OPTICAL RECORDING OF SYNAPTIC POTENTIALS FROM PROCESSES OF SINGLE NEURONS USING INTRACELLULAR POTENTIOMETRIC DYES

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ABSTRACT To record post synaptic potentials or electrical activity from processes of single cells in a central nervous systm (CNS) preparation in situ, voltage sensitive dyes can be injected intracellularly, thereby staining only the cell under investigation. We report the structure, evaluation, and synthesis of 11 fluorescent styryl dyes developed for iontophoretic injection. The optical signals that represent small synaptic potentials from single processes of iontophoretically injected cells are expected to be very small and, therefore, such measurements are not easy. We report the methodology that permitted the optical recording of action potentials from a 3-μm axon and the recording of small synaptic potentials from the processes of single cells in the segmental ganglia of the leech. The same dyes also proved useful for optical recording of action potentials of anterogradely labeled axons, following local extracellular injection at a remote site in a mammalian CNS preparation.

INTRODUCTION

Information processing by neuronal integration is often accomplished in fine neuronal processes that are too small to be impaled with microelectrodes and too far, electrically, from the somata to be reflected there without distortion. These include the dendritic trees and axonal terminals of vertebrate central neurons, the secretory endings of neuroendocrine cells, neuroglia, and the neuropile jungles of invertebrates. The time course of the membrane potential changes that occur in these less accessible reaches of the nervous system and the effects of the cable properties of these structures, as well as their nonlinear elements, on integration are becoming amenable to study using optical methods that employ linear potentiometric probes (for reviews, see Cohen and Salzberg, 1978; Waggoner, 1976, 1979; Salzberg, 1983; Grinvald, 1985; Cohen and Lesher, 1986).

In isolated systems, optical recording from neuronal processes has already been reported. Electrical events in the processes of single cells in dissociated cell cultures are readily monitored by optical methods (Grinvald et al., 1981; Grinvald and Farber, 1981; Grinvald et al., 1982, Grinvald et al., 1983) after incubation in a medium containing a low concentration of an impermeant probe molecule that binds to the plasma membrane and changes its absorption or fluorescence emission within microseconds of a change in membrane potential. In this case, there is no uncertainty as to the source of the optical signal. In situ, the situation is usually far more complex, since the determination of the origin of an optical signal from a heterogeneous structure is not straightforward. Salzberg et al. (1983) took advantage of the homogeneity of the vertebrate neurohypophysis to record optically from populations of synchronously active fine nerve terminals and to investigate their ionic conductances. However, in general, the central nervous systems (CNS) of both invertebrates and vertebrates are so densely packed and heterogeneous that the optical isolation of specific neuronal processes within, for example, the neuropile tangle of many invertebrates is feasible only in limited situations. For example, Ross and Krauthamer (Ross and Krauthamer, 1984; Krauthamer and Ross, 1984) employed impermeant voltage-sensitive dyes in the conventional fashion (Salzberg et al., 1973), i.e., applied extracellularly, and then exploited the fact that when the cell body is driven repetitively with a microelectrode, and the optical signal from the neuropile is averaged extensively (200–400 trials), only signals (absorption changes) related to potential changes in the processes of the driven cell will sum coherently. They found that some of the fine processes in the barnacle supraesophageal ganglia are excitable whereas others are not. They also detected the spike initiation zone along a large axon and estimated the space constant of various neuronal processes. Thus, this technique has certain advantages, including relative ease of application, but it is unlikely to be of much use for recording postsynaptic events from fine

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neuronal processes in the neuropile or to study the electrical behavior of these processes if more than one cell is active.

A possible solution to this problem would be to limit the application of the molecular probe to the cell of interest by intracellular staining of that cell with an impermeant dye. The optical behavior of potentiometric probes when applied intracellularly has been investigated previously in voltage-clamped squid giant axons. Impermeant, charged dyes have been microinjected (Davila et al., 1974; Cohen et al., 1974) and internally perfused (Salzberg, 1978; Gupta et al., 1981; Loew et al., 1985), and their absorption or fluorescence changes in response to step changes in transmembrane voltage studied. Theoretical considerations suggest that fluorescent rather than absorption probes are likely to provide a better signal-to-noise ratio for the recordings from fine processes (Rigler et al., 1974; Waggoner and Grinvald, 1977; Agmon, 1983). For single CNS neurons, iontophoretic injection of suitable probes seems to be the method of choice. Requirements for intracellular probes are considerably more demanding than for extracellular probes. A successful molecular indicator for this purpose should be very sensitive to changes in membrane potential; should act from the inside of the cell; should diffuse readily, or be actively transported throughout the axoplasm of the fine processes of the neuron; should be retained within the cell without appreciable leakage; should not bind selectively to intracellular organelles; and should be without toxic effect. It should also have a large extinction coefficient, a very low quantum yield in aqueous media, but a high quantum yield when bound to the external membrane, and, of course, a fast linear response to changes in membrane potential.

We report here the synthesis and evaluation of several probes with many of these properties, and the use of one of these dyes to record action potentials from fine neuronal processes in the neuropile of segmental ganglia of the medicinal leech, *Hirudo medicinalis,* following intracellular injection. Further, we report the optical recording of post-synaptic potentials in neuronal processes close to the site of initiation. Some of these results have appeared in preliminary form (Agmon et al., 1982; Obaid et al., 1982).

