

Fluorescence Monitoring of Membrane Potentials: the Spatio-Temporal Resolution in Isolated Neurons of *Helix pomatia*

Thomas Knöpfel and Peter Fromherz

Abteilung Biophysik der Universität Ulm, D-7900 Ulm-Eselsberg, Bundesrepublik Deutschland

Z. Naturforsch. **42c**, 986–990 (1987); received December 29, 1986/February 16, 1987

Potential Sensitive Dye, Fluorescent Probes, Styryl Dyes, *Helix pomatia*

Using amino-styryl-pyridinium dyes as fluorescent probes we have studied the membrane potential in spheroidal neurons isolated from the circumoesophageal ganglia of the snail *Helix pomatia*. A spatial resolution of 10 μm is attained at a time resolution of 2.5 ms with a typical signal-to-noise ratio of 10/100 mV as referred to an average sensitivity of the probes of 3%/100 mV and to a quantum yield of the detector of 0.13. The linear response of fluorescence, the effect of staining, the variation of sampled area and of illumination are characterized.

Introduction

Little experimental data are available on the spatiotemporal behavior of voltage transients within highly arborized neurons. The situation is unsatisfactory in the light of advanced theoretical considerations on signal processing in dendrites [1–3].

In principle a potential map of a neuron may be obtained from an optical indicator in the cell membrane [4]. A successful application of this method requires a suitable probe with negligible perturbation of the cell, a high signal-to-noise ratio of the optoelectrical set-up and a fast image processing system. Scores of dyes have been screened as fluorescent probes by Cohen *et al.* [5, 6]. Amphiphilic amino-styryl-pyridinium dyes have been described as most sensitive probes of fast potential changes with low pharmacological and photodynamical effect [7–9]. These dyes have been applied to sample time dependent potential distributions in isolated neurons using a coarse photodiode array [10], considering in particular the optimization of the signal-to-noise ratio [8, 11].

As a first step towards a spatio-temporal mapping of the membrane potential in a single neuron at high resolution we characterize in the present paper the features of amino-styryl-pyridinium dyes using spheroidal neurons. We consider the optimization of the fluorescence signal with respect to a spatial resolution of 10 μm at a time resolution of 1 ms.

Methods

Signal-to-Noise

The resolution of a potential map as probed by a fluorescent dye is limited by the shot noise caused by photons [8, 12] according to Eqn. (1) where S_T is the total photoelectric signal at a power bandwidth of $(4 \tau_D)^{-1}$ (time constant τ_D).

$$N_T = \sqrt{S_T/2 \tau_D} \quad (1)$$

The signal S_T depends on the intensity of illumination I_O , on the absorbance of the probe per unit area a_P and its quantum yield q_P , on any background b of stray light and inactive fluorescing dye, on the membrane area A_S sampled, on the fraction of light f_C collected and on the quantum yield q_D of the detector according to Eqn. (2). The response of a dye to a change of membrane potential ΔV_M is attributed to a relative change of its optical properties and is expressed by a sensitivity S_P according to Eqn. (3) [12].

$$S_T = I_O \cdot A_S \cdot (a_P \cdot q_P + b) \cdot f_C \cdot q_D \quad (2)$$

$$S_P \Delta V_M = \Delta(a_P \cdot q_P) / a_P \cdot q_P \quad (3)$$

$$\Delta \tilde{S}_T / \tilde{S}_T = \Delta V_M \cdot S_P \cdot (1 - f_B) = \Delta V_M \cdot S_P^* \quad (4)$$

From Eqn. (2) and (3) a relation of the membrane potential ΔV_M and the change of the average total signal is obtained according to Eqn. (4), where $f_B = b / (a_P \cdot q_P + b)$ is the fraction of background. We define S_P^* as an effective sensitivity. Combining Eqn. (1) to (4) we obtain the signal-to-noise ratio according to Eqn. (5).

$$\Delta \tilde{S}_T / N_T = \Delta V_M \sqrt{A_S 2 \tau_D} \cdot S_P \cdot \sqrt{(1 - f_B) \cdot a_P \cdot q_P} / \sqrt{I_O f_C q_D} \quad (5)$$

Reprint requests to P. Fromherz.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0700–0986 \$ 01.30/0



Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition “no derivative works”). This is to allow reuse in the area of future scientific usage.

