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Presynaptic Imaging of Projection Fibers by In Vivo Injection of Dextran-Conjugated Calcium Indicators

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Abstract

Dextran-conjugated calcium indicators are stably retained within neurons. As a result, they are well suited to measuring presynaptic calcium at physiological temperatures. In addition, dextran indicators can be used to label neurons and their presynaptic boutons in vivo. This has allowed measurements of calcium in the presynaptic boutons of projection fibers that cannot be stably loaded with other types of indicators. This protocol describes a technique for in vivo loading of the climbing fiber projection to the cerebellum with dextran-conjugated indicators for subsequent presynaptic calcium imaging in brain slices. This technique is applicable to studies of projection fibers in many species from which brain slices can be prepared. The dextran indicator is injected into the inferior olive using a stereotaxic device. After a period of 1–3 d, cerebellar slices are prepared and presynaptic calcium transients are measured at physiological temperature in labeled climbing fibers. The protocol also discusses important considerations for using dextran-conjugated indicators to measure presynaptic calcium.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Animal of choice, anesthetized

Dextran-conjugated calcium indicator (see Step 1 and the Discussion for considerations regarding the selection of calcium indicators)

Mineral oil

Equipment

Equipment for preparation of brain slices

Fluorescence excitation light source

This should have an aperture in an image plane conjugate with the specimen to enable adjustment of the field of illumination. The timing of illumination is restricted with a computer-controlled shutter.

Imaging setup

For presynaptic calcium measurements in brain slices, use an upright microscope with standard epifluorescence optics. Water-immersion objectives with high numerical aperture (NA) are important for efficient visualization and detection of

fluorescence (e.g., Olympus 60 $\times$, 0.90 NA). The microscope should also have a 5 $\times$ air objective (NA 0.15). For detection of fluorescence, a photodiode or photomultiplier tube provides high temporal resolution and a high signal-to-noise ratio. Alternatively, a cooled charge-coupled device (CCD) camera may also be used. The signal-to-noise ratio of the fluorescence signal can be optimized by positioning an aperture in the excitation light path to restrict illumination to the region of the labeled fiber of interest.

Injection apparatus (use a 1- μ L Hamilton syringe or an oocyte injector [e.g., Drummond Scientific Nanoject])

Micromanipulator (for positioning the stimulus electrode)

Micropipettes

Stereotaxic instrument (with a fine micromanipulator for positioning the injection apparatus)

Stimulus isolation unit (e.g., World Precision Instruments A365)

METHOD

Preparation of Loading Solution

- 1 For indicators with sufficient fluorescence for visualization at resting levels of calcium (e.g., Calcium Green dextran), combine 5 μ L of a 20% stock solution of dextran-conjugated indicator and 5 μ L of H₂O.

To prepare the stock solution of dextran-conjugated indicators, add 250 μ L H₂O to a 5-mg vial of the indicator. Vortex for 30 sec and sonicate for 1 min. Several dextran-conjugated calcium indicators have extremely low resting fluorescence (e.g., Fluo-4 dextran and Rhod dextran) and must be combined with a calcium-insensitive dextran-conjugated fluorophore to visualize labeled fibers. In this case, combine 5 μ L of a 20% stock solution of the calcium indicator with 5 μ L of a 20% solution of a calcium-insensitive dye. For green calcium indicators, dextran conjugates of Alexa 568, Alexa 594, or Texas Red can be used. For red calcium indicators, use Alexa 488 dextran.

In Vivo Injection of Dextran Indicators

- 2 Prepare an injection pipette by breaking the tip of a micropipette so that it has an open tip diameter of 20–50 μ m.
- 3 Place an anesthetized animal in the stereotaxic instrument.
- 4 Attach the injection pipette to the injection apparatus (Hamilton syringe or oocyte injector) and attach the injection apparatus to the stereotaxic instrument.
- 5 Backfill the injector with mineral oil. Fill the tip with 1–3 μ L of injection solution.
- 6 Advance the injection pipette into the brain and position at the desired loading site.
- 7 Deliver a volume of 0.2–0.3 μ L of dextran injection solution.
- 8 Leave the syringe in place for 5–10 min after injection to prevent leakage of dye from the electrode track.

- 9 Slowly withdraw the pipette and allow the animal to recover from surgery.

