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Simultaneous optical measurements of electrical activity from multiple sites on processes of cultured neurons

(tissue culture/neurons/membrane potential/fluorescence/growth cone)

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ABSTRACT **Optical methods using changes in fluorescence and absorption of voltage-sensitive dyes were developed to record electrical activity from processes of nerve cells grown in monolayer culture. For transmission measurements, a merocyanine dye was discovered that was more sensitive than others previously tested on cultured neurons. Action potentials from the somata of these cells were detected without averaging, with a signal-to-noise ratio of 20:1. With this dye, electrical responses were simultaneously recorded from many points along the arborization of neuroblastoma cells by using a 10 × 10 array of photodiodes positioned in the microscope image plane. Frequently different processes had different shapes of electrical responses, suggesting regional specializations. Fluorescence measurements with an oxonol dye proved to be more sensitive than transmission measurements, particularly when recording from small processes. By changing the position of the cell relative to a laser microbeam while recording electrically from the cell body, it was possible to monitor the membrane potential in the cell body and in the process simultaneously. From the delay in reponse in the process, a lower limit for the mean conduction velocity of 0.2–0.6 m/sec was found for 2- to 7-μm processes. The mean space constants of processes were estimated by comparing the amplitudes of passive voltage responses in the cell body and growth cone. A lower limit of 400–950 μm was obtained for 4- to 7-μm processes.**

Many questions in neurobiology require an understanding of the regional properties of neurons. For example, the integration of multiple synaptic inputs by single nerve cells requires a knowledge of the passive and active electrical membrane properties of the dendritic branches, soma, and axon (1–4); the space constants of each of the processes and the location and nature of specialized segments with localized excitability must be determined. Also, the role that given ions play in the regulation of neuronal development requires knowledge of the electrical properties of the developing neurites (5). Yet, the electrical events in the growth cone are not known.

Nerve cells maintained in tissue culture offer a simplified system for studying these electrical parameters, yet these questions are difficult to approach by current techniques because it is hard to record intracellularly from small processes. Extracellular recording from small processes of cultured neurons has been reported (6–8). However, this technique is inadequate when studying graded potentials or regional variations in the form of the action potential (9, 10). Furthermore, simultaneous measurements of events in different regions within one neuron are very hard with classical recording methods. Some of the limitations inherent in these methods were recently discussed (10).

Optical methods for measuring membrane potential by using voltage-sensitive dyes have been developed by Cohen and coworkers (11–14) and by Waggoner (15) and colleagues. These dyes act as molecular transducers which transform changes in membrane potential into changes in optical properties of stained neurons. (For recent reviews see refs. 15–18.)

The optical signals are relatively small and, therefore, are difficult to detect. Considerable effort has been expended to optimize the apparatus and to select the best probes. Almost 1000 dyes have been already tested on squid giant axons (11, 12). A few of those are sensitive enough to be used to record the neuronal activity of several invertebrate central nervous systems (19). However, Ross and Reichardt (20) have reported that there was a species-specific difference in the optical response of a number of absorption dyes; the best dyes were not sufficiently effective when tested on dissociated cells from vertebrate neuronal preparations.

In this paper we report the discovery of sensitive transmission (absorption) and fluorescence probes for cultured neurons and used both methods to record from multiple sites on a single nerve cell and its processes. In addition, we implemented a laser microbeam to provide localized illumination for fluorescence experiments. The experiments show that optical techniques can be applied to the determination of conduction velocity in the processes, to the measurement of space constants, and to the detection of regional variations in electrical properties which are not easily determined with conventional techniques.

MATERIALS AND METHODS

Cell Cultures. Mouse neuroblastoma clone N1E-115 and the glioma neuroblastoma hybrid NG-108-15 were originally obtained from M. Nirenberg. The cells were grown as described (21, 22). Dishes were used 6–35 days after reseeding.

Apparatus. Transmission and fluorescence experiments were done with an apparatus built around a Zeiss Universal microscope rigidly mounted on a vibration isolation table. (For details see Fig. 1 and ref. 19.)

Electrophysiology. For physiological experiments, the culture media were changed just before use to a solution of Dulbecco's modified Eagle's medium (GIBCO H-21) modified to contain 5.4 mM K^+, 120 mM Na^+, 4.1 mM Ca^{2+}, 0.8 mM Mg^{2+}, 10 mM glucose, 20 mM sucrose, and 10 mM Hepes buffer (pH 7.4). Total osmolarity was 340 milliosmol/liter.