It depends on the microscopical technique (I_O , f_C , q_D), on the nature of the sample (S_P , a_P , q_P , f_B) and on the conditions of the actual experiment (primary signal ΔV_M and spatio-temporal resolution $A_S \tau_D$).

During an action potential we cannot evaluate directly the signal S_T at constant V_M with respect to its signal-to-noise ratio. Instead we fit Eqn. (4) to the actual voltage-dependent signal by a least-square-procedure. The RMS-noise $N_T = \sqrt{\overline{S_T^2} - \overline{S_T}^2}$ is evaluated then from the difference of the average linear response and the actual signal. The signal-to-noise ratio per unit voltage is defined as $S/N = -S_P^*/N_T$.

Cells

To attain a well defined variable sample area A_S and a low background f_B we use freshly prepared neurons from the circumoesophageal ganglia of *Helix pomatia* [13]. They are dissociated and stripped from connecting tissue by repeated pipetting in saline and by trypsin treatment. Using polylysine they are fixed on a cover slide forming the bottom of a dish. Intact cells exhibit in phase contrast a fine granular cytoplasm surrounded by a smooth circular membrane of a diameter of 30–40 μm . Electrical contact is made by impaling the cells by micropipettes (40–60 M Ω , 3 M KCl, Ag/AgCl) attached to a micromanipulator (Leitz). The resting potential is around –50 mV. A bridge circuit is used to pass current through the electrode. Action potentials are induced by injecting current pulses up to .5 nA.

Probes

High sensitivity S_P with high photostability is attained with the amino-styryl-pyridinium dyes RH-160 and RH-237 (see Fig. 1) (gift of Dr. A. Grinvald). Sensitivities of $S_P^* = -3\%$ to $-12\%/100$ mV have been reported [8]. The cells are stained by bath application of the dyes dissolved in ethanol/saline (bath concentration of probe $c_P = .2 - 5 \mu\text{M}$, ethanol $< 0.1\%$). The bath is not exchanged during the experiments. Values of a_P and q_P are not available. We have checked the pattern of fluorescence by densitometry of microphotographs. It corresponds to the projection of a homogeneously stained spheroid membrane indicating a low background f_B due to staining of plasma and connecting tissue.

Optics

A high stable intensity I_O is attained by imaging the central part of the arc of a Hg-lamp (Osram HBO

200) onto the cell, through an shutter (Compur), a heat filter (10 cm water), an interference filter (Schott-AL 541 nm, width 20 nm) and an oil immersion objective (Zeiss planapo 100 \times , N.A. 1.3 Pol) in a Zeiss-Axiomat. The total cell is illuminated. Efficient light collection f_C with low stray light is provided by the high aperture of the objective, a dichroitic beam splitter (Zeiss FT-570) and a cut-off filter (Schott RG 610). The background is typically $f_B < .02$. I_O and f_C are not calibrated. The sample area A_S is defined by circular diaphragms of diameter d_S in the first image plane. The photocathode of a multiplier (RCA C31034) with a quantum yield of $q_D = .13$ at 700 nm is adjusted closely behind.

Electronics

The output of the photomultiplier S_T is amplified with negligible electronic noise, bandpass filtered, digitized and sampled by a microcomputer (Apple II). Sufficient resolution of the small signal ΔS_T is obtained by digitizing directly the difference of S_T and a reference S_{TO} taken just before each measurement. In a standard cycle the computer controls the illumination time (100–250 ms), the stimulation (depolarization for about 20 ms) and the sampling of fluorescence ΔS_T and of potential V_M (8 bit at 1–10 kHz). The bandwidth of the filter corresponds to $\tau_D = 2.5$ ms. Dye bleaching is compensated by subtracting a fluorescence signal as obtained without stimulation. V_M is folded with the filter function of the fluorescence detection system.

