Periodic Current Injection (PCI) – A New Method to Image Steady-State Membrane Potential of Single Neurons *in situ* Using Extracellular Voltage-Sensitive Dyes

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Z. Naturforsch. **50 c,** 435–438 (1995); received March 21, 1995

Membrane Properties, Imaging, Optical Recording

A new method is described which allows to image the steady-state distribution of membrane potential of single neurons *in situ*. The method consists of staining the tissue with an extracellular voltage-sensitive dye (Di-4-ANEPPS) and impaling a single neuron with a microelectrode. After focusing the imaging system onto the cell a large series of images are taken with a CCD camera at the appropriate excitation wavelength of the voltage-sensitive dye while the neuron's membrane potential is shifted by a periodic current injection (PCI). Afterwards two groups of images are averaged separately: those images while the cell was at rest and those images while the cell was hyperpolarized. After subtraction of these averaged images, the resulting difference image shows only the membrane potential of the cell which was altered periodically. The success of the method is demonstrated on leech cells in intact ganglia. If applied to cells with a basically two-dimensional arborization pattern, the decrease of activity in the difference image in areas further away from the injection site should relate to a decrease in membrane potential according to the passive electrotonic properties of the cell under study.

Introduction

With the advent of voltage-sensitive dyes and other optical recording methods the spatial domain was opened for studies of the nervous system (Cohen and Salzberg, 1978). In particular voltage-sensitive dyes allow to record the spatio-temporal activity in large ensembles of nerve cells such as the vertebrate visual cortex (Grinvald *et al.,* 1994) or invertebrate ganglia (Kleinfeld *et al.,* 1994). However, due to the inherent lipophilic property of these dyes, iontophoretic injection into single neurons is reported in only a few cases (Grinvald *et al.,* 1987). Thus, voltage-sensitive dyes are applied to single nerve cells mostly in culture systems (Parsons *et al.,* 1989; Fromherz and Vetter, 1991; Staub *et al.,* 1995) where the dye again can be applied by perfusion and residual dye which is not bound to the nerve cell membrane can be washed out after staining. These methodological problems have hindered the analysis of membrane potential spread in single neurons using voltage-sensitive dyes. On the other hand, there is a growing interest in just these problems because it is a well established fact that nerve cells are not isopotential. In contrast, the spatial distribution of membrane potential is determined by various factors amongst which are the geometry of the cell, the passive membrane and internal resistivity, the state and spatial distribution of voltage-gated ion channels and the activity of synaptic input. So far, the spatial voltage distribution of neurons could only indirectly be assessed by punctual microelectrode measurements feeding into compartmental model simulations of neurons (Segev *et al.,* 1989). No method has been available allowing an immediate visualization of the spatial voltage distribution within single neurons *in situ*. In the following I will describe a new way allowing for that. In principle, the method should be applicable to any neurons provided that they arborize in a more or less flat manner and are optically accessible.

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Material and Methods

Set-up

The set-up consists of an upright microscope (Zeiss Axioplan) with epifluoresence illumination (HBO 100 mercury arc lamp) and the appropriate

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filter combination (Rhodamin Filter set #15 from Zeiss with BP 546/12 excitation filter, FT 580 dichroic mirror and LP 590 barrier filter). A stage movable in all three axes carries the preparation and the micromanipulator. Images are acquired by a CCD camera (Photometrics System 250A with a Thompson chip TH 7883, 384×576 pixel) connected with a computer (MacQuadra 840 AV, Apple). For current injection standard electrophysiology equipment is used (amplifier Axoclamp 2A from Axon Instruments, oscilloscopes TDS 320 and TAS 455 from Tectronics). Electrodes were pulled on a Flaming/Brown micropipette puller (Model P-87, Sutter Electronics) and had a resistance of about 30 MΩ when filled with 1 M KCl.

