IMPROVED FLUORESCENT PROBES FOR THE MEASUREMENT OF RAPID CHANGES IN MEMBRANE POTENTIAL

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ABSTRACT To improve the quality of fluorescent voltage-sensitive probes twenty new styryl dyes were synthesized. Some of the new probes are significantly better than any used in the past. A signal-to-noise ratio of 90 root mean square (rms) noise was obtained for an optical recording of action potentials from neuroblastoma cells maintained in monolayer culture. The fluorescence fractional change of the optical signal is as large as 14%/100 mV. Photodynamic damage and bleaching are much less significant with the new probes. These fluorescent probes can be used to measure small and rapid changes in membrane potential from single cells maintained in monolayer cultures, from single cells in invertebrate ganglia, from their arborization, and from other preparations. The optical measurement can be made with a standard fluorescent microscope equipped with DC mercury illumination. Guidelines for the design of even better fluorescent probes and more efficient instruments are suggested.

INTRODUCTION

The membrane potential is an important property of many cells and organelles. Changes in membrane potential control or accompany numerous biological processes, such as information transfer in neuronal networks, muscle contraction, and energy transduction during photosynthesis or metabolism. The measurement of such changes in membrane potentials, however, is often difficult or impossible because of the small size of the object under investigation or for other technical reasons.

Transmission and fluorescence signals of voltage-sensitive molecular probes have provided a new and sometimes powerful method for measuring changes in membrane potential in a wide variety of preparations (Cohen et al., 1978; Cohen and Salzberg, 1978; Waggoner, 1979).

Generally, fluorescence measurements are more sensitive than transmission measurements when the number of probe molecules is small (i.e., a small object), and provided that background fluorescence is negligible (Hirshfeld, 1977; Rigler et al., 1974). Thus, fluorescence rather than transmission is the method of choice for measuring membrane potential changes in small single cells and in segments of cells such as nerve processes. These theoretical predictions (Waggoner and Grinvald, 1977) have been realized in practice (Grinvald et al., 1981 *b;* Grinvald and Farber, 1981).

Much progress has been achieved in the development of voltage-sensitive optical probes for the measurement of rapid changes in membrane potential (Cohen et al., 1974; Ross et al., 1977; Gupta et al., 1981). However, for several applications, certain physicochemical properties of the optical probes make the optical experiments difficult, thus limiting their general usefulness. The most important of the properties of optical probes that have required improvement are voltage sensitivity, absence of photodynamic damage, bleaching, and pharmacological side effects induced by the binding.

Styryl dyes are a subgroup of the cyanine dyes. They were first found to give rather small optical signals (Cohen et al., 1974). However, the successful synthesis of better analogues led to the discovery of much larger signals (Grinvald et al., 1978; Gupta et al., 1981). Other styryl dyes have been designed by Loew and his colleagues (Loew et al., 1978; Loew and Simpson, 1981), who introduced a theoretical approach into the design of electrochromic (voltage-sensitive) probes and thereafter studied the dyes on artifical bilayers.

In this work we report the design and properties of 20 new styryl dyes, including the most sensitive fluorescent probes currently available. Dyes were tested on both mammalian and invertebrate neurons. In addition, we describe improvements in a simple apparatus that can be used to measure membrane potential changes in small cells or in neuronal processes. Preliminary results have been published (Grinvald et al., 1980; Grinvald et al., 1982 *a*).

METHODS

Cell Cultures

Mouse neuroblastoma clone N1E-115 was obtained from M. Nirenberg. The cells were grown as described (Kimhi et al., 1976). Dishes were used 3–20 days after reseeding.

Leech Ganglia

Leeches (*Hirudo medicinalis*) were obtained from Ricarimpex (France). The segmental ganglia were dissected as described (Nicholls and Baylor, 1968).

Electrophysiology

For the optical experiments with tissue culture cells, the culture medium was changed just before use to a balanced salt solution containing 144 mM Na^+, 5.5 mM K^+, 5 mM Ca^{+2}, 0.8 mM Mg^{+2}, 20 mM HEPES buffer, 0.25% glucose, and ~10 mM sucrose. The pH was adjusted to 7.2 and the osmolarity to 340 mosmol/liter. Leech physiological solutions contained 115 mM Na^+, 4 mM K^+, 8 mM Ca^{+2}, and 10 mM Tris maleate adjusted to a pH of 7.4.

Cells were impaled with 20- to 25-MΩ thin-wall glass microelectrodes (Frederick Haer, Brunswick, Maine). A W.P. Instrument electrometer was used (New Haven, CT.).

Apparatus

The fluorescence experiments were performed with an apparatus built around a Zeiss Universal microscope (Carl Zeiss, W. Germany) rigidly mounted on a vibration isolation table (Fig. 1). The general features of the optical apparatus are described elsewhere (Salzberg, et al., 1973; Grinvald et al., 1981 *b*). Recent modifications are described below. An HBO-100 mercury lamp (Osram, W. Germany) was used in the present experiments, rather than a xenon lamp or a He-Ne laser. The lamp was operated with an ATE 75-15M-regulated power supply (Kepco, Inc., Flusing, N. Y.).

