In Vivo Ca$^{2+}$ Dynamics in a Cricket Auditory Neuron: An Example of Chemical Computation

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Fura-2 calcium imaging in the cricket omega neuron revealed increased intracellular free calcium ion concentration in response to simulated cricket calling songs and other sound stimuli. The time course of the increase and decrease in intracellular calcium coincided with the time course of forward masking, a time-dependent modulation of auditory sensitivity. The buffering of calcium transients with high concentrations of a kinetically fast calcium buffer eliminated the post-stimulus hyperpolarization associated with forward masking, whereas the uncaging of calcium inside the neuron produced a hyperpolarization. The results suggest that sound-stimulated intracellular calcium accumulation acts by means of a calcium-activated hyperpolarizing current to produce forward masking. These findings underscore the importance of chemical dynamics in neural computation by demonstrating a behaviorally relevant role of calcium dynamics in vivo.

With the advent of techniques for measuring the spatial and temporal dynamics of chemical species in neurons (1), it is possible to ask how the dynamics of chemical activity (such as ion concentration) contribute to neural computation. Here we report how one form of chemical activity, the temporal dynamics of intracellular free calcium ion concentration, [Ca$^{2+}$], in a cricket auditory interneuron, underlies forward masking, a psychophysical and electrophysiological phenomenon in cricket hearing. Forward masking is a form of temporal inhibition in which a loud sound suppresses the response to subsequent sounds (a temporal analog of lateral inhibition). This temporal inhibition may be used for automatic gain control or background subtraction, allowing female crickets to "focus" on the loudest caller in the presence of multiple calling males and background noise. Because this ability would enable the cricket to home in on an individual chirping male, it has been referred to as a form of selective attention (2) in analogy to the "cocktail party phenomenon" observed in human auditory psychophysics (3). An electrophysiological correlate of forward masking is observed in the omega neuron, one of the first interneurons in the cricket auditory pathway. This correlate appears as a long-lasting, post-stimulus hyperpolarization and concomitant reduction in action potentials after sound stimulus (2).

The response of an omega neuron to a simulated calling song and the forward masking effect are shown in Fig. 1 (4). The response to chirps of a single calling song presented at 60-dB sound pressure level (SPL) is shown in Fig. 1A and Fig. 1B shows the response to two simultaneous calling songs. The chirps of the 90-dB song precede the chirps of the 60-dB song and suppress the number of action potentials elicited by the 60-dB song, compared with the number of action potentials evoked by the 60-dB song by itself. This effect is accomplished by a small hyperpolarization that occurs between chirps. The masking increases from the first to the fourth chirp in the sequence, at which point the action potentials evoked by the quieter calling song are completely eliminated. The hyperpolarization that follows each chirp also accumulates with each successive chirp. Figure 4C summarizes this forward masking effect, showing the number of spikes per chirp in the absence of masking and with two different masking intensities. As the masking intensity is increased the curves shift to the right, showing that the cell's auditory threshold is temporarily raised by the masking stimulus. The amount of masking produced by the 90-dB song on the 60-dB song increases with each subsequent chirp (Fig. 1B), suggesting that the masking effect takes longer to decay than the inter-chirp interval. As a result, the response to later chirps is affected by the residual masking from earlier chirps. This observation and the data in Fig. 1C suggest that the physiological variable that controls masking increases with sound intensity and decays slowly (several seconds), compared to the inter-chirp interval. Forward masking cannot be explained by inhibition from the known connections onto the omega neuron (5). Therefore, we hypothesized that a calcium-activated hyperpolarizing current produces forward masking and, thus, that the dynamics of forward masking are determined by the dynamics of [Ca$^{2+}$].

To investigate this hypothesis, we mea-
Assured changes in $[\text{Ca}^{2+}]_i$ in response to sound stimuli in the omega neuron in the semi-intact cricket with the fluorescent calcium indicator fura-2 (Fig. 2A). Calcium accumulation was observed during presentations of simulated cricket calling songs at naturally occurring intensities (50- to 100-dB SPL) (Fig. 2B)—the same stimulus conditions that produce the forward masking effect shown in Fig. 1. With the onset of each chirp, there was an influx of calcium, most likely through voltage-sensitive calcium channels or ligand-gated channels, which did not completely decay before the next chirp. Consequently, calcium accumulated over the course of the calling song and decayed back to resting level when the calling song ended. Louder chirping produced larger calcium accumulations. This observation suggests that under conditions encountered by a cricket in the wild (multiple chirping males for several hours), $[\text{Ca}^{2+}]_i$ in the omega cell approaches a steady state that depends on the recent average sound intensity. A possible role of this elevated calcium in modulating the auditory neuron’s threshold, as in forward masking, is suggested by our finding that the elevated calcium is correlated with a decreased response to sound (Fig. 3A).

