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Monitoring Presynaptic Calcium Dynamics in Projection Fibers by In Vivo Loading of a Novel Calcium Indicator

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Summary

Fluorometric calcium measurements have revealed presynaptic residual calcium (Ca_{res}) to be an important regulator of synaptic strength. However, in the mammalian brain, it has not been possible to monitor Ca_{res} in fibers that project from one brain region to another. Here, we label neuronal projections by injecting dextran-conjugated calcium indicators into brain nuclei in vivo. Currently available dextran conjugates distort Ca_{res} due to their high affinity for calcium. Therefore, we synthesized a low-affinity indicator, fluo-4 dextran, that can more accurately measure the amplitude and time course of Ca_{res}. We then demonstrate the utility of fluo-4 dextran by measuring Ca_{res} at climbing fiber presynaptic terminals. This method promises to facilitate the study of many synapses in the mammalian CNS, both in brain slices and in vivo.

Introduction

Presynaptic calcium plays a fundamental role in synaptic transmission. High local levels of calcium (10–100 μM) near open calcium channels (Ca_{local}) trigger the release of neurotransmitter (Matthews, 1996). Many neuromodulators alter synaptic strength by inhibiting influx through voltage-dependent calcium channels (Anwyl, 1991), which in turn reduces Ca_{local}. It is difficult to measure Ca_{local}, but a smaller, longer lasting residual calcium signal (Ca_{res}) can be measured with fluorometric methods and used to quantify changes in calcium influx (Regehr and Atluri, 1995). Ca_{res} affects release on longer time scales through processes such as delayed release, facilitation, posttetanic potentiation, and recovery from depression (Magleby, 1987; Zucker, 1999). Despite the importance of calcium in controlling synaptic strength, it is not possible to monitor Ca_{res} at many synapses of interest.

Accurate measurements of Ca_{res} require low-affinity indicators with fast kinetics that are capable of resolving the large and rapid presynaptic calcium transients typical of the mammalian CNS (Regehr and Atluri, 1995). GFP-based calcium sensors (Miyawaki et al., 1997), although promising for many applications, are currently based on the interaction of calmodulin with calmodulin binding protein, resulting in slow calcium binding kinetics. However, low-affinity calcium-sensitive fluorophores based on BAPTA (Tsien, 1980) have rapid kinetics and are better suited for measurements of Ca_{res}.

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Loading BAPTA-based indicators into neurons is usually accomplished in one of two ways. The most direct approach is to dissolve the acid form of an indicator into the internal solution of micro- or patch pipettes, which can then be used to gain intracellular access (Eilers and Konnerth, 2000). Acid indicators are negatively charged and remain in cells for hours after loading. Another loading technique takes advantage of the membrane-permeant AM dye form (Tsien, 1981), which can be focally applied in brain slices to a homogeneous fiber tract, such as the parallel fibers in the cerebellum or the mossy fibers in the hippocampus (Regehr and Tank, 1991). Once inside the cells, endogenous esterases cleave the AM groups, leaving the acid form of the indicator.

Unfortunately, these methods are not adequate for labeling presynaptic terrminals arising from axons that project in heterogeneous fiber tracts over long distances, such as projections from the lateral geniculate nucleus to the visual cortex, or the inferior olive to the cerebellum or the modulatory fibers originating in the raphe nuclei. It is usually not possible to load selectively the presynaptic terminals for such synapses, because the axons and boutons are typically too small for direct microinjection. Furthermore, selective AM loading is impractical, because fiber tracts usually contain axons from many types of cells. One possible solution is to load cell bodies in vivo. However, it can take many hours or days for molecules to diffuse from the cell body to the distant presynaptic terminals of projection neurons, and acid indicators leak from cells or are sequestered into organelles on this timescale (Blatter and Wier, 1990; Roe et al., 1990).

Calcium indicators conjugated to dextrans provide a promising alternative for monitoring presynaptic calcium transients from these types of synapses. Dextrans are biologically inert, high–molecular weight polysaccharides. Conjugation of dextrans to indicator dyes prevents their extrusion from cells (Haugland, 1996). In zebra fish, *Xenopus* tadpoles, and lamprey, neurons have been successfully labeled in vivo with such indicators (Fetcho and O'Malley, 1995; McPherson et al., 1997; Edwards and Cline, 1999). Calcium indicator dextran conjugates have also been used to label and record activity from motor neuron cell bodies in the rat spinal cord in vitro (Lev-Tov and O'Donovan, 1995). In mammals, in vivo injections of dextran conjugates have not been used to label presynaptic terminals with calcium indicators, despite their widespread use in tracing neuronal morphology (Veenman et al., 1992). One limitation is that currently available dextran-conjugated calcium indicators are high affinity and poorly suited to measuring Ca_{res} in the mammalian CNS.

