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Intracellular long-wavelength voltage-sensitive dyes for studying the dynamics of action potentials in axons and thin dendrites

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Abstract

In CNS neurons most of synaptic integration takes place in thin dendritic branches that are difficult to study with conventional physiological recording techniques (electrodes). When cellular compartments are too small, or too many, for electrode recordings, optical methods bring considerable advantages. Here we focused our experimental effort on the development and utilization of new kinds of voltage-sensitive dyes (VSD). The new VSDs have bluish appearance in organic solvents, and hence are dubbed "blue dyes". They have preferred excitation windows for voltage recording that are shifted to longer wavelengths (~660 nm). Excitation in deep red light and emission in the near-infrared render "blue VSDs" potentially useful in measurements from fluorescent structures below the tissue surface because light scattering is minimized at longer wavelengths. Seven new molecules were systematically tested using intracellular injection. In comparison to the previously used red dye (JPW-3028) the blue dyes have better sensitivity ($\Delta F/F$) by approximately 40%. Blue dyes take little time to fill the dendritic tree, and in this aspect they are comparable with the fastest red dye JPW-3028. Based on our results, blue VSDs are well suited for experimental exploration of thin neuronal processes in semi intact preparations (brain slice). In some cases only six sweeps of temporal averaging were needed to acquire excellent records of individual action potentials in basal and oblique dendritic branches, or in axons and axon collaterals up to 200 μm away from the cell body. Signal-to-noise ratio of these recordings was ~10. The combination of blue dyes and laser illumination approach imposed little photodynamic damage and allowed the total number of recording sweeps per cell to exceed 100. Using these dyes and a spot laser illumination technique, we demonstrate the first recording of action potentials in the oblique dendrite and distal axonal segment of the same pyramidal cell.

Keywords: Voltage-sensitive dye; Cortex; Pyramidal neuron; Action potential backpropagation; Dendritic imaging; Laser illumination; Blue dyes

1. Introduction

During an ordinary behavioral task pyramidal neurons of the mammalian cerebral cortex are engaged in massive processing of electrical signals (Gilbert, 1977; Leventhal and Hirsch, 1978; Reinagel et al., 1999; London et al., 2002; Elston, 2003). Conventional laboratory tools, such as glass electrodes and calcium imaging are not ideally suited to study the integration of electrical signals in thin dendritic branches, where the majority of synaptic inputs actually impinge on pyramidal neurons (Larkman, 1991). The small diameters of basal, oblique and apical tuft dendrites prevent routine recordings beyond 140 μm from the cell body (Nevian et al., 2007). Also, single-site patch-clamp recordings cannot capture the spatial aspect of signal initiation and propagation. Calcium imaging, on the other hand, is a very indirect way of looking into the neuronal electrical activity (Regehr and Tank, 1990; Miyakawa et al., 1992). Calcium signals in many cases do not correlate well with electrical transients (discussed in Milojkovic et al., 2004). One alternative for recording electrical signals from structures that are too small or fragile for electrode recording is based on voltage-sensitive dyes (VSD). VSD provide a direct, fast, and linear measure of the change in membrane potential of the stained membranes, with time courses that are rapid compared to the rise time of an action potential (Ross et al., 1977; Loew et al., 1985). The development of water-soluble dyes (Antic et al., 1992; Loew, 1988, 1994) has made it possible to inject VSDs into individual cells of interest and analyze the dynamics of electrical transients in dendritic branches that have never been probed with electrodes

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(Antic et al., 1999). Using this method, action potentials and postsynaptic potentials have been characterized in apical tuft branches of the mitral cell (Djurisic et al., 2004), apical and oblique dendrites of the hippocampal CA1 pyramidal neuron (Canepari et al., 2007; Gasparini et al., 2007) and basal dendrites of the neocortical pyramids (Antic, 2003; Kampa and Stuart, 2006). Voltage-sensitive dye imaging is a method of choice in cellular compartments with submicron diameter, such as axon (Palmer and Stuart, 2006) or dendritic spine (Nuriya et al., 2006), reviewed in (Stuart and Palmer, 2006). In spite of these successes, single-cell voltage-imaging technique is burdened with several serious problems, including poor sensitivity, slow diffusion, and high toxicity of currently available VSDs.