To investigate quantitatively the temporal correlation between $[\text{Ca}^{2+}]_i$ accumulation and the post-stimulus hyperpolarization that accompanies forward masking, we measured $[\text{Ca}^{2+}]_i$ in response to sounds of long duration that elicit larger hyperpolarizations than individual chirps (Fig. 3B). These sounds evoke a large depolarization and brisk firing of action potentials for the entire duration of the sound. Immediately after the sound, the cell shows a deep hyperpolarization that decays back to resting potential with a time course of several seconds (4.3 ± 1.0 s) (6). These sounds also produced a large increase in $[\text{Ca}^{2+}]_i$ that returned to resting level with a time course of 4.5 ± 1.4 s (7), thus matching the time course of the electrophysiologically observed post-stimulus hyperpolarization. In addition to having a similar time constant, both peak calcium concentration and hyperpolarization increased to asymptotic levels as sound intensity and duration were increased.

The tight correspondence among $[\text{Ca}^{2+}]_i$, the hyperpolarization produced by sound, and reduced omega cell excitability during stimulation supports the hypothesis that a calcium-activated hyperpolarizing current is the mechanism by which calcium accumulation leads to the forward masking effect. The most likely

![Graph](image-url)
candidate for such a current is a calcium-activated potassium current such as those demonstrated in Drosophila (8), in the large monocular cells of the fly optic lobe (9) as well as in cockroach motor neurons (10).

To test the involvement of a calcium-dependent potassium conductance in the post-stimulus hyperpolarization, we injected the omega cell with a high concentration of fura-2. Moderate concentrations of kinetically fast, high-affinity affinity buffers like fura-2 are known to block calcium-activated potassium channels in other preparations (11). Omega neurons filled with a high concentration of fura-2, unlike normal omega cells, continued to fire for several seconds after the termination of the sound stimulus and showed a reduced post-stimulus hyperpolarization (Fig. 4, A and B). This behavior suggests that a calcium-activated current is necessary for the production of the hyperpolarization observed in normal cells. Additional evidence of a calcium-activated current was obtained with DM-Nitrogen, a caged calcium compound, which releases calcium upon photolysis (12) and transiently raises [Ca$^{2+}$]. When calcium was released inside omega neurons through this method, a transient hyperpolarization and decreased sensitivity to sound were observed (13) (Fig. 4, C and D).

The omega cell responds to sound stimuli including species-specific sounds with an accumulation of [Ca$^{2+}$], that depends on the intensity and duration of sound and that decays slowly (3- to 5-s time constant). The accumulation of [Ca$^{2+}$], reflects the average environmental sound level through its "leaky integrator" dynamics. Our data suggest that the integration and decay times of [Ca$^{2+}$], accumulation give rise to the temporal dynamics of forward masking by activating a hyperpolarizing current, temporarily raising the neuron's threshold and shifting its input-output function (action potentials per decibel of sound). The dynamics of [Ca$^{2+}$], accumulation and decay have a neuroethological significance because hyperpolarization of individual omega neurons in crickets (Grillus bicinctatus) that are actively engaged in tracking calling songs reduces the crickets' tendency to turn toward calling songs on the hyperpolarized side (14). Our findings provide a simple example of chemical computation in central nervous system function by illustrating how the dynamics of a chemical species can be used to perform a useful transformation of information.

**REFERENCES AND NOTES**


4. Crickets (Acheta domesticus) were waxed ventrally side up with their forelegs inserted into leg holes (H. U. Klein, D. A. Cooper, D. W. Wohlers, J. Comp. Physiol. A 141, 2831 (1981)). The cuticle over the prothoracic ganglion was removed. The ganglion was desheathed and supported as described in (14). Intracellular recording and dye filling were made with glass capillary microelectrodes (3.0 mm by 0.58 mm borosilicate glass, filled at the tip with 28 mM fura-2, 2% pentaethylene glycol (Molecular Probes) in 150 mM potassium acetate and backfilled with 150 mM potassium acetate). The preparation was perfused with oxygenated saline containing a bicarbonate buffer. Sound stimuli were generated by a synthesized function generator gated by a pulse generator and passed through a cosine switch and precision attenuators. Simulated calling songs consisted of a 4.8-Hz sine wave amplitude modulated into chirps composed of three pulses (syllables), each with a 25-s duration and a 3.5-s period with rise and fall times of 5 ms. Sound intensities were calibrated in decibel SPL (G. E. Hewlett-Packard 4136 microphonic and HP3566A digital signal analyzer).