Here, we synthesize a novel low-affinity indicator, fluo-4 dextran, which reports the time course of presynaptic calcium without significant distortion. Once loaded, this indicator remains in cells for many days. We demonstrate the utility of this indicator by injecting fluo-4 dextran into the inferior olive in vivo, enabling us to measure Ca_{res} in cerebellar climbing fiber presynaptic terminals. This method extends quantitative measurements of Ca_{res} to projecting fiber synapses and expands

Table 1. Indicator Dissociation Constants for Calcium

Indicator	K_D (μM)	Reference
Fura-2	0.16	(Kao and Tsien, 1988)
Magnesium green	7	(Zhao et al., 1996)
Fura dextran (10,000 MW)	0.52	(Konishi and Watanabe, 1995)
Calcium green dextran (3,000 MW)	0.54	(Haugland, 1996)
Fluo-4 dextran (10,000 MW)	3.1	

Dissociation constants (K_D) were determined by in vitro calibration. The K_D for fluo-4 dextran was determined as described in the Experimental Procedures.

the possibilities for in vivo imaging of presynaptic activity and calcium influx.

Results

Testing Dextran-Conjugated Indicators in Parallel Fibers

Due to chemical constraints in conjugation, the choice of indicators coupled to dextrans is severely limited. Those that are commercially available have a high affinity for calcium and slow kinetics, and these properties distort the measurement of Ca_{res}. Therefore, a novel low-affinity, dextran-conjugated calcium indicator with fast kinetics was needed.

We synthesized such a probe by attaching the calcium indicator fluo-4 (Gee et al., 2000) to a 10,000 MW dextran. The dissociation constant (K_D) for fluo-4 itself is 345 nM. Dextran conjugation of calcium indicators normally reduces the binding affinity by a factor of ~2. The binding affinity of fluo-4 dextran was further reduced by using an electron-withdrawing acrylate moiety at the 5′ position of fluo-4 to couple the indicator to aminodextran, giving a calcium K_D of 3.1 μM.

We first characterized fluo-4 dextran in cerebellar parallel fibers, where it is possible to load focally calcium indicators in brain slices (Regehr, 2000). Parallel fiber boutons are small, and stimulus-evoked changes in Ca_{res} are large and rapid (several hundred nanomolars with a half decay time of ~35 ms), typical of many synapses in the mammalian CNS. The ability of calcium indicators to track Ca_{res} transients depends upon their kinetics and K_D (Table 1), as well as the magnitude of the calcium signal to be measured (Regehr and Atluri, 1995; Sabatini and Regehr, 1995, 1998). Indicators such as fura-2 do not accurately report the time course of Ca_{res} in parallel fiber presynaptic terminals due to their slow off rates and high affinity (Figure 1A, left) (Regehr and Atluri, 1995; Atluri and Regehr, 1996). In contrast, low-affinity indicators with fast off rates, such as magnesium green, provide accurate time course measurements (Figure 1A, left).

Previous studies have shown that two closely spaced stimuli produce very similar incremental increases in Ca_{res}, which is reflected in similar increases in fluorescence for low-affinity indicators (Figure 1A, right). As a result, the fluorescence changes for indicators such as magnesium green are linearly related to changes in calcium influx. For high-affinity indicators, the incremental increase in fluorescence evoked by a second stimulus is greatly attenuated due to saturation of the indicator, and, consequently, there is not a linear relationship between calcium influx and fluorescence intensity (Regehr and Atluri, 1995). Low-affinity indicators therefore provide a means of both quantifying changes in calcium entry and accurately determining the time course of presynaptic calcium transients.

The currently available dextran conjugates, calcium green dextran and fura dextran, are not well suited to measurements of the time course and amplitude of Ca_{res} (Figures 1B and 1C) due to their high affinity. The time course of the fluorescence transient after a single stimulus is much slower than that reported by the low-affinity indicator magnesium green. Furthermore, the incremental fluorescence increase for two closely spaced stimuli indicates saturation of these high-affinity indicators.

By contrast, fluo-4 dextran provided a linear measure of presynaptic calcium influx, as expected from the in

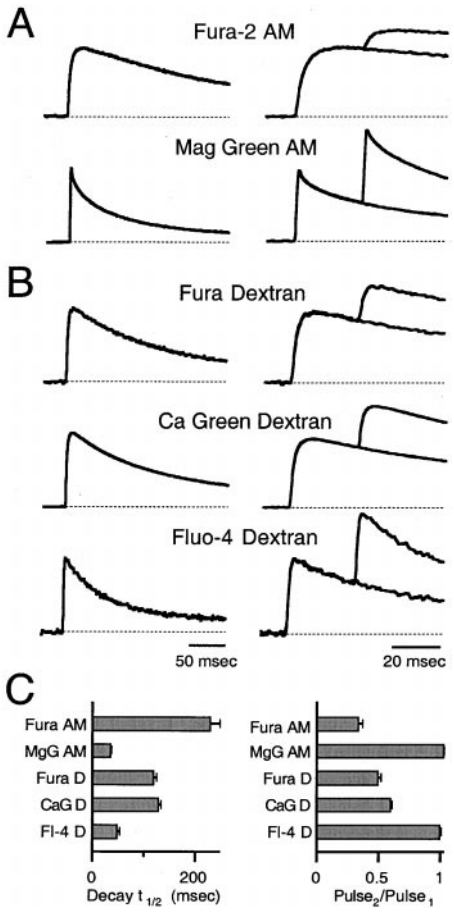

Figure 1. Characterization of Dextran-Conjugated Calcium Indicators in the Parallel Fibers