Results

We have studied the change of fluorescence $\Delta S_T/S_T$ as a function of the membrane potential V_M . Fig. 1 shows the result as obtained during an action potential. We have applied RH-160 and RH-237 at concentrations of 2 μM . The total cell membrane (50 μm diaphragm) was sampled at a relatively low illumination with repetitive detection. The linear dependence of $\Delta S_T/S_T$ and V_M is apparent. The slight hysteresis referring to up- and downstroke of a spike is not significant. The effective sensitivity in these two experiments is $S_P^* = -2.8\%/100$ mV for RH-160 and $S_P^* = -3.2\%/100$ mV for RH-237. The signal-to-noise ratio for a single measurement is $S/N = 12/100$ mV for RH-160 and $S/N = 7.5/100$ mV for RH-237.

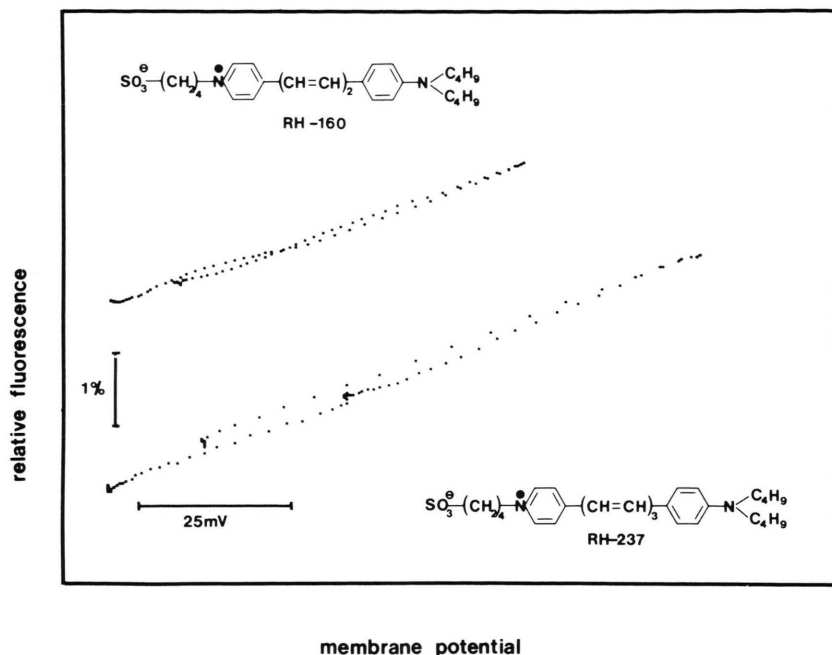


Fig. 1. Relative change of the fluorescence $-\Delta S_T/S_T$ of the aminostyryl-pyridinium dyes RH-160 and RH-237 as a function of the membrane potential during an action potential in a ganglial cell of *Helix pomatia*. The number of repetitions is $N=56$ for RH-160 and $N=18$ for RH-237.

We have not observed a unique sensitivity in our experiments as shown in Fig. 2. The sensitivity of both probes scatters around $S_p^* = -3\%/100 \text{ mV}$. A correlation to concentration is not found. The insert in Fig. 2 shows a histogram of the signal-to-noise ratio for the same data. Apparently we have obtained quite frequently $S/N > 10/100 \text{ mV}$ (best value $S/N = 18/100 \text{ mV}$). A correlation of S/N to S_p^* is found for sets of data obtained at identical illumination.