Cells were impaled with 50- to 90-MΩ microelectrodes filled with 3 M KCl. A WPI electrometer was used. The electrodes were controlled by a M-103 Narishige hydraulic micromanipulator mounted on a moveable stage. With this arrangement, the cell could be moved relative to the fixed microbeam while maintaining a stable electrode penetration. The criteria for mature,

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Abbreviation: S/N, signal-to-noise ratio.

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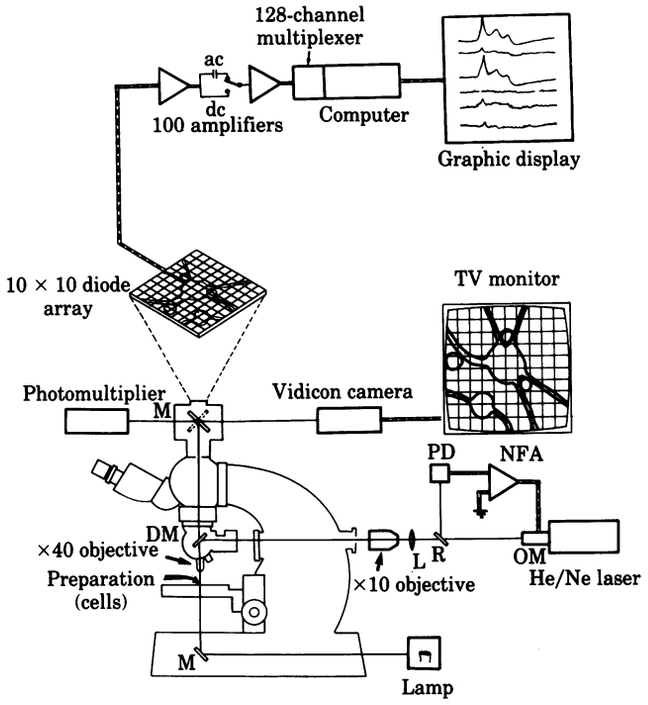

FIG. 1. Schematic diagram of the apparatus used for transmission and fluorescence experiments. A Zeiss Universal microscope was rigidly mounted on a vibration isolation table (Newport Research, Fountain Valley, CA). In transmission experiments a 12V/100W tungsten/halogen lamp was used. Cells were viewed through a long-working-distance, ×40, water-immersion objective (Zeiss, 46-07-15). Transmitted light was detected by a 10 × 10 square array of photodiodes, each 1.4 × 1.4 mm (Centronix, Croyolon, England). Each photodiode received light from a 45 × 45 μm area of the microscope object field. Each photodiode was coupled to a current-to-voltage converter and amplifier operated either ac or dc. In transmission experiments a coupling capacitor with a time constant of 100 msec was used before the second amplifier (×4000). The output of each amplifier was multiplexed through two 8-bit analog-to-digital converter cards (Adac, Woburn, MA) and deposited in the PDP-11/34 computer memory. The time resolution obtained was about 0.8 msec in each of the 100 channels. The recordings from each channel were then displayed on the screen of a Tektronix 4010 display terminal. In the fluorescence experiments, a 50-mW He/Ne laser (Spectra Physics, Mountain View, CA) tuned to 632.8 nm was used. Laser light fluctuations were reduced by utilizing a 3033 electro-optical modulator (Lasermetrics, Teaneck, NJ) in a negative feedback system with a portion of the beam as a reference (23). The beam was focused onto the target by adding a ×10 objective and a 5-cm-focal-length lens in front of the standard epi-illumination system of the microscope (24). By adjusting the lens, the diameter of the microbeam could be varied from 1.5 μm to 120 μm. Fluorescence was detected by an EMI 9658R phototube. To record the absolute level of fluorescence, 12-bit conversion was used. NFA, negative feedback amplifier (Tektronix 5031 oscilloscope); PD, PV444 photodiode; R, reflector made from a coverslip; m, mirror; L, 5-cm-focal-length lens; DM, dichroic mirror or half-silvered mirror; BM, Zeiss three-way beam splitter.

healthy cells were a resting potential lower than 50 mV and an action potential larger than 40 mV. Optical and electrophysiological experiments were done at 37°C.