Preparation of Brain Slices

- 10 Prepare brain slices from the experimental animal after allowing sufficient time for diffusion of dextrans from the injection site to presynaptic terminals of projection fibers.

The time required for indicator diffusion must be empirically determined. For labeling of cerebellar climbing fibers by injections into the inferior olive of a juvenile mouse, 1–3 d is sufficient.

Presynaptic Imaging

- 11 Identify a labeled climbing fiber. Restrict the area of fluorescence illumination to the region of interest.

See Troubleshooting.

- 12 Stimulate the climbing fibers using a glass electrode placed near the ascending axon in the granule cell layer. Gate the fluorescence illumination and measure the stimulus-evoked change in fluorescence.

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 11): No dye is present in climbing fibers of interest.

Solution: Consider the following:

1. First, verify that the injection has been made into the correct site. This can be performed by adding Fast Green (final concentration 0.1%) to the injection solution. (To prepare a 1% stock solution, dissolve 100 mg of Fast Green in 10 mL of H₂O. Vortex for 30 sec and sonicate for 2 min.) Perform an injection and immediately section the brain to determine the location of the loading site. This method can provide useful feedback for determining the correct stereotaxic coordinates for in vivo injections.
2. If the dye has been injected into the correct site but no labeled fibers are visible, try allowing more time for the dextrans to diffuse.
3. If fibers are still not visible after longer postoperative survival times, add Triton X-100 (final concentration 0.1%) to the loading solution.

Problem (Step 12): Stimulus-evoked calcium transients have small amplitudes or slow decay times.

Solution: This is a symptom of overloading. Because of the variable uptake of dextran dyes into neurons, there will often be a wide range of indicator concentrations in labeled fibers from a single animal. Consider the following:

1. Often the basal fluorescence of the calcium indicator, or the fluorescence of a colabeled fluorophore, can be used to select fibers that are appropriately labeled (enough indicator to provide a reasonable signal-to-noise ratio recording without significantly perturbing calcium signaling).
2. If signals are consistently small or slow, increase the dilution of the dextran-conjugated calcium indicator in the loading solution.

DISCUSSION

Calcium ions entering a nerve terminal evoke neurotransmitter release and regulate several forms of synaptic plasticity (Zucker and Regehr 2002). Many critical determinants of presynaptic function are temperature dependent, such as calcium channel gating kinetics, calcium extrusion mechanisms, and endogenous buffering properties. To understand the roles of calcium in presynaptic function, it is therefore important to perform calcium measurements at physiological temperature.

Unfortunately, calcium indicators in their widely used free-acid form have limitations with regard to measuring presynaptic calcium at physiological temperatures. Low-molecular-weight, acid-based indicators are extruded from neurons in a temperature-sensitive manner (Roe et al. 1990). For indicators that are loaded into a cell with a pipette, the continual replenishment of indicator from the pipette can offset the indicator that escapes the cell. For the cell-permeant acetoxymethyl (AM)-conjugated indicators, the acid form is released following cleavage of the AM group by intracellular esterases (Tsien 1981). Following AM loading, there is a limited amount of indicator within the cell and the temperature-dependent extrusion of the indicator can be particularly pronounced for fine neuronal processes (Beierlein et al. 2004). This greatly limits the experimental time period during which calcium measurements can be made. To address this limitation, dextran-conjugated calcium indicators have been developed; these molecules are stably retained in neurons at physiological temperature for prolonged periods of time and have properties favorable for the measurement of presynaptic calcium (Fig. 1) (Beierlein et al. 2004; Martin et al. 2004).

As with any calcium indicator, dextran-conjugated indicators compete with endogenous calcium buffers and can perturb intracellular calcium transients when the added buffer capacity of the indicator is significant relative to the endogenous calcium buffering capacity of the neuron. Because of their high molecular weights (3–10 kDa), however, dextran-conjugated indicators are less mobile and may therefore have less effect on localized calcium microdomains than the low-molecular weight acid forms of indicators.

