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Research report

Optical recording from cerebellar Purkinje cells using intracellularly injected voltage-sensitive dyes

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Abstract

We evaluated several techniques for their ability to record membrane potential changes with voltage-sensitive dyes introduced into CNS neurons in the brain slice preparation. Using a probe designed for intracellular application, JPW1114, we found that iontophoresis or pressure pulses could not push the lipophilic dye through electrodes whose resistance was sufficiently high to produce good electrical recordings in cerebellar Purkinje neurons. However, properly selected patch electrodes could introduce the dye into the cell and still give good electrical records. Using this technique we recorded depolarizing and hyperpolarizing transients and climbing fiber responses using either a single photodiode or a fast, cooled CCD camera. While these results are promising, there are still problems due to the slow diffusion of the dye in the dendrites and a low sensitivity which requires signal averaging to acquire traces with a good signal to noise ratio.

Keywords: Purkinje cell; Voltage sensitive dye; Imaging; Fluorescence

1. Introduction

To understand the details of synaptic integration in neurons it would be useful to accurately measure the dynamics of membrane potential at many locations in the dendrites, including sites on the fine arbors where most synaptic inputs are located. Microelectrode recordings, usually from the soma or a single site on a thick dendrite do not give a complete picture of the integrative process. Consequently, other methods, especially the use of noninvasive optical techniques, have been used in the study of this problem. High speed imaging of $[Na^+]_i$ and $[Ca^{2+}]_i$ changes [5,10–13,16] has been a particularly rewarding approach. However, this technique reveals the consequences of voltage changes rather than the voltage changes themselves. The more direct approach of recording with voltage-sensitive dyes [2,3,7] from dendrites has been tried with moderate success. The main limiting factor is the modest signal-to-noise ratio (S/N) obtainable with currently available dyes when used for in situ measurements. In addition, many dyes respond differently on different preparations [15] and differently when applied inside instead of outside the neuron e.g. [4]. These difficulties have slowed progress towards this goal.

Grinvald et al. [6] first used voltage-sensitive dyes to record from the processes of neurons in culture. By signal averaging the absorbance changes from cells stained in the dish they could detect a spectrum of voltage responses in different processes. Ross and Krauthamer [14] recorded voltage signals from neurons in a barnacle ganglion using bath applied absorbance dyes and a photodiode array. Signal averaging locked to the stimulation of a single cell improved the S/N and eliminated signals from other stained neurons. However, this approach could not be used when more than one cell was synchronously active, as in synaptically activated preparations. To overcome this limitation, impermeant intracellularly injected dyes have been used to selectively stain individual neurons. Grinvald et al. [9] tested 11 fluorescent styryl dyes in neurons from the leech ganglion. They were able to detect action potentials and synaptic potentials from the neuropil. But signal averaging was required and light had to be integrated from a

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large fraction of the neurites to obtain records with a decent S/N. In an effort to improve on this performance Antic and Zecevic [1] tested styryl dyes that were specifically designed for intracellular injection. When tested in snail neurons the best of these dyes, JPW1114, showed improved sensitivity by a factor of about 50 compared with previously examined indicators. With this dye Antic and Zecevic were able to detect action potentials and hyperpolarizing potentials from long axonal branches of these cells.

It is of considerable interest to apply this same technique to recording from the dendrites of CNS neurons in brain slices. Several aspects of this preparation make it more of a technical challenge than recording from snail neurons. First, the cells are smaller and more delicate than the invertebrate neurons. Therefore, higher resistance electrodes are required, making it more difficult to eject these hydrophobic dyes from the electrodes. Second, it is almost impossible to reimpale a neuron in a slice and make good electrical recordings. Therefore, the technique used on molluscan neurons [1] where the preparation was incubated overnight to allow dye to diffuse to the ends of the processes, cannot be used. Third, brain slice preparations have generally been found to be more sensitive to pharmacological and photodynamic injury than invertebrate preparations [8]. To assay the extent of these difficulties we tested several procedures for recording voltage-sensitive dye signals from Purkinje cells in the rat cerebellar slice preparation. Using patch electrodes to inject high concentrations of dye we were able to record voltage transients elicited by intrasomatic pulses and climbing fiber synaptic activation from small spots on the soma and dendritic arbor. These results are promising but improvements still are necessary before this technique can be used routinely.

