, cricket song; acoustic stimuli, sound pulses; Meso Nv 3A, activity of mesothoracic nerve 3A. Vertical scale bar: intracellular, 25 mV; extracellular, 10 mV; wing, 1 mm. Horizontal scale bars, 250 ms.

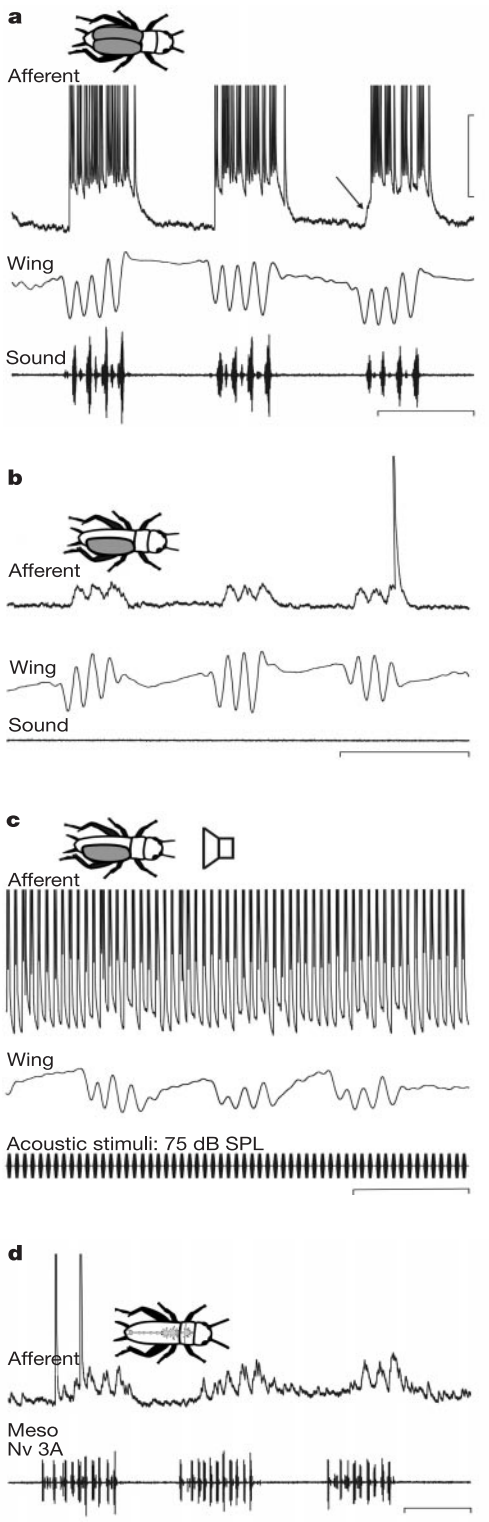

Figure 2 Presynaptic inhibition of auditory afferents during singing. **a**, Auditory afferents respond in phase with sound production. Arrow indicates a primary afferent depolarization (PAD) at the start of a chirp. **b**, During silent singing, PADs are present in auditory afferents during the rhythmic wing movements. They increase in amplitude from $+2.6 \pm 0.5$ mV at the start of the chirp to $+3.3 \pm 0.7$ mV at the end ($n = 6$ crickets). **c**, The spike pattern of an auditory afferent presented with a sequence of 4.5 kHz, 75 dB SPL sound pulses is not modulated during silent singing. **d**, PADs are also present during fictive chirps which increase in amplitude from 1.9 ± 0.3 mV at the start of the fictive chirp to 3.5 ± 0.1 mV at the end ($n = 6$ crickets). Afferent, intracellular recording of an auditory afferent, the spikes have been truncated. Vertical scale bar: intracellular, 15 mV; extracellular, 10 mV; wing, 1 mm (**a**), 0.25 mm (**b**), 0.5 mm (**c**). Horizontal scale bars, 250 ms.

the IPSPs in ON1. The PADs did not affect spike production in the auditory afferents, as they spiked consistently to acoustic stimulation throughout silent singing (Fig. 2c). PADs were also present in six fictively singing crickets (Fig. 2d), even when their ears were removed ($n = 2$ crickets). In many sensory systems, PADs have an inhibitory function[17] as they reduce synaptic efficacy at the afferent terminals. The ultrastructural and histochemical prerequisites for presynaptic inhibition have been described in the cricket[18].