MATERIALS AND METHODS

Dyes for Iontophoretic Injections and Their Synthesis

A series of charged styryl dyes were synthesized in an effort to optimize a fluorescent probe for iontophoretic injection. 11 probes were evaluated for this purpose: RH-292, RH-355, RH-414, RH-415, RH-425, RH-437, RH-460, RH-461, RH-527, RH-528, and RH-685. Their structures are shown in Table I. RH-461 [4-(4'-*P*-diethyl aminophenyl-buta-1':3'-dienyl) 1-γ-*N*, *N*, *N*-trimethyl ammonium propyl pyridinium bromide] proved to be the most suitable for use with leech segmental ganglia. Its synthesis is given below.

Synthesis of RH-461. 3-Bromopropyltrimethyl ammonium bromide (prepared as described by Gray et al. (1955), and 4-picoline (1 eq of each) were heated at 120°C for 5 min. The resulting solid was crystallized in methanol ether and was used without further purification. *N*, *N*-diethyl aminocinnamaldehyde was prepared according to a procedure previously described by Jutz (1958) and Grinvald et al. (1982). The aldehyde was identified by mass spectrometry: m/e 203 (molecular peak) and m/e (M-CH_3) + (yield 11%).

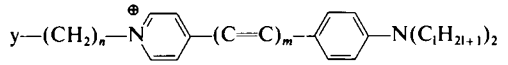
The quaternary salt and *N*, *N*-diethyl aminocinnameldehyde (1 mM of each) were dissolved in 5 ml methanol and 0.5 ml of piperidine was added as a condensing agent. The mixture was stirred at room temperature for 1 h, then precipitated with ether. Pure dye RH-461 was obtained after several precipitations with ether from methanol (yield 85%). It is an amorphous hygroscopic solid, with an absorption maximum at 534 nm in absolute ethanol. The purity of the dye was assessed by thin layer chromatography. The dye was found to be very stable if kept in the cold and in the dark (a few years). Frozen concentrated solutions of the dye were stable for at least three months.

Ganglion Preparation and Injection Procedures

Leech (*Hirudo medicinalis*) segmental ganglia were dissected, singly or in pairs, from the ventral nerve cord and maintained in a leech Ringer's solution containing (in millimoles): NaCl, 115; KCl, 4; $CaCl_2$, 8; Tris maleate, 10; pH adjusted to 7.4. Ganglia were mounted in a specially constructed chamber designed for use with an inverted microscope. The bottom of the chamber (facing the objective) consisted of a replaceable No. 0 coverslip, clamped between lucite retainers having circular holes in the light path. Above this was a sheet of Sylgard (Dow Corning, Inc., Midland, MI), ~1 mm thick, through which two holes ~1 mm in diameter and 6 mm apart were bored. In most experiments, a pair of ganglia joined by their connective were pinned out on the Sylgard using 1-mm lengths of etch sharpened 50-μm tungsten wire, in such a way that the ganglia were suspended below the two holes. The Sylgard sheet, with ganglia attached, was fixed lightly with vaseline to the coverslip bottom of the chamber with the ganglion against the glass but exposed for microelectrode penetration from above through the two bored holes in the Sylgard.

Neurons were impaled under direct visual control, using a swing-in stereomicroscope (Wild Heerbrug Instruments, Inc., Farmingdale, NY), which replaced the swing-out condenser of the inverted microscope (model IM 35; Carl Zeiss, Oberkochen, Germany). Visualization was

TABLE I
STRUCTURE OF INTRACELLULAR PROBES



	RH	Y	n	m	l
1*	355	$(Me)_3N^+$	3	2	1
2	461	$(Me)_3N^+$	3	2	2
3	437	$(Me)_3N^+$	3	2	3
4	425	$(Me)_3N^+$	3	2	4
5	460	$(Me)_3N^+$	3	2	Di-isobutyl
6	685	$(Me)_3N^+$	4	2	2
7	414	$(Et)_3N^+$	3	2	2
8	415	$(Et)_3N^+$	3	2	3
9*	292	$(Et)_3N^+$	3	2	4
10	527	PO_3^{2-}	4	2	2
11	528	PO_3^{2-}	4	2	3

*Properties of these probes were already described elsewhere (Grinvald et al., 1982).

improved by means of a darkfield condenser mounted on the revolving objective nosepiece. Thin-walled microelectrodes were backfilled with approximately 100 mM solutions of RH-461 dissolved in distilled water. The stock solution was centrifuged for 15 min before electrode fillings. Dye electrodes used 3–5 h after filling provided the best injection performance. The iontophoretic injection and the intracellular recordings could be accomplished through the same electrode, which typically had a resistance of 80–160 MΩ (20 MΩ if filled with 3 M KCl). Optimization of injection procedures to maximize filling of the cells' fine arborizations in the neuropile is important, since these differ from those best suited for optical recording from the somata. For cell body recording, the dye was injected with an intermittent depolarizing current of 0.25 nA for 1–3 min (50% duty-cycle). Further injection resulted in degraded signals because of increased cytoplasmic dye fluorescence, compared with that from the plasma membrane. For optical recording from processes within the neuropile, longer injections (10–20 min) were preferred, and the ganglia were then incubated in the cold (8°C) for 1–3 h to allow diffusion of the relatively slowly moving dye.