Excitation wavelengths corresponding to the 546.1 nm or 577.0 and 579.0 nm mercury lines were selected with interference filters. A KG3 heat filter (Schott Optical Glass Inc., Duryea, Pa.) was inserted in front of the interference filter. An HPL (Zeiss) semisilvered mirror was used to direct the excitation light to the objective from the epi-illuminator. Scattered and reflected light was blocked with Schott glass OG-590 or with RG-610 cut-on filters. An EMI 9658R phototube was used to measure the fluorescence intensity.[1] A pinhole of variable size was placed in front of the photomultiplier to block the light from the field of view, except for a circle 20–120 μm in diameter in the object plane. To increase the resolution of the digitized signal, an additional amplifer having an automatic offset circuit was used: the output of the photomultiplier was first amplified and recorded by the computer. In addition, a sample-and-hold circuit sampled the amplified output for ~10 ms (immediately after the excitation shutter was opened) and a second differential amplifier further amplified the output of the first amplifier minus the average DC level obtained from the sample-and-hold at the sampling time. This high-gain signal also was stored by the computer (Fig. 1). Thus, a 12-bit analog-to-digital converter, having a range of ±10 V could digitize "fully blown" small signals, "ignoring" the "DC" part of the signal (Senseman and Salzberg, 1980).

A multiplexer/analog-to-digital converter (Model 600 11/AD, Adac, Woburn, Mass.) was used to record 10 different inputs, including electrophysiological data, the DC fluorescence intensity, the amplified fluorescence offset, and the reference monitor of the excitation intensity. The sampling interval per channel was 160 μs for each of the channels and could be slowed down under software control. The response time-constant of the photomultiplier amplifier combination was either 0.3 or 1.5 ms. The data was stored on RL01 hard disks for further analysis.

[1]A photodiode-amplifier combination is inherently noisier than a photomultiplier, and therefore inadequate to measure small signals if the light level is low. (At present, lower than 10^7 to 10^8 photons/ms.) However, a photodiode could be used to detect fluorescence signals (Salzberg et al., 1973), because the fluorescence intensity from a single cell in a leech ganglion was at least 10 times higher than that presently obtained from single isolated cells in dissociated cultures.

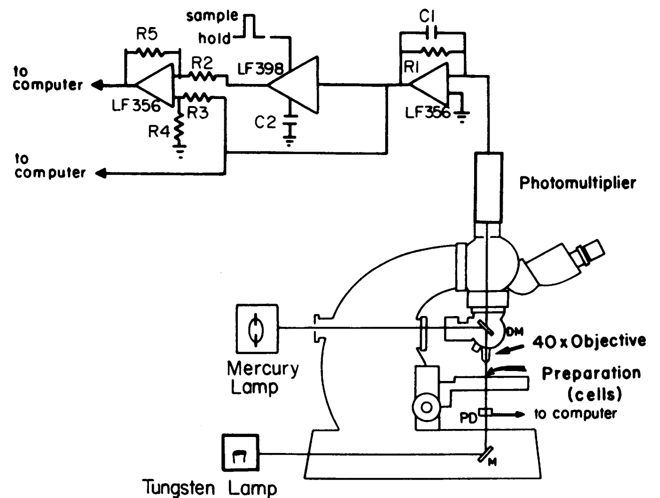

FIGURE 1 Schematic diagram of the apparatus used for fluorescence experiments. Fluorescence was excited with an HBO 100 mercury lamp and detected with an EMI 9658R phototube. The scheme for the electronic offset circuit is shown at the top. The resistors values were $R_1 = 20$ kΩ, $R_2 = R_3 = 1$ kΩ, $R_4 = R_5 = 22$ kΩ. The capacitors values were C_1 = 15 nF or 75 nF, C_2 = 0.47 μF. *DM* is either a dichroic mirror ar a semisilvered mirror. *PD* is a swing-in reference photodiode that measures the fluctuations in the excitation beam. (An alternative convenient position for the reference photodiode is just behind the dichroic mirror.)

Performance of HBO-100

Arc lamps are notoriously noisy due to arc wandering, but we found that if the HBO-100 was operated with a stable power supply (current of 5 A) a relatively stable light output could be obtained. When the power supply was used in the voltage mode, slow light fluctuations were large. However, utilizing the current limit mode the slow fluctuations were dramatically reduced. On the average, when 10 consecutive trials each lasting 500 ms were tested, in 8 trials the largest peak-to-peak noise was smaller than 5×10^{-4} (line frequency noise), and, typically, in only two trials were larger light fluctuations observed (occasionally as large as 10^{-2}). During averaging experiments, such poor trials can be rejected. The decision whether to include them in the accumulating average was made automatically by the computer after the reference trace was tested[2] (Fig. 1). A trial was rejected if $0.997 < Y_i/Y_{i+3} < 1.003$ for every fourth channel (Y_i is the light intensity detected by the reference detector at the ith time interval). The light intensity of the 546.1-nm mercury line is 50–100 times higher than that obtained with a 100 W tungsten halogen lamp and a 540-nm interference filter (70% peak transmission, 30 nm wide).

Bleaching Correction

Relatively strong light intensity was used, and even with these improved new probes 3–20% bleaching of the dye per 1 s of illumination was

[2]Note that the reference detector is positioned below the objective, where the intensity of the excitation light is at least four orders of magnitude larger than that of the fluorescence signals. Voltage-dependent transmission signals are not larger than $2–5 \times 10^{-4}$ and therefore also do not interfere with the rejection procedure. In fact, the same reference trace can be used for ratio recordings, which would correct the systematic line frequency noise in the excitation source up to the level of 10^{-4}.

observed. The bleaching time course is a complex function that can be fitted to a multiexponential decay or a polynomial. When the dye was left in solution, the recovery of the fluorescent intensity was rapid, with almost complete recovery in 10 s. Under these conditions, the time course of the bleaching was usually reproducible within ~0.5%.

Clearly, the bleaching of the dye distorts the time course of the voltage-dependent fluorescence change. Each fluorescence record was therefore corrected for the bleaching. In each experiment the exact time course of the bleaching was measured by repeating the experiment without electrical stimulation. The details of the correction procedure are described in the Appendix.