Because of their stable retention within neurons, dextran dyes can be injected into the brain of an intact animal with a stereotaxic instrument to label specific populations of neurons and their associated axons and presynaptic boutons. It is not known how hydrophilic dextran-conjugated dyes are taken up by neurons, although mechanisms such as pinocytosis or endocytosis and membrane damage near the injection site may all play roles. The application of some compounds, such as the nonionic detergent Triton X-100, may enhance loading by locally permeabilizing membranes (Helenius and Simons 1975; van de Ven et al. 2009). Following stereotaxic injection, the dye is taken up nonspecifically by neurons in the vicinity of the injection site, and because of its long-term intracellular stability, the dextran indicator can diffuse throughout neuronal processes over periods from hours to days. Using this method, axonal projections extending several millimeters from the injection site can be labeled. This approach permits calcium imaging in presynaptic terminals of projection fibers present in brain slices subsequently prepared from these animals. This method has been applied to measurements of presynaptic calcium in cerebellar climbing fibers that originate in the inferior olive (Fig. 2) (Kreitzer et al. 2000; Kreitzer and Regehr 2001). In vivo injection of dextran-conjugated calcium indicators has also been used in studies of presynaptic calcium in several systems. The in vivo loading technique described here allows calcium measurements in presynaptic terminals that are not suitable for loading with other techniques and is widely applicable to synapses throughout the brain.

Advantages of Dextran-Conjugated Calcium Indicators

A detailed analysis of the temperature dependence of indicator stability for AM- and dextran-conjugated indicators has been performed by Beierlein et al. (2004). These experiments, performed by loading parallel fiber bands in transverse cerebellar slices, provide quantitative information about indicator diffusion and extrusion that cannot be obtained with *in vivo* injections (Fig. 1). After focal application of the low-affinity calcium indicator MagFura-5 AM to parallel fibers at room temperature, steady-state indicator concentrations were reached in <1 h at distances of 600–900 μm from the loading site, and indicator levels were stable for at least 4 h at room temperature. However, when the temperature was shifted to 35°C, the indicator was rapidly extruded and about half of the indicator was lost after 20 min. Such rapid extrusion severely limits the time during which presynaptic calcium measurements can be made at elevated temperatures in brain slices. Moreover, *in vivo* loading of projection fibers with AM indicators is impractical because of the time required for diffusion. It should be pointed out that extrusion appears to be most rapid in small fibers such as the parallel fibers, and acid forms of indicators may be extruded more slowly from larger structures such as cell bodies, perhaps because of their lower surface area to volume ratio.

In sharp contrast to the rapid loss of indicator in AM-loaded fibers at elevated temperature, indicator levels in dextran-loaded parallel fibers were stable for several hours of incubation at 35°C with no evidence of extrusion (Beierlein et al. 2004). Other studies in which dextrans were injected *in vivo* also establish that the dextran indicators are retained in neurons for days (Fritzsche et al. 1990; O'Donovan et al. 1993; Rogers et al. 2006). Although diffusion of the dextran dyes along axons proceeds more slowly at room temperature than in fibers loaded with AM dyes, at a temperature of 35°C diffusion of the dextrans was more rapid. These findings illustrate that dextran-conjugated indicators are stably retained in parallel fiber axons, a property that makes them extremely useful for presynaptic calcium measurements at physiological temperatures. No detrimental effects of dextran loading are apparent. Synaptic responses evoked by dextran-labeled fibers are identical to responses from unlabeled fibers (Kreitzer et al. 2000), and in parallel fibers calcium transients have similar kinetics with long-term dextran loading compared to short-term loading with AM indicators (Beierlein et al. 2004).

Besides their improved stability at physiological temperatures, dextran-conjugated indicators are not prone to compartmentalization within internal organelles, in contrast to the more commonly used acid-based indicators that with time often enter intracellular compartments. Indicator compartmentalization is problematic for quantification of fluorescence signals because dye that has entered an intracellular compartment is not responsive to changes in cytosolic calcium, thus increasing the fluorescence background (Steinberg et al. 1987; Blatter and Wier 1990; Roe et al. 1990; Zhao et al. 1997). A further advantage of dextran dyes is that their affinity for calcium is not altered by the intracellular environment (Luby-Phelps et al. 1986). This stands in contrast to indicators in the acid form that can interact with proteins in the cytoplasm, thereby decreasing the calcium affinity of the indicator by three- to fivefold (Konishi et al. 1988).