Experimental protocol

Animals (*Hirodu medicinalis*) were obtained from a local pharmacy. Leech ganglia were dissected and stained for 30 min at 4 °C in a solution containing the extracellular voltage-sensitive dye 1-(3-sulfonatopropyl)-4-β-2-(di-*n*-butylamino)-6-naphthylvinylpyridinium betaine (Di-4-ANEPPS, Molecular Probes). The dye is diluted from a stock solution containing 5 mg of the dye dissolved in 2 ml dimethylsulfoxide (DMSO, Serva) and 0.4 ml pluronic acid just before use in leech saline by a factor of 100. Leech saline contains 115 mM NaCl, 1.8 mM $CaCl_2$, 4 mM KCl and 10 mM Tris maleate (pH 7.4) (after Muller, Nichols and Stent, 1981). After rinsing off the dye solution with ringer the ganglion was placed on the microscope stage for imaging and intracellular recording. After impaling the cell of choice, a periodic hyperpolarizing current (−5 nA, 6 s period, 50% duty cycle) was applied. Simultaneously, the preparation was imaged with a CCD chip using a frame rate of 3 Hz. Only half of the chip was exposed to the preparation, while the other one is used for intermediate storage. Before A/D-conversion, the images were binned by a factor of 2 in *x*- and *y*-direction resulting in an image resolution of 192×144 pixels. One series lasted about 33 s and consisted of 100 images.

Data evaluation

The images of each series first are split in two parts, one half being those images taken while the cell was at rest (the control images C_i), and those images, while the hyperpolarizing current was applied (the stimulus images S_i). These images are then converted into their relative change in fluorescence $\Delta f/f$ with respect to the first image of the control series (the reference image R). The resulting series of $\Delta f/f$ images are averaged separately, and the average images are subtracted from each other. This procedure is equivalent to the following formula:

$$D = \frac{1}{nR} \sum_{i}^{n} (S_i - C_i). \tag{1}$$

Noise in the difference image can be further reduced by replacing the reference image R, which is just the first image of all C_i, with $\frac{1}{n} \sum_{i}^{n} C_i$. The difference image D then becomes:

$$D = \frac{\sum_{i}^{n} (S_i - C_i)}{\sum_{i}^{n} C_i}. \tag{2}$$

The resulting image shows the spatial steady-state activity distribution with a DC hyperpolarizing current applied at the injection site. Image acquisition and processing is done using IPLab software from Signal Analytics, Vienna, USA.

Results

The data shown in Fig. 1 are obtained by injecting a periodic hyperpolarizing current of −5 nA into a Retzius cell of the intact leech ganglion stained with the voltage-sensitive dye Di-4-ANEPPS. While the averaged control (Fig. 1, top middle) and stimulus (Fig. 1, top right) images (shown as relative fluorescence changes with respect to the reference image) are still contaminated by a lot of noise arising from various factors, the resulting difference image (Fig.1, bottom, calculated according to formula (1)) clearly outlines the cell body of the Retzius cell while the noise in the surrounding tissue is significantly reduced. This is due to the fact that for each change in relative fluorescence in a stimulus image S_i a control image C_i exists with the only difference corre-