Staining with Voltage-sensitive Probes

Staining solutions were freshly prepared before each experiment from a stock solution of ethanol saturated with the dye. The dye was diluted into the physiological solution, and a concentration of 0.5–5.0 μM of the dye was used. Ethanol was diluted by at least 1,000-fold. The dye in solution was much less fluorescent than the membrane-bound dye, thus it was possible to leave it in the bath during the measurement. If the dye was washed out, the cells remained stained for several hours, depending on the hydrophobicity of the dye. The permeability of the dye was tested by visual inspection of the fluorescent cells. Initially, the stained cells exhibited "fluorescence rings" (only the membrane is fluorescent), indicating that the styryls do not quickly permeate the membranes of live neurons. However, with some dyes, after half an hour the cell interior was also bright, indicating that the dye had penetrated the cell membrane. The permeation was slowed even further (1–3 h) if the dye was washed away after the staining period. Therefore it is recommended to wash the dye out. Such washing also reduce background fluorescence from dye binding to the bottom of the dish. Dye permeation rate depends on the preparation and the dye structure. Dead cells are heavily stained. If they are present in the pinhole field of view, their bright fluorescence results in a degradation of the signal.

Synthetic Procedures

The dyes were synthesized according to the general procedure described by Brooker et al. (1951). The synthetic procedures for anhydro-4-(4′-*p*-dibutylaminophenyl-buta-1′,3′-dienyl)-1-δ-sulfobutylpyrydinium hydroxide (RH-160) are described below as an example.

The dye was synthesized by condensing a quaternary salt with an aldehyde. The quaternary salts were prepared according to the procedure described by Brooker et al. (1951). γ-Picoline and butane sultone (1, 4) were heated at 120°C for 15 min. After cooling, the white precipitate was washed with ether.

The synthesis of *N,N*-dibutylaminocinnamaldehyde involves the substitution of *N,N*-dibutylaniline with methylanilinopropen-1-al-3, through a Vilsmeier-type reaction, using $POCl_3$ to yield the dibutylaminocinnamaldehyde (Jutz, 1958). The resulting aldehyde was purified by column chromatography on alumina equilibrated with a mixture of 95:5 hexane-ethyl acetate and eluted with a 9:1 mixture of the same solvents and then used without further purification. It was identified by mass spectroscopy (*m/e* 259) NMR and visible spectrum (λ_{max} = 402 nm in ethanol).

The quaternary salt and *N,N*-dibutylaminocinnamaldehyde (1 mM of each) were suspended in 5 ml of methanol. Piperidine (0.5 ml) was used as a condensing agent. The mixture was heated at 110°C for 30 min and then kept at 0°C for 2 h. The resulting red crystals were washed with ether. Pure dye (200 mg) was obtained by two recrystallizations from methanol.

The purity of the dye was assessed by thin-layer chromatography. It appears as a single red spot after development with a 9:1 acetone:water mixture. Iodine staining revealed no impurity. The melting point of the pure dye is 275°C. Its absorption maximum is 526 nm, the value of ϵ is 51,000 $mol^{-1} \times cm^{-1} \times$ liter, and its fluorescence peaks at 685 nm in ethanol solution (uncorrected).

RESULTS AND DISCUSSION

The Fluorescence Signal

Fig. 2 illustrates a comparison between electrical recordings and fluorescence recordings using anhydro-4-(4′-*p*-dibutylaminophenyl-1′,3′-dienyl)-1-δ-sulfobutylpyridinium hydroxide dye (RH-160). Evidently, the time-courses of the electrical and the fluorescence recordings are the same.

The sensitivity of the dye can be characterized by the value of the normalized fractional change in the fluorescence. In this experiment the normalized fractional change is 12%/100 mV potential change. This is the largest fractional change observed thus far in measurements from single cells in culture, and is about twice as large as the corresponding value for the oxonol dye, WW 802 (Grinvald et al., 1981 *b*). (The true fractional change of the membrane-bound dye is probably even larger, because an unknown amount is bound to surface membrane proteins and other nonspecific binding sites covering the sticky surfaces of these cells.)

The signal-to-noise ("typical" peak-to-peak noise) ratio obtained in this experiment was 30:1 for a single sweep, again the largest value obtained thus far for this type of experiment. (The signal-to-noise [rms] ratio was better than 90.)

Side Effects

In this experiment, to investigate the pharmacological side effects of the dye, the cell was impaled with a microelec-

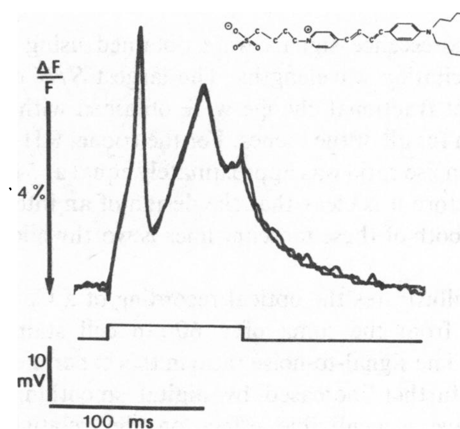

FIGURE 2 Comparison of electrical and fluorescence recordings. The cell was impaled with a microelectrode and stained for 5 min with 5 μM of RH-160. The cell was then stimulated with a current pulse (lower trace), and the fluorescence change was recorded simultaneously with the electrical recording. The time constant of the electrical and optical detection system was 1.5 ms. The thin trace is the electrical recording. The noisy trace is the optical recording. Averaging was not used. The structure of the dye is shown at the top. The arrow in each figure shows the fractional change of the fluorescence. Its direction indicates that upon depolarization the fluorescence decreases. The normalized fractional change in this experiment was 12%/100 mV.

trode before staining. During the staining with a 2-μM concentration of the dye, the membrane potential was usually hyperpolarized by 2–4 mV, and the shape and size of the action potential remained almost unaltered. Turning the bright illumination onto the cells induced a fast 2- to 4-mV reversible hyperpolarization of the membrane potential. A fast depolarization was also observed with other types of dyes, and the direction of the voltage change was very sensitive to the dye structure (Farber and Grinvald, unpublished observation). Photovoltaic effects were measured in artificial membranes (Ullrich and Kuhn, 1972; Huebner, 1975; Baker et al., 1979). However, more experiments are required to elucidate the mechanism of the present small light-induced potential change. Pharmacological side effects on synaptic transmission remain to be evaluated for every given preparation.

