J. Membrane Biol. 123, 43–47 (1991)
002226319100105V

The Journal of
Membrane Biology

Extrusion of Calcium from a Single Isolated Neuron of the Snail *Helix pomatia*

A.V. Tepikin, P.G. Kostyuk, V.A. Snitsarev, P.V. Belan
Department of General Physiology of Nervous System, A.A. Bogomoletz Institute of Physiology, Kiev-24, GSP 252601, USSR

Summary. Simultaneous optical measurements of extra- and intracellular Ca^{2+} concentrations were carried out on isolated snail neurons injected iontophoretically with Ca^{2+}. The fluorescent indicator Fura-2 was used to measure intracellular concentration of free Ca, and the absorbant indicator Antipyrylazo III to measure changes in extracellular calcium concentration in the microchamber containing the cell. The velocity of Ca^{2+} extrusion from a single cell has been shown to be in accordance with the level of free Ca in the neuronal cytoplasm. After an increase in intracellular free Ca by iontophoretic injection from a microeletrode to 0.2–0.5 μM, the velocity of Ca^{2+} extrusion from the neuron was approximately 0.3–4.6 μM/sec per cell volume. During caffeine-induced calcium-dependent calcium release of Ca^{2+} from intracellular stores a stimulation of calcium extrusion took place, reaching the velocity of 5.0 μM/sec per cell volume.

Key Words *Helix pomatia* neurons · calcium extrusion · Fura-2 fluorescence · Antipyrylazo III absorbance

Introduction

Calcium ions are the most universal secondary messengers, and changes in their intracellular concentration trigger a whole spectrum of cellular responses to external stimuli (Berridge & Irvine, 1989). Considerable progress has been achieved during recent years in the development of experimental methods for direct determination of the level of free calcium in the cytoplasm; most important was the synthesis by Tsien and coworkers of fluorescent calcium probes Fura-2, Indo-1, etc. (Grinkiewicz, Poenie & Tsien, 1985).

The methods for measurements of calcium extrusion from cells are much less developed. Usually only relatively slow extrusion processes from multicellular preparations or proteoliposomes are studied directly using radioisotope techniques (Dawson & Comerford, 1989). Therefore, we made an attempt to develop a system which would enable measurement of calcium efflux from a single cell over a wide time range, and used this system for a comparison between changes in intracellular free calcium level and rate of calcium extrusion from the cell.

Materials and Methods

For the measurements of calcium extrusion we used a microchamber with the volume of extracellular solution of about 4–7 nl; this volume was approximately ten times greater than that of the cell. The microchamber (Fig. 1) was, in fact, a drop of hemispheric form with a radius of 110–140 μm. The drop was formed on siliconized (Silicon solution, Serva) glass using a set of special plastic micropipettes. An isolated snail neuron with diameter of 100–120 μm was put into the center of the drop. Then the drop, containing the cell, was covered with a layer of nonfluorescent oil to avoid evaporation of the extracellular solution. Methods of neuronal isolation and loading with Fura-2 indicator are described in our previous papers (Tepikin, Belan & Mironov, 1987; Kostyuk et al., 1989).

The extracellular solution contained (in mM): 1 $CaCl_2$, 6 Antipyrylazo III, 1 $MgCl_2$, 5 KCl, 35 TrisCl, 100 NaCl. Under these conditions the free calcium concentration in the extracellular solution was about 50 μM, which is approximately one to two orders of magnitude above the level of free Ca in the cytoplasm of the cell. The records of Antipyrylazo III absorbance were carried out at two wavelengths (710 and 780 nm). Changes in extracellular calcium concentration were obtained from the Antipyrylazo III differential absorbance signals (details about the indicator properties can be found in Scarpa, Brinley & Dubyak, 1978; Blinks, et al., 1982; Pizarro, Cleemann & Morad, 1987), and details concerning the procedure of estimations of the extracellular calcium concentration and calibration experiments have already been described in our previous paper (Tepikin, Snitsarev & Belan, 1990). The spectral range, in which absorbance measurements were carried out, is far from the emission and fluorescence maxima of Fura-2; thus, we managed to make simultaneous measurements of the extra- ($[Ca]_{out}$) and intracellular ($[Ca^{2+}]_{in}$) calcium concentrations.