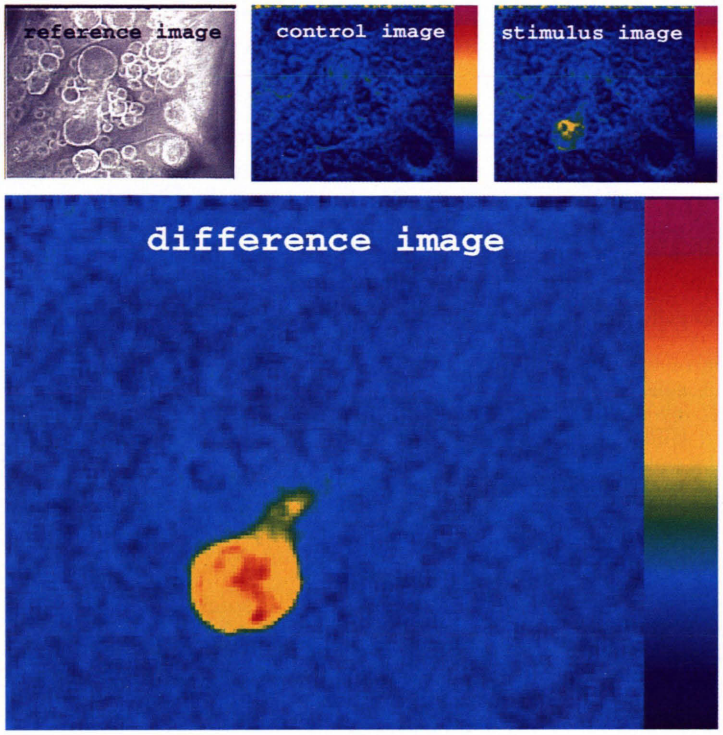

Fig. 1. A Retzius cell of the intact leech ganglion, stained with Di-4-ANEPPS, has been impaled and periodically injected with a hyperpolarizing current of −5 nA resulting in a shift of membrane potential of about 43 mV from rest. Simultaneously, images were taken with a CCD camera using epifluoresence illumination and a Rhodamin filter set. The raw fluorescence image is shown in the upper left ('reference image'). Next to it is shown the average image of relative fluorescence change during the period of no current injection ('control image'). On the right is shown the average image of relative change of fluourescence during the period of injection of the hyperpolarizing current ('stimulus image'). The difference between the stimulus and control image is shown below ('difference image'). Apart from the reference image, all images are displayed in a false-color code using warmer colors to indicate increases of the fluorescence and, thus, more negative membrane potentials. The color scale bar reaches from −2.5 (black) to +7.5 (red) permille relative change of fluoresence. Note how background noise contaminating both the control and the stimulus image in the same way is reduced significantly in the resulting difference image.

sponding to the impaled cell. Due to averaging a total of 50 images in each series, an improvement of the S/N-ratio is achieved by a factor of approximately 7. Thus, with a 12 bit camera system averaging allows for an improvement of detection of relative changes of fluorescence from .25 permille to .035 permille. However, the fading of the signal further away from the soma in the difference image (Fig. 1) is not due to the electrotonic decay of the membrane potential but, in this case, generated by the processes of the Retzius cell diving deep into the neuropile.

Discussion

The method described above is an easy-to-use way to obtain a direct visualization and measure of the steady-state membrane potential distribution within a single neuron *in situ* for a point-like injection of hyperpolarizing current. The signal-to-noise ratio improves significantly by the square-root of the number of images taken within an experimental session. Through the use of periodic current injection, drift noise contaminating control and stimulus images in approximately the same way is further reduced in the resulting difference image.

The example shown in Fig. 1 demonstrates both the validity and the limitation of the method described herein. Clearly, a spatial signal reduction can be produced by two factors, one which one would like to measure i.e. the electrotonic decay of membrane potential according to cellular geometry and passive internal and membrane resistances, and the other which one would like to correct for i.e. the variation in depth of the cellular processes. Within the limits that the cell remains visible at least to some extend a potential correction procedure can be applied by filling the cell with an intracellular dye which has to contrast sufficiently with the extracellular voltage-sensitive dye so that the cell is well visible at the excitation wavelength of the intracellular dye. One can then use the image of the dye-filled cell to determine i) the bounderies within which the difference image of relative change of fluorescence should be calculated and can be interpreted as being due to electrotonic spread and decay of membrane volt-

age, and ii) to correct within these bounderies for changes due to variation of the processes in depth. The voltage change at the injection site can serve for calibrating the optical signal provided that there is no systematic spatial inhomogeneity in the staining pattern of the cell with the voltage-sensitive dye. Experiments demonstrating this on nerve cells *in situ* with a sufficiently two-dimensional arborization pattern are currently being done.

Acknowledgements

I am grateful to T. Brotz, K. G. Götz and J. Haag for stimulating discussions and for carefully reading the manuscript.

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