Presynaptic Calcium Measurements at Physiological Temperatures Using a New Class of Dextran-Conjugated Indicators

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INTRODUCTION

Presynaptic calcium (Ca_{pre}) both triggers neurotransmitter release and regulates release through several forms of synaptic plasticity (Zucker and Regehr 2002). The measurement of presynaptic calcium levels has therefore become an important tool in studying synaptic transmission. Because of the technical difficulties associated with performing measurements of Ca_{pre} at physiological temperatures (35–37°C), most recordings of Ca_{pre} have been conducted at temperatures well below the physiological range. However, presynaptic calcium signals are controlled by a number of highly temperature-dependent processes including: the opening of presynaptic voltage-gated calcium channels (McAllister-Williams and Kelly 1995; Noble et al. 1990), extrusion via calcium pumps (Zenisek and Matthews 2000), and intracellular buffering (Edmonds et al. 2000). Thus there is an important need to measure Ca_{pre} at physiological temperatures.

Anecdotal reports suggest that the methods widely employed to measure Ca_{pre} at room temperature are not readily applied at elevated temperatures. For room temperature studies, the preferred approach is to measure Ca_{pre} with fluorescent 1,2-bis(2-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA) derivatives of small molecular weight (Tsien 1980). In contrast to green fluorescent protein (GFP)-based indicators that are slow and nonlinear (Higashijima et al. 2003; Ikeda et al. 2003; Miyawaki 2003; Miyawaki et al. 1999; Miyawaki et al. 1997), these indicators have rapid kinetics, they can provide a linear measure of Ca_{pre} and they are available with a range of optical properties and calcium sensitivities. The acid form of these fluorophores can be loaded directly into cells with a pipette (Eilers and Konnerth 2000). Alternatively they can be derivatized with acetoxyethyl (AM) esters to make them membrane-permeant (Tsien 1981). Once inside cells, hydrolysis by intracellular esterases releases the ion-sensitive indicator. Although these indicators have been used to measure calcium transients within cell bodies at physiological temperatures (Stosiek et al. 2003), measurements of Ca_{pre} have been much more limited under these conditions. It is possible that these indicators, whether loaded as an acid or AM form, are pumped from cells at a rapid rate at high temperatures (Di Virgilio et al. 1990; Roe et al. 1990; Tombal et al. 1999).

In addition, Ca_{pre} can be measured with indicators conjugated to dextrans, which are long-chain polysaccharides of high molecular weight. Dextran dyes are not broken down by intracellular enzymes and are not extruded from the cytoplasm (Haugland 1996). Therefore this class of indicators has been widely used for loading cell bodies or synapses in vivo (Edwards and Cline 1999; Fetcho and O’Malle 1997; Kreitzer et al. 2000; McPherson et al. 1997; O’Donovan et al. 1993; Takahashi et al. 2002; Wachowiak and Cohen 2001) where large distances between the loading site and presynaptic terminals necessitate transport times of up to several days. Although there is currently a somewhat limited range of commercially available dextran-conjugated calcium indicators, their properties appear particularly promising for measurements of Ca_{pre} at physiological temperatures.

Here we directly compared AM and dextran-conjugated indicators in their ability to report stable measurements of Ca_{pre} at physiological temperatures. We found that under those conditions only dextran-conjugated indicators permit long-term measurements of Ca_{pre}. To address the limitations in the properties of available dextran-conjugated dyes, we synthesized three new red indicators with a range of calcium affinities that are excited at wavelengths of 540–580 nm. These dyes can be used at excitation and emission wavelengths that do not
conflict with other dextran-conjugated calcium indicators or with GFP and are ideally suited for measurements where tissue autofluorescence is particularly problematic. Moreover, we show that they are well suited to the measurement of $[Ca^{2+}]_{\text{pre}}$ from individual synaptic terminals.

**METHODS**

Slice preparation and indicator loading of parallel fibers

Rats (P17–P19) were anesthetized with halothane and decapitated, and transverse cerebellar slices (300 μm) were obtained as described previously (Atluri and Regehr 1996). Slices were cut in a sucrone solution consisting of (in mM) 81.2 NaCl, 23.4 NaHCO$_3$, 69.9 sucrose, 23.3 glucose, 2.4 KCl, 1.4 NaH$_2$PO$_4$, 6.7 MgCl$_2$, and 0.5 CaCl$_2$. Slices were incubated at 32°C for 30 min and then transferred to a saline solution at 32°C consisting of (in mM) 125 NaCl, 26 NaHCO$_3$, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 25 glucose, 2 CaCl$_2$, and 1 MgCl$_2$. Experiments were performed at either 22 or 35°C from a small area.

