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Species-Specific Effects on the Optical Signals of Voltage-Sensitive Dyes

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Summary. The absorption changes of two merocyanine dyes in response to membrane potential changes were measured on several nueronal preparations to see whether the dyes would be useful in recording from these cells.

We were able to record large signals without averaging from barnacle and leech neurons. The greatest signal with WW375 was seen at 750 nm. Much smaller increases in transmitted light intensity were seen at all other wavelengths between 500 and 780 nm. In contrast, vertebrate neuronal preparations produced much smaller signals with an entirely different action spectrum. Essentially the same spectrum was seen in cells of the sympathetic ganglion of the bullfrog, *Rana catesbiana,* dissociated chick spinal cord neurons, or dissociated rat superior cervical ganglion neurons. In each case an action potential was accompanied by increases in transmitted light intensity between 500 and 600 nm and 730 and 780 nm, and decreases in intensity between 600 and 730 nm with the dye WW375, the best dye tested. Similar results were obtained with dye NK2367 on both vertebrate and invertebrate preparations, except that the spectral properties were shifted 30 nm towards the blue. Both dyes caused some photodynamic damage to the cultured neurons after a few minute's exposure to the illuminating light. Several analogues of these dyes were also tried, but did not produce larger signals.

Voltage-sensitive dyes suitable for detecting action potentials in neuronal tissue have been developed and studied during the past few years (reviewed in Cohen & Salzberg, 1978; Conti, 1975). These dyes offer the possibility of recording independently from large numbers of neurons or from very small neurons and neuronal processes. Neither of these kinds of measurements would be easy using microelectrodes.

Most of the recent research has been directed towards finding sensitive dyes (Cohen *et al.*, 1974; Ross *et al.*, 1977) or towards developing the technology to exploit them (Salzberg *et al.*, 1977). Currently, the best

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dyes change their absorption at a particular wavelength in proportion to changes in membrane potential. Synaptic potentials of 5–10 mV have been detected in 50 μm diameter cells and a simple apparatus has been constructed which could detect action potentials in 14 separate cells simultaneously. However, these dyes have not yet been used to examine neurophysiological questions and, consequently, their behavior has been tested on only a few preparations.

There are a number of interesting physiological problems involving dissociated neurons in culture which might be approached using this technique to record changes in membrane potential: e.g., a study of the circuits made by large groups of these cells, or a study of the development of excitability in the processes and growth cones of these cells. Experiments directed towards the first problem would use the dyes to record from large numbers of cells; the second would exploit the possibility of recording from small membrane areas. Our work was designed to see if the current level of development was adequate to explore these problems.

Our initial experiments used two of the best absorption dyes on dissociated neurons from the superior cervical ganglion (SCG) of the newborn rat. We found that these dyes behaved somewhat differently on these cells than had been previously reported on the squid giant axon (Ross *et al.*, 1977). Consequently, we examined a number of other preparations and found that there appeared to be a systematic variation in the behavior of these absorption dyes on neurons from different sources.

Some of these results have been reported in abstract form (Ross & Reichardt, 1977).

Materials and Methods

Preparations

Five different preparations were examined. Most experiments used neurons from the SCG of newborn rats. These cells were dissociated and cultured according to the methods of Mains and Patterson (1973). Saline for the electrophysiological and dye experiments consisted of 154 mM NaCl, 3 mM KCl, 7 mM $CaCl_2$, 4 mM $MgCl_2$, 3.5 mM Tris-HCl, adjusted to pH 7.6. The high divalents facilitated electrode penetrations. In one experiment, the full physiological saline (O'Lague *et al.*, 1974) was used with no change in results. Typical cell diameter was 40 μm.

Dissociated neurons from embryonic chick spinal cord were cultured according to the procedures of Fischbach (1972) and kindly provided to us by David Farb. Although there are several different kinds of neurons in the cultures (Farb, Choi & Fischbach, 1977), no attempt was made to distinguish among them. No variation relevant to these

experiments was noticed. Physiological saline for these cells was the same as for the SCG neurons.