Staining with Voltage-Sensitive Probes. Staining solutions were freshly prepared before each experiment. For absorption experiments, cells were stained for 5 min with 0.04–4 mM dye and then washed twice in physiological medium. When low concentrations of dye were used, the dye was left in the bathing solution during the experiment. For fluorescence experiments, concentrations of 5–30 μM dye were typical. The dye in solution was much less fluorescent than membrane-bound dye and, therefore, it was possible to leave it in the bath during the measurements without increasing background fluorescence intensity.

Dyes are available from A. S. Waggoner (Dept. of Chemistry, Amherst College, Amherst, MA), from sources cited in ref. 12 and from us.

Pharmacological Effects, Photodynamic Damage, and Bleaching. Because binding of large amounts of probe molecules to the cell membrane might modify its properties (12, 19), cells were first impaled with microelectrodes and then stained. Thus, the effects of a given dye could be observed with intracellular recordings. The present experiments were carried out with low dye concentrations that led to negligible effects on the resting potential and the shape of the action potential. Pharmacological effects were observed at relatively high dye concentrations (unpublished data).

Bleaching of the dye is not a serious problem in transmission measurements. During a typical experiment, which lasted 2 min, the illumination bleached half of the bound dye, reducing the optical signal by 50%. However, the preparation could be restained to regain the original signal size. In fluorescence experiments, where a higher intensity of illumination was used, bleaching distorted the time course of the optical records. This distortion was variable. Therefore, each optical recording of a stimulated action potential was immediately followed by a measurement without stimulation, which was then used for the correction. Control experiments showed that the time course of these corrected fluorescence records was identical with the electrical recordings.

Photodynamic damage (12, 25) was the factor limiting the duration of fluorescence experiments. Therefore, no more than four trials were averaged in these experiments. Frequently we verified that the result obtained in the first trial was identical with the one obtained in the last trial.†

RESULTS AND DISCUSSION

Search for Sensitive Optical Probes. We have tested most of the sensitive optical probes which have been developed and tested on squid axons (11, 12). For transmission experiments we have discovered that a merocyanine-rhodanine dye, 5-{[1-γ-sodium sulfopropyl-4(1*H*)-quinolylidene]-2-butenylidene}-3-propylrhodanine, designated WW 401, is 2–3 times more sensitive on cultured neuroblastoma cells than any other dye we have tested. It is 5 times more sensitive than the dye 5-{[1-γ-sodium sulfopropyl-4(1*H*)-quinolylidene]-2-butenylidene}-3-ethyrhodanine, designated WW 375 (12, 20). The action spectra of the transmission signals have the same wavelength dependence for both dyes. These triphasic spectra were similar to those observed with dissociated neurons from the rat and chicken, but very different from the spectra obtained with invertebrate neurons (20). This finding suggests that neuroblastoma cells may be a good model system for evaluating the sensitivity of voltage-sensitive dyes for vertebrate neurons.

In fluorescence experiments the best results were obtained with the barbituric acid pyrazolone oxonols, a recently designed family of fluorescent dyes (23).

Typical optical recordings obtained from transmission and fluorescence experiments with the two different dyes are illustrated in Fig. 2. A comparison of the optical and electrical measurements indicates that the optical recordings closely resemble

† When better resolution is required, higher illumination intensities can be used and photodynamic damage can be reduced by at least a factor of 100 by removing oxygen from the saline (12). Oxygen-free and oxygen-enriched solutions can be alternately superfused to prevent anoxia.