2. Materials and methods

2.1. Animals, solutions, and tissue preparation

Sagittal slices from the vermis of 3–5-week-old Sprague-Dawley rats were prepared after the animals were anesthetized with sodium pentobarbital and decapitated. The dissection of the cerebellum was made in 4°C Krebs solution composed of (in mM): NaCl 124, KCl 5, NaH_2PO_4 2.4, $NaHCO_3$ 26, $MgSO_4$ 1.3, $CaCl_2$ 2.4, and glucose 10. Slices were typically 300 μm thick and were stored at room temperature in the same solution. All solutions were bubbled continuously with 95% O_2/5% CO_2.

2.2. Electrophysiology

Slices were mounted in a chamber on the stage of a Zeiss Universal Microscope [12] and were superfused with the same oxygenated Krebs solution at 30°C. Microelectrodes were pulled from 1.5 mm o.d., thick-wall, omega-dot capillary tubing (#1511-M, Friderick and Dimmock, Millville, NJ). Tips were filled with 1 mg/ml JPW1114 (a positively charged styryl dye, gift of Leslie Loew; see [1] for structure) in 140 mM K-Gluconate by capillary action, and backfilled with 3M KAc. Patch electrodes were pulled from the same glass on a Flaming/Brown P-97 puller (Sutter Instruments, Novato, CA). Their tips were also filled by capillary action with solutions of 1, 2, or 4 mg/ml JPW1114 in a solution of (in mM): NaCl 4.0, K-Gluconate 140.0, Hepes 10.0, EGTA 5.0, $CaCl_2$ 0.5, Mg-ATP 4.0 (pH = 7.2).

Both microelectrode penetrations and patch recordings were made under direct visual control using a $\times 40$ (NA = 0.75) water-immersion lens (Zeiss, Thornwood, NY). Before patching, cell bodies of Purkinje cells could be viewed easily with brightfield optics when the substage diaphragm and condenser aperture were reduced to small openings.

Depolarizing and hyperpolarizing stimuli were given through the same electrode used to deliver the dye and to record the electrical response. Climbing fibers were activated with a bipolar electrode placed on the white matter below the Purkinje cell of interest.

2.3. Optical recording

The fluorescence of JPW1114 was detected using a 520 ± 45 excitation filter, 565 nm dichroic mirror, and an RG610 emission filter. The light source was a 75 W xenon lamp. Time dependent measurements were made with either a single PIN-3-D photodiode or a cooled CCD camera (Photometrics, Tucson, AZ) operated in the frame transfer mode [10]. The photodiode had a 500 MΩ feedback resistor and a response time constant of 3 ms. It was used to make high speed measurements from a single location. With the $\times 40$ lens this area was about a 25 μm diameter spot in the plane of the slice. Signals from the cell body or dendrites could be recorded by moving the preparation. To maximize sensitivity for the time dependent events the intensity signal was high-pass filtered with an RC circuit having a time constant of 60 ms. This process introduced some distortion in the optical records shown in the figures. The signal was further amplified, digitized and stored in a computer. S/N was further improved with averaging (usually 15–25 sweeps).

The camera was operated in a high speed mode, with frame intervals of 10–20 ms. This reduced the spatial resolution but signals from different regions of the cell could easily be resolved. The same camera was operated in a high spatial resolution mode to take pictures of the dye-filled neurons.

In a typical experiment data acquisition began with the shutter closed. After a short interval (typically 80 ms) the shutter was opened, giving the resting fluorescence. About 500 ms later the cell was stimulated. Most sweeps had a duration of 1.2 s. Background fluorescence was deter-

mined by measuring the intensity from a location in the field away from the stained neuron.

3. Results

Following previous work [1], we began our experiments using sharp microelectrodes. To get good electrical recordings we had to use high resistance electrodes (70–100 MΩ when filled with 4 M KAc) instead of the much lower resistance pipettes used in snail neurons. Unfortunately, with these electrodes we could not inject the somewhat lipophilic dye into the cell using either depolarizing current or pressure pulses.

