brief communication

Optical recording of the electrical activity of synaptically interacting *Aplysia* neurons in culture using potentiometric probes

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ABSTRACT We used multiple-site optical recording methods, in conjunction with impermeant molecular probes of the cell membrane potential, to record the electrical activity of model neural circuits in vitro. Our system consisted of co-cultured pairs of left upper quadrant neurons from the abdominal ganglion of the marine gastropod *Aplysia*. These neurons interact via inhibitory synapses in vitro. Photodynamic damage to the neurons was essentially eliminated over the time course of the measurements, ≤ 30 s, by removing oxygen from the recording solution and replacing it with argon. This procedure did not affect the synaptic interactions. We observed repetitive spiking activity in single-trace optical recordings with a maximum signal-to-noise ratio per detector of ~ 50. Individual optical signals that corresponded to either the activity of the presynaptic neuron or that of the postsynaptic neuron were clearly identified. This allowed us to monitor the activity of synaptically interacting neurons, observed as a reduction of the firing rate of the postsynaptic cell after activity of the presynaptic cell. Our results demonstrate that optical methods are appropriate for recording prolonged, asynchronous activity from synaptically interacting neurons in culture.

INTRODUCTION

The study of model systems in vitro has contributed to our understanding of neurophysiology (for review see Fischbach and Nelson, 1977). Recent work has focused on in vitro systems constructed from identified invertebrate neurons (Ready and Nicholls, 1979; Kaczmarak et al., 1979; Dagan and Levitan, 1981; Wong et al., 1981). The identity, spatial location, and growth conditions of the neurons in these circuits can be experimentally controlled in a relatively precise manner. Thus, questions that could not be adequately addressed in vivo can be examined with these culture systems. Examples include the development of electrical connections between neurons (Hadley et al., 1983), the development and physiology of chemical synapses (Fuchs et al., 1982; Camarado et al., 1983; Haydon, 1988), short-term and long-term aspects of synaptic modification (Rayport and Schacher, 1986; Montarolo et al., 1986), and the analysis of circuits with multiple, stable output states (Kleinfeld et al., 1988c).

Our interest in model systems concerns the patterns of electrical activity that may occur in neural circuits as a result of the synaptic coupling between cells. Large in vitro networks, comprised of highly interconnected neurons, are expected to exhibit a rich repertoire of dynamical behavior. One possibility is the emergence of multiple, temporally stable patterns of activity in networks with symmetric connections (Hopfield, 1982; Amit et al., 1985a and b). Another possibility is the existence of widespread, temporally incoherent activity in networks with asymmetric connections (Sompolinsky et al., 1988).

In this work we examine the feasibility of using potentiometric probes (e.g., Cohen and Salzberg, 1978) and multiple site optical recording techniques (Salzberg et al., 1977; Grinvald et al., 1981a) to measure the activity of co-cultured *Aplysia* neurons. Co-cultures of identified neurons were prepared using the procedures developed by Schacher and Proshansky (1983). We focused our efforts on co-culturing left upper quadrant (LUQ) cells from the abdominal ganglion, L2 through L6, either among themselves or with interneuron L10. The function of the LUQ cells in vivo is uncertain (Kandel, 1976), yet they form robust, relatively slow inhibitory connections in vitro (Kleinfeld et al., 1988a). The use of inhibitory synapses avoids difficulties that may occur in a circuit containing excitatory connections, e.g., confusing excitatory chemical synapses with excitation via electrotonic connections.

Preliminary reports of this work and related experiments have appeared (Obaid et al., 1988; Parsons et al., 1988a and b).

METHODS

Culture techniques

Juvenile *Aplysia californica* (1–10 g, stage 12), raised in mariculture, were a gift of the Howard Hughes Medical Institute, Woods Hole, MA. Identified neurons were isolated from the abdominal ganglion and maintained in cell culture as described earlier (Schacher and Proshansky, 1983). The central region of the culture dishes were replaced with a

No. 00 glass cover-slip (50–80 μm thick) to facilitate the optical studies.

Solutions

Artificial sea water (ASW) consisted of 460 mM NaCl, 10 mM KCl, 10 mM Hepes, 10 mM glucose, 55 mM $MgCl_2$, and 10 mM $CaCl_2$; pH 7.6. Deoxygenated ASW was prepared by bubbling argon through a 500-ml volume for at least 15 min. The final oxygen tension, monitored with an electrochemical probe (Transidyne General Corp., Ann Arbor, MI), was $Po_2 \lesssim 0.02$ atmospheres.

