RESEARCH COMMUNICATIONS

A LARGE CHANGE IN DYE ABSORPTION DURING THE ACTION POTENTIAL

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Recently, we began a search for easily detected changes in the optical properties of neurons that occur during excitation (1, 2). Until now, changes in extrinsic fluorescence were the most sensitive indicators of neuronal activity. More than 500 fluorescent dyes have been examined on giant axons from squid and a number were found (2) which gave fluorescence changes during single action potentials that were significantly larger than the noise in the measurements. Although it was subsequently demonstrated that one of these fluorescence signals was large enough to allow optical monitoring of action potentials in individual neurons of a leech segmental ganglion (3), larger signals would facilitate more complex experiments of this kind. Previous results (4–8) had suggested that there might be changes in light absorption of dyed axons during activity. We have now found absorption changes during the action potential using a number of dyes and several of these could be measured with a signal-to-noise ratio considerably larger than fluorescence changes obtained under the same conditions.

Giant axons (diameters 320–500 μm) from the squid, *Loligo pealii,* were cleaned of small fibers and placed horizontally in a chamber containing two pairs of platinum electrodes for stimulating and extracellular recording. The chamber was mounted on the stage of a Leitz Ortholux II microscope (E. Leitz, Inc., Rockleigh, N.J.). Light from a quartz-halogen tungsten-filament lamp was collimated, made quasi-monochromatic with a bandpass interference filter, and focused on the axon by means of a bright-field condenser. Light collected by a 20 × (NA 0.4) objective was passed through a slit in the objective image plane which limited the light reaching a SGD-444 photodiode (E G & G, Inc., Salem, Mass.) to that originating from a 150 μm length of axon. The axons were incubated with a solution of dye in seawater (2) for 20 min and then bathed in seawater bubbled with argon. In a few axons the intracellular potential was measured by impaling the axon with a glass microelectrode (filled with

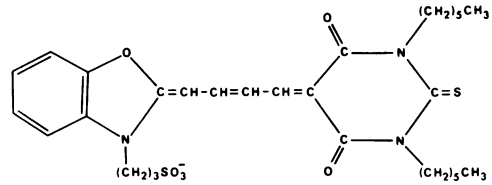

FIGURE 1 The structure of the merocyanine dye used in the experiments illustrated in Figs. 2 and 3.

4 M potassium acetate) connected to a WPI electrometer (W-P Instruments, Inc., Hamden, Conn.).

Staining the axon for 20 min with a 0.2 mg/ml solution of a merocyanine dye (Fig. 1) resulted in a decrease in transmission at 570 nm of 30–50%. When the axon was stimulated, the result shown in the top trace of Fig. 2 was obtained. This trace is an inverted record of the intensity change recorded in a single sweep following a single stimulus; the bottom trace is the action potential recorded with a microelectrode under the same conditions. During the action potential the light intensity reaching the photodiode decreased; evidence presented below indicates that the intensity decrease resulted from an absorption increase; an increase in absorption is plotted upward in Figs. 2 and 3. As no signal could be measured with the light off, the intensity change in Fig. 2 was not the result of some electrical coupling between the action potential and the light-measuring system, and, since no signal could be measured before adding the dye, the signal was dye-related and not some other optical effect such as light scattering (9). When a second interference filter, also having a maximum transmission at 570 nm, was placed between the axon and photodiode, the change in intensity divided by the

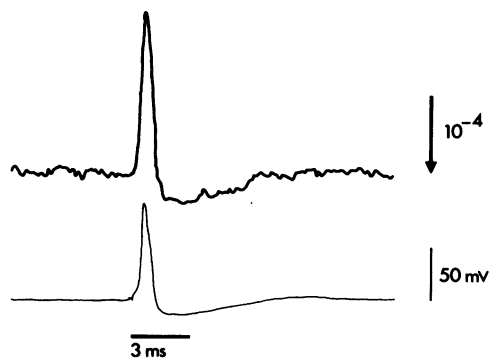

FIGURE 2 The absorption change (top trace) of a dyed axon during the action potential (bottom trace). The absorption change, measured in a single sweep, is large enough to allow optical monitoring of action potentials in a 150 μm length of giant axon. The direction of the vertical arrow to the right of the trace indicates the direction of an increase in intensity reaching the photodiode; the size of the arrow indicates a $\Delta I/I_{tr}$ of 10^{-4}. The interference filter had a peak transmission at 570 nm and a width at half-height of 30 nm. The response time constant of the light-measuring system was 170 μs. Temperature, 21°C.