The experiments were performed with iontophoretic Ca^{2+}-injection under microelectrode voltage clamp; for this purpose we used a three-barrel intracellular microelectrode (*see* Fig. 1) (details concerning iontophoretic injection can be found in Kostyuk et al., 1989). A second microelectrode, introduced into the extracellular solution in the drop and filled with 2.5 M KCl, was used as the reference one (Fig. 1); it had a resistance of 3-5 MΩ.

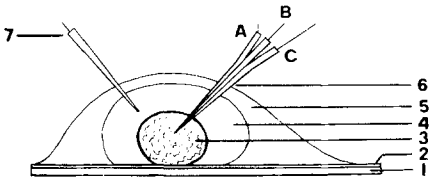

Fig. 1. Microchamber: (*1*) glass plate; (*2*) silicon layer; (*3*) neuron; (*4*) extracellular solution; (*5*) nonfluorescent oil; (*6*) three-barrel microelectrode with barrels: (*A* and *B*) filled with 2.5 M KCl for voltage clamp and (*C*) filled with 0.2 M Ca- Cl_2 for Ca^{2+} injection; (*7*) reference microelectrode with 2.5 M KCl.

Results

During injection, the intracellular concentration of free Ca increased, and immediately after the end of the injection it started to return toward the baseline (Fig. 2*A*). The extracellular calcium concentration also started to increase during the injection and reached a steady level within a few minutes after its end (Fig. 2*B*). The velocity of calcium extrusion was calculated as the derivative of extracellular calcium concentration changes multiplied by the relation of the drop volume to the cell volume: $V = (d[Ca]_{out}/dt) \cdot (V_d/V_c)$ (Fig. 2*c*). The dependence of the velocity of calcium extrusion on time was similar to the dependence on time of the intracellular level of free Ca (*compare* curves *A* and *C* of Fig. 2).

In the experiments described, the holding potential was maintained between -45 and -50 mV, which was close to the resting potential level. During injection, a small outward current, less than 1 nA, was sometimes recorded; after the end of injection this current decreased gradually (*see* Fig. 2*D*).

During prolonged increase of the level of free Ca in the neuron by means of iontophoretic injection, the velocity of calcium extrusion stabilized and essentially did not change for a long time (Fig. 3). The stabilized velocity of calcium extrusion in this case was about 0.7 μM/sec per cell volume. In the same cell, maintaining the intracellular Ca^{2+} concentration at a higher level (0.76 μM) resulted in a higher velocity of extrusion (1.9 μM/sec per cell volume). Analogous values of extrusion rate (0.3-4.6 μM/sec per cell volume; $n = 10$) were obtained on other cells with changes in free Ca^{2+} in the physiological range (0.2–0.5 μM).

If the intracellular level of free Ca was maintained at a constant level by means of constant iontophoretic injection from the microelectrode, the velocity of calcium extrusion became stabilized during the whole period of injection. This velocity was directly related to the elevated level of intracellular calcium (in the range of $[Ca^{2+}]_{in}$ between 0.1 and 0.5 μM), as can be seen from examples presented in Fig. 4.

The system was used also for an attempt to determine the fate of calcium released from intracellular stores in the neuron. A well-known activator of calcium release is caffeine (Palade, 1987*a*, *b*; Kostyuk et al., 1989). Added to the extracellular solution at a concentration of 5 mM (by replacement of an equivalent amount of NaCl), caffeine by itself did not evoke spontaneous calcium release. However, if in parallel calcium has been injected into the cell iontophoretically, an additional calcium transient appeared, sometimes after termination of the injection (Fig. 5). As the $[Ca]_{out}$ was kept at a quite low level, such transients could occur only from release of ions from intracellular stores. In parallel with such a transient, a rapid rise of calcium extrusion occurred (Fig. 5). After the return of $[Ca^{2+}]_{in}$ to the base level, extracellular calcium stabilized at a higher level as compared to the level before injection. These data indicate that a Ca-dependent caffeine-induced release of Ca^{2+} from intracellular stores induces extrusion of Ca^{2+} from the neuron into the extracellular space, and the velocity of such extrusion can be quite high (up to 5.0 μM/sec per cell volume). During the whole period of caffeine-triggered release the neuron could lose a considerable amount of (50–100 μM; $n = 5$) of stored calcium.