Electrophysiology

Standard current-clamp methods were used. Neurons were penetrated with single-barrel microelectrodes filled with 3 M KCl ($R_E \simeq 20$ MΩ) before the optical trials to document the presence of synaptic connections. All measurements were performed in ASW at room temperature. Co-cultures were examined 3–6 d after the plating of the second neuron.

Optical recording

Experiments were performed using a modified version of an optical system described previously (Salzberg et al., 1983). The modified system was built around an inverted microscope (model IM 35; Carl Zeiss, Inc., Thornwood NY) to allow simultaneous intracellular and optical recording from cells in culture. Light from a 100-W tungsten-halogen lamp (No. 64625; Osram, Berlin, FRG) was collimated, band-limited by a heat filter (model KG-1; Schott Glass Technologies Inc., Duryea, PA) and an interference filter (700 ± 25 nm) (Omega Optical, Inc., Brattleboro, VT), and focused with a bright-field condenser (0.6 NA) onto the preparation. Transmitted light was collected with a high numerical aperture objective (25 × 0.8 NA plan neofluor; Carl Zeiss, Inc.) and projected onto a 12 × 12 array of silicon photodiodes (model MD 144-0; Centronic, Newbury Park, CA) located in the image plane of the microscope. The photocurrents generated by the central 124 detectors of the array were amplified by AC-coupled (0.4–300 Hz bandwidth) current-to-voltage converters and subsequently digitized, as described (Salzberg et al., 1977; Grinvald et al., 1981a).

Two sources of noise were present in our optical system, shot-noise and technical noise caused by vibrations. At relatively low intensities of illumination, the noise current at the output of each detector was dominated by shot-noise, and the signal-to-noise ratio increased with increasing intensity. At relatively high intensities of illumination, the noise current was dominated by technical noise, and the signal-to-noise ratio was essentially independent of the intensity. We illuminated our preparations with sufficiently intense light, 1.6 W/cm^2, to insure that we achieved the maximum signal-to-noise ratio at the majority of the detectors.

The microscope stage was fixed to an air table (model RS-46-12; Newport Corp., Fountain Valley, CA) and the optics were mounted on a massive X-Y translator with micrometer control (Calvet and Calvet, 1981). This arrangement allowed the optical system to be moved relative to the culture dish and intracellular electrodes without loss of focus or disruption of the intracellular recordings. It also provided vibration isolation and mechanical stability. The spatial alignment of cells relative to the photodiode array was determined by photographing the preparation through a reticule, imprinted with an outline of the detector matrix, that was located at an intermediate image plane of the microscope.

The cultures were purged with ASW before recording in an effort to remove as much precipitate and cellular debris as possible from the culture dish. Floating particles, as well as poorly adherent cells, resulted in unacceptable levels of optical noise. The neurons were then vitally stained for 10 min with a solution (0.1–0.2 mg/ml in ASW) of the pyrazo-oxonol potentiometric dye RH155 (Grinvald et al., 1980) (No. NK3041; Nippon Kankohshikiso Kenkyusho, Okayama, Japan) and perfused with ASW for 15 min to remove unbound dye. The cells were stained using ASW, as opposed to culture media, to avoid precipitation of the dye by an unknown component in the media. We often observed that the resting potential of the cell hyperpolarized by as much as 25 mV during the staining process, but recovered after removal of excess dye. The use of the dye RH155 at concentrations below 0.1 mg/ml markedly reduced the size of the optical signal, whereas concentrations of the dye above ~0.2 mg/ml caused irreversible changes in the electrophysiological properties of the cells.

The oxygen tension in the culture was lowered immediately before the optical measurements by perfusing the recording chamber with deoxygenated ASW. The chamber was sealed, except for small ports to allow passage of the intracellular electrode, and superfused with argon gas to minimize exchange with oxygen. The oxygen tension in the chamber was monitored continuously during the experiments.

RESULTS

We first examined the basic optical response of individually cultured LUQ neurons in normal, oxygen equilibrated, ASW. Electrical excitation of the cell resulted in changes in the optical absorption, ΔA, determined from the observed change in transmitted intensity, as shown in Fig. 1 B.[1] Relatively large signals, corresponding to action potentials, were observed throughout the cell soma (Fig. 1 C) and the initial segment (Fig. 1 D). The activity in the neurites could also be resolved in a single sweep (Fig. 1 E), but signals from the most distant neurites were unresolved (Fig. 1 F). Similar results were observed with other cells ($n = 25$).