Fluorescence transients measured in response to one and two stimuli in the parallel fibers using fura-2 AM and magnesium green AM (A), and fura dextran, calcium green dextran, and fluo-4 dextran (B). Traces are averages of 5–50 trials and are normalized to facilitate comparison. (C) gives summary data showing decay time (left) and indicator saturation (right).

vitro determination of the K_D, and allowed for the time course of presynaptic calcium transients to be determined without significant distortion (Figures 1B and 1C). This is a major improvement over calcium green dextran and fura dextran, and it suggests that quantitative measurements of Ca_{res} may be possible in projection fibers using in vivo loading techniques.

Loading Climbing Fibers with Fluorescently Labeled Dextrans

The climbing fiber to Purkinje cell synapse is an example of a system in which it has not been possible to measure presynaptic calcium levels despite many compelling reasons to do so. In adult animals, each Purkinje cell is innervated by a single input originating in the inferior olive (Ramon y Cajal, 1911; Eccles et al., 1966; Sugihara et al., 1999). This fiber forms hundreds of synapses onto the thick proximal dendrites of a Purkinje cell (Palay and Chan-Palay, 1974), resulting in a powerful excitatory connection (Eccles et al., 1966). Because facilitation is not prominent at these synapses, climbing fibers are particularly useful in the study of depression and calcium-dependent recovery from depression (Dittman and Regehr, 1998; Hashimoto and Kano, 1998; Silver et al., 1998). Climbing fibers also undergo significant changes during development (Crepel et al., 1976; Crepel, 1982), and Ca_{res} measurements would be invaluable in understanding how this synapse is regulated.

One reason why it is difficult to measure Ca_{res} at this synapse is that the inferior olive is located in the brainstem, many millimeters from Purkinje cells. This separation and the geometry of the olivocerebellar projection make it impractical to cut brain slices that contain inferior olive neurons contacting Purkinje cells. Synaptic inputs can still be studied, because the climbing fiber remains intact and displays characteristics in the slice that are similar to those displayed in vivo, despite the absence of a cell body (Konnerth et al., 1990; Perkel et al., 1990). Because the cell body is not present, and the climbing fiber is too small for direct microelectrode recording, it is not practical to label climbing fibers with microinjections. It is also impossible to label cells with AM indicators in the slice, because the anatomy of the cerebellum does not allow selective loading of these fibers. The difficulties associated with measuring Ca_{res} in climbing fibers are representative of those encountered at many other synapses. Thus, it is likely that the development of a method to measure Ca_{res} in climbing fibers will be widely applicable.

We therefore tested the feasibility of loading presynaptic fibers with dextran–calcium indicator conjugates in vivo. The method employed is shown in Figure 2. Dextran-conjugated dyes were injected into the inferior olive of anesthetized rats (Figure 2A). After allowing 48–72 hr for the dyes to be taken and transported (Figure 2B), sagittal brain slices of the contralateral cerebellum were cut. In each slice preparation, a number of climbing fibers were labeled, one example of which is shown in Figure 2C. This confocal image stack of a single calcium green dextran–labeled climbing fiber shows the relatively thin axon passing through the granular layer and then thickening and branching in the molecular layer (Figure 2C). The Purkinje cell innervated by this climbing fiber was visually identified using bright-field microscopy and labeled with a fluorescent dye, Alexa Fluor 568 hydrazide, via a patch pipette (Figure 2D). The overlay of the climbing fiber and the Purkinje cell, with regions of overlap appearing as yellow, shows that a vast majority of the climbing fiber synaptic contacts are on the smooth proximal dendrites of the Purkinje cell (Figure 2E). This is consistent with classical descriptions of climbing fiber morphology (Palay and Chan-Palay, 1974). Therefore, it is possible to introduce fluorescently labeled dextrans into individual climbing fibers and to visualize them days later in a brain slice.