Giant barnacles, *Balanus nubilus,* were supplied by David King of Friday Harbor, Washington, and the supraesophageal ganglion was dissected out. The ganglion was pinned to the bottom of a Sylgard (Dow Corning Corp.) coated petri dish and treated with 3 mg/ml Pronase (Sigma) for 5 min to soften the connective tissue (Hudspeth & Stuart, 1977). This procedure did not affect the optical measurements. Experiments were carried out on several of the large (40–80 μm) neurons of the ganglion. Physiological saline consisted of 461.5 mM NaCl, 8 mM KCl, 20 mM $CaCl_2$, 12 mM $MgCl_2$, 10 mM Tris-HCl, adjusted to pH 7.6 (Brown *et al.*, 1970).

Segmental ganglia from the leech, *Hirudo medicinalis* (kindly supplied to us by Ken Muller), were dissected out and pinned to the bottom of the same dishes as barnacle ganglia. Most of the experiments used the N and T sensory neurons (Baylor & Nicholls, 1969). Physiological saline consisted of 115 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 10 mM Tris-HCl adjusted to pH 7.4.

Some experiments were performed on principal cells from sympathetic ganglia of the bullfrog, *Rana catesbiana* (Blackman, Ginsborg & Ray, 1963) which were pinned out in similar Sylgard coated chambers. Physiological saline consisted of 115 mM NaCl, 2 mM KCl, 3.6 mM $CaCl_2$, 1 mM Na-HEPES, adjusted to pH 7.1.

Apparatus

The main equipment used to measure the optical signals is shown in Fig. 1 and is, in principle, the same as that described in Salzberg *et al.* (1977). All preparations were mounted in chambers fixed to the stage of a Zeiss WL microscope modified to have a fixed stage and a movable body. Constant illumination was provided by a 12V–100W tungsten-halogen lamp controlled by a regulated power supply (Kepco, JQE 0–25V, 0–10A). Cells were viewed through a long-working distance, water-immersion, 40×, phase objective (Zeiss 46-17-03). The long working distance allowed us to impale cells under the same objective used to carry out the experiments. Water-immersion was employed to eliminate the fluctuations in light intensity due to the vibrations of the air-water interface. Phase contrast improved the visability of the cultured neurons during impalement. Prior to optical measurements the condenser was switched from phase to bright field to maximize light intensity (the phase ring in the objective, caused only about 15% loss of light). A photodiode (UV-100B, E.G.G., Salem, Mass.) was mounted in the objective image plane above the microscope. It was connected to a manipulator so that the image of the cell could be centered on the diode. Accurate positioning was accomplished by using a trinocular body with a 75/25 beam-splitter which enabled us simultaneously to see the cells and focus an image of the substage diaphragm on the photodiode. Three apertures were used to restrict light reaching the detector to include only light passing through the cell and to reduce light scattering. The substage diaphragm was adjusted to just fill the field under 40×. The condenser iris was closed until a reduction in light intensity was just observed at the photodiode. The aperture in front of the photodiode was selected to conform to the magnified diameter of the cell of interest. This arrangement of apertures gave the maximum signal-to-noise ratio. In this configuration, the light power at the photodiode was 4×10^{-6} W at 750 ± 15 nm with an aperture selected for a 40 μm diameter cell. The fractional shot noise level at this wavelength (peak-to-peak at 1 kHz) was typically 5×10^{-5}. Vibrational noise increased this to about 10^{-4}.

The wavelength of illumination was determined by interference filters. These usually had a band width of 30 nm, blocked to 1.3 μm unless stated otherwise, and were obtained from Karl Feuer Associates, Upper Montclair, N.J. For wavelengths shorter than 690 nm an additional heat filter (Oriel #G-766-7100) was included.