The highest signal-to-noise ratio was consistently observed with detectors that imaged the proximal end of the initial segment (Fig. 1 D). For these detectors, the ratio of the signal to the peak-to-peak noise (S/N) ranged between a typical value of 20 to a maximum value of 50. The corresponding increase in the optical absorption was $\Delta A_{max} \lesssim 1 \cdot 10^{-3}$.

Our results with isolated cells demonstrate the feasibility of recording activity from multiple sites on identified neurons without the need for signal averaging. They also suggest that the shape of the action potential was essentially invariant along the major neural processes (cf. Fig. 1, C–E). However, these experiments also indicated that

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[1]The optical absorption of the sample, A, is related to the intensity of the incident light, I_{IN}, and the intensity of the transmitted light, I_{OUT} by $A = -\log_{10}(I_{OUT}/I_{IN})$. A change in the absorption of the sample, ΔA, will cause a change in the transmitted intensity, ΔI_{OUT}, that can be expressed as $\Delta A = -\log_{10}(1 + \Delta I_{OUT}/I_{OUT})$. For the relatively small changes in intensity that we observed, i.e., $|\Delta I_{OUT}/I_{OUT}| \ll 1$, this becomes $\Delta A \simeq -2.3(\Delta I_{OUT}/I_{OUT})$.
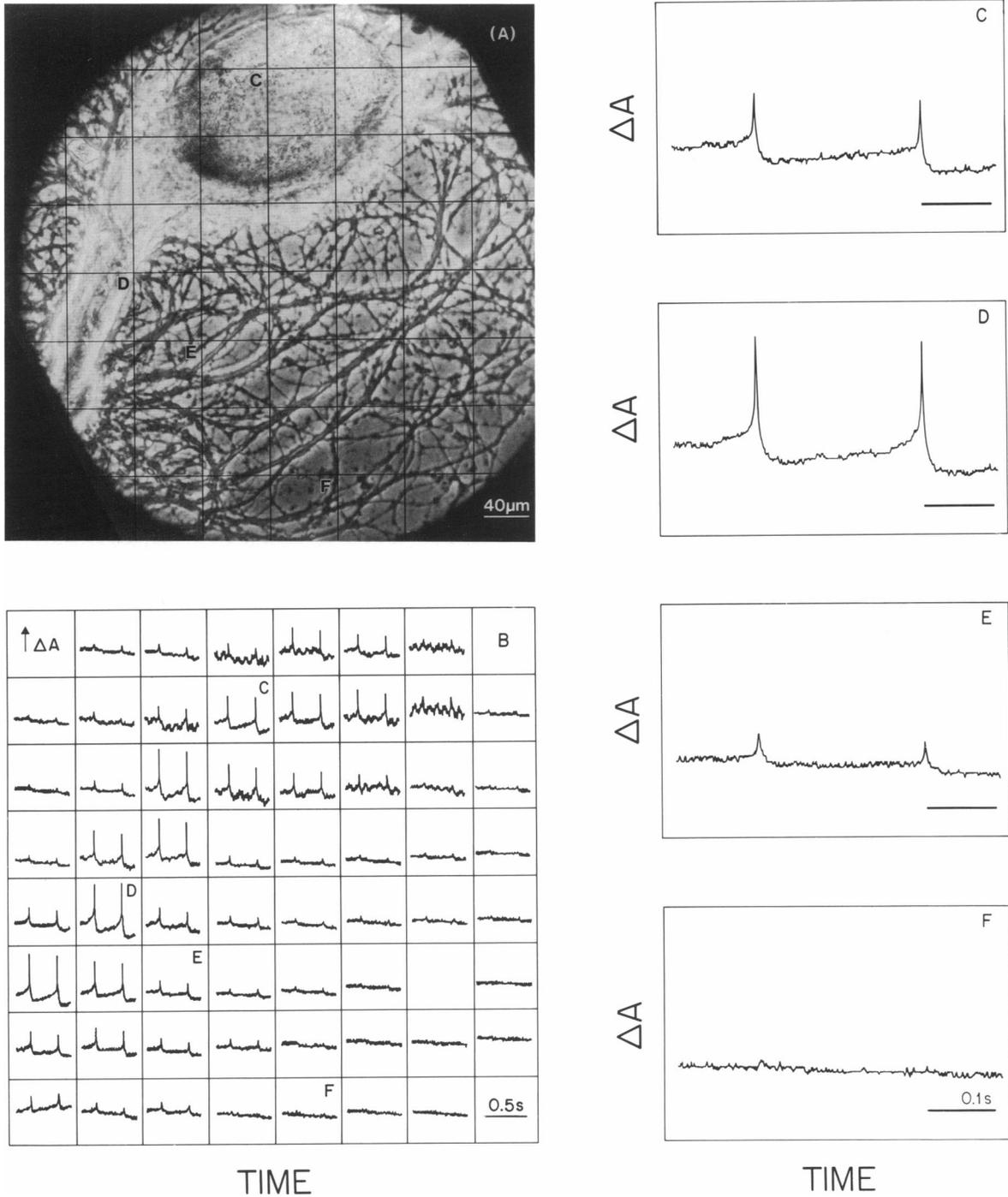
FIGURE 1 Optical recording of consecutive action potentials in an LUQ neuron, 3 d in vitro. The cell was bathed in normal ASW. (*A*) Photograph of the neuron using phase-contrast optics. The total field corresponds to an area measured by 64 detectors of the photodiode array. Each small square corresponds to the field observed by one of the detectors. (*B*) Optical recording