Discussion

The stabilized Ca^{2+} extrusion rate estimated in our experiments corresponds to the extrusion rate calculated for snail neurons indirectly by Hermann and Hartung (1982). However, the system allowed us also to determine the kinetics of such extrusion over a wide time range; on the other hand, its disadvantage is that the small droplet surrounding the cell cannot easily be changed. The direct comparison of the intracellular concentrations of calcium ions and calcium flux through the membrane has shown that at physiological changes of $[Ca^{2+}]_{in}$ (0.1–0.5 μM) the extrusion rate of calcium in snail neuron is dependent on $[Ca^{2+}]_{in}$ (Figs. 2 and 4).

Since the absolute values of the transport number for calcium in calcium-injecting microelectrodes varied considerably (from 2 to 8%), it was difficult to estimate precisely the relation between the amounts of injected calcium ions absorbed by intracellular depots and extruded from the neuron. An approximate comparison indicates that at least 30% of Ca^{2+} injected during constant iontophoresis is extruded into the extracellular solution, and the rest

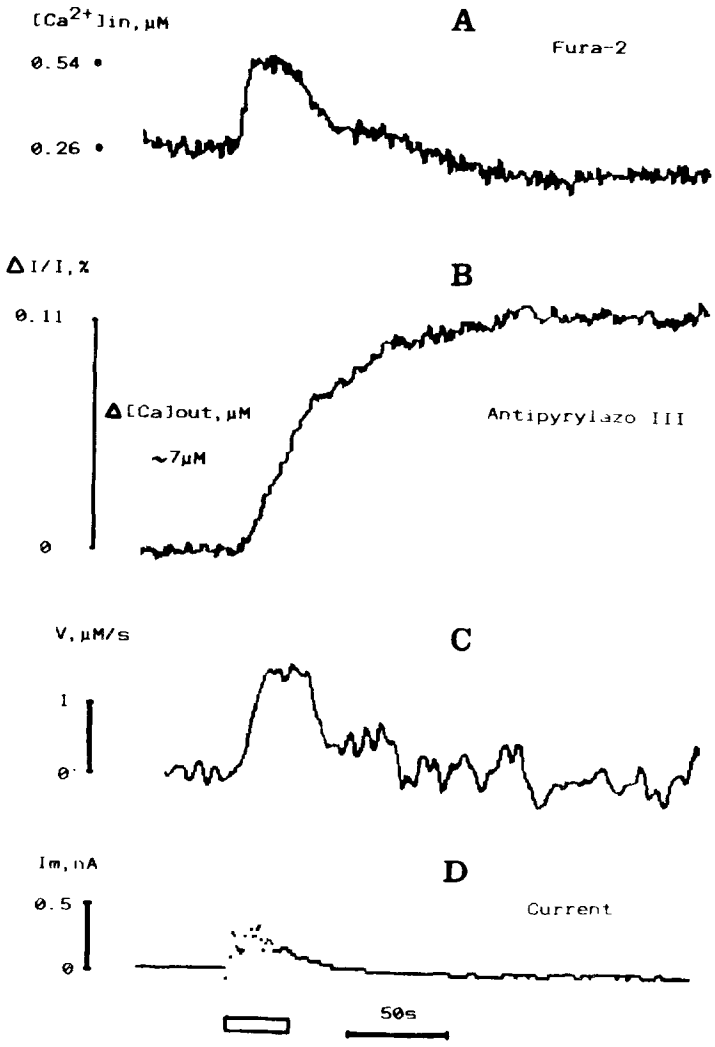

Fig. 2. Short injection of Ca^{2+} into a neuron: (*A*) concentrations of free Ca in the cytoplasm; (*B*) changes in extracellular calcium concentration; (*C*) rate of calcium extrusion (calculated from *B*); (*D*) transmembrane current. The open bar indicates the period of the injection of Ca^{2+}. Volume of the neuron V_c was 0.5 nl; volume of the extracellular solution $V_d$was 5 nl; the injection current was 15 nA; the membrane potential was −50 mV. In this figure and others $\Delta I/I$ shows the relative changes of absorbance signals at λ = 710 nm

may be immobilized inside the cell. It should be mentioned that all experiments were carried out at quite low concentration of free extracellular calcium (about 0.5×10^{-4} M, owing to the strong buffer ability of Antipyrylazo III to bind calcium ions), and this may result in an increased velocity of calcium extrusion from the neuron in comparison with the normal extracellular solution.

