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Separation of voltage- and ligand-gated calcium influx in locust neurons by optical imaging

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Abstract

Calcium ions can enter neurons through either ionotropic transmitter receptors or through voltage-gated calcium channels. Thus, an observed rise in intracellular calcium concentration upon synaptic stimulation can be due to either one of these mechanisms or to both of them. We analyzed the individual contribution of transmitter- and voltage-gated calcium entry in non-spiking somata, acutely dissociated from thoracic ganglia of the locust *Locusta migratoria*. By optically recording the calcium signal following different stimulation protocols, we isolated the voltage- and the transmitter-gated component and found that these components indeed summate to the total rise in calcium observed under control conditions.

Keywords: ACh-receptors; Calcium channels; Calcium imaging; Insect; Primary culture; *Locusta migratoria*

The nicotinic acetylcholine receptor (nAChR) of vertebrate neurons is known to have a high permeability for calcium ions [10,16,18]. In comparison to the state of knowledge about vertebrate nAChRs, little is known about the permeability of insect nAChRs. While one study on cultured Kenyon cells of the bee reports a calcium-to-sodium permeability ratio of 6.4 [5], in another study on cultured neurons from embryonic cockroach brains calcium influx through nAChRs was below the detection threshold [15]. To examine the calcium permeability of nAChRs on locust somata and to determine its observed contribution to the total calcium signal after synaptic stimulation, it would be advantageous to block all voltage-gated calcium channels (VGCCs) prior to stimulation. Divalent heavy metal ions, although reliably blocking VGCCs [12,17], might also influence nAChRs, besides being highly toxic and able to quench the fluorescence of calcium sensitive dyes. A reliable high efficacy organic blocking agent for insect VGCCs has not been found yet [3,6–9,11,12,17]. As an alternative, we depolarized the cells by the same amount that was the case following synaptic stimulation, but without agonist application. In complementary experiments we applied the agonist while keeping the cells from depolarizing. We used ratiometric measurements of the intracellular calcium concentration in isolated locust somata, an established preparation which allows for perfect control of the membrane potential by single electrode discontinuous voltage clamp. The preparation procedure has been described in detail by Suter and Usherwood [14]. The composition of the perfusion solution was (in mM) NaCl 180, KCl 10, $MgCl_2$ 15, $CaCl_2$ 7.5, HEPES 5 (pH = 6.8). For low calcium experiments, $CaCl_2$ was replaced by an equimolar amount of $MgCl_2$.

The cells were kept in a perfusion chamber mounted on an inverse microscope and filled with the fluorescent calcium indicator fura-2 by an intracellular microelectrode. To determine the time course of the intracellular calcium concentration, pictures of the cell were taken by a cooled CCD camera (CH250, Photometrics) at a rate of 1 Hz, alternating the wavelength of excitation between 340 and 380 nm. To stimulate the cells, we used iontophoresis with retaining current rather than pressure injection to avoid desensitization and displacement artifacts. Application of the cholinergic agonist carbachol gave the most reproducible depolarizing responses in trial experiments. The iontophoresis electrode filled with 0.1 M carbachol was placed upstream in the perfusion chamber at a distance of about 30–50 μm from the cell. The pharmacology of locust

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somatic AChRs has been already examined in detail [1,2,13,14].

In Fig. 1A, a typical control experiment is shown. In this experiment, the dye-filled electrode was retracted and replaced by a KCl-filled electrode to avoid artifacts by dye injection. As the carbachol was ejected (gray bar), the cell depolarized from the resting potential of −50 to about −20 mV (blue curve) and showed a strong increase in $[Ca^{2+}]_i$ (red curve). By the following two experiments, this bulk calcium signal was now dissected in its individual components. To isolate the calcium influx through VGCCs, we activated the voltage gated channels in exactly the same way as in the control experiment, but without carbachol application. To do so, we recorded the time course of the membrane potential in the control experiment and used it as a command voltage for the 'replay' experiment (Fig. 1B). Now, the calcium signal was smaller than in the control experiment. As the depolarizing current injection was the only stimulus in this experiment, the only possible mode of calcium entry was through VGCCs. To isolate the calcium component through nicotinic AChRs, we prevented the activation of VGCCs by clamping the membrane potential to −60 mV (Fig. 1C). When the cell was stimulated with carbachol under these conditions, we observed a calcium signal of about 25% of the control stimulation (red curve). Spatial analysis of the calcium signal revealed that the highest calcium concentrations occurred on the side of the cell which was directed towards the carbachol electrode (Fig. 1C; top). In the control experiment without voltage clamp, the calcium signal started synchronously from all directions (Fig. 1A; top). When the time courses of the calcium influx through ligand gated and voltage gated calcium channels were summed, the resulting curve matched the calcium signal in the control experiment quite well (Fig. 1A; blue curve in bottom panel). Thus, calcium-induced calcium release did not amplify the calcium signal in a significant way within the range of intracellular calcium concentrations from 50–500 nM.