Optical imaging

Parallel fiber tracts were stimulated extracellularly, and epifluorescence was measured with a photomultiplier tube (H7422-40, Hamamatsu Photonics, Hamamatsu City, Japan) from a small area 400–700 μm from the loading site. Excitation was generated with a monochromator (Polychrome IV, Till Photonics, Gräfelfingen, Germany). The excitation wavelength for magnesium green and calcium green dextran was 470 nm. The filter set used was 510DRLP dichroic, and 535DF35 for emission. The excitation wavelength used for the dyes rhod dextran (high and low affinity) was 540 nm, and the filter set was 560DRLP dichroic and 595AF60. The excitation wavelength for Texas Red and x-rhod dextran was 570 nm and the filter set was 590DRLP dichroic, and 600ALP for emission. The excitation wavelength for mag fura-5 AM was 370 nm and the filter set was 400 DRLP dichroic, and 525AF45 for emission (Omega Optical, Brattleboro, VT).

Time-lapse image sequences of dye-loaded parallel fiber bands were acquired with a 4× (0.28 NA) dry objective (Olympus, Tokyo) and a SensiCam CCD camera (PCO Computer Optics, Kelheim, Germany). Exposures ranged from 200 to 400 ms.

**Imaging individual mossy fiber boutons**

Parasagittal cerebellar slices (250 μm) of transgenic mice (P20–P25) expressing GFP in cerebellar mossy fibers (Feng et al. 2000) were prepared (Regehr and Mintz 1994) and incubated as described in the preceding text. Mossy fibers were labeled with Texas Red dextran by focal application to white matter tracts. Two-photon laser-scanning images of mossy fiber boutons were obtained with a modified Olympus confocal microscope equipped with a Ti-sapphire pulsed laser (Coherent Laser Group, Santa Clara, CA). GFP and Texas Red dextran were simultaneously excited at 810 nm and image stacks were collected using Fluoview software (Olympus, Melville, NY).

For calcium measurements of individual synaptic boutons, mossy fibers were loaded with x-rhod dextran as described in the preceding text. To facilitate visualization, we included Texas Red dextran in the loading solution. Presynaptic mossy fibers were activated using glass electrodes (5–10 μm diam) placed in the granule cell layer. Calcium transients were measured at room temperature with a photomultiplier tube as described in the preceding text. Excitation was restricted to a single labeled bouton.

**Data acquisition and analysis**

All signals were digitized with a 16-bit A/D converter (ITC-16, Instrutech, Port Washington, NY). FMT currents were digitized at 20 kHz and digitally filtered at 400 Hz. All analysis was performed using custom macros written in Igor Pro (WaveMetrics, Lake Oswego, OR) or Vision Software (Till Photonics). Averages are given as means ± SE.

**Synthesis of dextran-conjugated indicators**

The synthesis involved condensation of two equivalents of 3-dimethylanilinophenol (to generate the rhod dextrans) or 8-hydroxyjulolidine (to generate x-rhod dextran) with the BAPla aldehyde (Fig. 1). The resulting dihydrorhodamines were oxidized with chloranil, then trifluoroacetic acid (TFA) was used to selectively remove the benzhydryl ester. The resultant carboxylic acid was activated as its N-hydroxy-succinimide ester, followed by reaction with 10,000 MW aminodextran. Finally, the BAPla methyl esters were cleaved by saponification with KOH.

The calcium dependence of indicator fluorescence was measured with a spectro-fluorimeter (Aminco-Bowman AB2, SLM Aminco, Rochester, NY). Calcium calibration solutions were prepared as described previously (Tsien and Pozzan 1989). The dissociation constants ($K_{d50}$) for each dye (Table 1) were obtained by fitting the Hill equation to the data points of each dye with the Hill coefficient set to 1.
the transport and retention of dextran dyes within presynaptic terminals (Fig. 3, A–C). At room temperature, Texas Red dextran was transported much more slowly within parallel fibers than were AM indicators. At 22°C, peak fluorescence levels at distances of 600 μm from the loading site were reached only after 4–6 h (Fig. 3D, cf. Fig. 2D), and sites more distal did not reach steady-state levels after 6 h. In separate experiments when the bath temperature was raised to 35°C after 2 h, redistribution of the dye along the fiber tract occurred with a faster time course that led to a smaller fluorescence gradient between sites proximal and distal to the loading site compared with incubation at room temperature (Fig. 3C, cf. Fig. 3D). Furthermore there was little evidence for dye extrusion. Similar results were obtained with calcium green dextran \((n = 4, \text{data not shown})\). These data suggest that dextran-conjugated dyes are not extruded from parallel fibers and travel in axons via active, temperature-dependent transport.