Introducing the dye via patch electrodes was more successful. These electrodes were selected to have resistances of 15–18 MΩ. If the resistance was lower, too much dye leaked out of the tip staining the tissue. Because the dye is absorbed by membranes, it was difficult to wash this excess away. Higher resistance electrodes made it difficult to break into the cell after a seal was made. With optimal electrodes the positively charged dye leaked into the cell even when hyperpolarizing current was passed to maintain resting potential.

Using fluorescence optics we could see that the cell body was rapidly filled with dye. However, the voltage-sensitive dye diffused slowly into the dendrites – much more slowly than the water-soluble calcium sensitive indicators injected into the same cells [12]. Fig. 1 shows a fluorescence image of one of our best cells injected with JPW1114. The picture was taken 55 min after break in. The dye has diffused only about 150 μm into the dendrites, which extend well beyond this distance. Thus, even at the higher temperature of this preparation (compared with the snail), slow diffusion limits the ability to record from the active dendritic arbor.

The photodiode and CCD camera detect light by exciting electrons in a thin silicon layer in the front of the

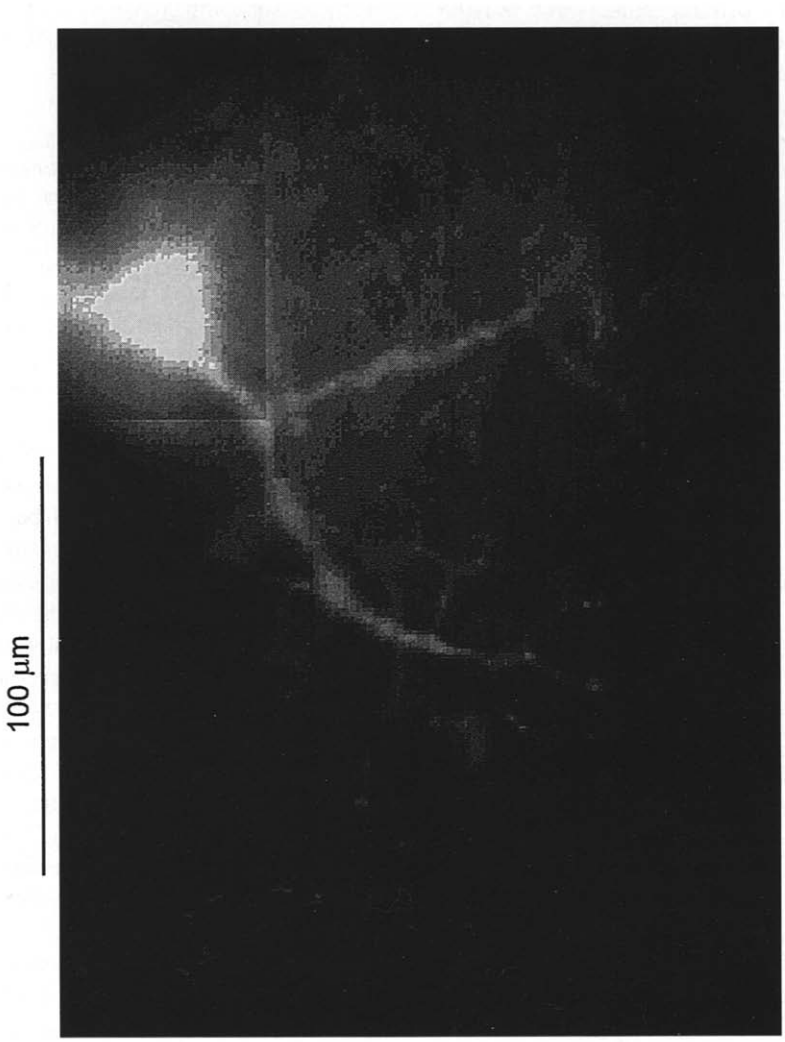

Fig. 1. Fluorescence image of a Purkinje neuron from the rat cerebellum filled with JPW1114 from a patch pipette. Even though the picture was taken about one hour after breaking into the cell, the dye has only diffused about 150 μm into the dendrites.