Fig. 1 shows a motorneuron ("nut") injected with RH-461 for 20 min. The ganglion remains in the chamber but was cleared with a glycerol-Ringer's mixture (50:50). The soma, main process with its bifurcation, and arborization within the neuropile are all clearly visible and closely resemble camera lucida drawings prepared from horseradish peroxidase (Muller and McMahan, 1976) or Lucifer Yellow (Stewart, 1978) fills of these cells. This figure also illustrates a difficulty that attends such measurements. The somata of noninjected neurons are faintly visible in the ganglion as a result of their autofluorescence (such autofluorescence was observed also from uninjected ganglia). This could have been a serious problem when measuring small changes in fluorescence intensity related to membrane potential, since autofluorescence contributes to the

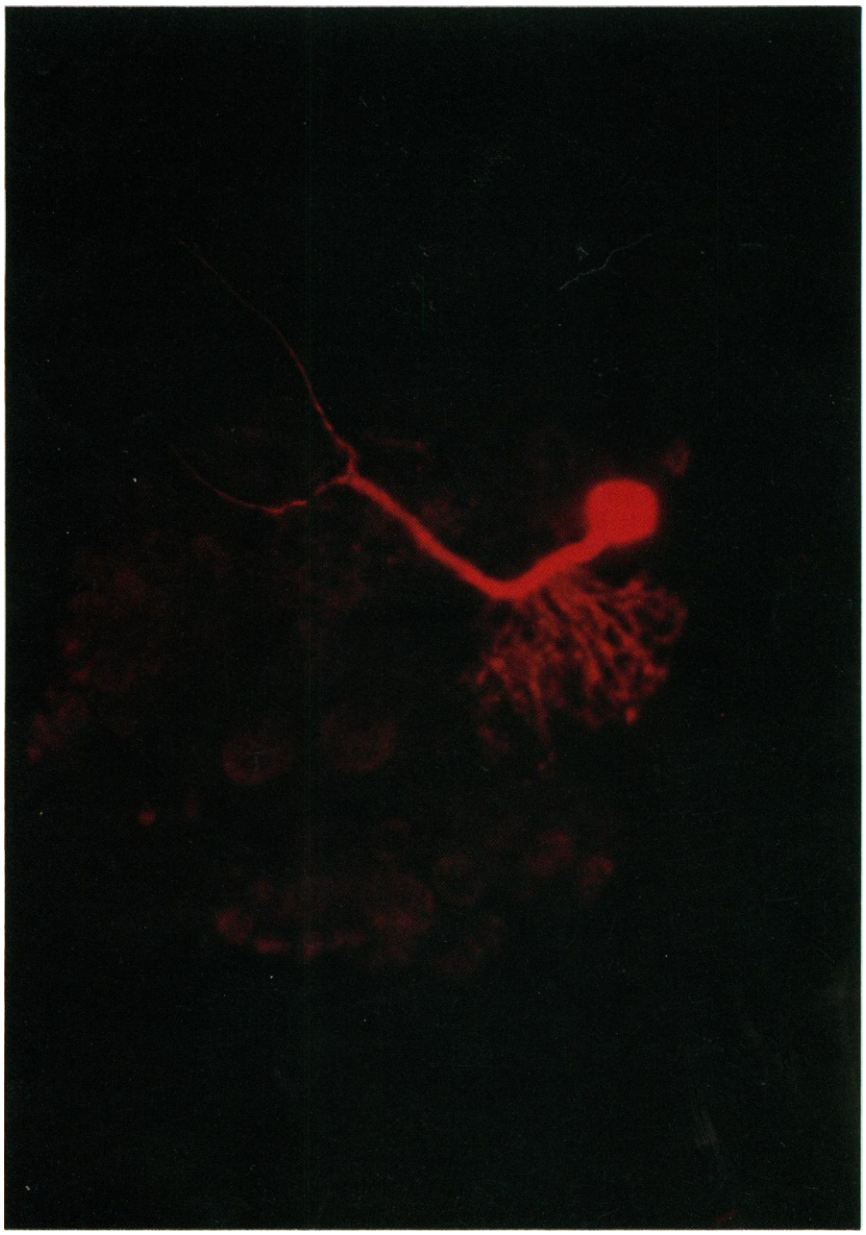


FIGURE 1 Leech motorneuron ("nut") following a 20 min of iontophoretic injection with the styryl dye, RH-461. The soma, main process with its bifurcation, and its arborization within the neuropile are all clearly visible.

background and will degrade the signal-to-noise ratio. We found that we were able to solve the problem of autofluorescence conveniently by taking advantage of the fact that the pigments responsible for its production may be bleached irreversibly by exposure to intense light. A 5 min exposure to the full output of a 100-W mercury arc lamp (model HBO/100; Osram Sales Corp., Newburgh, NY) filtered only with a heat filter (model KG3; Schott Optical Glass, Inc., Duryea, PA) reduced the autofluorescence at wavelengths longer than 610 nm by more than 95%. This procedure apparently had no effect upon either action potentials or synaptic transmission within the ganglion. The remaining fluorescence background was traced to fluorescence emission by the dichroic mirror (model FT580, Zeiss) in the epi-illumination system of the microscope.