of the electrical activity monitored in each of the detectors. (*C*) The activity monitored by a detector over the cell soma. (*D*) The activity monitored over the initial segment. Action potentials recorded by this detector had the largest S/N. (*E*) The activity monitored in a typical neurite. (*F*) The activity monitored in the finest neurites. Action potentials could not be observed in a single trace with this detector.
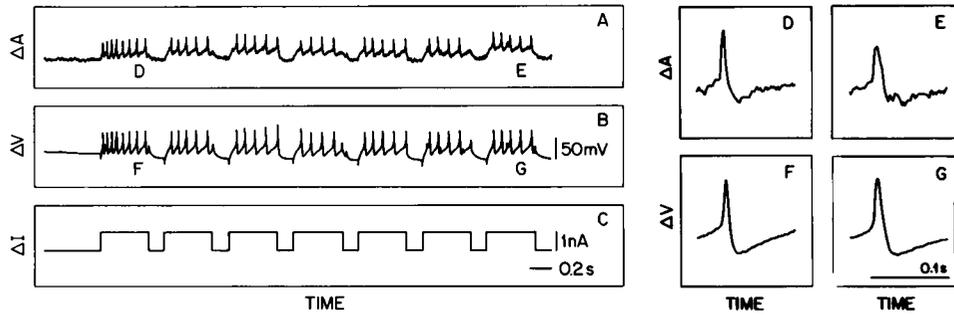
FIGURE 2 Simultaneous optical and intracellular recordings of repetitive action potentials in a cultured LUQ neuron. The cell was bathed in deoxygenated ASW. Action potentials were induced by injecting intracellular pulses of current. (A) Optical record, ΔA, of the first 7 s of activity. Note that the decrease in the number of action potentials during the first current pulse is caused by spike adaptation. (B) Intracellular record, ΔV, of the first 7 s of activity. (C) Record of the intracellular current, $\Delta I(t)$. Pulses were 2 nA in amplitude, 600 ms in duration and repeated every 800 ms. (D–G) Comparison of the records for individual action potentials occurring at the beginning and end of the measurements. This illustrates the integrity of both the optical and the electrical signal after sustained firing. The optical record for the seventh action potential (D) is to be compared with the concomitant intracellular record (F). The optical record for the 34th action potential (E) is to be compared with the concomitant intracellular record (G). A small degree amount of spike broading is observed between the seventh and 34th action potential. Note that the amplitude of the spikes in all records may be truncated due to undersampling of the data.