For all cells measured ($n = 6$), we evaluated the peak and the integral of the calcium signal in the 10 s after stimulus

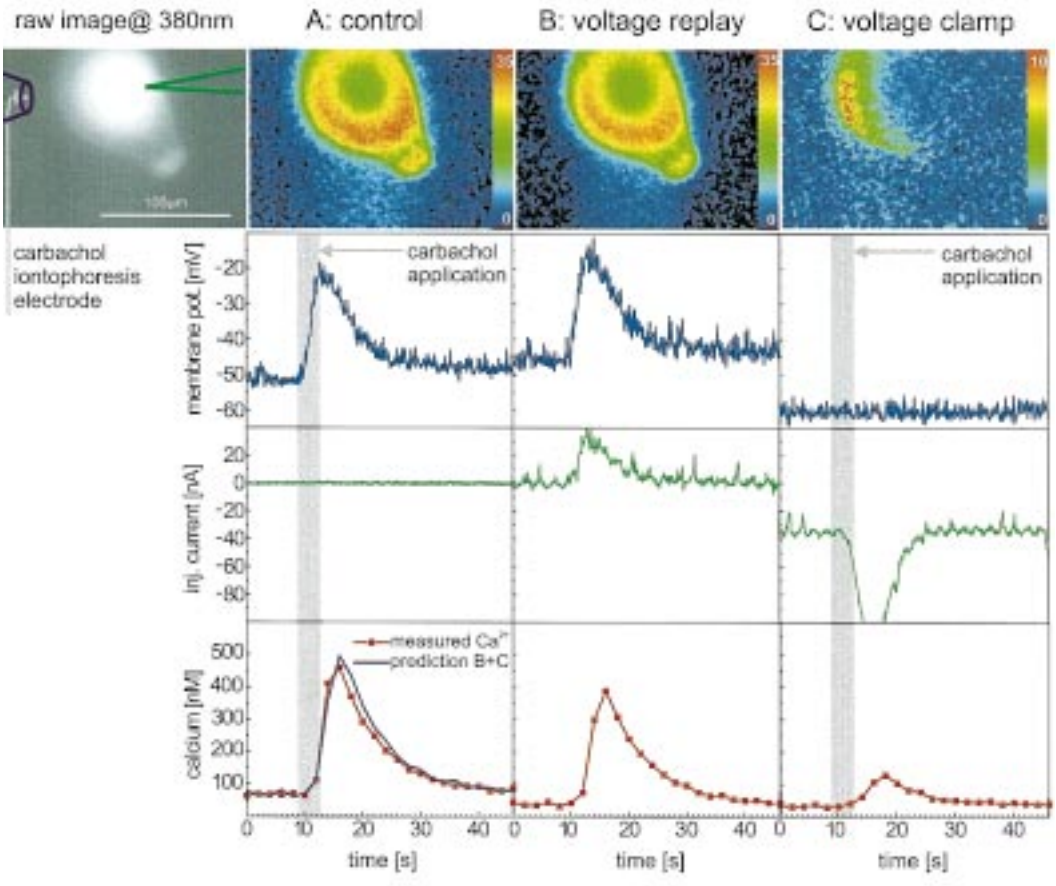

Fig. 1. A typical set of experiments on an isolated nerve cell soma filled with fura-2. (A) Control experiment: carbachol stimulation under current clamp conditions. Top: color coded change in $[Ca^{2+}]_i$ 5 s after stimulation onset. Background fluorescence has not been subtracted prior to calculating the ratio 340/380 nm. Curves show the time course of the membrane potential (blue), the injected current (green), the measured $[Ca^{2+}]_i$ (red squares) and the sum of experiments (B,C) (blue). The gray bar indicates the time of carbachol application. (B) Voltage replay experiment in voltage clamp without carbachol stimulation. (C) Membrane potential clamped to − 60 mV and carbachol stimulation as in (A). Note different scale bar in false color picture to enhance weak lateral calcium signal. All $[Ca^{2+}]_i$ time courses have been measured in the left half of the cell with background subtraction in the raw images.