The new technique also produced an opportunity to follow the transitions of calcium ions released from intracellular stores during the combined action of caffeine and injected Ca^{2+}. In this case the low concentration of Ca^{2+} in the extracellular solution was of great advantage as the intracellular stores were practically the only source of the elevation of intracellular calcium level (all experiments, in which we tried to elevate $[Ca^{2+}]_{in}$ owing to Ca influx from the extracellular solution, failed). During caffeine-induced calcium-dependent calcium release, the cell lost a considerable amount of its calcium (up to 100 μM), obviously due to a remarkable increase in the velocity of extrusion from the cell (up to 5.0 μM/sec per cell volume).

It has been shown by several authors that under the action of caffeine periodic oscillations of $[Ca^{2+}]_{in}$ may start in nerve cells (Lipscombe et al., 1988; Kostyuk et al., 1989). Now it is possible to estimate the amount of Ca^{2+} that has to enter the neuron in order to support such oscillations. Their period in *Helix pomatia* neurons was usually about 100 sec (Kostyuk et al., 1989). As shown above, the neuron lost from 50 to 100 μM of Ca during one caffeine-induced Ca-dependent Ca release, which may be considered as one period of such oscillations. On dividing the amount of lost Ca by the period of oscillations, we can obtain the average calcium influx

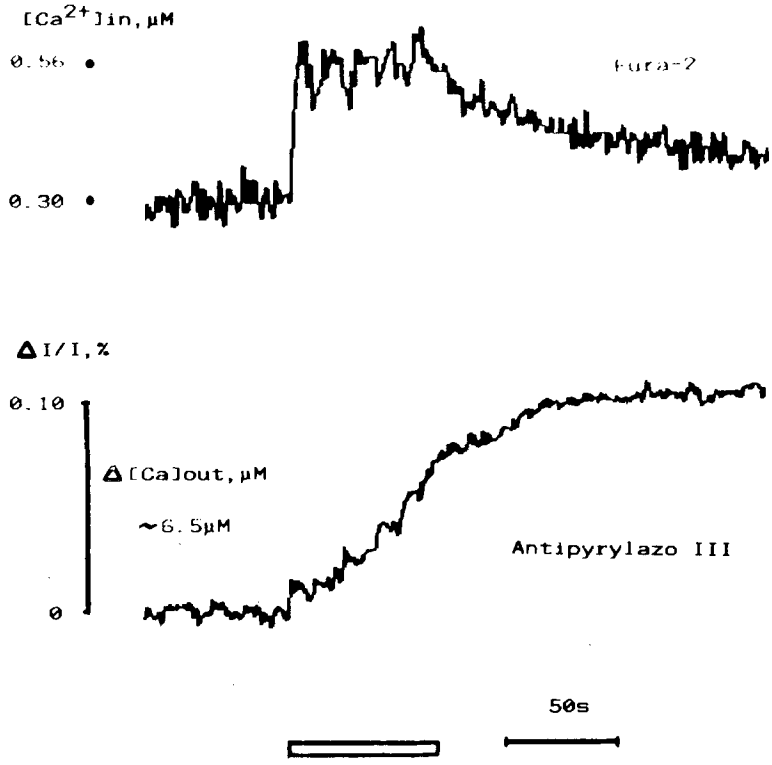

Fig. 3. Prolonged maintenance of a stable increased level of free Ca in the cell: the upper trace shows concentrations of free Ca in the cytoplasm; the lower one changes in extracellular concentration. The open bar indicates the period of the injection of Ca^{2+}. The injection current was 10 nA; the membrane potential was −50 mV