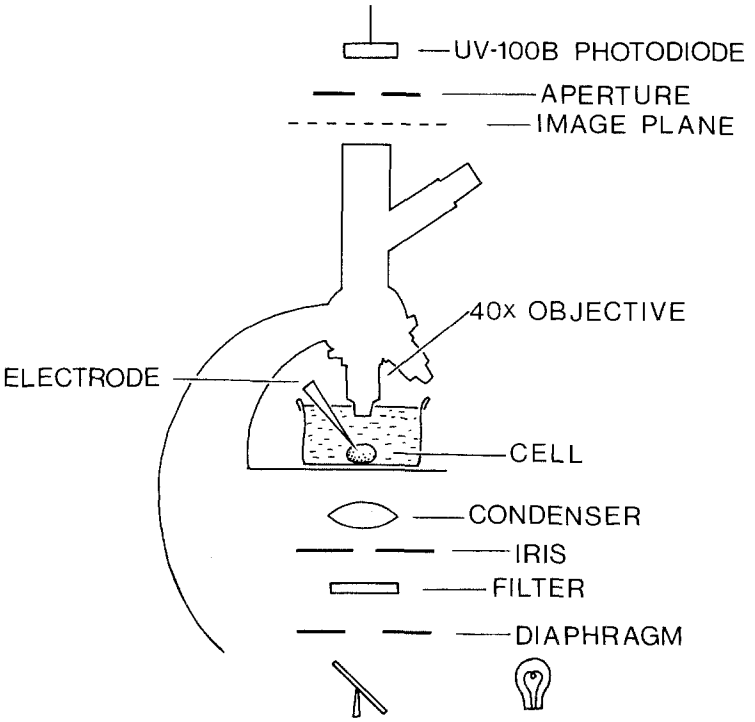


Fig. 1. Schematic diagram of the experimental apparatus. All components except the microelectrodes were mounted on a Zeiss WL microscope. The stage with heating and cooling devices is not shown. Also not shown are manipulators for positioning the photodiode assembly and the microelectrode

The microscope, micromanipulators, and the photodiode assembly were all bolted to the top of a vibration isolation table (EPOI, Inc., Garden City, N.Y.). This procedure reduced the vibrational component of the optical noise which was often large (Salzberg *et al.*, 1977).

The current output of the photodiode was converted to voltage and the dc component was capacitatively removed before it entered a Tektronix 3A9 amplifier and a Biomac-1000 signal averager. The amplifier restricted the bandwidth of the signal (usually to 1 kHz), and the signal averager stored or further processed the records (Cohen *et al.*, 1974).

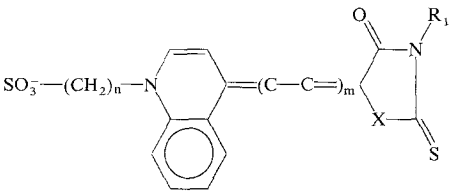
Cells were impaled with microelectrodes filled with 3 M KCl and stimulated through a bridge circuit in the WPI M4A electrometer. Principal cells of the frog sympathetic ganglion were excited by stimulating the preganglionic nerve using a suction electrode. Electrode resistances were 100–150 MΩ for the cultured neurons and 50–100 MΩ for neurons in ganglionic preparations.

When experiments were carried out at nonambient temperatures (4–37 °C), heating or cooling coils built into the stage were used to regulate the temperature.

Dyes

Most of the experiments described in this paper used dyes WW375 (a merocyanine-rhodanine, dye XVII of Ross *et al.*, 1977) or a close analogue, NK2367 (a merocyanine-

Table 1. Dyes Tested



Dye	N	M	R1	X
375	3	2	C_2H_5	S
NK2367	3	2	C_2H_5	O
433	4	2	C_2H_5	S
444	3	1	C_6H_{13}	S
448	3	1	C_6H_5 (phenyl)	S

oxazolone, Grinvald, Salzberg & Cohen, 1977). The two dyes are similar except for a shift of 30 nm to the blue for the peak of absorption spectrum of NK2367. NK2367 also bleaches faster and was therefore more difficult to use. Several other analogues were tested. The structures of all these dyes are indicated in Table 1. NK2367 was obtained from Nippon Kankoh-Shikiso Kenkyusho Co., Okayama, Japan. All other dyes were synthesized and kindly supplied to us by Dr. Alan Waggoner, Amherst College, Amherst, Mass. Solutions of these dyes were made immediately before use.

Protocol

The preparation or culture dish was positioned on the microscope stage with the cell of interest in the center of the field. The optics, including the photodiode, were aligned, and the microelectrode was positioned over the cell. The preparation was dyed for 10–20 min in a solution containing 1 mg/ml dye in physiological saline. None of the results of the experiments reported in this paper were very sensitive to variations in dye concentration (Fig. 5) or dyeing time (up to 40 min). The preparation was washed in physiological saline to remove excess dye and the cell penetrated under phase optics, using blue light to minimize bleaching and photodynamic damage (Pooler, 1972).