Photodynamic Damage and Bleaching

The photodynamic damage induced by this dye depends on the concentration. Fifty trials each lasting 250 ms led to 15-mV depolarization and widening of the action potential. This level of photodynamic damage is about five times lower than that of the best oxonol dye used (WW 802). It is about 200 times lower than that of some merocyanine dyes, which give large signals but kill the cells in a single trial (e.g., M540 or WW 591, Ross and Grinvald, unpublished results). The extent of dye bleaching was about 3% in 350 ms, i.e., three to four times smaller than that obtained with the oxonol, WW 802.

Wavelength Dependence of the Fluorescence Signals

Large fluorescence signals were obtained using two different excitation wavelengths. The largest S/N ratio and the largest fractional change were obtained with 546-nm excitation for all of the dienes. For the triene, RH-237, the signal-to-noise ratio was approximately equal at 546 or 578 nm; therefore it is clear that the design of an interference filter for both of these mercury lines is worthwhile for this dye.

Fig. 3 illustrates the optical recording of a Ca^{2+} action potential from the soma of a 60-μm cell stained with RH-237. The signal-to-noise ratio in this experiment could be even further increased by digital smoothing, which would have a negligible effect on the relatively slow time-course of this signal. Evidently when such slow signals are measured, slower RC filters can also be used, and the illumination intensity can be significantly reduced to minimize photodynamic damage and bleaching.

Sensitivity of other Analogues

Twenty different styryl dyes were synthesized. We tested most of the dyes on the cultured cells. The dye structures and the fractional changes of the fluorescence signals obtained in these experiments appear in Table I.

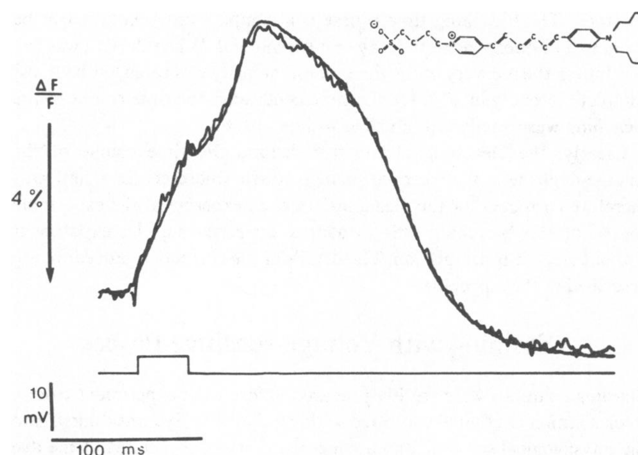

FIGURE 3 Comparison of electrical and fluorescence recording of a Ca^{2+} action potential. As described in Fig. 2, except that a 2-μM concentration of RH-237 was used for the staining. The structure of the dye is shown at the top. The experiment was carried out in normal medium containing 10 mM tetraethyl ammonium and 5×10^{-6} M tetrodotoxin. The lower trace shows the timing of the stimulating current. Four sweeps were averaged. The fractional change was 12%/100 mV.

TABLE I
STRUCTURE AND PROPERTIES OF NEWLY SYNTHESIZED STYRYL DYES

$$y-(CH_2)_n-\overset{\oplus}{N}\langle\text{pyridinium}\rangle-(C{=}C)_m-\langle\text{phenyl}\rangle-N(C_lH_{2l+1})_2$$

	RH	Y	n	m	L	λ^{*}_{max}	$\Delta F/F$(%)‡
1	246	SO_3^-	5	2	4	524	4
2	237	SO_3^-	4	3	4	544	14
3	330	SO_3^-	4	3	1	520	1
4	160	SO_3^-	4	2	4	526	12
5	293	SO_3^-	4	2	3	516	3
6	295	SO_3^-	4	2	2	516	2
7	270	SO_3^-	4	2	1	506	7
8	364	SO_3^-	4	1	4	500	—
9	369	SO_3^-	4	1	1	510	—
10	162	SO_3^-	3	2	4	526	4
11	332	SO_3^-	3	2	1	506	—
12	365	SO_3^-	3	1	4	500	—
13	348‖	SO_3^-	CH_2-ϕ	2	4	528	2
14	347‖	SO_3^-	CH_2-ϕ	2	1	512	>1
15	370‖	SO_3^-	CH_2-ϕ	1	4	512	—
16	376	PO_3^-	4	2	4	524	6
17	371	PO_3^-	4	2	1	502	0
18	292	$(Et)_3N^+$	3	2	4	550	7
19	355	$(Me)_3N^+$	3	2	1	520	2
20	46§	SO_3^-	4	2	4	572	>1

*Peak absorption in absolute ethanol
‡Normalized fractional change in fluorescence %/100 mV
‖Alkyl chain was replaced by benzyl groups
§Pyridinium chromophor was replaced by quinolinium

The most sensitive dye for the cultured cells is RH-237, but many analogues also give large signals. Uncharged zwitterionic dyes (e.g., RH-237, RH-160) and doubly positively charged dyes (RH-292) or negatively charged dyes (RH-376) all give signals of similar size. Loew and Simpson (1981) reported a similar observation in artificial bilayers, where zwitterionic and singly positively charged dyes gave nearly equal signals. However, a rather poor correlation was found between the characteristics of their cationic dyes (which have a delocalized charge) in artificial bilayers and the results obtained with the same dyes in voltage-clamp experiments on squid giant axons (Cohen, Grinvald, Salzberg, and Loew, unpublished results). A good correlation was obtained between the sensitivities of the newly synthesized dyes in neuroblastoma cells and in squid giant axons (Cohen and Grinvald, unpublished results). However, a poor correlation was observed for *Aplysia* neurons, rat dorsal-root-ganglia cells, and chick ciliary ganglion neurons (Farber, Bodmer, Gilbert, Pine and Grinvald, unpublished results). These results stress the importance of testing the dyes on the relevant biological preparation.