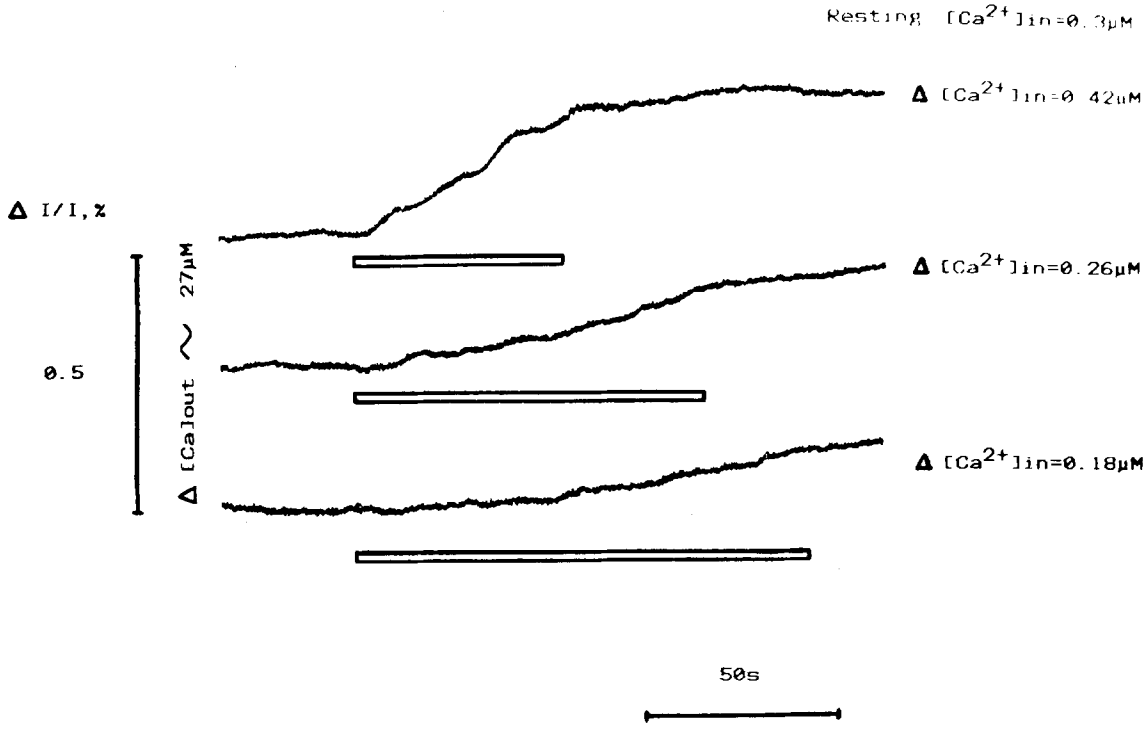

Fig. 4. Changes in extracellular calcium concentration caused by intracellular iontophoretic calcium injections of different intensities. Maximum velocities of calcium extrusion are 4.6, 1.8 and 1.5 μM/sec for the upper, middle, and lower traces, respectively, and corresponded to injection currents of 35, 15, and 10 nA. Values of deviation from the basal level of $[Ca^{2+}]_{in}$ (0.3 μM) are shown near the traces. Open bars indicate the periods of Ca^{2+} injection. The membrane potential was −45 mV