We have studied the response of the dyes for decreasing area of the sampled membrane. Fig. 3 shows the fluorescence change of RH-237 ($1.2 \mu\text{m}$) at a relatively low intensity I_0 observed through diaphragms of decreasing diameter $d_s = 50 \mu\text{m}$, $20 \mu\text{m}$ and $10 \mu\text{m}$ with an adequately enhanced number of signal repetitions. The total signal intensity S_T drops as $21:5.7:1$. Modelling the cell as a sphere of diameter $35 \mu\text{m}$ with central location of the diaphragms, we estimate a reduction of the area A_s sampled by the diaphragms as $24:4:1$, correlating to the intensity. The signal-to-noise ratio per 100 mV for a single measurement is found to be 7.5 , 5 and 2.5 respectively. The drop at a ratio $3:2:1$ correlates with the square-root of the area sampled.

We have improved the signal-to-noise ratio for the highest spatial resolution by application of a higher

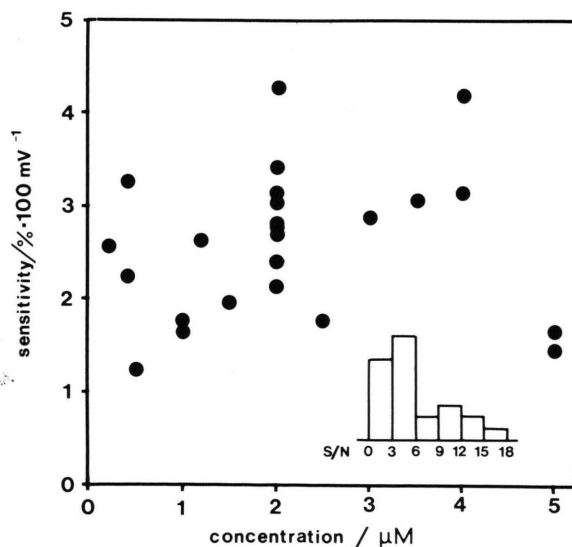


Fig. 2. Effective sensitivity $-S_p^*$ of the probes RH-160 and RH-237 in a ganglial cell for various concentrations c_p of the dyes in the bath medium (diameter of diaphragm $50 \mu\text{m}$). Each point refers to different cell. The insert characterizes the distribution of the signal-to-noise ratio.

light intensity I_0 . Fig. 4 shows the fluorescence change of RH-160 at $4 \mu\text{m}$ with a diaphragm of $10 \mu\text{m}$ at an arbitrarily enhanced I_0 without repetition. The signal-to-noise ratio is $S/N = 9/100 \text{ mV}$. However, in