Results

When 40–60 μm diameter barnacle neurons which had been stained for 20 min in the merocyanine-oxazolone dye NK2367 were stimulated, we were able to detect changes in the absorption of light by the cells at 720 ± 25 nm. With our apparatus these "optical action potentials" could easily be seen without signal-averaging (Fig. 2). In some preparations changes in intensity corresponding to 5 mV IPSP's could be detected above the noise level. With WW375, signals of about the same amplitude

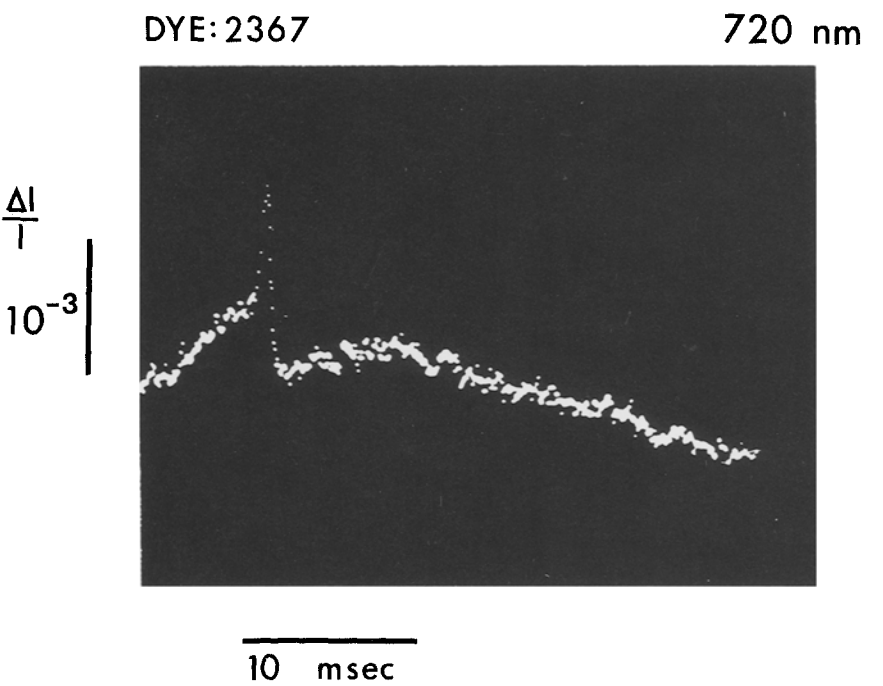


Fig. 2. Fractional change in transmitted light intensity during an action potential of a barnacle neuron stained with NK2367. A single sweep was photographed from the Biomac cathode ray tube; no averaging was used. The amplitude of the action potential measured with the WPI electrometer was 75 mV. The wavelength of the filter was 720±25 nm. The bandwidth of the 3A9 amplifier was set at 1 kHz

were obtained. The intensity changes seemed to correspond to the amplitude and time course of the potential change recorded intracellularly, although no detailed comparison was made (these properties have been tested on the squid axon for WW375 (Ross *et al.*, 1977) and NK2367 (L.B. Cohen, A. Grinvald, K. Kamino, S. Lesher, B.M. Salzberg, *unpublished observations*). These results confirm the results of Grinvald *et al.* (1977) and Salzberg *et al.* (1977). The action spectrum for dye WW375 is shown in Fig. 3. During the action potential there is a decrease in absorbance at all wavelengths tested with the maximum change at 750 nm. With dye NK2367 the action spectrum was shifted 30 nm towards the blue (peak at 720 nm).