Optical Recording

For most experiments, a large Zeiss inverted microscope (model IM-35) was used, with a Zeiss Neofluor 63×, 1.25 numerical aperture oil immersion objective (working distance, 500 μm). A few experiments (Obaid et al., 1982), however, were carried out using a large upright compound microscope (Reichert Zetopan, American Optical Co., Buffalo, NY) modified to incorporate a focusing head and stage. In all instances, the photodetector was a PV-444 silicon photodiode (E.G.&G., Electro-Optics Div., Salem, MA), mounted in the camera tube. With the inverted microscope, an intermediate image was formed on an adjustable aperture that replaced the graticule, and a final real image was projected onto the photodiode. The adjustable aperture was used to limit the detected light to that coming from a chosen region of the image. To provide a reference light intensity for use in the processing of the optical signals (see below), a second silicon photodiode was situated behind the beamsplitter in the beamsplitter housing and was used to monitor a small fraction (5%) of the incident intensity. A Silicon Intensified Target (SIT) camera was located in the inverted microscope's second camera port so that the filling of the cell could be observed under low light level conditions to minimize dye bleaching and phototoxicity (see below). The inverted microscope was mounted on an X-Y positioner (Calvet and Calvet, 1981) and its stage replaced with a large flat platform rigidly attached to the top of a vibration isolation table (Newport Corp., Fountain Valley, CA). This arrangement is optimal for combining optical measurements with intracellular microelectrode recording.

Illumination was provided by the 546 nm line of a 100-W mercury arc lamp, power by a DC source and stabilized with a negative feedback circuit designed by J. Pine (personal communication). Details of the apparatus have been described elsewhere (Grinvald et al., 1983). To minimize exposure time, the light was shuttered and the detection system incorporated an analogue sample-and-hold circuit (Grinvald et al., 1982). The shutter assembly turned out to be a significant source of noise in these measurements, and three important steps were taken to minimize this problem. First, the electromechanical shutter (Uniblitz, Rochester, NY) was mechanically isolated from its housing by "floating" it with small pieces of foam rubber. Second, the pulse that triggered the shutter opening was filtered to slow its rise time. Third, the aperture of the shutter was sealed from both sides with round KG1 heat filters to minimize the acoustical noise which resulted in vibrations of the intracellular electrodes. These precautions reduced the mechanical artifact significantly.

Processing of Optical Signals

Optical detection of synaptic potentials from fine processes of single cells required special procedures. The voltage changes are small and often the membrane area is small. In addition, the relative size of the signals ($\Delta F/F$) from intracellularly applied dyes are smaller than those obtained from extracellularly stained processes in dissociated neuronal cultures because intracellularly applied dye is also bound to cytosolic proteins and internal membrane, and this significantly increases the background fluorescence. For example, in tissue culture the fractional change for a recording from a fine neuroblastoma cell process was ~2% (Grinvald et al., 1983); in leech segmental ganglia, the fractional change in fluorescence intensity from a single neuron was ~0.1% (Salzberg et al., 1973) and, in the present work, we anticipated fluorescence signals having a fractional change of ~0.01%. In the hope of improving the signal-to-noise ratio, we decided to employ a relatively bright light source in the present application. This resulted in fast disappearance of the dye fluorescence (bleaching), and the optical signals were, in fact, minute compared with drift due to bleaching and noise associated with the arc lamp.

Fig. 2 illustrates the rather elaborate procedures that we implemented for on-line automatic processing of the raw optical data. In this experiment, the L motor neuron was filled with RH-461 2 h before the measurement of the synaptic potential evoked by electrical stimulation of the P sensory cell. The medial P cell in one segmental ganglion was stimulated with a microelectrode that also recorded the resulting action potential (trace *1*, labeled P). The injected current monitor is shown below in trace *2*. The contralateral L motorneuron in the next (posterior) ganglion was impaled with a second microelectrode. When the medial P-cell fired an action potential, the resulting excitatory postsynaptic potential (EPSP) was recorded electrically in trace *3* (labeled L). Trace *4* (labeled a) shows the DC output of the silicon diode photodetector positioned over the fluorescence image of the neuropile arborization of the L motorneuron ("sample" photodetector), and trace *5* (labeled b) shows the DC output of the reference diode. Trace *6* (labeled (a) × 50) is the amplified output of the sample-and-hold circuit monitoring the fluorescence intensity emitted by the injected L motorneuron. It represents, of course, the time varying portion of the DC output of the "sample" photodetector. It exhibits several noteworthy features, perhaps the least prominent of which is the optical signal itself. First, the early portion of the trace shows the residual vibrational noise triggered by the opening of the shutter. The slow, approximately exponential downward course of the signal is largely due to the disappearance of fluorescence resulting from the rapid bleaching of the injected probe. This trace will also exhibit any "droop" introduced by the analogue sample-and-hold circuit. A small

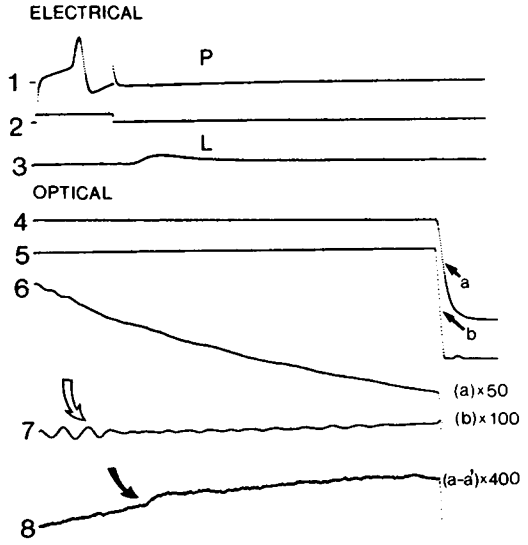


FIGURE 2 Recovery of optical signal from raw data. Trace *1* shows the action potential recorded electrically from the P sensory neuron, the presynaptic element in a monosynaptic pathway. Trace *2* is the record of the current monitor (pulse duration: 40 ms). Trace *3* is the excitatory post synaptic potential (EPSP) recorded from the axonal arborization of the L-motor neuron, the postsynaptic element in this pathway. Traces *4* and *5* are the DC outputs of the sample and reference photodiodes, respectively. Traces *6* and *7* represent the outputs of the sample-and-hold circuits monitoring the sample and reference photodiodes, respectively, and trace *8* is the optical signal obtained as the average of 14 pairs of trials in which sweeps without stimulus are subtracted from sweeps with stimulus (see Methods).