there was photodynamic damage to the neurons. This damage was manifested as a depolarization of the resting potential, a broadening of the action potential and an alteration in the excitability of the neuron. The severe nature of this damage limited the duration of the recording to ~2 s in normal ASW.

In an attempt to alleviate the phototoxicity, the cultures were bathed in deoxygenated ASW for the duration of the recording sessions. We observed a marked decrease in the extent of damage. This allowed us to record repetitive activity from the cultured neurons, as shown in Fig. 2. In these records, action potentials were elicited in an LUQ cell by injecting periodic current pulses through an intracellular electrode. Examination of both the optical signal and the electrical signal during an episode of firing lasting ~8 s (cf. Fig. 2, C and D, and Fig. 2, E and F) revealed a slight degree of broadening of the action potential and a small decrease in the amplitude of the optical signal. This decrease appears to result from photobleaching of the dye (see also Salzberg et al., 1977). It is important to note that the photolytic products had essentially no deleterious effects on the spiking characteristics of the cells (Figs. 1 and 2). Similar results were obtained with other cultured cells (n = 13).[2]

We next examined the consequences of low oxygen tension on synaptic interactions between cultured LUQ

neurons. These cells form slow, inhibitory connections in vitro with a decay-time (full width at half-maximal response) of τ = 3–5 s.[3] The basic synaptic response between LUQ neurons in normal ASW ($Po_2 \simeq 0.20$ atmospheres) is shown in Fig. 3 A. We observed that there was essentially no change in the synaptic response, for times at least up to 10 min, after the recording solution was exchanged for deoxygenated ASW ($Po_2 \leq 0.02$ atmospheres); see Fig. 3 B. Similar results were found with other cell pairs (n = 11), although the amplitude of the synaptic response in some preparations decreased by up to 50% when normal ASW was replaced by deoxygenated ASW. The full amplitude of the response was restored after the return to normal ASW. These results imply that synaptically interacting LUQ cells remained viable and functionally coupled in deoxygenated ASW for periods that were long in comparison with the typical time of an optical recording session.

The above results allowed us to use optical methods to monitor the electrical activity of synaptically interacting neurons in culture. This is shown in Fig. 4 for a LUQ/L10 pair that formed an inhibitory connection from the LUQ to the L10, similar to that shown in Fig. 3. The presynaptic cell was initially quiescent, whereas the postsynaptic cell was biased to fire repetitively. A 2-s train of action potentials was subsequently elicited in the presynaptic cell by injecting a depolarizing current (Fig. 4 C). The output from a selected detector shows the resulting cessation of activity in the postsynaptic cell (Fig. 4 A). The

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[2]Our results are based on 4–8 s continuous recording sessions. However, by performing repeated measurements on the same cell we observed a decrease in the signal-to-noise ratio that occurred over a data collection period of ~30 s. Comparison of the optical record with simultaneously recorded intracellular signals indicated that the reduction resulted from photobleaching of the potentiometric dye, and not from photodynamic damage to the neurons.

[3]An example of the synaptic response between LUQ neurons is shown in Kleinfeld et al. (1988a). The time scale for the figure shown in this reference was mislabeled. It should range from 0–20s.
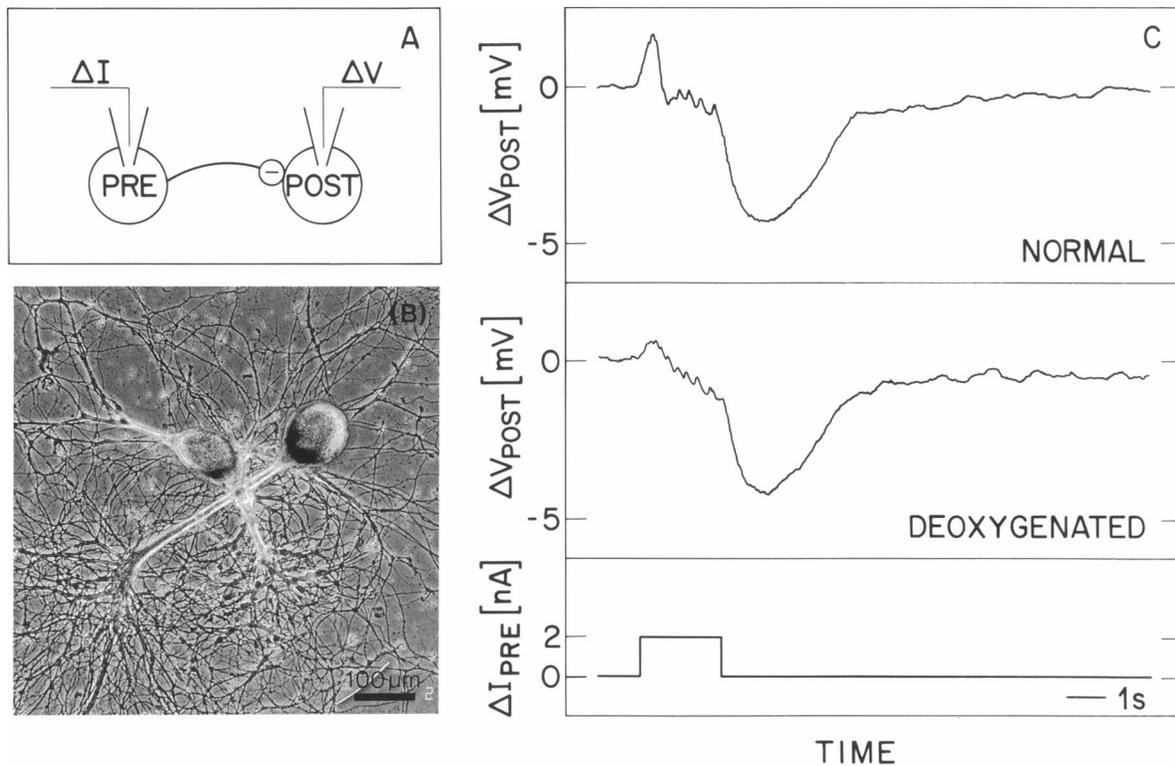
FIGURE 3 The effect of deoxygenated ASW on the synaptic interaction between a pair of co-cultured LUQ neurons. (*A*) Schematic diagram of the intracellular recording scheme. The inhibitory postsynaptic response, $\Delta V_{post}(t)$, was measured in response to a burst of action potentials in the presynaptic cell. The burst was induced by injecting a pulse of current, ΔI_{pre}, that was a 2 nA in amplitude and 2 s in duration. The oxygen tension in the culture dish was continuously monitored. (*B*) Photograph of an LUQ/LUQ pair after 3 d in vitro using phase-contrast optics. The cell on the left is putatively an L2 neuron and the cell on the right is putatively an L4 neuron. (*C*) The postsynaptic response between LUQ cells. The response was observed in normal ASW ($PO_2 = 0.21$ atmospheres) and after 12 min of perfusion with deoxygenated ASW ($PO_2 \leq 0.02$ atmospheres). The initial depolarization was due to electrical coupling between the cells; $V_{post}/V_{pre} = 0.3$ in normal ASW. The amplitude of the coupling decreased in deoxygenated ASW.