Optical signals from dyed SCG neurons had a different character, however. Figure 4 shows that we were able to detect changes in absorbance which corresponded to the action potential stimulated in these

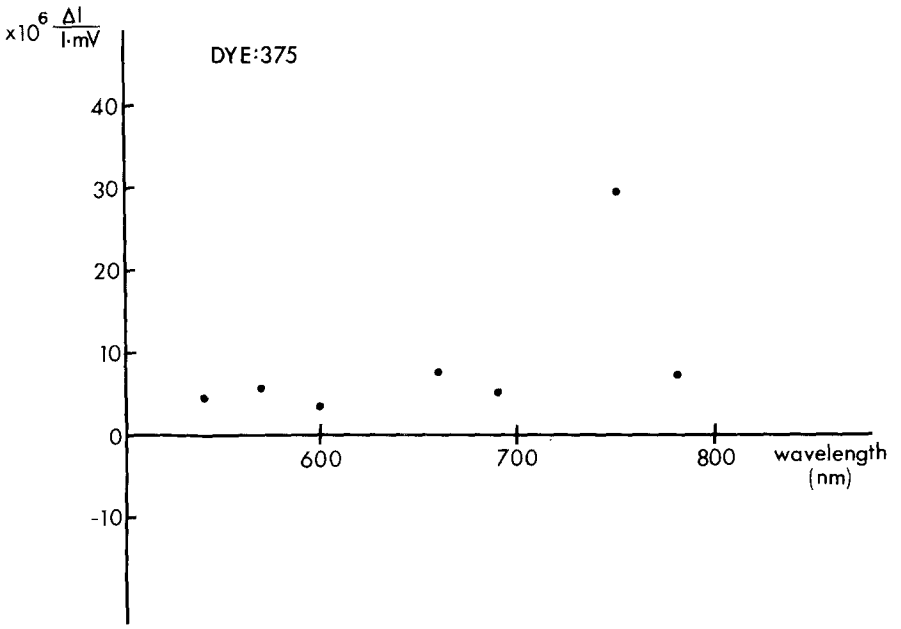


Fig. 3. Wavelength dependence of the fractional change in intensity of light transmitted through a stained barnacle ganglion while a single neuron was stimulated to fire and action potential. The ganglion was dyed for 15 min with 1 mg/ml of WW375. Staining caused a decrease in intensity of 35% at 750 nm. All filters were 30 nm wide (FWHM). The intensity increased at all wavelengths during the action potential with the maximum change at 750 nm. A similar spectrum was obtained with NK2367 except that the peak was at 720 nm

cells. However, unlike the barnacle neurons, we were rarely able to detect these signals without signal averaging. In addition, the action spectrum with dye WW375 was radically different (Fig. 5*A*), with absorption increasing at some wavelengths and decreasing at others in response to an action potential in the cell. Maximum signals were obtained at 690 nm instead of the 750 nm measured in the barnacle neurons, and the change in absorption was of the opposite sign. At 750 nm, only a weak signal could be detected. The triphasic character of the action spectrum was insensitive to the concentration of dye used (Fig. 5*A*) or to the length of time the cells were dyed (up to 40 min). The resting absorption spectrum is shown in Fig. 5*B* for comparison. Changing the temperature from room level (20–22 °C) to 37 °C also had no effect on the spectrum. These experiments were repeated with dye NK2367. The optical signals with this dye were approximately of the same size and had a similar action spectrum, but shifted 30 nm to the blue. Again