6. The characteristic decay times determined from single exponential fits (22 measurements from seven crickets) were averaged to produce the reported time constant. The optical recording methods used in these experiments are similar to those reported by W. G. Regehr and D. W. Tank (ibid. 12, 4202 (1993)). The omega neuron was filled with the calcium indicator fura-2 by the passing of a 0.72- to 1.0-nA hyperpolarizing current for ~10 min. The electrode was removed and the fura-2 was allowed to diffuse for at least 5 s before the cell was imaged through an upright epi-fluorescence microscope (Zeiss, Axioskop; 100-W Hg lamp, 385-nm excitation, 450-nm dichroic mirror, long-pass barrier filter). Fluorescence was imaged with a microcomputer-controlled cooled CCD camera (Chiron, Photometrics). For Fig. 2, a photodiode (Hamamatsu, S167-S1) was used in place of the camera for increased temporal resolution. The field of view of the photodiode was 65 μm by 65 μm centered on the body of the neuron in the left lower quadrant of Fig. 2A. The photodiode output was amplified, low-pass-filtered (25-ms time constant), and sampled (5-Vs-20 ns, rate) by a microcomputer. An additional digital Gaussian filter (=70 ms) was applied to the data. In CCD data, background fluorescence from an area adjacent to the cell was subtracted before the percent change was computed. In photodiode experiments, background subtraction was not performed. Increased calcium leads to a decrease in fluorescence with 385-nm excitation. Both CCD and photodiode data were corrected for bleaching (~1% per 15 s) by linear extrapolation. The change in 385-nm fluorescence measured by the photodiode was reduced by high autofluorescence in the tissue, lack of background subtraction, and spatial averaging over regions of neuropil other than the filled dendrites of the omega cell. Changes observed with the CCD camera were larger because background subtraction was performed and the fluorescence measurements could be restricted to the fura-2–filled neurites of the omega cell.

7. The characteristic decay times determined from single exponential fits (53 measurements from seven crickets) were averaged to produce the reported time constant.


11. For example, see W. M. Roberts, Nature 363, 74 (1993).


13. Omega neurons were impaled with electrodes filled at the tip with 100 mM DM-Nitrogen (Cylbochromo) and 10 mM Sulfothiodamine (Sigma) (for identification of filled neurons). The neurons were filled with a current ranging from 0.5 to 1.0 nA of current for 5 to 10 min. To photolyze the DM-Nitrogen, the ganglion was exposed to light from a 100-W mercury arc lamp band-pass-filtered between 340 to 400 nm. Light exposures were 1 s long. Hyperpolarization or decreased response to sound was not observed in response to short flashes in the absence of DM-Nitrogen and was not observed after two or three flashes, at which point all of the caged calcium was probably released. After the hyperpolarization accompanying the uncaging of calcium, a slow depolarization was observed that lasted for several seconds. This depolarization did not appear to be due to Ca$^{2+}$ because it could be elicited repeatedly after all of the Ca$^{2+}$ was presumably uncaged and was most likely an artifact of the light flash. The photolysis products of the DM-Nitrogen, or both [A. M. Gunney, in Fluorescent and Luminescent Probes for Biological Activity: A Practical Guide to Technology for Quantitative Real Time Analysis, W. T. Mason, Ed. (Academic Press, London, 1993), pp. 335-348].

14. In most experiments, imaging was performed.
Prevention of Vertebrate Neuronal Death by the crmA Gene

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Interleukin-1β converting enzyme (ICE) is a mammalian homolog of CED-3, a protein required for programmed cell death in the nematode Caenorhabditis elegans. The activity of ICE can be specifically inhibited by the product of crmA, a cytokine response modifier gene encoded by cowpox virus. Microinjection of the crmA gene into chicken dorsal root ganglion neurons was found to prevent cell death induced by deprivation of nerve growth factor. Thus, ICE is likely to participate in neuronal death in vertebrates.

The survival of sensory neurons during development of the vertebrate nervous system depends on neurotrophic factors produced by the neuronal targets (1). In the absence of these factors, the neurons are thought to undergo programmed cell death, an active process requiring RNA and protein synthesis (2). Little is known about the genes regulating cell death in vertebrates. A gene essential for cell death in the nematode C. elegans, ced-3, was recently shown to share sequence similarity with a mammalian gene, ICE, which encodes a cysteine protease involved in the processing of interleukin-1β (3). Overexpression of ICE induces programmed cell death in Rat1 fibroblasts and this death can be suppressed by the cowpox virus crmA gene, a specific inhibitor of ICE, and by the BCL-2 protooncogene (4, 5). To determine whether ICE is involved in neuronal death, we investigated the effect of crmA on the survival of chicken dorsal root ganglion (DRG) neurons cultured in the absence of nerve growth factor (NGF), a condition that would normally lead to cell death.

We microinjected an expression vector (pHD1.2) containing a crmA complementary DNA (cDNA) under the control of the chicken β-actin gene promoter, or in vitro transcribed crmA RNA (6), into cultured DRG neurons along with rhodamine-isothiocyanate-labeled dextran (7). About 90% of the injected neurons retained a normal morphology, and, in the presence of NGF, about 85% survived through day 6. Within 3 days of NGF deprivation, however, more than 80% of the control neurons (injected with dye alone, vector alone, or antisense crmA cDNA) died, with fewer than 10% surviving to day 6 (Fig. 1). In contrast, more than 60% of the crmA-injected neurons survived through day 6 in the absence of NGF (Fig. 1).

Expression of crmA protein was detected in almost all of the injected neurons by immunofluorescence staining with an affinity-purified rabbit polyclonal antibody to crmA (Fig. 2) (8). The crmA protein could be detected in neurons 9 days after injection, suggesting that it is very stable. The crmA-injected DRG neurons cultured in the absence of NGF were round and often devoid of extensive neurites, a morphology similar...