We tested the effectiveness of the inhibition mediated by the corollary discharge (sum of presynaptic and postsynaptic inhibition) on auditory processing in ON1 by playing acoustic stimuli that were similar to the natural calling song during silent singing (Fig. 3a). At rest and during the chirp intervals, each sound pulse evoked a depolarization and a burst of spikes in ON1 with an average maximum spike frequency of 376 ± 50 Hz ($n = 5$ crickets). During silent chirps ON1 responded to the stimuli with bursts of only 123 ± 29 Hz, which was significantly lower than the response during the intervals (two-tailed paired t-test: $P < 0.002$, $t = 7.71$, degrees of freedom (d.f.) $= 4$; Fig. 3b).

We then tested the effect of the inhibition on the sensitivity of ON1. Intense acoustic stimulation causes ON1 to spike, but it also causes a graded hyperpolarization that reduces its sensitivity to sound[19]. The hyperpolarization is thought to be due to Ca^{2+}-dependent K^+ channels that are activated by the spikes[20]. The response to self-generated sound should therefore desensitize the response of ON1 to external sounds in the absence of a corollary discharge. We mimicked the effects of the animal listening to its own

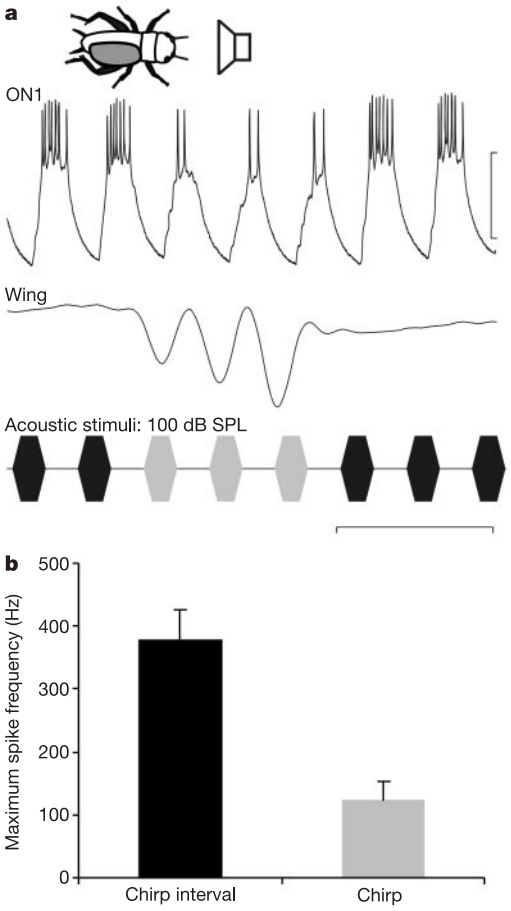

Figure 3 Testing the efficacy of inhibition during silent singing. **a**, A typical ON1 response to 100 dB SPL sound pulses during the chirp interval (in black) and during a silent chirp (in grey). **b**, Averaged maximum spike frequency to 150 sound pulses presented to five silently singing crickets during the chirp interval (in black) and the chirp (in grey). Vertical scale bar: intracellular, 25 mV; wing, 1 mm. Horizontal scale bar, 100 ms.

song and examined its responses to quieter test stimuli ($n = 8$ crickets). First a series of test sound pulses was presented at 80 dB SPL that each elicited a burst of spikes with an average maximum frequency of 203 ± 24 Hz (Fig. 4a). When normal singing was mimicked with 100 dB SPL chirps, the response to the following 80 dB SPL test stimuli was reduced so that very few spikes were elicited (Fig. 4b) with an average maximum spike frequency of 30 ± 11 Hz. This was significantly different to the response in the control situation (two-tailed paired t-test: $P < 0.001$, $t = 8.70$, d.f. = 7; Fig. 4a, b, d). The reduction was greatest just after the 100 dB SPL chirp but persisted for the rest of the artificial chirp interval.