break in the exponential shape may (or may not) be recognizable about a third of the way through the trace (the optical signal). Trace *7* (labeled (b) × 100) is the amplified sample-and-hold output of the reference photodector, which also exhibits the vibration artifact introduced by the shutter. (Of course, there is no bleaching here, as the light does not come from the sample.) The systematic distortions can be cancelled by a subtraction procedure. Trace *8* (labeled (a − a') × 400) is the average (14 pairs of trials) of subtracting the result of one trial without stimulation of the P cell (a') from another trial in which the P-cell was stimulated (a). Evidently, this procedure dramatically reduced much of the distortion accompanying the optical recording of the membrane potential change. A perfectly flat base line would indicate that all of the distortions were exactly reproducible. These conditions are seen to hold only approximately, since the bleaching time course varied systematically during the experiment, being always smaller for the subtracted trace. This systematic distortion could usually be corrected; the same paired experiment was repeated without any stimulus, and the small residual base line drift was subtracted from trace *8* to obtain the final optical record (seen in Fig. 5). As a control for this procedure, several such double experiments were performed but without any stimulation. The corrected traces were flat to less than $5 \cdot 10^{-5}$. After completing this set of experiments we found that if the order of the no-stimulus/stimulus sequence was reversed after each pair, then trace *8* is flat and does not require any correction.

This computer-controlled processing of the optical signal was useful, but it could not by itself ensure against rapid artifactual fluctuations in light intensity being amplified and "processed" into a "signal." On-line monitoring of trace *8* provides some safeguard, however, since fluctuations at the level of the light source are detected, and bad subtractions caused by arc wander, for example, may be rejected during the signal averaging either automatically by using preset rejection criteria, or under manual control.

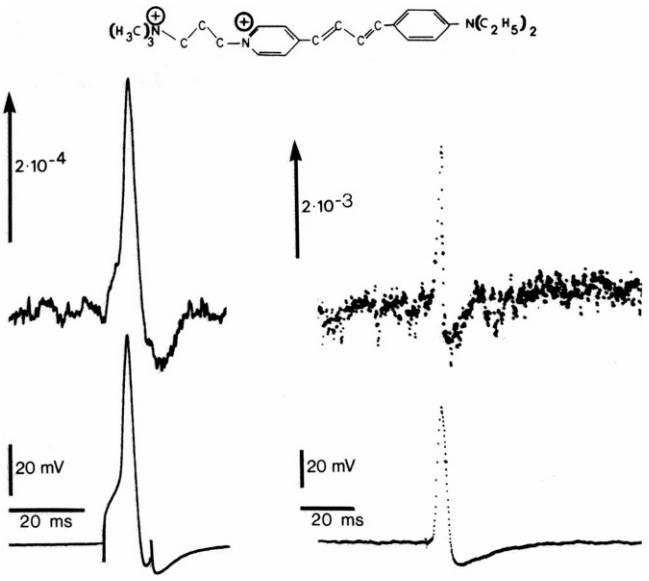


FIGURE 3 Two examples of optical recording of the action potential from cell bodies of leech central neurons injected with the potential-sensitive styryl dye, RH-461. (*Inset, top*) The structure of this red-fluorescing dye. (*Bottom traces*) Action potentials recorded with microelectrodes from the cell bodies of P sensory cells in segmental ganglia of leeches after intracellular staining with RH-461. (*Upper* traces) Optical recordings with the vertical calibration bars representing the fractional changes in fluorescence emission. The record on the left is the average of five trials; the record on the right shows a single sweep. (see Results for details).

RESULTS

Evaluation of the Intracellular Probes

Fig. 3 illustrates action potentials recorded optically (*top traces*) from the cell bodies of P-cells in segmental ganglia of leeches following iontophoretic injection with the styryl dye RH-461. (The dye's structure is shown in the *inset*.) In each experiment, the simultaneous microelectrode recording of the action potential is shown in the lower trace. The vertical calibration bar next to each optical signal represents the fractional change in fluorescence emission. The record on the left is the average of five pairs of trials after a lateral P cell was filled for 2 min. This cell was stimulated by passing current through the recording electrode by means of a bridge circuit. The record on the right was obtained in a single sweep in a slightly different fashion, and, in this case, the optical signal required no processing whatsoever. Here the lateral P cell was injected for 5 min. Stimulation was provided by a suction electrode positioned on the ipsilateral posterior root of the cell. The discrepancy in the sizes of the signals (and in the noise) is probably the result of differences in the effectiveness of the injections. The record on the right shows a larger fractional change in fluorescence, although the total fluorescence was much smaller. Most likely this reflects a higher proportion of membrane associated dye. (This record is dominated by detector noise [see e.g., Salzberg, 1983; Grivald, 1985; Cohen and Lesher, 1986]). Numerous experiments in which insensitive dyes failed to exhibit any signals provide an important control against light scattering as the origin of our optical signal.