Many of the dyes listed in Table I are sparingly soluble in water and give large signals in the micromolar concentration range (e.g., RH-160, 162, 237, 246). Other analogues are hygroscopic and highly soluble in water (e.g., RH-270, 292, 376), as a result of their more hydrophilic substituents (Table I).

The availability of a set of voltage-sensitive probes should be helpful in optimizing the probe selection for a given preparation in terms of maximizing the signal size and minimizing undesirable side effects. In addition, probes with different charges should be helpful as a control for nonspecific charge effects in situations in which other controls are difficult to use. Charged styryls have also proved useful for iontophoretic injections into neurons (Grinvald et al., 1982 *b*). It is possible that a mixture of positive and negative dyes would minimize pharmacological side effects that may originate from the addition of fixed charges to the membrane (Krasne, 1977).

Absorption and Fluorescence Spectra

The values of λ_{max} (Table I) indicate that the absorption spectrum is sensitive both to the conjugate chain length and to the alkyl substituents on the anilino nitrogen. Fig. 4 shows the absorption and the fluorescence spectra of three analogues with a variable conjugate chain length. The absorption spectra of the dyes are relatively wide, probably reflecting a high degree of sensitivity of the spectra to the molecular microenvironment. The shift of the fluorescence spectra with respect to the absorption spectra is 170 nm, which is very large in comparison with the shift of 20 nm observed for cyanine dyes (Sims et al., 1974). The large shift indicates that the electronic structure of the relaxed excited state is very different from that of the ground state.

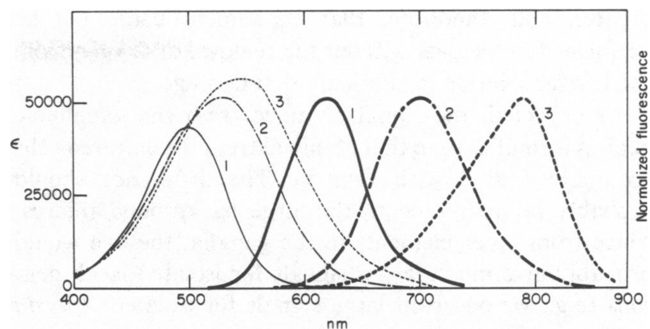

FIGURE 4 Absorption and fluorescence spectra of three styryl analogs with different chain lengths. (*1*) RH-364; (*2*) RH-160; (*3*) RH-237. Solvent: absolute ethanol. Each additional vinyl group shifts the peak of the absorption spectra 20 nm to the red. The fluorescence is shifted by ~40 nm. The absorption spectra were measured with a Bausch and Lomb spectronic 500 spectrophotometer. Uncorrected fluorescence spectra were measured with a Perkin-Elmer A44 fluorimeter. (The absorption and fluorescence of these dyes is different when they bind to membrane rather than ethanol.)

In such cases, for the design of fluorescent voltage-sensitive probes, theoretical approaches which deal with the excited state and the fluorescence should be more useful than those which deal only with the ground state, the Frank-Condon excited state, or absorption.

Measurements from Invertebrate Ganglia

Fluorescence recordings of action potentials for a P cell in the segmental ganglia of the leech are shown in Fig. 5. The size of the normalized fractional change was 0.8%/100 mV. This value is approximately six times larger than that obtained in the pioneering experiments of Salzberg et al. (1973) in which merocyanine 540 was used. (It should be considered a lower limit because the size of the pinhole that blocked the background light from other cells was larger than the image of the P cell.) Moreover, the photodynamic damage with merocyanine 540 was at least 200 times

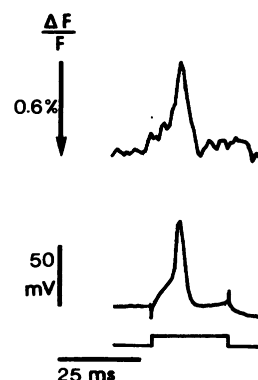

FIGURE 5 Fluorescence recordings of action potential from a P cell in the segmental ganglion of the leech. The ganglion was stained with 5 μM RH-237. The cell was positioned in the center of the field, and a pinhole (equivalent to 100 μm on the ganglion) at the objective image plane blocked the fluorescence from the rest of the ganglion. Four trials were averaged. The response time of the optical detection system was 1.5 ms.

greater, and, therefore, that experiment could not be performed in the past without the removal of oxygen from the Ringer solution to eliminate that damage.

As expected, the signal obtained from the ganglionic neuron is smaller than that obtained from the cultured cells (compare Fig. 2 with Fig. 5). The difference should probably be attributed to the large background fluorescence from other elements in the ganglia; the dye would probably give much larger signals for isolated leech neurons (e.g., we observed large signals for isolated *Aplysia* neurons maintained in culture).