detector. Therefore, their quantum efficiencies and wavelength sensitivities are similar. However, the electronics used for processing the signals in the two detectors are very different. In addition, the detector in the camera is cooled to about −30°C, reducing the dark noise. Therefore, it was of interest to compare the responses of these two detectors to the fast, low amplitude optical transients produced by the voltage sensitive dyes when the cell was stimulated with typical experimental protocols. Fig. 2 shows electrical and optical responses in the soma to alternating depolarizing and hyperpolarizing pulses in two cells. To measure the response with the CCD camera we averaged the signals from all the pixels over the soma and used a frame interval of 20 ms (Fig. 2, left). After signal averaging the response to the depolarizing and hyperpolarizing pulses could be clearly detected. The signal from the photodiode, in response to a similar stimulation, was much noisier (Fig. 2, right, top trace). This reflected the higher bandwidth of a detector with a 3 ms response time constant. Due to this noise we could not resolve individual transients from the fast Na^+ spikes (typically less than 0.5 ms wide) evoked by the depolarizing pulse, even though most voltage-sensitive dyes can follow voltage changes with the speed of action potentials [2]. Consequently, we reduced the bandwidth of the detector to follow only the slower depolarizing and hyperpolarizing pulses. Fig. 2, right, middle trace, shows the same signal, filtered by averaging all points in 20 ms intervals. This signal has about the same S/N as the record taken with the CCD camera at the same bandwidth. Therefore, it appears that both detectors, when operated at similar bandwidths and detecting from similar areas, produce records of comparable S/N.

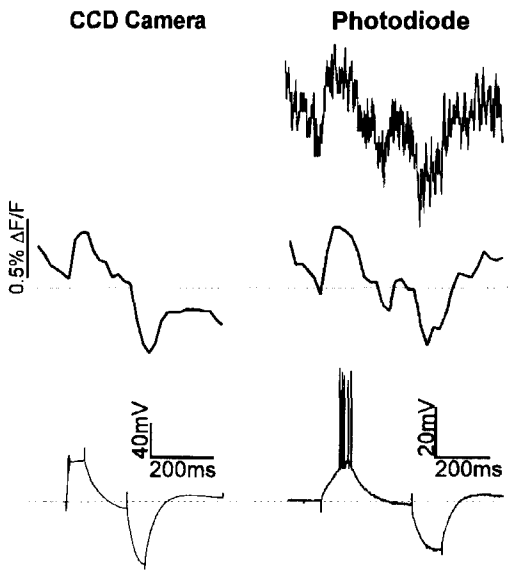

Fig. 2. Comparison between the signals measured with a single photodiode and with the CCD camera in response to a depolarizing pulse followed by a hyperpolarizing pulse. A: camera signal detected from an area of 25×25 μm^2 over the soma. The peak-to-peak intensity change ($\Delta F/F$) is less than 1% after subtraction of autofluorescence from F. Frame interval 20 ms; 16 trials were averaged. B: signals detected from another Purkinje neuron with the photodiode. The top (noisy) trace is the unfiltered optical signal. The bandwidth of the detector and the noise prevent the detection of the individual spikes on the depolarizing pulse. The lower trace shows the same signal filtered by averaging points in 20 ms intervals to correspond to the camera frame interval. Nineteen trials were averaged. Electric traces are from one of the trials in the sequences.