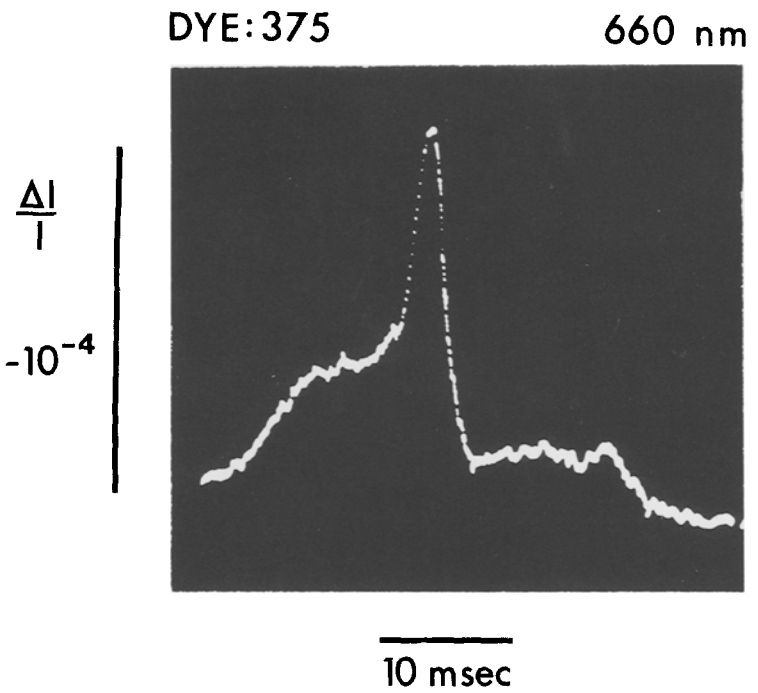


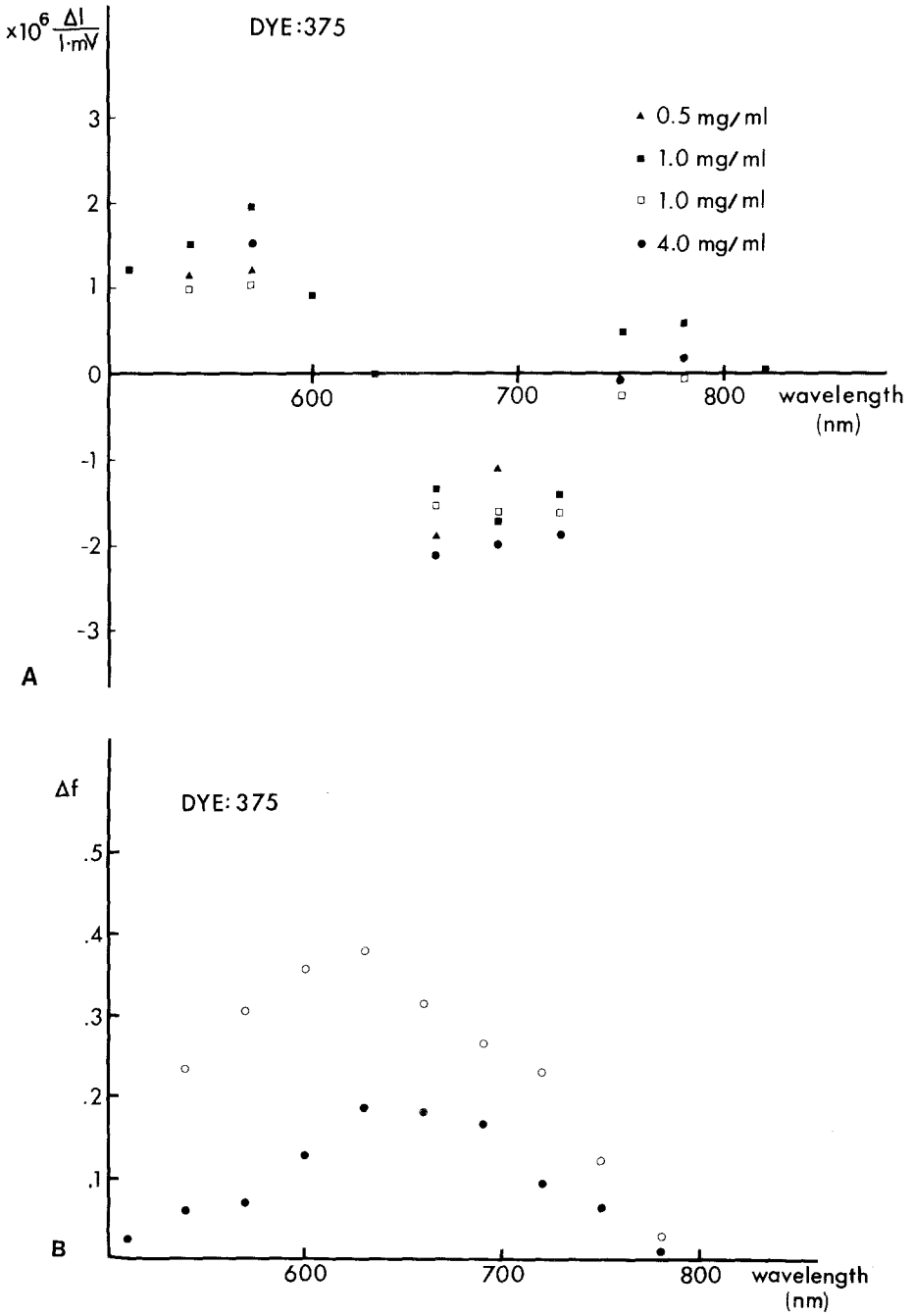
Fig. 4. Fractional intensity change during an action potential of an SCG neuron stained with WW375. 64 sweeps were averaged; the action potential amplitude was 65 mV. The filter was 660±15 nm and the 3A9 bandwidth was 1 kHz. A slight jitter in the firing time and the low temperature (20 °C) are probably responsible for the increased width compared with that normally recorded from these cells (O'Lague *et al.*, 1974). Note that the intensity decreased during the action potential, the opposite of Fig. 2