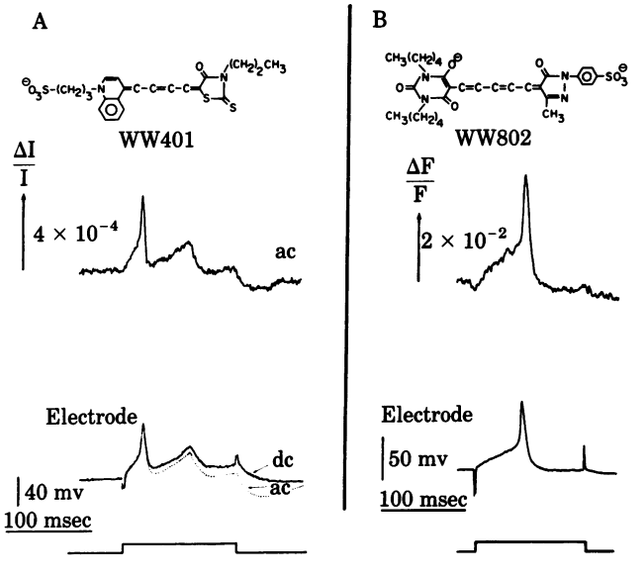

FIG. 2. Transmission and fluorescence signals detected without signal-averaging from stained N1E-115 neuroblastoma cells. (*A*) Change in transmission recorded by a square photodiode positioned over the image of a 45 × 45 μm^2 area in the center of a 120-μm-diameter cell stained with 0.4 mM WW 401. The simultaneous electrical measurement is shown below. The shape of the undershoot following the optical action potential is distorted by the coupling capacitor (100-msec time constant). When the electrical recording was ac-coupled with the same time constant as the optical recording, both records had identical time courses. Illumination wavelength was 690 ± 15 nm. The arrow shows an increase in absorption (decrease in transmission). The structure of WW 401 is shown at the top. (*B*) Change in fluorescence detected from a 90-μm-diameter cell stained with 30 μM [1,3-dipentylbarbituric acid-(5)]-[1-*p*-sulfophenyl-3-methyl-5-pyrazolene-(4)]-pentamethinoxonol, designated WW 802 (top). The fluorescence was excited by a 120-μm-diameter laser microbeam. Excitation light was blocked with a Schott RG665 post-filter. The fluorescence record was corrected for bleaching. The arrow shows an increase in fluorescence. Note that the fractional change in transmission is only 5×10^{-4}/100 mV, whereas in fluorescence it is two orders of magnitude larger.

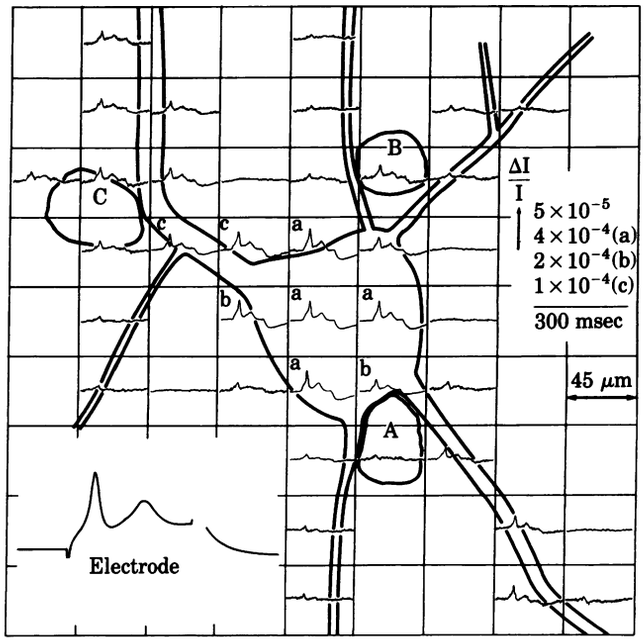

FIG. 3. Simultaneous transmission changes recorded by a 10 × 10 photodetector array positioned over the real magnified image of a stained N1E-115 neuroblastoma cell. The image of the cell has been superimposed over the array outline (positioned to an accuracy of about 10 μm). The soma was stimulated with a microelectrode and 50 sweeps were averaged. Traces in the individual boxes show the optically detected electrical activity. Some records that are not from the cell or its processes are shown as a control at high gain to show the noise level. Occasionally, as in *C*, there is a large signal over another small cell in the field. This may have resulted from electrical coupling between cell *C* and the large cell or from a nonvisible growth cone extending under this cell. [Whereas Moolenar and Spector did not find electrically coupled cells in N1E-115 cultures (26), such coupling was found by Harris and Dennis in another line of neuroblastoma cells (27)]. The gain of the optical recordings was usually 5×10^{-5} as shown on the scale, but different records may have different gain. The lower case letter indicates the corresponding calibration on the scale. Activity was detected only on the 9 × 9 elements shown (other elements received low-light levels and were not useful).

intracellular electrical recordings, although the absolute value of the membrane potential is not readily obtained in optical measurements. No averaging was necessary for these records.