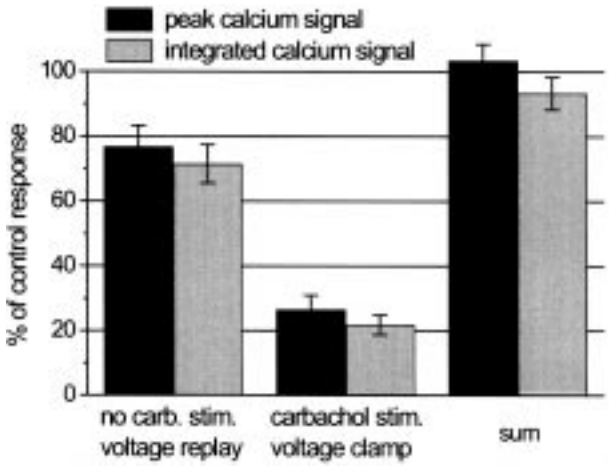

Fig. 2. Statistical analysis of all cells measured ($n = 6$). Changes in $[Ca^{2+}]_i$ were normalized to the control experiment (=100%). We evaluated the peak change in $[Ca^{2+}]_i$ (black bars) and the integrated change in $[Ca^{2+}]_i$ (light gray bars) during the 10 s following the onset of stimulation. The sum was calculated for each experiment individually, prior to calculating the SEM. (error bars). The sum of voltage- and ligand-gated calcium signal (third pair of columns) and the calcium signal under control conditions are from the same distribution ($P \geq 0.95$, Wilcoxon matched pairs signed rank test).

onset (Fig. 2). For both measures the total calcium signal observed under control conditions fitted a linear summation of the two currents described above almost perfectly. The ratio of voltage-gated to ligand-gated calcium influx was surprisingly constant in all measured cells (Fig. 2). Thus, our selection criteria seemed to result in a rather homogenous population of cells: Only cells of 60–80 μm diameter with a visible dendritic stump were recorded from. Spiking cells, cells not responsive to carbachol, and cells with a membrane potential above −45 mV were rejected.

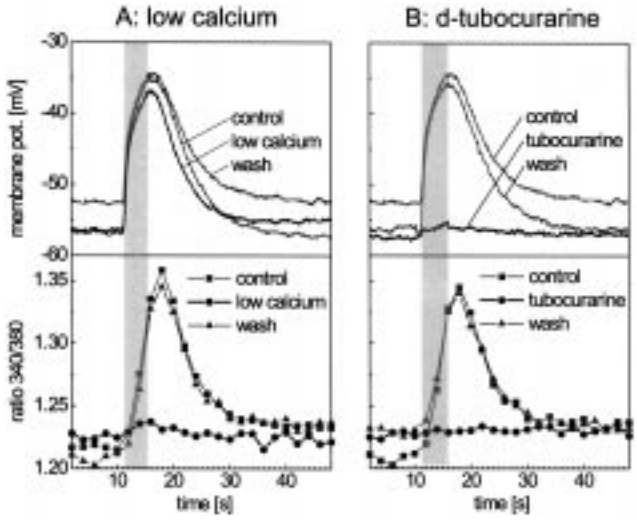

Fig. 3. (A) Carbachol stimulation in low calcium saline only slightly reduced the amplitude of depolarization (top). The calcium signal, however, was completely and reversibly abolished (bottom). (B) The addition of 0.1 mM D-tubocurarine to the perfusion solution blocked the electrical response upon carbachol stimulation (top) as well as the calcium signal (bottom). After 10 min wash in standard saline, both signals were restored to their original amplitude.

In principle, the calcium signal under hyperpolarized voltage clamp (Fig. 1C) could also be caused by stimulated muscarinic AChRs that triggered calcium release from intracellular stores. To test for calcium release, we stimulated the cells with carbachol after 5 min of perfusion in low calcium saline. The drop in membrane potential was hardly altered under low calcium conditions, suggesting sodium as the main charge carrier (Fig. 3A; top). The calcium signal, however, was completely abolished under these conditions (Fig. 3A; bottom). We conclude that calcium ions entered the cell from outside. The nicotinic pharmacology of the calcium signal was further confirmed by blocking experiments (Fig. 3B). Bath application of 100 μM D-tubocurarine, a specific nicotinic receptor blocker known to be effective in *Locusta* [1], completely blocked the electrical response as well as the calcium signal upon carbachol stimulation ($n = 3$). The block was reversible after 10 min perfusion in standard saline. We therefore attribute the calcium signal observed upon carbachol stimulation under hyperpolarized voltage clamp to calcium influx through nAChRs. This influx contributed about 25% to the total calcium signal as measured in current clamp. In nerve cells, a large change in calcium concentration can be caused by a very small current, because of the very low calcium concentration at rest. However, the precise calcium-to-sodium permeability ratio of the receptor channel cannot be readily derived from our results without further assumptions.

Electrophysiological methods of determining the calcium permeability of ligand-gated receptors involve measuring the shift in reversal potential in solutions with different external calcium concentrations. High external calcium concentrations are needed for reliable measurements, but carry the risk of unwanted interactions of calcium with other ions. For example, calcium might bind inside the channel pore and hinder the permeation of other ions. There might be also regulatory binding sites for calcium on AChRs, as has been demonstrated for the neuronal $\alpha 7$ nicotinic AChR [4]. The optical method described in this report is not dependent on changes in the extracellular ion concentration or selective blocking agents and might be applicable to a wide variety of cell types. The high sensitivity of calcium sensitive dyes make it possible to demonstrate calcium influx that might be below the detection limits of purely electrophysiological methods.

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