we found that this result was insensitive to dye concentration or dyeing time.

Other Preparations

Several other preparations were tested to see if the action spectrum of the dyes showed any systematic behavior. When WW375 was applied to sensory neurons of the leech segmental ganglion, action potentials could be detected without signal averaging. The wavelength of maximum

Fig. 5. (*A*): Wavelength dependence of the fractional change in intensity of light transmitted through stained SCG neurons during the action potential. The cells were dyed for 15 min with the indicated concentrations of WW375. At all concentrations a triphasic action spectrum with the maximum change at 690 nm was obtained. A similar spectrum was obtained with NK2367 except that the peak was at 660 nm. (*B*): Wavelength dependence



of the fractional change in intensity of light transmitted through unstimulated SCG neurons when they are stained. Spectra from two cells from two culture dishes are shown, each dyed for 15 min with 1 mg/ml of WW375. The different amplitudes reflects the variability of the procedure. The slight shift in peak position may be a consequence of aggregation of the dye (West & Pierce, 1965)

Table 2. Classification of preparations according to the type of action spectra measured with dye WW375[a]

Monophasic	Triphasic
Barnacle neurons	Frog sympathetic neurons
Leech neurons	Frog muscle fibers[d]
Squid axons[b]	Dissociated rat SCG neurons
Aplysia neurons[c]	Dissociated chick spinal cord neurons

[a] These spectra were classified according to measurements made at 540, 570, 600, 660, 690, 720, 750, and 780 nm. For those species whose spectra are not shown in the text or published elsewhere, the sign of the absorbance change at each wavelength and the wavelength of maximum change were noted.

[b] Ross *et al.*, 1977.

[c] J.C. Woolum & F. Strumwasser (*personal communication*).

[d] Nakajima & Gilai, 1977.

absorbance change was 750 nm, and there was an increase in absorption at all wavelengths. For dissociated chick spinal cord neurons and principal cells of the undissociated frog sympathetic ganglion, a triphasic action spectrum was obtained with this dye. In neither of these latter two cases could the signals be detected without signal averaging. These results are included in Table 2.

Other Dyes

Because the signals with WW375 and NK2367 were too small to be detected without signal averaging on SCG neurons, several analogues of these dyes (Table 1) were evaluated for signal size. None of these dyes gave larger signals at any wavelength when applied to SCG neurons than were obtained with WW375.

Discussion

The main conclusion of this paper is that some voltage-sensitive dyes have different properties when applied to different preparations. With WW375 and NK2367 we found that the ability to follow the action potential with a proportional change in absorption was maintained when they were applied to different kinds of neurons. However, the action spectrum of this effect was clearly different on SCG neurons when com-

pared with that measured on barnacle neurons. Also, the magnitude of the effect, i.e., the percentage change in intensity for a specific change in voltage, was much smaller on the cultured neurons (this result also holds for the percentage change in absorption although the presence of glial cells and connective tissue makes a quantitative statement difficult).

Table 2 shows that the preparations tested could be grouped into two distinct classes based on their measured action spectra. In the first class action potential signals could be detected without signal averaging, the absorption increased at all wavelengths (monophasic), and the maximum change was at 750 nm (for WW375) or 720 nm (for NK2367). In the other group the signals were much smaller (usually requiring signal averaging to be detected); the absorption increased at some wavelengths and decreased at others (triphasic action spectrum). The peak absorption was usually at 690 nm (WW375) or 660 nm (NK2367). In this table we have included results from other published and unpublished work.

Examination of the table shows that several possible explanations for the two classes of optical signals can be eliminated.