duration of the inhibition was in accord with the observed recovery of the inhibitory interaction (Fig. 3 *C*). Further, the information contained in the optical record compares faithfully to that observed in the coincident intracellular record (cf. Fig. 4, *A* and *B*).

We were able to resolve simultaneously the activity of the presynaptic and postsynaptic neurons using multiple site optical recording techniques. This is illustrated in Fig. 5 for a pair of synaptically interacting LUQ cells. In this example, the presynaptic current pulses led to a decrease in the firing rate of the postsynaptic cell. The activity measured by a detector that imaged a region containing the proximal axonal segments of both cells is shown in Fig. 5 *B*. A second detector measured activity in a portion of the soma of the postsynaptic cell (Fig. 5 *C*), and a third measured activity in a portion of the soma of the presynaptic cell (Fig. 5 *D*). Activation of the presynaptic cell (Fig. 5, *D* and *E*) was observed to cause a reduction in the rate of firing of the postsynaptic cell (Fig. 5 *C*). The compound optical signal in Fig. 5 *B* clearly shows contri-

butions from both cells. In all pairs examined ($n = 5$), the rate of firing of the postsynaptic cell returned to its initial value after the decay of the inhibitory postsynaptic potential.

DISCUSSION

The use of molecular probes and optical techniques to record the activity of individual neurons began with studies on isolated invertebrate ganglia (Salzberg et al., 1973). Optical recordings with a relatively large signal-to-noise ratio and relatively little evidence of photodynamic damage were obtained using ganglia from a variety of species (Salzberg et al., 1977; Cohen and Salzberg, 1978; Ross and Reichardt, 1979; Grinvald et al., 1981a; London et al., 1987), including *Aplysia* (Woolum and Strumwasser, 1978; Wu et al., 1987). The extension of optical techniques to cultured cells was subsequently pursued by a number of investigators. Grinvald et al.
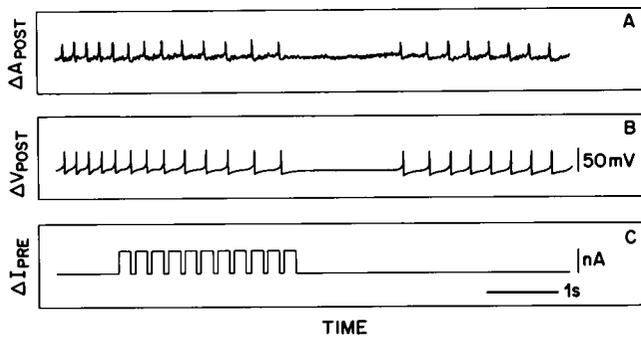
FIGURE 4 Optical recording of the activity of a synaptically interacting pair of LUQ and L10 neurons. These cells were coupled by an inhibitory synapse similar to that shown in Fig. 3. The basal activity of the postsynaptic cell was set by an intracellular bias current and a burst of action potentials were elicited in the presynaptic cell by injecting a train of current pulses. (*A*) Optical recording of the activity monitored in the initial segment of the postsynaptic neuron. Note the cessation of activity after stimulation of the presynaptic cell. (*B*) Simultaneous recording of the intracellular voltage in the postsynaptic cell. (*C*) Record of the current-pulse, ΔI, injected into the presynaptic cell; 2 nA in amplitude, 150 ms in duration at 180-ms intervals.