Regional Variation in the Form of Optical Responses from Different Segments of a Single Cell. The large absorption signals obtained from the soma with WW 401 suggested that this dye could be used to record from small processes. A large N1E-115 cell with five major processes was positioned in the center of the field, stained with WW 401, and stimulated at a rate of 1 Hz with a microelectrode in the soma. Fig. 3 shows that the transmission changes were detected simultaneously by all of the photodiodes on which an image of any part of the cell fell. The signals from the processes were noisier than those from the soma because there was less membrane area. However, it is clear that the action potential can be detected along all of the processes. Furthermore, the signals from several photodiodes over a particular process could be combined by the computer to give signals with an improved signal-to-noise ratio (S/N). From such records, regional variations in the response could be determined. For example, using the experiment shown in Fig. 3, we calculated the ratio of the fast action potential and the slower depolarization that follows it. A value of 2.4 was obtained for the cell body and the lower process, and values of 2.2, 2.3, and 1.8 were obtained for the upper left, middle, and right processes, respectively. Larger differences were observed when the recordings from the distal segments of the various processes rather than the average values for the whole processes were compared with those from the soma. For the thin processes, the ratios were clearly larger—in the range of 4–6 (compare trace 81 with trace 88 in Fig. 4*A*).

In the case of passive attenuation, fast responses should decay more rapidly than slow responses. In this experiment the opposite result was obtained. One explanation for this regional difference in the shape of the electrical responses is that the action potential propagated without attenuation along the process, whereas the slow response was subthreshold and decremented passively. Another explanation is that the slow depolarization was due to voltage-dependent Ca^{2+} channels (26) and that these were less active in some of the processes as has been reported for other neuronal types (9, 10). In both cases the observed differences are smaller than those which would have been detected had the process been electrically more isolated from the soma. In this experiment the large 400- to 950-μm space constants for such processes (see below) make the cell appear relatively uniform.

In other experiments the undershoot of the action potential recorded in 5-μm processes were much larger than those recorded in the cell bodies (Fig. 4*B*). Experiments to examine regional membrane specializations and mechanisms which might be responsible for these variations will be reported elsewhere (28).