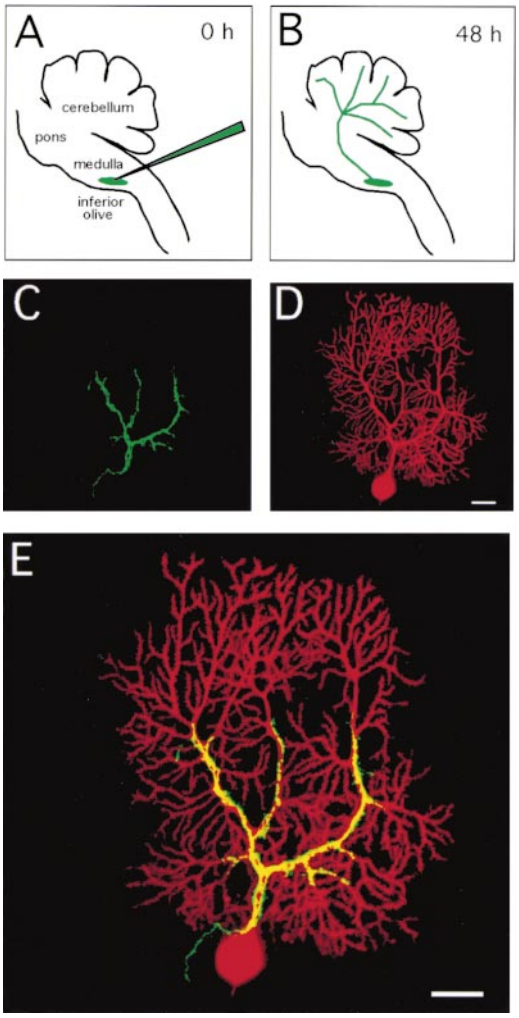

Figure 2. In Vivo Loading of Climbing Fibers

(A) Schematic of the in vivo loading method. Fluorescently labeled dextrans were injected into the inferior olive of neonatal rats.

(B) Following injection, the dye is taken up and labels projecting fibers to cerebellar cortex.

(C) Confocal image stack of a calcium green dextran–labeled climbing fiber in a sagittal cerebellar slice showing incoming axon and terminal arborization.

(D) Confocal image stack of the postsynaptic Purkinje cell innervated by the climbing fiber shown. The Purkinje cell is labeled with the fluorescent dye Alexa Fluor 568 hydrazide.

(E) Overlay of the images in (C) and (D) showing the climbing fiber terminal arborization on the thick proximal dendrites of the Purkinje cell.

Scale bars, 20 μm.

To establish that we could record presynaptic calcium transients from climbing fibers loaded in this manner, we placed a small glass stimulus electrode near the presynaptic axon of a labeled climbing fiber (Figure 3A) and stimulated at a range of intensities. In all cases,

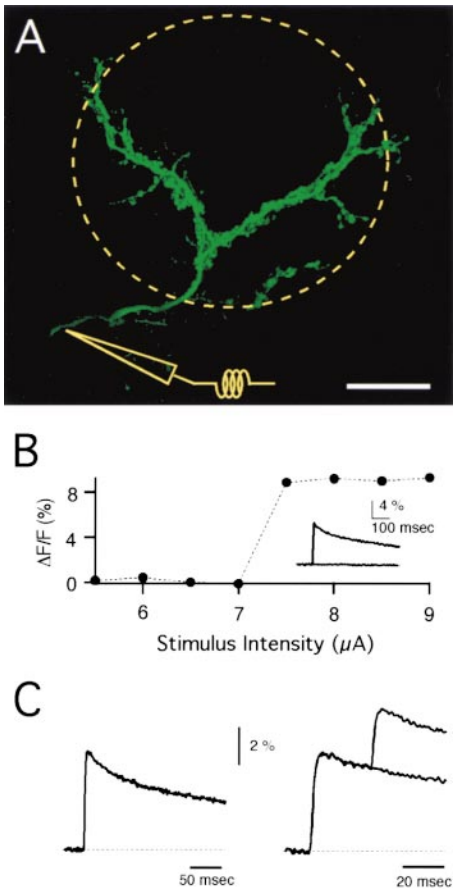

Figure 3. Characterization of the Climbing Fiber Response using Calcium Green Dextran

(A) Confocal image stack of a calcium green dextran–labeled climbing fiber showing stimulation and recording sites. Scale bar, 20 μm.
(B) All-or-none response of climbing fiber fluorescence transients in response to increasing stimulus intensity.
(C) Stimulus-induced changes in calcium green dextran fluorescence for one (left) and two (right) stimuli.

there was a well-defined threshold for a fluorescence response (Figure 3B), consistent with the all-or-none nature of climbing fiber responses seen in electrophysiological investigations.

We found a range of labeling in the climbing fibers within a slice. For calcium green dextran, the fluorescence transients recorded from very bright fibers remained elevated for extended periods, indicative of the introduction of sufficient indicator to buffer calcium, thereby slowing the decay of calcium. We therefore avoided such clearly overloaded fibers and only measured fluorescence changes in moderately labeled fibers.

Stimulation of the climbing fiber with single stimuli and with two closely spaced stimuli produced fluorescence transients reminiscent of those observed in parallel fibers when high-affinity indicators were used. Following single stimuli, calcium green dextran transients decayed with a half decay time of 168 ± 18 ms (mean ± SEM, n = 20). The incremental increase in fluorescence was smaller for the second of two closely spaced stimuli (Figure 3C). The ratio of the second fluorescence transient to the first was 0.59 ± 0.02 (mean ± SEM, n = 17). It is not clear, however, whether this reduction in the size of the second fluorescence transient is due to less calcium influx or saturation of calcium green dextran. Therefore, a low-affinity, dextran-conjugated indicator was required to determine accurately the time course and magnitude of calcium influx in the climbing fiber.