For this experiment (Obaid et al., 1982) an upright compound microscope (Reichert Zetopan) having focusable stage and head was used. Epi-illumination with a 40×, 0.75 numerical aperture water immersion objective (Zeiss) produced a real image above the trinocular tube. A single photodiode was positioned behind a set of four independent knife edges (Salzberg, 1978; Salzberg and Bezanilla, 1983), permitting optical isolation of a region of the preparation of arbitrary size, aspect ratio, and orientation. Excess noise from the arc lamp (model HBO/100; Osram) was reduced by means of a reference photodiode, located beneath the preparation, which sampled the transmitted intensity. The gain of the current-to-voltage converter was continuously variable, and its DC output could be matched, by transient nulling, with that of the fluorescence detector. The AC coupled outputs of the two photodetectors were then measured differentially. A response time constant of 600 μs and an AC coupling time constant of 100 ms were employed. This method had the advantage of simplicity but did introduce some AC distortion of the signal. (Obaid et al., 1982)

It is evident from these records that intracellular staining of an invertebrate central neuron permits optical recording of membrane potential changes from the somatic region. Staining was restricted to the injected cell, and there was no suggestion of significant dye leakage or of

optical signals from adjacent regions of the ganglion. In this experiment, the depolarization of the cell led to an increase of fluorescence whereas extracellular staining with the dye provided signals with opposite direction. The direction of an optical signal will depend partially upon the relation between the transition moment of the dye molecule and the electric field vector, and the transition moment will be oppositely aligned depending upon the side of the membrane to which the dye is applied. Therefore, it is not surprising that, in general, the response of impermeant dyes, applied to the inside surface of the axolemma, has been shown to be opposite in sign, but similar in time course, to the signal obtained when the dye was applied to the outside of the cell. The dye RH-160, a styryl dye, for example, exhibits very similar, but oppositely signed signals when added to squid giant axons by perfusion or superfusion (B. M. Salzberg, unpublished observations). Permeant dyes, on the other hand, such as Nile Blue A, may reorient upon crossing the membrane, and therefore exhibit fluorescence changes that do not depend upon the side of application (Salzberg, 1978).

Evaluation of the New Probes. To select the best probe for the present application, all of the probes in Table I were tested on the somata of single cells in the leech segmental ganglia (2–4 cells for each dye). The negatively charged styryls were injected with hyperpolarizing current. In the present preparation, however, they did not prove to be sufficiently sensitive. In addition, it seems that although injection of positively charged styryls with depolarizing currents might be less convenient, it did not present a problem here. The largest signals from cell somata were obtained with RH-437 (the dipropyl analogue of RH-461 (a diethyl)). However, this dye did not fill the fine processes within the neuropile even after 3 h. RH-355 (the dimethyl analogue) gave smaller signals than RH-461, but it diffused much faster than RH-461 and provided striking fluorescent images of the injected cells. In addition, it proved much easier to inject. The continuously variable behavior of these three dyes illustrates the manner in which the synthesis of a series of closely related chemical analogues permitted a "fine tuning" of the molecular properties that yielded the best compromise between conflicting requirements (fast diffusion requires hydrophilic probes, but larger sensitivity to changes in membrane potential usually requires hydrophobic probes [Wolf and Waggoner, 1986]). The remainder of the dyes provided signals which were not as large as those obtained with RH-461. In view of the considerable variability of results with a single dye, one should not necessarily conclude that RH-461 was much better than other dyes. In addition, it is well known that dye sensitivity is preparation and species dependent (Ross and Reichardt, 1979; and recent reviews). Therefore, it is strongly recommended that several dyes (Table I) should be tested when the present approach is applied to other preparations.

Recording from Processes. This method of intracellular staining was developed in order to monitor potential changes in otherwise inaccessible regions of neurons, either individual fine axonal processes in a nerve trunk, or neuronal elements within a central neuropile. Fig. 4 illustrates an experiment in which the action potentials were recorded from a 3-μm diameter axon of a lateral P cell within one of the nerve roots (selected by means of a rectangular slit in the intermediate image plane). The optical signal (*top trace*) is the average of 12 trials, and the electrical recording was obtained by means of a KCl electrode in the cell body. A 63×, 1.25 numerical aperture oil immersion objective was used, so that a 170-μm length of axon was monitored.

Recording of Postsynaptic Potentials. The most critical test of the method of optical recording from fine neuronal processes by means of intracellular probes is the detection of small synaptic potentials from processes in the neuropile. To accomplish this, we took advantage of the reliable interganglionic synaptic coupling of the lateral P cell to the contralateral L motorneuron. Since the presynaptic P cell is approached most readily from the ventral aspect and the postsynaptic L motorneuron is most easily impaled from the dorsal side, we mounted the pair of ganglia with a half twist in the interganglionic connective. The L motorneuron was filled with RH-461 by injecting it for 30 min using 0.25-nA depolarizing current. The ganglia were then incubated in the cold (8°C) for 2 h before optical recording was begun. Electrical recordings were obtained from pre- and post-synaptic somata by means of KCl filled microelectrodes. Fig. 5 illustrates one of several such experiments. The top trace shows the action potential in the medial P cell, and the middle trace shows the