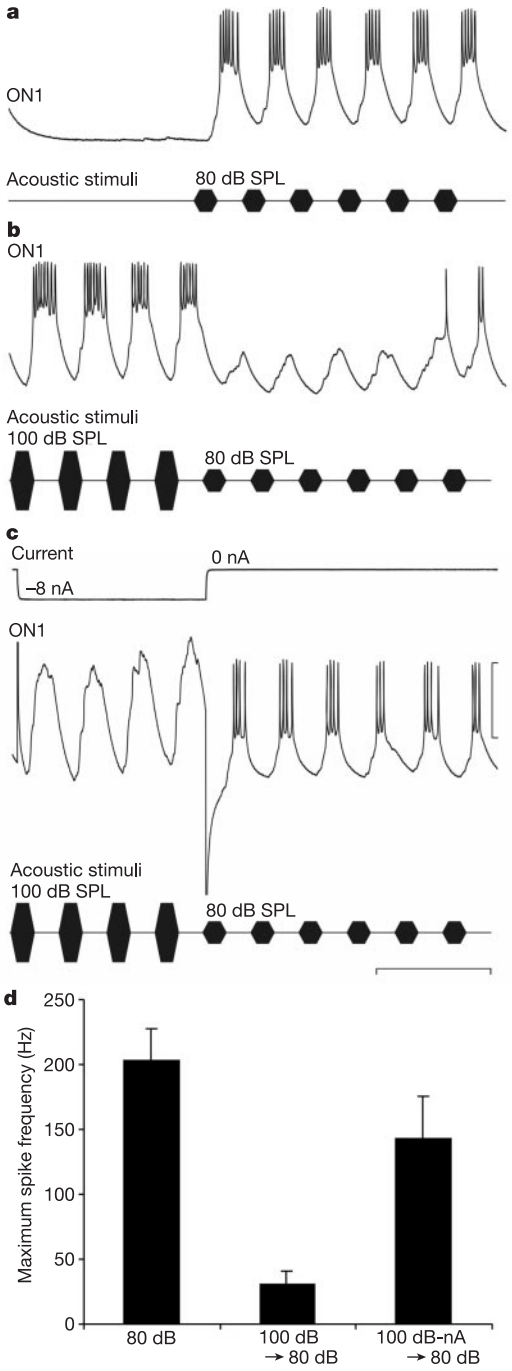

Figure 4 Inhibition of ON1 during acoustic stimulation prevents subsequent desensitization. **a**, In a control experiment, ON1 produces bursts of spikes at 203 Hz in response to 80 dB SPL pulses. **b**, Response of ON1 to 80 dB SPL sound pulses is reduced to 30 Hz if the sound pulses are preceded by a 100 dB SPL chirp. **c**, If spiking is prevented by hyperpolarizing current injection during responses to 100 dB SPL chirps, the response to the subsequent 80 dB SPL pulses is 143 Hz. **d**, Pooled, averaged maximum spike frequency of eight crickets to 150 consecutive presentations of each stimulus paradigm. Current, current injected into the cell. Vertical scale bar: intracellular, 25 mV; current, 20 nA. Horizontal scale bar, 100 ms.