Dye Structure, Signal Size, and Species-Specific Effect

Four dyes were tested on the leech central nervous system. The results indicated that RH-237 is the most sensitive. The signals obtained by its shorter dimethylamino analogue (RH-270) were six times smaller. RH-293 and RH-295 were similar to RH-270. When the same dyes were tested on the cultured cells all of these dyes gave large signals with fractional changes of similar size (Table I). In cultured *Aplysia* neurons, RH-237 and RH-160 exhibited poor sensitivity, but RH-376 was sensitive enough to permit single sweep recordings from the processes of these cells (Bodmer, Farber, and Grinvald, unpublished results). Evidently, the relationship between signal size and chemical structure is a function of the preparation. Loew and Simpson (1981) predicted that by using a theoretical approach it would be possible to design species-independent electrochromic probes. The present data, together with previous experiments with other preparations and other dyes (Ross and Reichardt, 1979; Grinvald et al., 1981 *b*), strongly emphasize the importance of the tedious semiempirical approach of designing many analogues of voltage-sensitive probes and of the search for the optimal analog for any given preparation. Evidently, many more presently unknown variables must be included in any theory to make it a powerful and practical tool in general probe design. It was our repeated experience that whenever a "kit" of voltage-sensitive dyes was available, the selection of the optimal probe was relatively easy: the recommended procedure is to start with visual inspection of the staining quality using a fluorescence microscope. At the second stage, only those dyes that nicely stained the cells (formed bright fluorescence rings) should be tested as illustrated in Fig. 2.

Possible Future Improvements in the Signal-to-Noise Ratio

It is important to analyze the prospects for even larger signals that would further simplify the use of the present technique. Our analysis briefly considers both spectral parameters and instrumental factors.

Spectral Parameters

The theoretical derivation of the expected signal-to-noise ratio as a function of spectral and instrumental parameters for a generalized two-state mechanism has been described (Waggoner and Grinvald, 1977):

$$\Delta F/F = (\epsilon_2 Q_2 - \epsilon_1 Q_1) \cdot \Delta n/F \quad \text{(fractional change)} \tag{1}$$

$$(S/N)_F = (\Delta F/F) \cdot (2\tau \cdot q)^{1/2} \cdot (g \cdot F)^{1/2}. \tag{2}$$

In Eqs. 1 and 2, ϵ_1 and ϵ_2 are the extinction coefficients for the dye in state 1 and state 2, and Q_1 and Q_2 are the corresponding quantum efficiencies of the dye, Δn is the number of molecules which move from state 1 to state 2 under the influence of the changing electrical field, τ is the rise time of the detector circuit, q is the quantum efficiency of the photomultiplier, g is the fraction of the fluorescence that the detector optics will capture, and F is the total value of the fluorescence, including the nonspecific background fluorescence.

A quantum efficiency of 0.3 for a membrane-bound styryl dye (with one double bond), was determined (L. Loew, personal communication). Our fluorescence measurements indicated that the quantum yield of the dienes and trienes are progressively smaller than that of the shorter analog (in ethanol). Thus, design of dyes with higher fluorescence quantum yields may improve the signal-to-noise ratio. The value of ϵ for these dyes is only 5.0×10^4 mol^{-1} cm^{-1} liter. ϵ values are frequently larger by a factor of up to 5 for other dyes. Another spectral property of this family of dyes is the relatively small value of $(\Delta\epsilon/\Delta\lambda)_{max}$. For example, styryl dyes have a value of only 750 mol^{-1} cm^{-1} liter/nm, whereas some merocyanine dyes have a value of 10,000 mol^{-1} cm^{-1} liter/nm.

In conclusion, it seems that from the spectroscopic point of view another family of dyes, having a higher quantum yield, a larger absorption coefficient, and a steeper absorption spectrum, may give rise to even better signal-to-noise ratios. Future synthetic efforts are therefore worthwhile.

Instrumental Factors

Optimalization of the instrumental factors largely depends on the preparation and the physiologic methods employed. A detailed discussion is beyond the scope of this paper. However, the points deserving careful consideration are discussed.

Photodiodes may not be suitable for measuring small changes in the relatively low-level fluorescence from single cells or cell processes. A photomultiplier was therefore the detector of choice for the present experiments. The photocathode of the EMI 9658R photomultiplier has a quantum efficacy of 8% at 700 nm. GaAs photocathodes (e.g., Hammamatsu R636) have a quantum efficacy of 14% at

700 nm. The use of such a photomultiplier would improve the signal-to-noise ratio.[3]

A useful improvement could be obtained if the illumination intensity and the collection efficiency were improved by substituting a dichroic mirror for the semisilvered Zeiss HPL mirror. An ideal dichroic mirror would increase the illumination intensity by a factor of ~2 and the fluorescence intensity by a factor of ~4. Yet another significant improvement could be realized by using a microscope objective with a higher numerical aperture (NA) whenever practical (e.g., an inverted microscope). Theoretically, using epi-illumination with a 1.3 NA × 63 objective rather than the 0.75 NA × 40 objective, the incident light intensity would increase by a factor of 3 and the fluorescence light intensity by a factor of 9 (an increase of 3 in signal-to-noise ratio). In preliminary experiments we measured 43 times more fluorescence from a stained cell by using a long working distance ×63 1.25 NA objective (Zeiss 461820) and a Zeiss dichroic mirror (FT590) rather than a ×40 objective and the HPL mirror.[4]

In the present experiment a signal-to-noise (rms) ratio of 90 was obtained for a 70-μm cell. If all the modifications described above can be implemented, the level of the detected fluorescence intensity will increase by a factor of 64, (1.75 × 4 × 9) improving signal-to-noise ratio by a factor of 8. Thus, a signal-to-noise ratio of >70 is expected for a 7-μm cell (with the same potential change, etc.). If photodiodes, having 6–10 times higher quantum efficiency, can be used the signal-to-noise ratio will be improved by 20 rather than 8. Small and rapid potential changes of a few millivolts could then be detected. Evidently, if larger cells and larger or slower potential changes are studied, both the light intensity and the concentration of the probe can be reduced, thus minimizing photodynamic damage, bleaching, and pharmacological side effects.