Here we present a new set of voltage-sensitive dyes designed for intracellular application. The new dyes (blue VSDs) are characterized with fast diffusion through the dendritic tree, better sensitivity, and less toxicity than previously used "red" intracellular dyes JPW-1114 and JPW-3028 (Antic et al., 1999; Djurisic et al., 2004; Dombeck et al., 2005; Palmer and Stuart, 2006). In addition, blue dyes give best signals when excited with longer wavelengths (658 nm) than red dyes (520 nm), which reduces the scattering of light by the brain tissue and improves the resolution of imaging (Denk et al., 1990; Denk et al., 1994; Shoham et al., 1999). One big challenge for cellular physiology is to carry out simultaneous multi-site recordings of electrical activity in the dendritic tree of pyramidal cortical neuron *in vivo*. In the last decade, *in vivo* dendritic calcium imaging was attained using longer wavelength and laser illumination, in particular two-photon excitation (Svoboda et al., 1997; Waters and Helmchen, 2004). Here we demonstrate the use of low-cost red-laser single-photon illumination (658 nm) for dendritic voltage imaging in brain slice preparation, and collection of photons in the near-infrared part of the spectrum (>710 nm) using a CCD camera at 2.7 kHz frame rate. The improvements in voltage-sensitive dye techniques described in this work, represent a significant expansion of the experimental tools for probing the dendritic function in semi-intact preparations (brain slice) and bring us one step closer to axonal and dendritic voltage-imaging *in vivo*.

2. Materials and methods

2.1. Dye synthesis

Voltage-sensitive dyes were synthesized according to the aldol condensation and palladium-catalyzed coupling strategies described in (Hassner et al., 1984; Antic et al., 1992; Wuskell et al., 2006). Specific details for the synthesis of the new PY dyes will be published elsewhere.

2.2. Brain slice and electrophysiology

Sprague–Dawley rats (P21–42) were anesthetized with isoflurane, decapitated, and the brains were removed with the head immersed in ice-cold, artificial cerebrospinal fluid (ACSF), according to an animal protocol approved by the Center for Laboratory Animal Care, University of Connecticut. Brain slices (300 μm) were cut from frontal lobes in the coronal plane. ACSF contained (in mM) 125 NaCl, 26 $NaHCO_3$, 10 glucose, 2.3 KCl, 1.26 KH_2PO_4, 2 $CaCl_2$ and 1 $MgSO_4$, pH 7.4. Whole-cell recordings were made from visually identified layer V pyramidal neurons on the medial part of the slice. Intracellular solution contained (in mM) 135 K-gluconate, 2 $MgCl_2$, 3 Na_2-ATP, 10 Na_2-phosphocreatine, 0.3 Na_2-GTP and 10 Hepes (pH 7.3, adjusted with KOH). Electrical signals were amplified with Multiclamp 700 A and digitized with two input boards: (1) Digidata Series 1322 A (Axon Instruments) at 5 kHz, and (2) Neuroplex (RedShirtImaging) at 2.7 kHz sampling rate. Only cells with a membrane potential more hyperpolarized than −50 mV, and action potential amplitudes exceeding 80 mV (measured from the base line) were included in this study. All experiments were performed on layer five cortical pyramidal cells at 34 °C.

2.3. Dye injections

The dye injection protocol was based on our previous work with red dyes (JPW-1114 and JPW-3028; Antic, 2003). Neurons were filled through whole-cell recording pipettes with voltage-sensitive dyes listed in Table 1. Blue dyes were stored in ethanol stock solution at −20 °C. On the day of the recording blue dyes were dissolved in standard K-gluconate based intracellular solution. Loading pipette was filled with two varieties of the same intracellular solution; one with and one without dye. Dye-free solution was occupying the very tip of the pipette, while the back of the pipette lumen was microloaded with dye-rich solution. The purpose of dye-free solution in the tip of the patch pipette was to prevent dye-leak during the maneuver through brain slice tissue. VSD styryl dyes are lipophilic and bind indiscriminately and irreversibly to all membranes around the neuron of interest. Even a small amount of dye that leaks out of the pipette during the formation of the gigaohm seal, can generate strong fluorescent background. Fluorescent light emanating from surrounding tissue has a devastating effect on dendritic optical signals. Elimination of the background fluorescence is critical for dendritic voltage imaging. How much dye-free solution one should put in the tip depends on time period between the submersion of the patch pipette into the recording chamber and formation of the gigaohm seal. The faster one can perform this maneuver the less dye-free solution she needs to achieve optimal staining.

Blue VSD were injected at room temperature for 25–60 min. The filling pipette was carefully pulled out (outside-out patch) and brain slices were left to incubate for 20–120 min at room temperature. Just before optical recordings the cells were re-patched with dye-free pipette at the physiological temperature (34 °C).

2.4. Optical measurements

Voltage-sensitive dye imaging was performed on a Zeiss Axioskope 2FS microscope equipped with NeuroCCD camera (RedShirtImaging). We used Zeiss 40× objective IR-Achroplan 0.80 W. The size of the square visual field captured by NeuroCCD camera was 384 μm × 384 μm. In the place of the arc lamp (normally used for epi-illumination), we inserted a 200 μm fiber optic guide with a collimator. Laser beam was