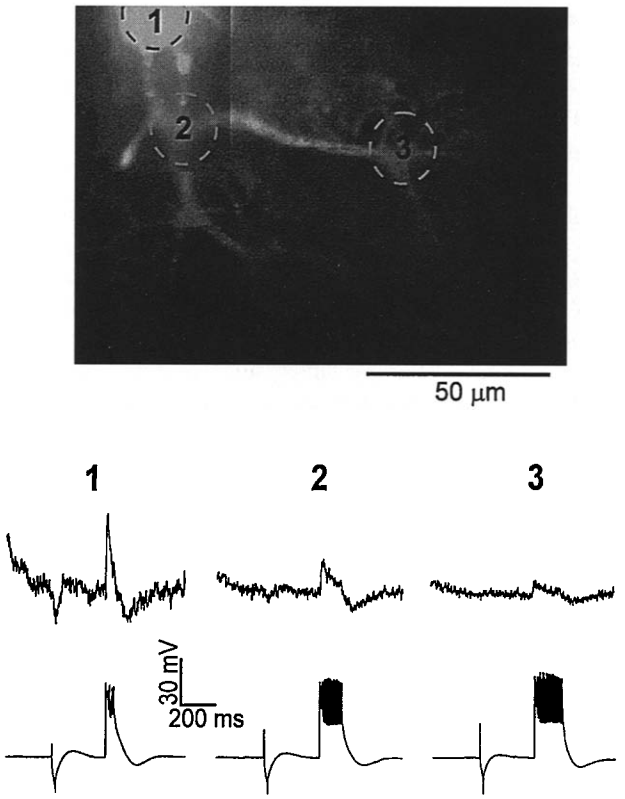

Fig. 3. Climbing fiber induced voltage transients detected with the photodiode from the soma and two dendritic locations. In each case the CF response is preceded by a 20 ms hyperpolarizing pulse. The CF responses are followed by a burst of action potentials riding on a plateau. The optical traces have not been filtered, but they reflect the 75 ms time constant of the AC coupling in the detector circuit. Fifteen trials were averaged for 1; 16 trials averaged for 2; and 25 trials averaged for 3. Note that there is little detectable signal from the hyperpolarizing pulse in the distal dendritic location (3).

Fig. 3 shows optical signals detected with the single photodiode from a cell that alternately responded to a hyperpolarizing pulse from the patch electrode and to climbing fiber activation. The field of view was moved several times during the experiment to detect light from the soma and two dendritic locations. From each location clear transients were detected. The shape of the somatic signal corresponded closely to the shape of the electrically recorded responses, allowing for the AC coupling in the detector electronics. These signals were detected using a dye concentration of 4 mg/ml in the tip of the patch electrode, the highest of the three concentrations we tested.

With this concentration we did not find major photodynamic damage after 2–3 min illumination using sets of 15–25 sweeps (1.2 s duration) with 10 s intervals between sweeps.

4. Discussion

This work records a partial success in the effort to use intracellularly applied voltage-sensitive dyes to record from neurons in the mammalian brain slice preparation. With patch electrodes we could rapidly load Purkinje cells with JPW1114, the currently favored intracellular indicator. Cell electrophysiology was normal and we could record optical signals within 15 min after breaking into the cell. The optical signals in the soma were a faithful reproduction of the somatically recording electrical response (after allowing for filtering of the optical trace). Pharmacological effects or photodynamic damage were not significant problems in these test experiments.

The most serious problems were the slow diffusion of this dye and the still insufficient S/N of the optical responses. Over the time course of an hour of recording from a Purkinje cell from a young rat the visible dye did not extend much beyond halfway to the tips of the dendrites. JPW1114 was designed to be lipophilic to bind to membranes to report membrane potential changes. However, this lipophilicity clearly reduced the mobility of the dye in the cell.

The S/N obtainable with this dye was insufficient to detect clearly the climbing fiber response in this cell in a single trial. This means that it will be difficult to follow the heterogeneous spatial and temporal activity in this cell as can be done with currently available calcium indicators [12]. However, with signal averaging clean optical responses could be recorded.

4.1. Possible improvements

These results suggest that with appropriately designed experiments it may be possible to follow synaptic integration and spike propagation in the dendrites of Purkinje cells using voltage sensitive dyes. In this effort some of the problems we encountered may be easier to overcome than others. The poor S/N, for example, could be compensated for by using higher concentrations of dye and higher light intensities, as we found no significant pharmacological or photodynamic effects in these experiments. On the other hand the slow diffusion problem can only be overcome by the synthesis of more mobile derivatives of the dye. Synthetic efforts in this direction are under way (L. Loew, personal communication).

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