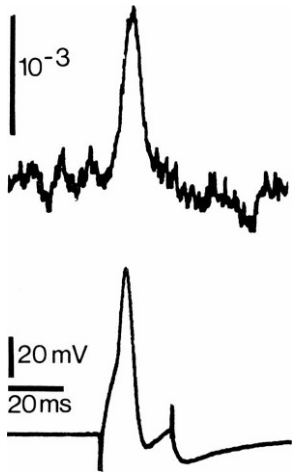


FIGURE 4 Optical recording of the action potential in a 3-μm diameter axonal process of a P-sensory neuron injected with RH-461. The recording was made from a 175-μm axonal segment in the anterior root. The slit in the graticule was appropriately positioned to block the fluorescence from the second process in the posterior root. 12 trials were averaged.

electrical recording of the small EPSP in the soma of the L-motorneuron in the next segmental ganglion. The *inset* shows the typical anatomy of the L-motorneuron's branching (from Muller and McMahan, 1976), and the dark circle indicates the area from which the fluorescence emission was collected by the microscope optics. The bottom trace shows the optical recording of the synaptic potential in the processes of the L motorneuron. It is the average of 14 trials, and is trace *8* of Fig. 2, corrected for the base line drift as discussed and considerably expanded. In several experiments a comparison of the time course of the electrical recording from the soma, with the optical recording from the neuropile, showed that the rising phase of the EPSP was faster in the neuropile. (This is evident also in Fig. 5, even though the response time constant for the optical recording of the synaptic potential was 2.7 msec, much slower than that for the electrode record.) Similar results were obtained in recording the synaptic potential from the AE (Annulus erector) or the "nut" motor neurons, in response to electrical stimulation of the P-cell.

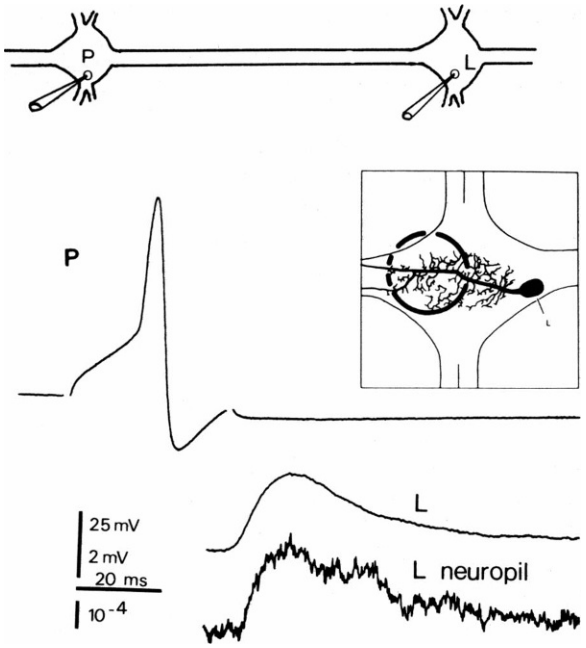


FIGURE 5 Optical recording of the excitatory postsynaptic potential in the arborization of the L-motorneuron. (*Top*) The experimental arrangement for interganglionic stimulation and recording. (*Inset*) The region of the neuropile that was monitored (after Muller and McMahan, 1976). (*Top traces*) the Microelectrode recordings of the presynaptic action potential in the P sensory neuron and the postsynaptic potential in the soma of the L motor neuron. The optical recording of the synaptic potential from the neuropile is shown at the bottom.

DISCUSSION

Difficulties and Refinements

Pharmacological Effects, Photodynamic Damage, and Bleaching. Extrinsic probes, added intracellularly, may have a greater possibility of perturbing the neuron's normal behavior than dyes added to the outside of the cell because of their superior access to the cell's machinery. Indeed, we found that some dyes, at high concentration, produced obvious broadening (200 ms) of the action potential and spontaneous firing. Some cells (e.g., the "nut" motorneuron), but not others (e.g., sensory cells or the L motorneuron), were clearly affected. Similarly, the susceptibility of an injected cell to photodynamic damage (Ross et al., 1977) limited the duration of most of our experiments to 20 paired trials.

Finally, the rapid bleaching of the fluorescence may introduce some slow distortion of the optical signal, despite the correction procedures (see Methods). Although these difficulties may appear formidable, we believe that the present state of the technique already permits its practical use for a variety of studies. Moreover, the design and synthesis of improved probes will certainly reduce the significance of these problems. At the same time, improvements in the signal-to-noise ratio by other means will achieve the same goal.

Signal-to-Noise Ratio

Inspection of the optical signals reported here indicates that shot noise was not the dominant noise source in these measurements. A careful reading of the extant reviews in the field suggested that some reduction of the illumination intensity (5–10-fold), combined with additional signal averaging, would have resulted in a significantly improved signal-to-noise ratio. Such a strategy can now be implemented because of the reduction in the amplifier dark noise recently achieved (J. Pine, personal communication). This strategy would also eliminate the slow distortion due to bleaching that may escape the correction procedures.

Most of the vibrational noise that, in fact, dominated our records could be attributed to the electromechanical shutter. A purely electrooptical device or another type of mechanically and acoustically silent shutter would have eliminated this problem. In this case, individual trials in many kinds of experiments could be abbreviated by as much as a factor of 10, thus permitting 5 to 10 measurements from the same cell, under different experimental conditions. On the other hand, reduction or elimination of arc wander, by substituting xenon for mercury, or by using a filament lamp would permit longer duration sweeps, which may be required for other kinds of experiments.