We then injected hyperpolarizing current into ON1 to prevent it from spiking during the 100 dB SPL chirp. The average maximum response to subsequent 80 dB SPL stimuli was a burst of spikes at 143 ± 32 Hz, which was significantly higher than the response without current injection (two-tailed paired t-test, $P < 0.004$, $t = -4.21$, d.f. = 7; Fig. 4a, c, d). The response after current injection did not reach the response to the test stimuli, because afferent adaptation occurs at high sound intensities[21]. This effect was still present after deafening the ear contralateral to the soma of ON1 and was therefore independent from inhibition from the contralateral ON1. In three one-eared crickets, the average spike frequency to 80 dB SPL stimuli was 263 Hz; this was reduced to 23 Hz when the stimuli were preceded by a 100 dB SPL chirp but increased to 168 Hz if the neuron was hyperpolarized during the chirp. Thus, a reduction in the spiking response of ON1 to intense sounds maintains the neuron's sensitivity to subsequent sounds.

To allow animals to distinguish between reafferent and external stimuli, the two types of sensory information must be treated differently. It was proposed 50 years ago that efferent neural signals, efference copies[22] or corollary discharges[23], from central motor networks alter the responsiveness of sensory pathways in phase with the generation of reafferent sensory information. Both mechanisms could prevent self-induced desensitization and help discriminate important features from the mix of self-generated and external sensory information. These mechanisms have now been identified in several sensory systems[24–27]. In auditory systems, inhibition of auditory neurons has been seen in many vertebrates[1–7], but intracellular recordings have never been obtained during sound production to establish the exact site and mode of the inhibition.

We have shown here that in the cricket an efferent signal modulates auditory information processing at two levels of the auditory system: it causes PADs in auditory afferent terminals and IPSPs in ON1. This efferent signal may be termed a 'corollary discharge' because it is generated in the nervous system and the strength of its inhibition is independent of sound production[28]. The neuron(s) that mediate the corollary discharge are unlikely to be the descending command neurons for stridulation, as they spike with little modulation of their firing rates during singing[29]. The most likely candidate neurons are those contained in the thoracic stridulatory motor network. The inhibition significantly reduces the response of ON1 during sound production and thereby maintains this neuron's sensitivity to external sounds in the chirp intervals. In this way, the corollary discharge prevents auditory desensitization and allows crickets to respond to auditory signals during singing. □

Methods

Crickets

We carried out experiments at 18–22 °C on adult male *Gryllus bimaculatus* from a colony maintained on a 12 h/12 h light/dark cycle. Crickets were fixed on a holder in a standing position to allow free wing movement. The holder was then rotated 180° for intracellular recording from prothoracic neurons and pharmacological stimulation of the brain. For silent singing the left wing was removed. Exposed nervous tissue was bathed in insect saline (ionic composition (mM): NaCl, 140; KCl, 10; $CaCl_2$, 4; $NaHCO_3$, 4; $NaHPO_4$, 6). The acetylcholine esterase inhibitor eserine salicylate (10^{-2} M) was pressure-injected into the anterior protocerebrum of the brain through a glass microcapillary to elicit singing.

Acoustic stimuli

Acoustic stimuli were generated by the software Cool Edit 2000 and had a frequency of 4.5 kHz with rising and falling ramps of 2 ms. Acoustic stimuli that mimicked natural singing had a duration of 21 ms, 42-ms intervals and were 100 dB SPL relative to 20 μPa root mean square (r.m.s.). To reveal the time course of the neuronal response during singing, we presented a sequence of 75-dB SPL (r.m.s.) acoustic stimuli with a short interval (15 ms) and duration (8 ms). Sound amplitude was calibrated with a Bruel and Kjaer measuring amplifier (type 2610) and a microphone (type 4191) positioned at the cricket's ears.

Data recording and neuron visualization

Auditory neurons were recorded and stained using thick-walled glass micropipettes filled with 5% Lucifer yellow and 0.5 M LiCl (resistance 100–150 MΩ). After recording, ganglia were processed conventionally and the stained neurons were identified under an ultraviolet fluorescence microscope. To record fictive motor activity, we placed a suction electrode on mesothoracic nerve 3A, which contains motor axons that innervate wing closer and opener muscles. A microphone (Audio-Technica AT853A) recorded sound produced by the cricket and an optoelectronic camera monitored wing movements. All data were transferred directly onto a computer through an AD board (Data Translation 2821 F8DI) with a sampling rate of 10 kHz per channel. We analysed data off-line using Neurolab[30] and Microsoft Excel 2000.