Conclusions

The present results indicate that highly sensitive fluorescent voltage-sensitive probes are available and that they are useful in obtaining reliable optical recordings from small cells. Simultaneous recordings from multiple sites are also feasible if a few photomultipliers, coupled to individual light guides, are used. With the predicted increase in fluorescence intensity, arrays of photodiodes can also be considered for fluorescence experiments. The fluorescence technique is particularly useful for studying the electrical properties of processes of neurons maintained in monolayer culture (Grinvald and Farber, 1981). It is likely that the study of the integrative properties of the neuronal arborization of single cells in intact tissue will also benefit from the same technique, with a suitable fluorescent probe being iontophoretically injected into the cell under investigation (Grinvald, et al. 1982 *b*).

APPENDIX

The dye bleaching and the voltage-sensitive response are independent processes. The overall function describing the time course of the fluorescence $F(t)$ is given by

$$F(t) = \alpha_1 I_1(t) \times B_1(t) \times V_1(t), \quad \text{(A1)}$$

where $I(t)$ is the excitation intensity, $B(t)$ is the bleaching time-course, $V(t)$ is the normalized voltage-sensitive function, i.e., when no voltage change occurs $V(t) = 1$. α is a constant depending on instrumental factors and the number of probe molecules. When the experimental bleaching time-course $B_{ex}(t)$ is measured, $V(t) = 1$ and then

$$B_{ex}(t) = \alpha_2 \times I_2(t) \times B_2(t). \quad \text{(A2)}$$

Evidently

$$V(t) = \frac{F(t)}{B_{ex}(t)} \times \frac{\alpha_2 B_2(t) \cdot I_2(t)}{\alpha_1 B_1(t) \cdot I_1(t)}. \quad \text{(A3)}$$

If the bleaching time course was nearly identical in the two measurements then

$$V(t) = c \cdot F(t)/B_{ex}(t) \quad \text{(A4)}$$

where c is a constant if we assume that the excitation intensity was constant. However, if the bleaching time-course varies slightly during the measurements, a distortion in the signal shape might occur and a nonlinear slope of the baseline is evident. Under such conditions, if one wishes the fluorescence signal to resemble the electrical recordings, a better approach is to use the boundary conditions that would cause the values of the first and the last data points for the corrected trace to be the same. (Sufficiently long sweeps must be recorded so that the potential change would settle back to zero.) At the first stage, $F(t)$ is corrected by using the incorrect assumption that the bleaching time-course obeys a monoexponential function. Its time constant τ is estimated from the first and last data points of $F(t)$. The approximated correction $V_c(t)$ is then given by

$$V_c(t) = F(t)/(e^{-t/\tau}). \quad \text{(A5)}$$

The result is usually a convex trace, which obviously requires further correction. A good estimate of the deviation from a proper multiexponential correction can be obtained by correcting the bleaching curve $B_{ex}(t)$ in the same way. Thus, the bleaching curve itself is corrected by the same approximation. The resulting convex curve, $B_{cor}(t)$, is a good estimate of the deviation. Therefore, it is then subtracted from $V_c(t)$:

$$V(t) = V_c(t) - B_{cor}(t). \quad \text{(A6)}$$

[3] If the light level is significantly increased by one order of magnitude (by using a laser illumination or a more efficient apparatus or when studying a more fluorescent preparation), a low noise photodiode can be used (e.g., EG&G HAV 4000A). Note that the quantum efficiency of a photodiode is ~70%; thus yielding 6-10 times higher detection efficiency at a significantly reduced cost as compared with photomultiplier detection system. A significant additional improvement of the signal-to-noise ratio is expected.

[4] If this improvement were used, a tungsten halogen lamp could replace the mercury lamp. (The stability of a battery-operated tungsten lamp is better than 10^6.) Alternatively, with that increased intensity of fluorescence, photodiodes can be used.

If the potential change is zero at the end of the fluorescence recording, the second type of correction is preferable because the experimental errors, if any, are scattered everywhere along the trace. A clear nonlinear "slope" results from the first type of correction (Eq. 4) if the bleaching kinetics varies in each experiment. The fact that the corrected fluorescence is identical with the electrical recording (Fig. 2) proves that the correction procedure works. Because the bleaching data is noisy, the correction procedure would increase the noise in the fluorescence by a factor of $\sqrt{2}$. We completely eliminated that effect by using a smoothed curve for $B_{cor}(t)$. Smoothing was obtained by a least-squares fit of that curve to a polynomial of four degrees.

NOTE ADDED IN PROOF

The predictions regarding the possible improvements in the signal-to-noise ratio were recently confirmed: more sensitive dyes were found and the sensitivity of the apparatus was also improved by a factor of 20; A signal-to-noise ratio of 60 was obtained for a single-sweep recording of an action potential in a 2 μm process in culture. (Grinvald Farber, Anglister, and Hildeshein, submitted for publication).

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REFERENCES

Baker, J. A., J. R. Duchek, R. L. Hooper, R. J. Koftan, and J. S. Heubner. 1979. Lipid and salt effects on carbocyanine dye-induced photo-voltages in bilayer membranes. *Biochim. Biophys. Acta.* 553:1–10.

Booker, L. G. S., G. H. Keyes, R. H. Sprague, R. H. Van Dyke, E. Van Lare, G. Van Zandt, F. L. White, H. W. J. Cressman, and S. G. Dent, Jr. 1951. Color and constitution. X. Absorption of merocyanines. *J. Am. Chem. Soc.* 73:5332.