Yet another way to improve the signal-to-noise ratio is to try to maximize the specific binding of the dye to the external membrane rather than to cytosolic binding sites. An alternative approach is to use a specific quencher that can exclusively quench the fluorescence of free or protein bound dye, rather than the membrane bound dye. Similarly, if a suitable oxygen scavenger (Valenzeno and Pooler, 1978, but see also Salzberg, 1978) can be found, it may help to minimize the photodynamic damage or bleaching associated with these experiments.

Spatial Resolution

The present experiments employed a single photodiode and this, not the microscope optics, limited the spatial resolution. An advantage of the use of an intracellular fluorescent dye is that, ideally, all of the collected light comes from the injected cell processes, however small. Since our experiments were not shot noise limited, a multiple detector system would offer considerable advantages (Salzberg, 1983; Grinvald, 1985; Cohen and Lesher, 1986) for investigating regional variation in the responses. In this case, it would be preferable to use a large (~10 GΩ) feedback resistor in the first stage of the amplifier system. This arrangement should minimize the dark noise in the photodetector-amplifier combination. Such low noise amplifiers are being designed (J. Pine, Personal communication). Another factor that may significantly limit the spatial resolution is the scattering of the fluorescence emission. Obviously, its extent depends on the transparency of the preparation (for quantitative estimates, see Orbach and Cohen, 1983; Ross et al., 1986).

Further Applications

It has been reported that styryl dyes that are applied extracellularly at high concentrations permeate the external membrane and accumulate intracellularly (Grinvald et al., 1982). If the dyes are positively charged they will equilibrate slowly according to the Nernst equation, i.e., much more dye will be accumulated inside. This property combined with their relatively fast diffusion along the cell's processes suggested that these dyes can be used as retrograde or anterograde fluorescent labels of specific pathways in the CNS, following a localized extracellular injection. For example, intra-ocular injection of RH-461 in the rat resulted in dye being accumulated in ganglion cells and its diffusion along the optic nerve far away from the injection site. Optical recording from such nerve stimulated in vitro exhibited the fast compound action potential exclusively from intracellularly stained axons. When the isolated nerve was stained extracellularly, the compound action potential, recorded optically, had an opposite polarity. In addition, a large slow component was also observed, presumably from glia (Lev-Ram and Grinvald, 1986).

Additional applications of intracellular voltage sensitive fluorescent probes may exist. These include (*a*) the use of such a dye along with Lucifer Yellow as a second, contrasting marker for interacting neuronal elements during development (e.g., Purves et al., 1986); (It has been observed, for example, that the dye was retained inside cells in rat cortex for a period of at least 3 wk (L. Katz, unpublished observation); (*b*) the demarcation of individual neurons in electrically coupled systems, since RH-461, for example, crosses gap junctions far less readily than does Lucifer Yellow (A. L. Obaid, B. Rose, and B. M. Salzberg, unpublished observations in *Chironomous* salivary glands); (*c*) it should be possible to attach additional side chains to the styryl chromophore so that the modified dye could be covalently linked to proteins during the fixation procedures (e.g., Stewart and Feder, 1986). This would prevent the observed rapid leakage of dyes from ganglia cleared with 50% glycerol solution.

Intracellular Versus Extracellular Staining

Optical recording of electrical activity from cell somata or cells processes has been demonstrated previously in invertebrate ganglia (Salzberg et al., 1973; Salzberg et al., 1977; Grinvald et al., 1981; Ross and Krauthamer, 1984). The results presented in Figs. 3–5 demonstrate that intracellular staining with potentiometric probes also allows one to monitor electrical activity by optical means selectively from individual cells or regions of cells within a highly complex neuronal matrix. However, here, the selective application of the fluorescent probe confers important advantages over indiscriminate staining. First, regions of the neurons of interest, away from the soma, may be immediately visualized, and their electrical behavior monitored optically, against the background (anatomical and electrical) of the tangled neuropile. Second, optical recording of action potentials from small processes is possible with far less signal averaging than is required for extrinsic absorption measurements (Ross and Krauthamer, 1984) following bath application of the dye. (The trade off, however, is enhanced photodynamic damage.) Third, the technique demonstrated here permits optical recording of synaptic potentials with relatively little signal averaging. In the barnacle central nervous system, inhibitory and excitatory postsynaptic potentials have been recorded optically under favorable circumstances (Salzberg et al., 1977; Grinvald et al., 1981). In the past, however, it has not been possible to detect postsynaptic potentials from single cells at or near the site of initiation, especially when the synaptic locus is within a complex neuropile. With intracellular staining, a variety of inherently difficult experiments in synaptic physiology become feasible. For example, the sites of synaptic inputs from different presynaptic cells may be determined and the integration and processing of these inputs and their transmission to the spike initiation zone(s) may be studied. Similarly, the invasion of signals, both input and output, into different regions of the neuronal arborization, may be investigated, and the effects of neurotransmitters, neuromodulators, drugs, as well as the pattern of activity examined. While these kinds of experiments are still difficult technically, it seems that at present there is no alternative approach for analyzing the detailed behavior of the basic computational elements of the nervous system in situ.

We thank A. Agmon, A. Fine, A. L. Obaid and H. Shimizu for their contributions.

This work was supported by U.S. Public Health Service grants NS 16824 and NS 14716, a grant from the U.S. Israel Binational Science Foundation and a grant from the U.S. Air Force.

Received for publication 9 July 1986 and in final form 1 December 1986.

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