Received 19 February; accepted 23 May 2002; doi:10.1038/nature00919.

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Acknowledgements

We thank M. Burrows, T. Matheson and S. Rogers for comments on the manuscript. This work was supported by a Biotechnology and Biological Sciences Research Council (BBSRC) studentship and grants from the Wellcome Trust and the Royal Society.

Competing interests statement

The authors declare that they have no competing financial interests.

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Mechanism of magnesium activation of calcium-activated potassium channels

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Large-conductance (BK type) Ca^{2+}-dependent K^+ channels are essential for modulating muscle contraction and neuronal activities such as synaptic transmission and hearing[1–5]. BK channels are activated by membrane depolarization and intracellular Ca^{2+} and Mg^{2+} (refs 6–10). The energy provided by voltage, Ca^{2+} and Mg^{2+} binding are additive in activating the channel, suggesting that these signals open the activation gate through independent pathways[9,11]. Here we report a molecular investigation of a Mg^{2+}-dependent activation mechanism. Using a combined site-directed mutagenesis and structural analysis, we demonstrate that a structurally new Mg^{2+}-binding site in the RCK/Rossman fold domain—an intracellular structural motif that immediately follows the activation gate S6 helix[12–15]—is responsible for Mg^{2+}-dependent activation. Mutations that impair or abolish Mg^{2+} sensitivity do not affect Ca^{2+} sensitivity, and vice versa. These results indicate distinct structural pathways for Mg^{2+}- and Ca^{2+}-dependent activation and suggest a possible mechanism for the coupling between Mg^{2+} binding and channel opening.

The energetically separate Ca^{2+}- and Mg^{2+}-dependent activation pathways suggest that each pathway may involve distinct structural components of the channel. Previous results suggest that a low-affinity, divalent cation-binding site that is responsible for Mg^{2+}-dependent activation may be located in the amino-terminal core of mouse Slo1 (mSlo1) subunits[9,16] (Fig. 1a). Thus, the Mg^{2+}-binding site is distinct from the high-affinity Ca^{2+}-binding site that has been proposed to reside in the carboxy terminal tail[17–19] (Fig. 1a). The RCK domain is an intracellular motif of the core that immediately follows the activation gate S6 helix[12–15] (Fig. 1a). It is conserved among BK channels, various prokaryotic K^+ channels and TrkA proteins (which regulate K^+ conductance[12]). The X-ray crystal structure of the RCK domain of the *Escherichia coli* Kch channel indicates that this domain may contain a ligand-binding site at its N-terminal half[12]. To examine whether the N terminus of the mSlo1 RCK domain contains the low-affinity metal-binding site, we first studied chimaeric channels between mSlo1 and its homologue mSlo3 (ref. 20)—the activation of which is insensitive to Mg^{2+}, although it also contains the RCK domain[9,12] (Fig. 1). Comparing the sequence of mSlo1 with mSlo3, it is obvious that differences scatter within the N-terminal region of the RCK domain (Fig. 2a). If these differences occur in the metal-binding site, they may result in the difference in Mg^{2+} sensitivity between these two channels. Figure 1c (left panel) shows that the conductance–voltage (G–V) relation of chimaera C31-I (see Methods for definition of chimaeras) shifted less than that of mSlo1 when intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) increased from 0 to 10 mM, whereas the increase of $[Mg^{2+}]_i$ caused no change in the G–V relation of C31-II. The total loss of Mg^{2+} sensitivity in C31-II is consistent with the idea that the sequence of mSlo3 in this region may have destroyed the metal-binding site. On the other hand, chimaera C13 was activated by Mg^{2+} (Fig. 1c, right panel), indicating that this region in mSlo1 is sufficient to restore Mg^{2+} sensitivity in mSlo3. Figure 1d