(1981*b*, 1982, 1983) obtained good signal-to-noise ratios in recording action potentials from mouse neuroblastoma cells. However, these measurements were accompanied by extensive photodynamic damage that precluded the observation of prolonged activity. Other investigators used potentiometric probes to record from a variety of

vertebrate neurons (Ross and Reichardt, 1979; Tank and Ahmed, 1985; Chien et al., 1987) and invertebrate neurons (Obaid and Salzberg, 1982; Ross et al., 1987) in culture, although the signal-to-noise ratios obtained were disappointing, i.e., S/N ~ 1. Signal averaging techniques were usually required in these prior studies to discern the shapes of individual action potentials.

The present work improves upon past attempts to measure the activity of cultured neurons in a number of significant ways: (*a*) We recorded prolonged spiking activity of individual neurons with a relatively large signal-to-noise ratio. (*b*) There was little indication of photo-induced damage over the time course of the measurements. (*c*) We showed that reliable recordings can be obtained from synaptically interacting neurons. This demonstrates that optical methods may be useful for recording the activity of in vitro circuits.[4]

The ability to record optical signals from synaptically

[4] Under optimum conditions the noise in the detector system is dominated by shot-noise. In this limit the signal-to-noise ratio per neuron scales as $S/N \propto \Delta I_{OUT} / \sqrt{I_{OUT}} \propto \sqrt{I_{OUT}} \propto NA \propto M$. The proportionality between the numerical aperture of the objective, NA, and its magnification, M, is generally obeyed, due to practical constraints, for $M \leq 50$. The number of cells, n, that are contained in a field of view will vary directly with the area of the field. Assuming that all neurons require roughly the same area for their outgrowth, we have $n \propto 1/M^2$ and thus $S/N \propto 1/\sqrt{n}$. We typically observed $S/N \simeq 20$ with a field of view capable of encompassing four neurons. This suggests that a field encompassing 100 neurons could be observed with a $S/N \simeq 4$.
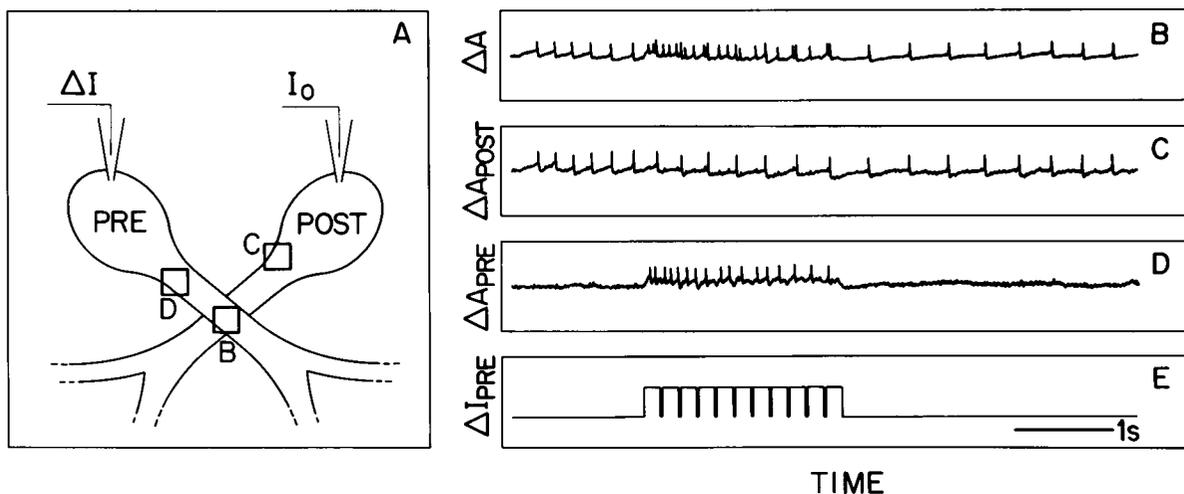