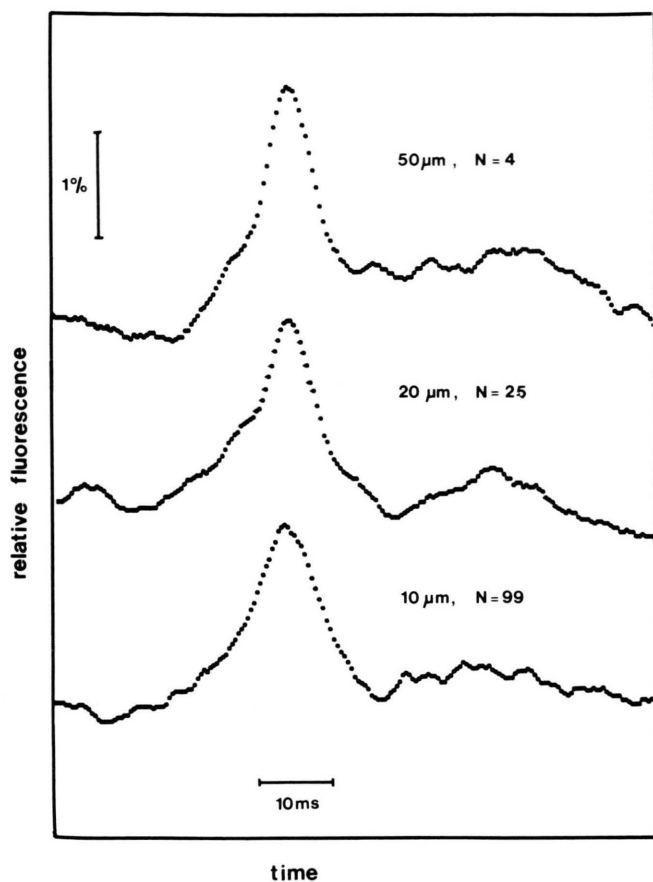


Fig. 3. Fluorescence change $-\Delta S_T/S_T$ of RH-237 in a ganglial cell as a function of time during stimulation at a relatively low intensity I_O of illumination at a concentration $c_p = 1.2 \mu\text{M}$ (time constant $\tau_D = 2.5 \text{ ms}$). The diameter of the sampling diaphragm is varied as indicated. The number of superpositions N is enhanced to balance the decreasing signal-to-noise ratio of a single measurement.

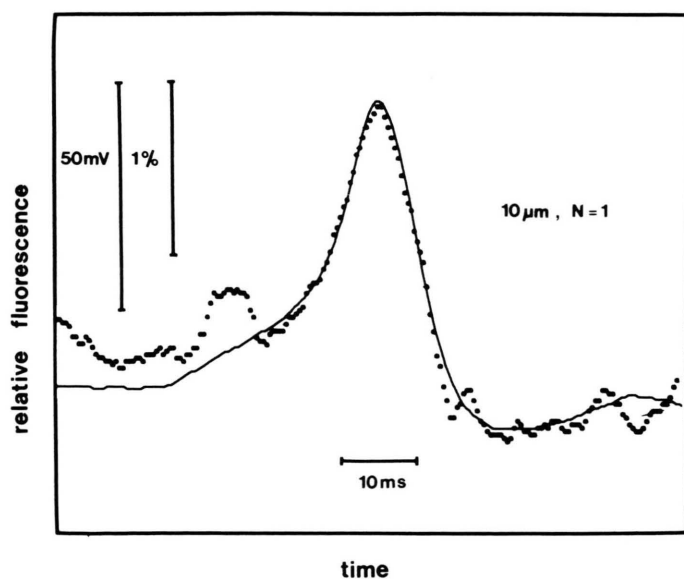


Fig. 4. Fluorescence change $-\Delta S_T/S_T$ of RH-160 in a ganglial cell as a function of time during a single action potential at a resolution of $10 \mu\text{m}$ at a high intensity I_O of illumination at a concentration $c_p = 4 \mu\text{M}$ and at a time constant $\tau_D = 2.5 \text{ ms}$. The time course of the membrane potential V_M is indicated.

this case the number of repetitions of an experiment is limited to $N < 5$ due to photodynamical destruction of the action potential.

Discussion

We have observed a perfect linearity of the fluorescence signal of the amino-styryl-pyridinium probes RH-160 and RH-237 as a function of the membrane potential. We have found a sensitivity in the range of $S_p^* = -3\%/100$ mV in the neurons of the circumoesophageal ganglia of *Helix pomatia*, scattering from -1% to -5% . The weak reproducibility may be assigned to the sensitivity S_p as due to an inhomogeneous cell population or to the background f_B as due to a variable staining of inactive cell material. Apparently an absolute calibration of the probes is rather inaccurate even for neurons of the same species.

The average sensitivity of the probes is lower than in neuroblastoma cells of line NIE-115. There an optimal sensitivity of $S_p^* = -12\%/100$ mV for RH-237 has been reported [8]. The difference may be due to species specific dye/membrane interaction affecting S_p or to the background f_B .

We have found good agreement of the experimental control of the signal-to-noise ratio with the theory with respect to spatial resolution, intensity of illumination and repetition. At a resolution of $10\text{ }\mu\text{m}$ (sample area about $A_s = 150\text{ }\mu\text{m}^2$) and a time constant $\tau_D = 2.5$ ms we have attained typically a signal-to-noise ratio of $S/N = 10/100$ mV. A substitution of the photomultiplier with $q_D = 0.13$ by a photodiode with $q_D = 0.7$ would enhance the signal-to-noise ratio to $S/N > 20/100$ mV.