1) Dissociating and culturing the SCG and spinal cord neurons cannot be the cause since similar spectra were obtained from the intact frog sympathetic ganglion and from frog muscle fibers. Therefore, the changes in the cell surface brought about by dissociation are not determining factors in the optical signals.

2) Ionic strength and composition of the physiological saline are not responsible since the squid axon and barnacle are essentially in seawater (osmolarity 960 mosmol) and the leech ganglion is in a low ionic strength saline (230 mosmol). These three preparations gave essentially the same optical signals. Yet the cultured neurons, in an intermediate strength saline (325 mosmol) had different spectra. Also, adding vitamins, glucose, and serum to the SCG saline did not affect the spectrum.

3) Temperature was not significant as the same spectrum was obtained from SCG neurons at 20 and at 37 °C. Also, the difference between cold blooded (frog) and warm blooded (rat and chick) vertebrates was not significant.

4) Cell geometry: On squid axons dyed with WW375, different action spectra were obtained from the center and edge of the axon using polarized light (Ross *et al.*, 1977), suggesting that geometrical factors might be significant in determining the action spectra on different neurons. However, two pieces of evidence indicate that geometry is not the main

determining factor of the difference noted in these experiments. First, different spectra were obtained from neurons of the barnacle ganglion and from the frog sympathetic ganglion; both have cells with approximately spherical somata. Also, spectra from the two classes were obtained from the squid axon and from frog muscle fibers, both having cylindrical geometries. [Nakajima and Gilai (1977) argue that the muscle T-system (Oetliker, Baylor & Chandler, 1975) cannot be the cause of the difference since the same spectrum was obtained from the edge or center of the fibers.] Second, the same triphasic action spectrum was obtained from dissociated SCG neurons using both an aperture which collected light from the whole cell and with a smaller aperture which only collected light from the center of the cell body, suggesting that there was no great difference between signals from the center and edge of these cells.

A correlation which remains after these explanations have been eliminated is that the monophasic spectra were all obtained from invertebrates (squid, barnacle, aplysia, and leech), while the triphasic spectra were obtained from vertebrate neurons (frog, rat, and chick)[1].

One possible explanation for the difference in the action spectra is that the dye exists in two different states on the membrane, each giving one of the observed responses. On some membranes the dye would have a preference for one state, on other membranes a preference for the other. If the membrane composition of neurons differed in a systematic manner, some support could be given to this idea. However, very little is known about the composition of neuronal membranes (Strichartz, 1977) leaving this explanation as only a suggestion. Since it is known (Waggoner, Wang & Tolles, 1977) that optical signals can be obtained from dyed artificial bilayer membranes, it should be possible to examine this question by putting dyes on membranes of varying composition.

Practical Consequences

This investigation began as an evaluation of several dyes as monitors of membrane potential in cultured SCG neurons. Clearly, optical signals can be obtained from these cells which are faithful representations of

[1] (However, aplysia neurons, dyed for 15 hr, show a triphasic action spectrum, although different from that seen on SCG neurons (J.C. Woolum & F. Strumwasser, *personal communication*). It is not known whether this change from the monophasic spectrum (seen after short dyeing times or in the presence of ascorbate) is a consequence of cell deterioration, slow penetration of these relatively impermeant dyes (Ross *et al.*, 1977), or some other cause.)

the potential change. However, since the signals can only be detected with signal averaging, the utility of these dyes would be limited. Nonrepetitive action potentials or synaptic potentials could not be detected, and signals from the processes and growth cones would require extensive averaging.

We also found that photodynamic damage was a problem with dyes WW375 and NK2367 when applied to SCG neurons. Although this problem is relatively unimportant with squid axons and barnacle neurons (Ross *et al.*, 1977; Salzberg *et al.*, 1977), we found that the action potential in dyed SCG neurons rapidly broadened and eventually disappeared (usually within 2 min) when constantly illuminated at either 660 or 720 nm (undyed cells were not damaged). The existence of significant photodynamic damage would make it difficult to improve the optical signal by increasing the light intensity.

A preliminary examination of several other dyes (Table 1) was made, but none gave larger signals than WW375 on SCG neurons. An extensive testing of other dyes will probably be required to find an adequate probe for these cells.

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