FIGURE 5 Optical recording of the activity of a pair of synaptically interacting LUQ neurons. These cells were coupled by an inhibitory synapse similar to that shown in Fig. 3. (*A*) Schematic diagram of the co-cultured neurons. The boxes correspond to the field observed by selected detectors in the photodiode array. A 2.0-s burst of action potentials were induced in the presynaptic cell by injecting intracellular current pulses, $\Delta I(t)$ (2 nA in amplitude, 150 ms in duration at 180-ms intervals). The basal activity of the postsynaptic cell was set by an intracellular bias current I_0. (*B*) Optical recording measured by an array element encompasses the neurites of both cells. The simultaneous activity of both neurons is seen. Note the decrease in firing rate of the postsynaptic neuron after stimulation of the presynaptic neuron. (*C*) Optical recording of the activity monitored in the initial segment of the postsynaptic neuron. (*D*) Optical recording of the activity monitored in the initial segment of the presynaptic neuron. (*E*) Time dependence of the current-pulse injected into the presynaptic cell.

TABLE 1 Comparison of available techniques for recording network properties of neurons in vitro

Recording method	Attribute				
	Record transmembrane potential	Simultaneously record from multiple sites	Record from an enclosed culture system	Inject constant current	Unconstrained cell placement
Intracellular microelectrodes[*]	Yes	No	No	Yes	Yes
Extracellular microelectrodes[‡]	No	No	No	No	Yes
Extracellular electrode arrays[§]	No	Yes	Yes	No	No
Potentiometric optical probes	Yes	Yes	Yes	No	Yes

[*]Purves, 1981. [‡]Forda et al., 1982; [§]Pine, 1980.

interacting neurons in vitro depended on the resolution of a number of technical issues. Photodynamic damage (Arvanitaki and Chalazonitis, 1961; Pooler and Oxford, 1973) was significantly reduced by bathing the cells in deoxygenated sea water during the optical measurements. Similar results are reported for the squid giant axon (Davila et al., 1973; Cohen et al., 1974). This suggests that photodynamic damage may result from the presence of mobile free-radicals, notably singlet oxygen, formed by photoexcited dye molecules. We were unable, however, to curtail the damage using a variety of both aqueous phase and lipid-soluble, free-radical quenching agents in normally oxygenated sea water (Parsons, T. D., A. L. Obaid, and B. M. Salzberg, unpublished observations). The relatively high signal-to-noise ratio achieved with cultured *Aplysia* neurons results in part from the large area of membrane imaged onto each detector. Furthermore, fluctuations in the optical signal caused by movement of the cell were minimized by the relatively strong adhesion between the neuron and the substrate. Lastly, the *Aplysia* cell membrane appears to bind a large concentration of the potentiometric probe (RH155; Methods) relative to cultured leech neurons (Ross et al., 1987) and cultured *Helisoma* neurons (Parsons, T. D., A. L. Obaid, and B. M. Salzberg, unpublished observations). The enhanced binding of dye by *Aplysia* neurons may result from the extensive invaginations of their plasma membrane (Graubard, 1975).

In the present study we were interested in resolving details of the action potentials. Thus we used a relatively intense light source as our monitoring beam to achieve the highest possible signal-to-noise ratio. The length of each record was subsequently limited by photobleaching of the potentiometric probes. In contrast, a much weaker light source can be used if only the occurrence of action potentials, but not their shape, are of interest. In this case the lower signal-to-noise ratio should be offset by an increase in recording time. Preliminary experiments, in which the activity of single cells were continuously monitored for up to 30 min, support this claim (Parsons et al., 1989).

How useful are nonintracellular recording techniques as a method for understanding the dynamics of networks in vitro? A comparison of the attributes of various recording techniques is shown in Table 1 (see also Regehr et al., 1988). Intracellular recording is the only technique that allows constant current to be injected into the cells. This is crucial for the study of some networks (Kleinfeld and Sompolinsky, 1988; Kleinfeld, D., F. Raccuia-Behling, and H. J. Chiel, submitted for publication). Potentiometric probes and extracellular surface electrodes (Thomas, 1972; Pine, 1980; Droge et al., 1986) are particularly suitable for recording activity at multiple sites in a culture system. The ability to record activity with arrays of surface electrodes, however, is highly dependent on the relatively exact, and difficult, positioning of the cells (Tank and Kleinfeld, 1986; Tank et al., 1986; Kleinfeld et al., 1988*b*). Thus we believe that multiple site optical recording, using potentiometric probes, is the most appropriate available technology for examining circuits containing many neurons.

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