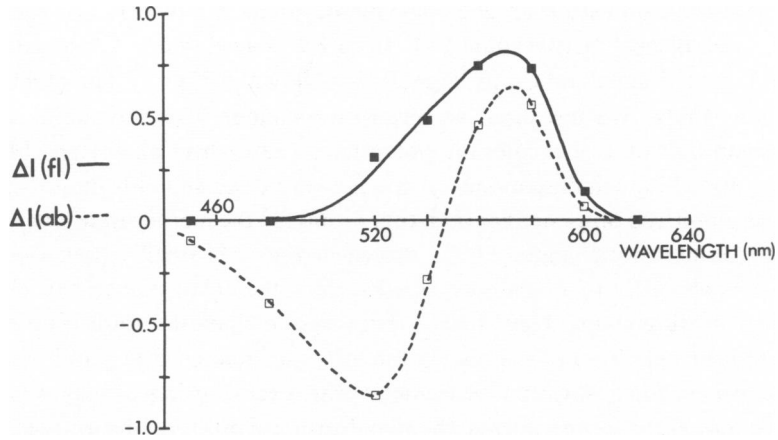

FIGURE 3 Intensity changes versus wavelength for fluorescence (filled squares) and absorption (open squares) measurements. The absorption increased during the spike at wavelengths longer than 550 nm and decreased at shorter wavelengths while there was a fluorescence increase at all wavelengths where a signal could be measured. The peak transmission of the interference filters is shown on the abscissa and the relative sizes of the changes in absorption, $\Delta I(ab)$, and fluorescence, $\Delta I(fl)$, are shown in arbitrary units on the ordinate. The experimental results were corrected for the variation in the efficiency of the apparatus as a function of wavelength. The filters had a width of 10 nm at half-height. The absorption changes were measured during action potentials; the fluorescence changes were measured during 50 mV depolarizing voltage-clamp steps using the fluorescence apparatus described in reference 2 with a Schott RG630 secondary filter (Schott Optical Glass Inc., Duryea, Pa.). The $\Delta I/I_{tr}$ for the absorption experiment was 2×10^{-4} at 580 nm, the $\Delta I/I_{fl}$ for the fluorescence experiment was 5×10^{-4} at 580 nm. The curves connecting the experimental points were drawn arbitrarily.

intensity transmitted by the resting axon, $\Delta I/I_{tr}$, was unaffected. This second filter blocked the fluorescence emission of the dye (emission maximum at 610 nm) but did not eliminate the intensity change. Additional evidence that the signal in Fig. 2 was the result of a change in absorption and not fluorescence was provided by the striking wavelength dependence of the signal. When wavelengths below 550 nm were used, the signal inverted (Fig. 3, dashed curve). The fluorescence change had a very different wavelength dependence; at all wavelengths where a signal could be measured there was an increase in fluorescence (Fig. 3, solid curve).

It is possible that the fluorescence and absorption signals are different expressions of a single alteration in the microenvironment of the dye. The membrane-bound dye might exist in equilibrium between two states having different absorption maxima and different quantum yields, for example a highly fluorescent monomer absorbing at about 570 nm and a slightly fluorescent dimer absorbing at about 520 nm (8, 10). If a depolarization caused a shift in the equilibrium toward the monomer, then wavelength dependences of the type found would be expected.

The dye that was used in the experiments illustrated in Figs. 2 and 3 was dye 138 in reference 2 and a close analogue of the merocyanine dye already reported (1). Similar experiments were carried out with an additional 27 dyes and it was possible to measure

a change in absorption with each one. The merocyanine dye used in previous experiments (1), available as "merocyanine 540" from Eastman Organic Chemicals (Rochester, N.Y.), gave a signal almost as large as that shown in Fig. 2. The other 26 dyes gave smaller signals. We measured an absorption change with rhodamine B, which was used as an indicator of membrane potential by Emrich et al. (5) on chloroplast membranes and Cd-arachidate monolayers. However, the signal-to-noise ratio with this dye was about 100 times smaller than that found for the merocyanine dye.

The relative intensity change, $\Delta I/I_{tr}$, shown in Fig. 2 is small, about one part in 10^4, and, it is an order of magnitude smaller than the relative intensity change in fluorescence measurements. Nevertheless, because the signal-to-noise ratio was proportional to the square root of the resting intensity (see reference 11), and because the resting intensity in the absorption experiments was several orders of magnitude larger than that in fluorescence experiments, the signal-to-noise ratios in absorption measurements were larger. For most dyes the signal-to-noise ratio for absorption changes was three to five times larger than that found for fluorescence measurements on the same apparatus with the same incident intensity. For the merocyanine dye (Fig. 1) this ratio was about 20 times larger in absorption measurements. We hope that the improvement in signal-to-noise ratio obtained with this technique will facilitate simultaneous optical monitoring of activity in many smaller neurons.

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