Cohen, L. B., B. M. Salzberg, H. V. Davila, W. N. Ross, D. Landowne, A. S. Waggoner, and C. H. Wang, 1974. Changes in axon fluorescence during activity: molecular probes of membrane potential. *J. Membr. Biol.* 19:1–36.

Cohen, L. B., B. M. Salzberg, and A. Grinvald. 1978. Optical methods for monitoring neuron activity. *Annu. Rev. Neurosci.* 1:171–182.

Cohen, L. B., and B. M. Salzberg. 1978. Optical measurements of membrane potential. *Rev. Physiol. Biochem. Pharmacol.* 83:35–88.

Grinvald, A., L. B. Cohen, S. Lesher, and M. B. Boyle. 1981 *a*. Simultaneous optical monitoring of activity of many neurons in invertebrate ganglia using a 124 element photodiode array. *J. Neurophysiol.* 45:829–840.

Grinvald, A. and I. Farber. 1981. Optical recording of calcium action potentials from growth cones of cultured neurons using a laser microbeam. *Science (Wash. D. C.).* 212:1164–1169.

Grinvald, A., R. Hildesheim, A. Agmon, L. B. Cohen, and B. M. Salzberg. 1982 *a*. Improved fluorescent probes for the measurement of rapid changes in membrane potential. *Biophys. J. (Abstr.).* 37:61*a*.

Grinvald, A., R. Hildesheim, A. Agmon, and A. Fine. 1982*b*. Optical recordings from neuronal processes and their utilization by iontophoretic injection of new voltage-sensitive dyes. *Neurosci. Abstr.* In press.

Grinvald, A., R. Hildesheim, R. Gupta, and L. B. Cohen. 1980. Better fluorescent probes for optical measurement of changes in membrane potential. *Biol. Bull. (Woods Hole).* 159:484.

Grinvald, A., K. Kamino, S. Lesher, and L. B. Cohen. 1978. Larger fluorescence and birefringence signals for optical monitoring of membrane potential *Biophys. J. (Abstr.).* 21:82*a*.

Grinvald, A., W. N. Ross, and I. Farber. 1981 *b*. Simultaneous optical measurements of electrical activity from multiple sites on processes of cultured neurons. *Proc. Natl. Acad. Sci. U. S. A.* 78:3245–3249.

Gupta, R., B. M. Salzberg, A. Grinvald, L. B. Cohen, K. Kamino, M. B. Boyle, A. S. Waggoner, and C. H. Wang, 1981. Improvements in optical methods for measuring rapid changes in membrane potential. *J. Membr. Biol.* 58:123–138.

Hirschfeld, T. 1977. The choice between absorption and fluorescence techniques. *Appl. Spectrosc.* 31:245.

Huebner, J. S. 1975. Photo-voltages of bilayer lipid membranes in the presence of cyanine dyes. *Biochim. Biophys. Acta.* 406:178–186.

Jutz, C. 1958. Darstellung ungesättigter Aldehyde nach Art der Vilsmeier-Reaktion. *Chem. Ber.* 91:850.

Kimhi, Y., C. Palfrey, I. Spector, Y. Barak, and U. Z. Littauer. 1976. Maturation of neuroblastoma cells in the presence of dimethylsulfoxide. *Proc. Natl. Acad. Sci. U. S. A.* 73:462–466.

Kranse, S. 1977. Cyanine dye-induced electrical and fluorescence effects in neutral and negative bilayer membrane. *Biophys. J.* (Abstr.). 17:214*a*.

Loew, L. M., G. W. Bonneville and J. Surow. 1978. Charge shift probes of membrane potential. Theory. *Biochemistry.* 17:4065–4071.

Loew, L. M., and L. L. Simpson. 1981. Charge shift probes of membrane potential. *Biophys. J.* 34:353–363.

Nicholls, J. G., and D. A. Baylor. 1968. Specific modalities and receptive fields of sensory neurons in the CNS of the leech. *J. Neurophysiol.* 31:740–756.

Rigler, R., C. R. Rabl and T. M. Jovin. 1974. A temperature jump apparatus for fluorescence measurements. *Rev. Sci. Instrum.* 45:580–588.

Ross, W. N., and L. F. Reichardt. 1979. Species-specific effects on the optical signals of voltage-sensitive dyes. *J. Membr. Biol.* 48:343–356.

Ross, W. N., B. M. Salzberg, L. B. Cohen, A. Grinvald, H. V. Davila, A. S. Waggoner and C. H. Wang. 1977. Changes in absorption fluorescence, dichroism and birefringence in stained axons: optical measurement of membrane potential. *J. Membr. Biol.* 33:141–183.

Salzberg, B. M., H. V. Davila and L. B. Cohen. 1973. Optical recording of impulses in individual neurons of an invertebrate central nervous system. *Nature (Lond.).* 246:508–509.

Senseman, D. M., and B. M. Salzberg. 1980. Electrical activity in an exocrine gland: optical recording with a potentiometric dye. *Science (Wash. D. C.).* 208:1269–1271.

Sims, J. S., A. S. Waggoner, C. H. Wang and J. F. Hoffman. 1974. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. *Biochemistry.* 13:3315–3330.

Ullrich, H. M., and H. Kuhn. 1972. Photoelectric effects in bimolecular lipid-dye membranes. *Biochim. Biophys. Acta.* 266:583–596.

Waggoner, A. S. 1979. Dye indicators of membrane potential. *Annu. Rev. Biophys. Bioeng.* 8:47–63.

Waggoner, A. S., and A. Grinvald. 1977. Mechanisms of rapid optical changes of potential sensitive dyes. *Ann. N. Y. Acad. Sci.* 303:217–242.