In neuroblastoma cells an optimal value of $S/N = 50/100$ mV has been reported with RH-237. This value refers to a membrane area of about $A_s = 1000\text{ }\mu\text{m}^2$ as selected by a $150\text{ }\mu\text{m}$ diaphragm from a cylindrical process of a diameter of $2\text{ }\mu\text{m}$ at a time constant $\tau_D = 1.5$ ms [11]. Normalizing this result to our sample with $A_s = 150\text{ }\mu\text{m}^2$ and $\tau_D = 2.5$ ms, it corresponds to $S/N = 25/100$ mV. Normalizing it with respect to our sensitivity $S_p^* = -3\%$ it corresponds to $S/N = 6/100$ mV.

With the present study we confirm, specify and extend the investigations of Grinvald *et al.* Although some of the parameters involved are not optimal we report for the first time a resolution of $10\text{ }\mu\text{m}$ at a time constant around 1 ms with fluorescent dyes [14]. For a sample with a sensitivity of $-21\%/100$ mV (as neuroblasts stained by RH-421 [8, 11]) we may attain with our set-up a signal-to-noise ratio distinctly above $100/100$ mV at a spatial resolution of $10\text{ }\mu\text{m}$. For such a sample we may consider a drastic reduction of the light intensity such that series of measurements may be performed with a single sample without repetition. The result indicates the feasibility to obtain a fine-scaled mapping of the spatio-temporal pattern of the electrical potential in a dendritic tree of even moderate size.

Acknowledgements

We thank A. Kutruff for expert help in the electronic set-up and the State Baden-Württemberg (Schwerpunktprogramm 24) and the Fonds der Chemischen Industrie for their generous support.

- [1] W. Rall, *Biophys. J.* **2** (suppl.), 145–167 (1962).
- [2] W. Rall and J. Rinzel, *Biophys. J.* **13**, 648–688 (1973).
- [3] C. Koch, T. Poggio, and V. Torre, *Phil. Trans. R. Soc. Lond. B* **298**, 227–264 (1982).
- [4] B. M. Salzberg, H. V. Davila, and L. B. Cohen, *Nature* **246**, 508–509 (1973).
- [5] L. B. Cohen and B. M. Salzberg, *Rev. Phys. Biochem. Pharmacol.* **83**, 35–88 (1978).
- [6] R. K. Gupta, B. M. Salzberg, A. Grinvald, L. B. Cohen, K. Kamino, S. Leshner, M. B. Boyle, A. S. Waggoner, and C. H. Wang, *J. Membrane Biol.* **58**, 123–137 (1981).
- [7] L. M. Loew and L. L. Simpson, *Biophys. J.* **34**, 353–365 (1981).
- [8] A. Grinvald, R. Hildesheim, I. C. Farber, and L. Anglister, *Biophys. J.* **39**, 301–308 (1982).
- [9] L. M. Loew, L. B. Cohen, B. M. Salzberg, A. L. Obaid, and F. Bezanilla, *Biophys. J.* **47**, 71–77 (1985).
- [10] A. Grinvald, W. N. Ross, and I. Farber, *Proc. Natl. Acad. Sci. USA* **78**, 3245–3249 (1981).
- [11] A. Grinvald, A. Fine, I. C. Farber, and R. Hildesheim, *Biophys. J.* **42**, 195–198 (1983).
- [12] A. S. Waggoner and A. Grinvald, *Ann. N.Y. Acad. Sci.* **303**, 217–241 (1977).
- [13] P. G. Kostyuk, O. A. Krishtal, and P. A. Doroshenko, *Pflügers Arch.* **348**, 83–93 (1974).
- [14] W. N. Ross and V. Krauthamer, *J. Neuroscience* **4**, 659–672 (1984).