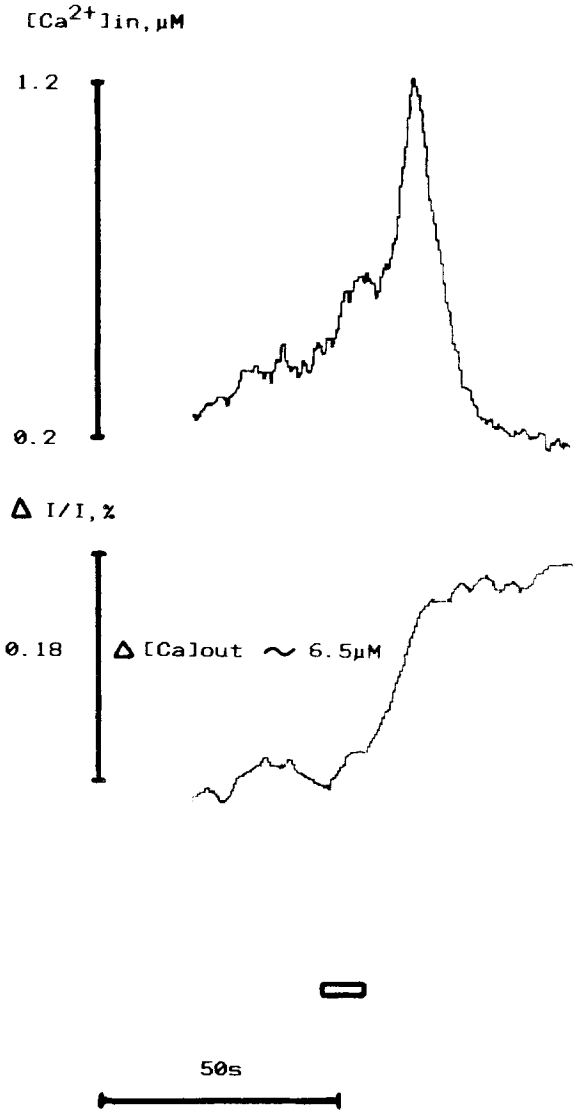

Fig. 5. Caffeine-induced calcium-dependent calcium release. The release is developing due to intracellular Ca^{2+} injection. Open bar indicates the period of Ca^{2+} injection. The upper trace shows changes in $[Ca^{2+}]_{in}$, the lower one, changes in extracellular calcium. The extracellular concentration of caffeine was 5 mM. The injection current was 30 nA; the membrane potential was −45 mV

rate. Thus, an influx of Ca^{2+} of about 0.5-1 μM/sec per cell volume must be necessary to support the stability of such oscillatory behavior.

We thank Dr. P. A. Doroshenko (Department of General Physiology of Nervous System of our Institute) for his helpful advice.

References

Berridge, M.J., Irvine, R.V. 1989. Inositol phosphates and cell signaling. *Nature* **341:**197–205

Blinks, J.R., Wier, W.G., Hess, P., Prendergast, F.G. 1982. Measurement of Ca^{2+} concentration in living cells. *Prog. Biophys. Mol. Biol.* **49:**1–114

Dawson, A.P., Comerford, J.G. 1989. Effect of GTP on Ca^{2+} movements across endoplasmic reticulum membranes. *Cell Calcium* **10:**343–350

Grinkiewicz, G., Poenie, M., Tsien, R.Y. 1985. A new generation of Ca^{2+} indicator with improved fluorescence properties. *J. Biol. Chem.* **260:**3440–3450

Hermann, A., Hartung, K. 1982. Properties of a Ca^{2+} activated K^+ conductance in *Helix* neurons investigated by intracellular Ca^{2+} ionophoresis. *Pfluegers Arch.* **393:**248–253

Kostyuk, P.G., Mironov, S.L., Tepikin, A.V., Belan, P.V., 1989. Cytoplasmic free Ca in isolated snail neurons as revealed by fluorescent probe Fura-2: Mechanisms of Ca recovery after Ca load and Ca release from intracellular stores. *J. Membrane Biol.* **110:**11–18

Lipscombe D., Madison D.V., Poenie, M., Reuter, H., Tsien, R.W. 1988. Imaging of cytosolic Ca^{2+} transients arising from Ca^{2+} stores and Ca^{2+} channels in sympathetic neurons. *Neuron* **1:**355–365

Palade, P. 1987*a*. Drug-induced Ca^{2+} release from isolated sarcoplasmic reticulum: I. Use of pirophosphate to study caffeine-induced Ca^{2+} release. *J. Biol. Chem.* **262:**6135–6141

Palade, P. 1987*b*. Drug-induced Ca^{2+} release from isolated sarcoplasmic reticulum: II. Release involving a Ca^{2+}-induced Ca^{2+} release channel. *J. Biol. Chem.* **262:**6142–6148

Pizarro, G., Cleeman, L., Morad, M. 1985. Optical measurement of voltage-dependent Ca^{2+} influx in frog heart. *Proc. Natl. Acad Sci. USA* **82:**1864–1868

Scarpa, A., Brinley, F.J., Jr., Dubyak, G. 1978. Antipyrylazo III, a "middle range" Ca^{2+} metallochromic indicator. *Biochemistry* **17:**1378–1386

Tepikin, A.V., Belan, P.V., Mironov, S.L. 1987. Changes in intracellular Ca^{2+} in isolated snail neurons revealed by fluorescent probe fura-2. *Biol. Membrane (Moscow)* **4:** 882–889

Tepikin, A.V., Snitsarev, V.A., Belan, P.V., 1990. Using Antipyrylazo III for study of calcium extrusion from isolated neurons. *Biol. Membrane (Moscow) (in press)*

Received 14 November 1990; revised 5 March 1991