Properties of Dextran-Conjugated Indicators

In comparison with AM-conjugated indicators, there are fewer calcium indicators available in dextran-conjugated form. However, several are well suited to presynaptic calcium measurements. Dextran-conjugated indicators that emit in the green (Fluo-4, Calcium Green, Oregon Green 488 BAPTA-1) and red (Rhod-2 and X-Rhod-1) portion of the spectrum are commercially available from Invitrogen. Also available is Fura-2 dextran, which is excited by ultraviolet wavelengths and allows for ratiometric measurements.

One of the primary considerations in selecting an indicator is its affinity for calcium; dextran indicators are available with dissociation constants that range from 240 nM to 5 μ M. For both Fluo-4 dextran and Rhod-2 dextran, high- and low-affinity versions are available. The low-affinity dextran conjugates of both Fluo-4 and Rhod-2 are suitable for linear measurements of presynaptic calcium transients in response to action potential trains and report rapid fluorescence decay times (Kreitzer et al. 2000; Beierlein et al. 2004). In the case of the dextran-conjugated calcium indicators, the calcium affinity can vary among different lots of the same dye, so it is important to obtain calibration data for each batch of indicator.

The red dextran indicators are particularly advantageous in tissues that show high levels of autofluorescence because there is generally lower tissue autofluorescence in the 500- to 600-nm wavelength range in which they are excited. Also, the availability of both red and green indicators allows for the possibility of simultaneous recording from different populations of labeled cells. Moreover, red dextrans can be used to make fluorescence measurements in neurons expressing green fluorescent protein because their excitation and emission wavelengths are sufficiently well separated that the two fluorophores can be independently imaged.

One property of many of the dextran-conjugated calcium indicators is that they have very low levels of fluorescence when they are not bound to calcium. For these indicators, particularly those with low affinity, the fluorescent signal at resting levels of calcium can be difficult to detect, posing difficulties for experimental identification of labeled fibers. For this reason, it is often desirable to label with two dextran-conjugated fluorophores, one calcium-sensitive and the other calcium-insensitive and of a different color. There are a variety of Alexa dextran conjugates available that are suitable for this purpose, such as Alexa 488 dextran for use with red calcium indicators and Alexa 594 dextran or Texas Red dextran for use with green calcium indicators.

Conclusion

Dextran-conjugated indicators allow presynaptic calcium measurements to be made at physiological temperatures and in the presynaptic boutons of projection neurons. Different color indicators are available with a range of calcium affinities. In vivo injection of dextran-conjugated dyes provides a powerful technique that can be used to measure calcium in presynaptic terminals of projection neurons.

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FIGURE 1.

Effects of temperature on loading with AM- and dextran-conjugated indicators. (*A,B*) Low-magnification images were taken of the parallel fiber band in a transverse cerebellar slice after focal loading of (*A*) MagFura-5 AM or (*B*) Texas Red dextran (MW 10,000) for 5 min at room temperature. Image acquisition times relative to the start of loading and incubation temperatures are indicated. (*C*) Fluorescence intensities as a function of time are shown for locations 300, 600, and 900 μm from the loading site for a slice loaded with MagFura-5 AM. Although some dye extrusion is evident at room temperature, the rate of dye loss greatly accelerates when the temperature is shifted to 35°C. (*D*) Fluorescence intensities are plotted as in *C* for a slice loaded with Texas Red dextran (MW 10,000). The data show that dextranconjugated indicators are not extruded from parallel fibers at elevated temperatures. (Modified, with permission, from Beierlein et al. 2004.)

FIGURE 2.

In vivo loading of climbing fibers with dextran-conjugated indicators. This diagram illustrates the in vivo loading method. (A) Dextran-conjugated indicator is injected into the inferior olive of a juvenile mouse. (B) Dye is taken up by neurons near the injection site and labels fibers that project to the cerebellar cortex. (C) Confocal image of a climbing fiber labeled with Calcium Green dextran from a sagittal cerebellar slice. Slices were prepared 2–3 d after stereotaxic injection. (D) Composite image of the labeled climbing fiber and the postsynaptic Purkinje cell loaded with Alexa Fluor 568 hydrazide. Scale bar, 20 μ m. (E) Stimulus-evoked fluorescent transient from a Calcium Green dextran-labeled climbing fiber in response to one (*left*) and two (*right*) stimuli. (Modified from Kreitzer et al. 2000, with permission from © Elsevier.)