Fluo-4 Dextran in the Climbing Fiber

We used the low-affinity, dextran-conjugated calcium indicator fluo-4 dextran to measure Ca_{res} in climbing fibers. Fluo-4 dextran is virtually nonfluorescent in the absence of calcium. This presents a difficulty for an in vivo loading approach, because it is necessary to visualize labeled fibers in order to stimulate and record from them. Therefore, we coinjected Texas red dextran with fluo-4 dextran. A confocal image stack of a Texas red–labeled fiber is shown in Figure 4A (upper left). The postsynaptic Purkinje cell is shown in Figure 4A (lower left) and was labeled with the fluorescent dye Alexa Fluor 488 via a patch pipette. The overlay of the climbing fiber and the Purkinje cell (Figure 4A, right) demonstrates that climbing fibers labeled with Texas red dextran exhibit identical morphology to fibers labeled with calcium green dextran (Figure 2). Texas red dextran–labeled climbing fibers were readily visualized with rhodamine optics, facilitating placement of the stimulus electrode near a labeled climbing fiber axon. Fluo-4 dextran transients could then be measured with fluorescein optics.

An example of the type of labeling observed at these two wavelengths is shown in Figure 4B. In this single confocal section, two climbing fibers are clearly labeled with Texas red dextran (left panel), but at rest, fluo-4 dextran fluorescence is difficult to resolve (middle panel). However, while selectively stimulating a single climbing fiber, the fluorescence of fluo-4 dextran becomes visible (right panel). We found that all fibers labeled with Texas red dextran were also labeled with fluo-4 dextran and showed clear fluorescence changes upon stimulation. The time course and fluorescence changes of fluo-4 dextran did not depend upon the intensity of labeling, suggesting that overloading was not a problem with this indicator.

Using fluo-4 dextran to measure Ca_{res} at the climbing fiber, we find that the incremental change in fluorescence for the first and second pulse remains constant. The ratio of the second fluorescent transient to the first was 0.99 ± 0.02 (mean ± SEM, n = 13). Moreover, we find that Ca_{res} decays rapidly at these terminals, with a half decay time of 33 ± 4 ms (mean ± SEM, n = 14) (Figure 4C). This suggests that, as in the parallel fibers, the incremental increase in calcium influx for the first and second pulse remains constant in the climbing fiber and that fluo-4 can accurately report Ca_{res} in these presynaptic terminals.

Responses of Dextran-Conjugated Calcium Indicators to Stimulus Trains

Two important applications of calcium indicators are to track presynaptic calcium during realistic patterns of activity and to allow presynaptic activity patterns to be faithfully monitored. Neurons in vivo often fire at high frequency, irregularly, and sometimes in bursts. We therefore tested the properties of both fluo-4 dextran and calcium green dextran by stimulating climbing fibers with trains (Figure 5).

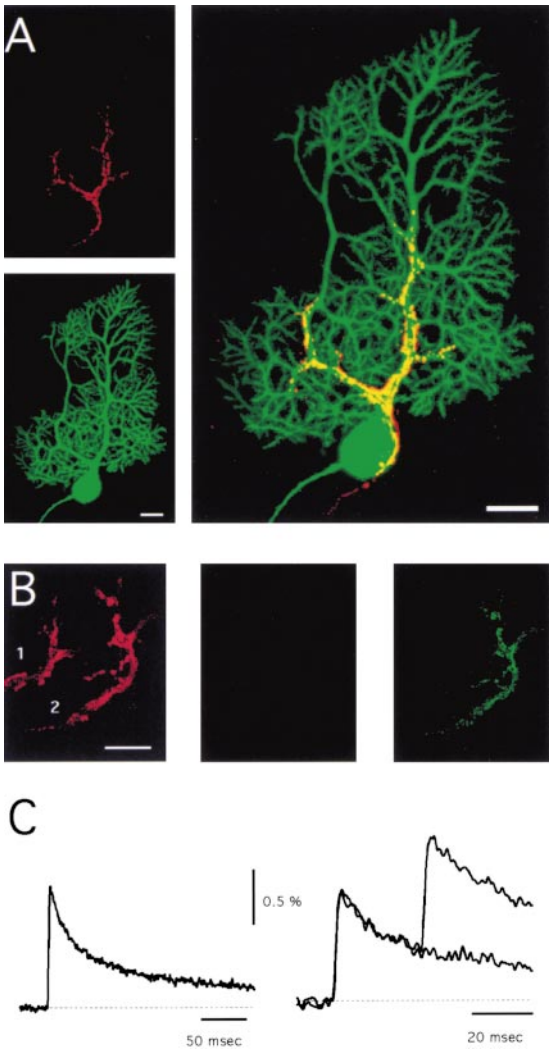

Figure 4. Quantification of Presynaptic Calcium Dynamics in Climbing Fiber Presynaptic Terminals Colabeled with Fluo-4 Dextran and Texas red Dextran

(A) A confocal image stack of a Texas red dextran–labeled climbing fiber (upper left) and the Purkinje cell onto which the climbing fiber synapses (lower left). The Purkinje cell is labeled with the fluorescent dye Alexa Fluor 488. The overlay of the climbing fiber and Purkinje cell shows the characteristic morphology of this synapse (right).