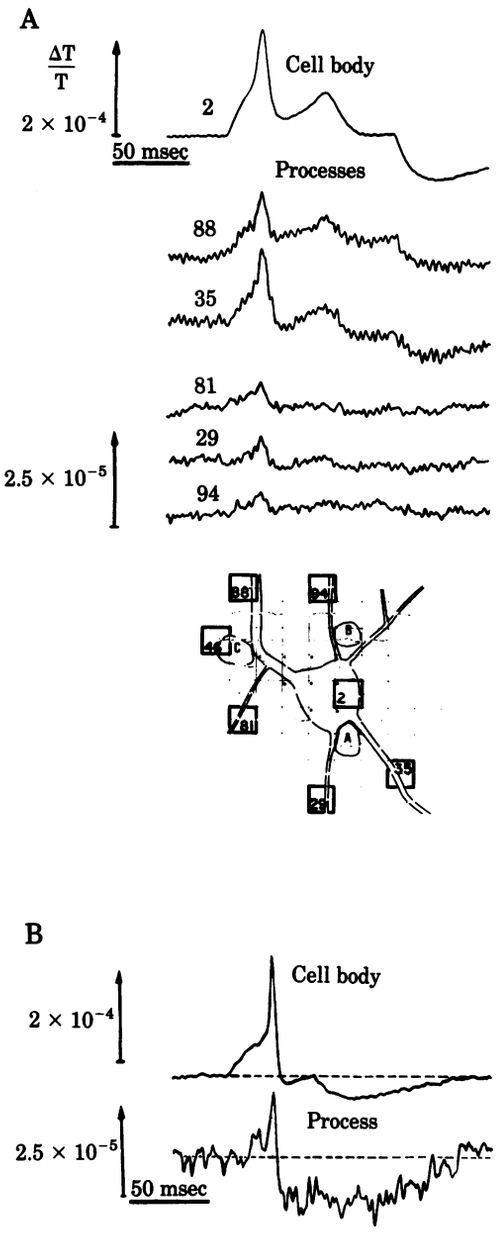

FIG. 4. Optical recordings from processes of neuroblastoma cells. (*A*) Records taken from the transmission experiment described in Fig. 3. The diagram indicates the parts of the neuron from which the recordings were taken. Trace 2 is the optical recording from the cell body. The excellent S/N shows the improved sensitivity due to averaging. Traces 88 and 35 are recordings from the large processes which are similar to those in the cell body. A different time course for the response is observed for the thin processes in records 81, 29, and 94. (*B*) The top trace is the recording from the soma; the bottom trace is the recording from a 4-μm process, 150 μm away. Note the larger hyperpolarization in the process; 50 trials were averaged.

Fluorescence Recording of Electrical Activity from Processes. Large fluorescence signals were obtained from the processes of these cultured cells (Fig. 5*A*). These signals could be observed in a single trial, unlike the absorption signals from the processes that required extensive signal averaging. The laser microbeam was reduced to a diameter of 30–50 μm, increasing the excitation intensity by a factor of about 10 relative to cell body measurements. This increased intensity improved the S/N. However, significant bleaching and photodynamic damage occurred with the present fluorescent probe under these conditions. Therefore, in the rest of the experiments presented in Fig. 5, the light level was reduced by a factor of 10 with a neutral density filter.

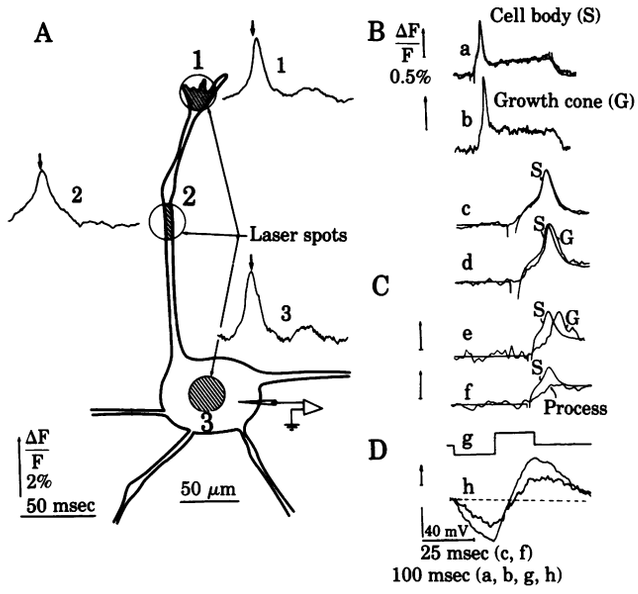

FIG. 5. (*A*) Fluorescence changes measured during stimulation of a stained N1E-115 cell at several points on the growth cone, processes, and cell body. For each record the stage was adjusted to position the appropriate point under the laser microbeam, which had a diameter of about 30 μm. Recordings were taken in the order 1, 2, 3. The vertical arrows on each record indicate the time of the peak of the action potential as recorded with an electrode in the cell body. There is a greater delay at points further away from the cell body. Dye concentration, 20 μM. Four sweeps were averaged for each record. (*B*) Measurement of conduction delay. Traces: a, electrical and corrected fluorescence recordings from the cell body (they appear to be identical, establishing the linearity of the dye response and the validity of the bleaching correction); b, fluorescence recording from the growth cone; c, trace a shown at an expanded time scale; d, comparison of electrical recording from the soma with the fluorescence recording from the growth cone b 300 μm away from the cell body. The delay is 0.8 msec. (*C*) Traces: e, same as d for another cell (the delay was 5.6 msec, and two separate trials were averaged); f, comparison of the electrical recording from the soma with the fluorescence record from the process leading to the growth cone. (*D*) Measurement of a space constant. The medium contained 1 μM tetrodotoxin and 15 mM tetraethylammonium. Traces: g, injected current pulses into the soma; h, fluorescence response from the cell body and the fluorescence response from the growth cone. The separation was 510 μm; the neurite diameter was 7 μm. The hyperpolarization signal is attenuated by a factor of about 1.7 in the growth cone. For experiments *B–D*, laser illumination was reduced to prevent possible photodynamic damage.