(B) A single confocal image section of two climbing fibers using rhodamine optics showing labeling with Texas red dextran (left). An image of these fibers at rest (middle) and during 20 Hz stimulation of fiber 2 (right) using fluorescein optics showing fluo-4 dextran labeling.

(C) Stimulus-induced changes in fluo-4 dextran fluorescence for one (left) and two (right) stimuli.

Scale bars, 20 μm.

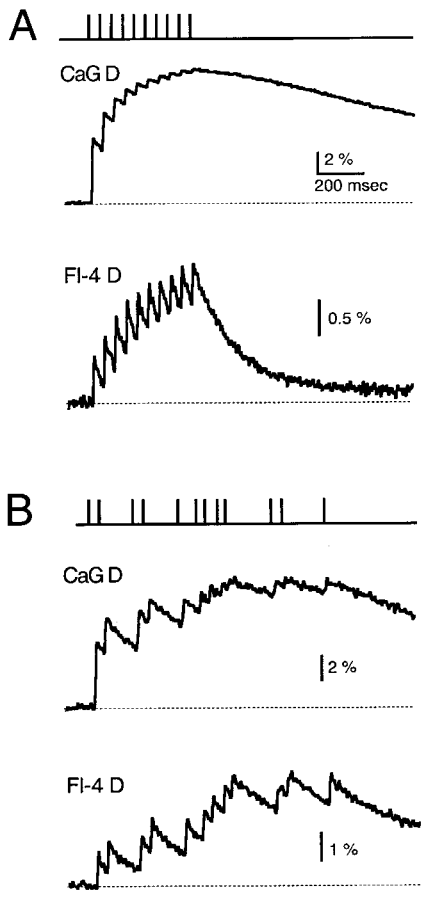

Figure 5. Calcium Green Dextran and Fluo-4 Dextran Fluorescence Transients in Response to Trains of Stimuli

(A) Stimulus-evoked fluorescence transients in response to ten stimuli at 20 Hz (top) for calcium green dextran (CaG D, middle) and Fluo-4 dextran (Fl-4 D, bottom). Traces are averages of five trials.

(B) Fluorescence transients in response to the indicated spike train (top) for calcium green dextran (middle) and fluo-4 dextran (bottom). Traces are single trials.

Stimulation with regular trains evoked very different responses for the high- and low-affinity indicators. For a 20 Hz ten pulse train, the fluorescence signal observed in calcium green dextran–labeled climbing fibers showed significant saturation, and by the tenth pulse, the stimulus-evoked increase in fluorescence was barely detectable (Figure 5A, top). In contrast, for fluo-4 dextran, stimulus-evoked increases in fluorescence remained similar throughout the train, and following stimulation, the fluorescence decayed quickly to baseline (Figure 5A, bottom). Similar results have been consistently observed for 20 Hz stimulation ($n = 4$ for both calcium green dextran and fluo-4 dextran). These records illustrate that high-frequency stimulation accentuates the distortion of calcium signals by calcium green dextran and that fluo-4 dextran overcomes such difficulties.

Another important application of dextran-conjugated calcium indicators is to detect presynaptic activity patterns. We tested these dyes with a Poisson stimulus train (Figure 5B, top). This pattern of stimulation is irregular and has high-frequency bursts, characteristic of the firing patterns of many neurons in vivo. The activity pattern of the presynaptic fiber cannot be determined with calcium green dextran (Figure 5B, top), because during high-frequency bursts, the indicator saturates. As a result, in this example, pulses 8, 9, and 11 do not evoke a discernible increase in fluorescence. Similar results were found in other fibers ($n = 4$ for both calcium green dextran and fluo-4 dextran). In contrast, the evoked responses measured with fluo-4 dextran are easily identified throughout the train (Figure 5B, bottom). Fluo-4

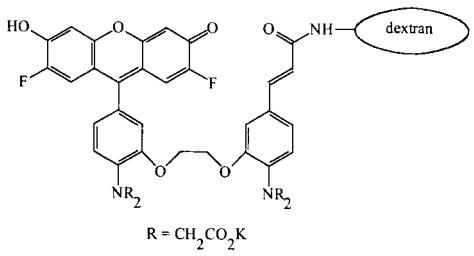

Figure 6. Structure of Fluo-4 Dextran

dextran is able to follow activity in the physiological range and therefore appears promising for in vivo studies.

Discussion

We have synthesized a novel low-affinity calcium indicator, fluo-4 dextran, and shown that it is well suited to measuring calcium transients in mammalian presynaptic terminals. Fluo-4 dextran remains in cells for many days, allowing neurons to be loaded in vivo. These properties enable a number of studies that were not previously possible.

Fluo-4 Dextran Enables the Study of Calcium-Dependent Processes at a Novel Class of Synapses

Loading fluo-4 dextran in vivo allowed us to more accurately resolve stimulus-evoked changes in Ca_{res} from projecting fibers that could not be labeled with standard techniques. Although we demonstrated the utility of this loading technique and novel indicator at the climbing fiber synapse, this approach could readily be applied to many other synapses. In preliminary experiments, we have labeled mossy fiber afferents to the cerebellum by injecting the lateral nucleus reticularis in the brainstem. In principle, it should be possible to label and record from many types of presynaptic projecting fibers, including those arising from the thalamus, and the inferior and superior colliculi, as well as the brainstem. Ascending brainstem projections, such as the serotonergic fibers of the raphe nucleus or the noradrenergic fibers of the locus ceruleus, play an important role in regulating circuit function in the CNS (Pape and McCormick, 1989), but the physiological properties of these fibers are not well understood. The ability to monitor presynaptic calcium in identified fibers originating in each of these nuclei promises to aid in the study of modulatory systems.

Fluo-4 dextran will facilitate the study of presynaptic modulation and calcium-dependent short-term plasticity at such synapses. Because of the low affinity and rapid kinetics of this indicator, quantification of changes in presynaptic calcium influx is possible. Such changes can occur, for example, in the presence of neuromodulators and can have a profound impact on the amount of neurotransmitter release. Measurements using fluo-4 dextran will also allow quantification of the time course of Ca_{res}, which is an important factor in many forms of use-dependent plasticity, including facilitation, recovery from depression, delayed release, and posttetanic potentiation. Quantitative study of the role of Ca_{res} in these forms of plasticity is not possible with high-affinity indicators, such as calcium green dextran.

Potential In Vivo Applications

Perhaps most exciting is the possible use of fluo-4 dextran for in vivo studies using two-photon microscopy (Svoboda et al., 1997). Fluo-4 dextran can resolve incremental increases in presynaptic calcium during repetitive neuronal firing, whereas currently available indicators, such as calcium green dextran, saturate and thus cannot distinguish individual events during repetitive activity (see Figure 5B). Moreover, fluo-4 dextran remains in cells for many days, and in vivo loading can be used to label many presynaptic fibers. This will allow the simultaneous monitoring of activity in a population of presynaptic fibers over extended periods of time.

One example of an important issue that could be addressed with fluo-4 dextran is the role of synaptic inputs in shaping receptive field properties in visual cortex (Reid and Alonso, 1996). Following injection of fluo-4 dextran into the lateral geniculate nucleus, two-photon imaging of thalamocortical afferents in layer 4 of visual cortex could be performed during visual stimulation. This would allow for a mapping of presynaptic activity patterns, which could, in turn, be compared with the receptive field properties of postsynaptic neurons.

These examples serve to illustrate the utility of in vivo loading with the low-affinity indicator fluo-4 dextran. The methods described here promise to be widely applicable for the study of synaptic transmission in the mammalian CNS.

Experimental Procedures

Synthesis of Fluo-4 Dextran

4-Fluororesorcinol (Sun et al., 1997) was condensed with 5-formyl-5′-benzyloxycarbonylethenyl-BAPTA, tetramethyl ester under strongly acidic conditions. The resulting 5′-acrylic acid derivative of fluo-4 tetramethyl ester was converted into its succinimidyl ester using disuccinimidyl carbonate, followed by reaction with aminodextran (10,000 MW, average of 3.1 amino groups/dextran molecule). The resulting conjugate was purified by gel filtration (Sephadex G-25) to separate the conjugate from the free dye. The BAPTA methyl esters were cleaved by treating an aqueous solution of the conjugate with sodium hydroxide overnight (pH $>$10.5), giving fluo-4 dextran (Figure 6), which was purified by dialysis. The calcium K_D (3.1 μM) was determined essentially as described by Minta et al. (1989), by monitoring fluorescence (490 nm excitation, 520 nm emission) in 100 mM KCl, 10 mM MOPS (pH 7.2), 0–10 mM Ca-EGTA at 22°C. In the absence of calcium, the probe is essentially nonfluorescent, undergoing a fluorescence increase of $>$100-fold upon saturation with Ca^{2+}. The degree of substitution (DOS) was determined to be 1.2 by measuring the optical density at λ_{max} of a pH 8 solution of a measured mass of fluo-4 dextran and assuming an extinction coefficient of 75,000 $mol^{-1}cm^{-1}$. Like other BAPTA-based indicators (Tsien, 1980), fluo-4 dextran is highly selective for Ca^{2+} over Mg^{2+} and is insensitive to normal pH changes in the physiological range.

Injections

Sprague-Dawley rats (postnatal day 9–12) were anesthetized with isoflurane and placed in a stereotaxic device (Kopf). A continuous flow of a 0.75%–1.5% isoflurane/oxygen mixture maintained anesthesia through the procedure, during which time heart and/or breathing rates were monitored continuously. A homeothermic heating system (Harvard Instruments) was used to keep body temperatures near 37°C. The brainstem was exposed by a dorsal–caudal approach. After the animals were deeply anesthetized, as determined by respiratory rate and lack of tail pinch response, a 2.5 cm incision was made along the dorsal midline at neck level. The muscles and overlying fascia were retracted, and the dura was exposed between the foramen magnum and C1. A small incision was made in the dura at the midline, exposing the dorsal brainstem.