Measurement of Conduction Velocity in a Process. By changing the position of a cell relative to the laser, spot recordings could be made from several positions along a process. The experiment shown in Fig. 5*A*, for example, allows us to estimate the mean conduction velocity of the action potential in the process. There was a delay of 1.6 msec between the fluorescence record of the peak of the action potential in the growth cone and the peak recorded electrically from the cell body, from which a mean conduction velocity of 0.2 m/sec is calculated.‡ Another conduction velocity experiment is shown in more detail in Fig. 5*B*. A delay of about 0.8 msec was found when the simultaneous electrical measurement from the cell body was compared with

‡ Part of the observed delay is due to the charging time of the growth cone and the large difference in geometry between the process and the growth cone (2, 3, 29). The velocity of propagation of an action potential in an "infinite" process would be faster than the mean conduction velocity.

the fluorescence record from the growth cone, situated 300 μm away. Thus, for this 6-μm process, the conduction velocity was 0.4 m/sec. These values fall in the range found for other vertebrate neurons (5, 6).

In one experiment a delay of 5.6 msec was observed for the action potential in the growth cone 520 μm away from the cell body. This long delay is probably not due to a slow conduction velocity of 0.1 m/sec. One possible explanation is that the response detected in the growth cone is due to passive spread from the soma. In this case the rising phase of the growth cone action potential should be slower than the somatic action potential. A significantly slower rise time was not observed, however. Another possibility is that the process is inexcitable and conducts only passively, yet the growth cone at the end of this process is excitable. We recorded from the process midway between the cell body and the growth cone. Record f in Fig. 5*C* shows that the peak of the action potential in the process is lower, suggesting that the depolarization in the cell body was indeed passively conducted. The noise in this experiment does not permit us to decide which explanation is correct.

Measurements of Space Constants. We were able to make an estimate of the amplitude ratio of the passive voltage responses in the cell body and growth cone by using the relative magnitudes of the fluorescence changes, ΔF, at different parts of the cell.[§] We found that the values of $\Delta F/F$ were approximately the same (±25%) for propagating action potentials in different parts of the cell (in two examples, Fig. 5 *A* and *B*, the differences are less than 10%). However, the magnitudes of responses to subthreshold stimulation were different. In one example a decrease in $\Delta F/F$ by a factor of 1.7 was found between the cell body and growth cone, which were 510 μm apart (Fig. 5*D*). If the decrease in $\Delta F/F$ is truly proportional to the voltage change, then a lower limit of 900 μm is determined for the space constant. In five other experiments the calculated space constants were 400–950 μm for 4- to 7-μm processes. These values are to be considered as lower limits because the values of the space constants are very sensitive to negative errors in the estimation of $\Delta F/F$.

CONCLUSIONS

At present the main advantage of transmission measurements is the capability of recording simultaneously with a large number of photodetectors. This is especially powerful for detection of regional variations in electrical properties and for the localization of postsynaptic neurons. The resolution limit with this method can be calculated from the single trial experiment shown in Fig. 2. Here the S/N is about 20 for a 70-mV potential change. Signal averaging can improve the resolution. Thus, 0.7 mV should be detected with a S/N of 2 in an experiment of 100 trials. Therefore, the sensitivity of the transmission measurements is sufficient to detect 1- to 2-mV synaptic potentials in cell bodies of postsynaptic cells (19).

The main advantage of fluorescence is its greater sensitivity, especially when recording from small areas. The change in intensity is typically 3–7% per 100-mV potential change, compared with 0.04% per 100 mV in transmission measurements. The S/N is also better when small areas with no background are examined. Fluorescence recordings with multiple detectors using light guides to individual photomultipliers or an image intensifier in front of the photodiode array should also be feasible.

Although improvements can be expected (28), currently available dyes and technology are adequate to investigate several problems in neurobiology that are difficult to approach by conventional electrophysiological techniques. Examples include the regional specialization of ionic channels and receptors and the study of the patterns of synapse formation in a large population of neurons. Of special interest for studying neural development is the capability of recording electrical events in the growth cone (28).

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[§] Values of $\Delta F/F$, measured from different sites, are expected to be proportional to the absolute membrane potential only if the nature of dye binding is identical and there is no fluorescence background from nonrelevant membranes. Space constants can also be determined by an analysis of the time course of voltage responses at different sites with cable theory (29). This method, when applicable, is preferred because it does not require knowledge of the corresponding amplitudes.