Dextran-conjugated calcium indicators were dissolved in water

at a concentration of 20%–25%. For fluo-4 dextran (and calcium green dextran, in some experiments), this solution was then mixed, in a 1:1 ratio, with a similar solution of Texas red dextran of the same molecular weight. Micropipettes were pulled and broken to a tip diameter of 40–50 μm. Pipette tips were attached to the end of a Hamilton syringe (model 7001) with epoxy. The syringe was filled with oil, and tips were then backfilled with 0.5 μl of the dextran solution. The syringe was then affixed to the stereotaxic device, and injections were made unilaterally at the midline, just rostral of the dorsomedian spinal vein at a depth of 1.8–1.9 mm. The injection needle was at an angle 65° from vertical and 5° from the midline. In some cases, two injections were made in the same animal to achieve better labeling. A volume of 0.2–0.3 μl was delivered over 15–20 min by tapping the plunger of the syringe periodically. The syringe was then left in place for 5–10 min to prevent dye leakage from the needle track.

One consideration in the application of this technique is the mechanism by which dextran conjugates are taken up into cells. In an initial study, damage to nerves increased the uptake of dextrans, but it was also found that dextrans were taken up at intact and functioning neuromuscular junctions (Glover et al., 1986), suggesting that other mechanisms, such as endocytosis or pinocytosis, could bring dextrans into cells. In our recordings, the climbing fiber morphology and threshold for stimulation are still normal, even 5 days after injections, whereas inferior olive lesions result in climbing fiber degeneration and removal within 3 days (Desclin, 1976), indicating that the fibers we record from are not damaged.

Slice Preparation

After a survival time of 2–5 days following injection, rats were anesthetized with isoflurane and decapitated. Using methods described previously (Regehr and Mintz, 1994), sagittal slices (300 μM thick) were cut from the cerebellum and superfused with an external saline solution containing (in mM): NaCl, 125; KCl, 2.5; $CaCl_2$, 1.5; $MgCl_2$, 1; $NaHCO_3$, 26; NaH_2PO_4, 1.25; and glucose, 25 bubbled with 95% O_2/5% CO_2. Slices were incubated in the dark for 1 hr at 32°C and stored in the dark at room temperature for up to 6 hr before recording.

Optical Imaging

Climbing fibers were labeled as described above. Parallel fibers, made up of granule cell axons and presynaptic terminals, were labeled in vitro with dextran dyes using a high-pressure stream, as previously described for AM dye loading (Regehr and Atluri, 1995). The solution for focally loading parallel fibers consisted of 0.1% calcium indicator dye, 0.1% Texas red dextran, and 0.1% Triton X-100 in dH_20. The inclusion of Triton X-100 improved the loading obtained with brief focal applications of the dye but was not used for in vivo loading. Fast green was included in this solution to visualize the loading area. Loading times were 1–2 min for high-affinity dyes and 3–4 min for low-affinity dyes.

Climbing fibers were stimulated with a glass electrode placed near each of their ascending axons in the granule cell layer. Fluorescence was measured from the climbing fiber terminal arborization with a photodiode. In the parallel fibers, tracts were stimulated extracellularly, and epifluorescence was measured with a photodiode from a spot several hundred micrometers from the loading site, where nearly all of the fluorescence signal arises from parallel fiber presynaptic boutons that synapse onto Purkinje cells (Regehr and Atluri, 1995).

Purkinje cells were labeled by diluting microinjection-ready Alexa Fluor 488 hydrazide and Alexa Fluor 568 hydrazide dyes (Molecular Probes, Eugene, OR) into an internal patch pipette solution and obtaining the whole-cell recording configuration using 2–3 MΩ pipettes. After 5–10 min, the patch pipette was slowly withdrawn, thereby forming an outside-out patch and sealing the Purkinje cell.

The filter set for magnesium green, calcium green dextran, and fluo-4 dextran was 470DF20 (Zeiss) for excitation, 510DRLP dichroic, and OG530 for emission. The filter set for Texas red dextran was 580DF15 for excitation, 600DRLP dichroic, and 610LP for emission. The filter set for fura and fura dextran was 380DF15 for excitation, 435 DRLP dichroic, and 455LP for emission (Omega Optical, Brattleboro, VT).

Confocal Microscopy

An Olympus Fluoview confocal imaging system was used to acquire images from ACSF-perfused cerebellar slices. An Olympus 60× water immersion objective was used in combination with standard Olympus fluorescein/rhodamine filter sets. Calcium green dextran, fluo-4 dextran, and Alexa Fluor 488 were excited at 488 nm by an argon laser. Texas red dextran and Alexa Fluor 568 were excited at 568 nm by a krypton laser.

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