**Presynaptic calcium imaging at physiological temperatures**

The data in Fig. 2 indicate that AM dyes are rapidly extruded from the axons of parallel fibers. To more directly relate dye extrusion to the measurement of \(\text{Ca}_{\text{pre}}\) we measured fluorescence transients generated by the low-affinity calcium indicator magnesium green AM (Fig. 4A). Parallel fiber tracts were loaded and slices were incubated for 2–3 h at room temperature prior to imaging. We then measured fluorescence transients evoked by single stimuli to parallel fibers once every min. At 22°C, fluorescence intensity decreased slightly while the amplitude of the fluorescence transients remained stable (Fig. 4A, left). On average, fluorescence levels decayed to 89 ± 2% \((n = 3)\) after 120 min. Amplitudes of fluorescence transients at 120 min remained virtually unchanged (101 ± 4%). In a different set of experiments, we raised the bath temperature to 35°C after 30 min. This led to an initial increase in fluorescence intensity (Fig. 4A, right). It is likely that this increase reflected increased binding of magnesium to the indicator as magnesium levels are the main determinant of magnesium green fluorescence under resting conditions (Haugland 1996). After this initial increase fluorescence levels then gradually declined. On average, fluorescence intensities 30 min after raising the bath temperature were 81 ± 4% of control, 30 ± 4% after 60 min, and 10 ± 3% after 90 min \((n = 10)\) compared with fluorescence intensity levels prior to the increase in temperature. The amplitude of the fluorescence transient immediately decreased to 39 ± 4% of control, after the increase in bath temperature. This decrease likely reflected a reduction in calcium influx due to changes in presynaptic waveform and faster calcium channel kinetics at higher temperatures (Sabatini and Regehr 1996, 1999). Fluorescent transients remained relatively constant for ∼30 min and then began to decrease further. After ∼90 min fluorescence transients could no longer be evoked (inset, Fig. 4A, right). On average, peaks were 96 ± 3% after 30 min, 49 ± 3% after 60 min, and 13 ± 2% after 90 min compared with the values immediately after the temperature increase. Thus while calcium transients remained stable for ∼30 min in spite of significant dye loss, long-term measurements were not feasible with AM indicators.

We then tested whether dextran-conjugated calcium indicators can be used to obtain stable responses at physiological temperatures. For these measurements, we loaded parallel fi-

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**Table 1. Properties of red dextran-conjugated calcium indicators**

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Excitation Maximum, nm</th>
<th>Emission Maximum, nm</th>
<th>(K_{\text{eq}}), μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>x-rhod dextran</td>
<td>583</td>
<td>602</td>
<td>1.4</td>
</tr>
<tr>
<td>Rhod dextran</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High affinity</td>
<td>556</td>
<td>577</td>
<td>1.4</td>
</tr>
<tr>
<td>Low affinity</td>
<td>556</td>
<td>577</td>
<td>3.9</td>
</tr>
</tbody>
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Indicator properties were measured in vitro at room temperature.

**RESULTS**

**AM-dye extrusion at high temperatures**

AM indicators have been widely used to measure \(\text{Ca}_{\text{pre}}\) at room temperature \((20–25°C)\) or moderate temperatures \((30–32°C)\) at a variety of synapses in mammalian brain (Atluri and Regehr 1996; Dittman and Regehr 1996; Mintz et al. 1995; Regehr et al. 1994; Sabatini and Regehr 1996-1998; Wu and Saggau 1994). Here we tested whether they are also appropriate for long-term measurements of \(\text{Ca}_{\text{pre}}\) at physiological temperatures of 35–36°C. We first assessed whether AM indicators are retained in axons and presynaptic terminals by performing time-lapse measurements of fluorescence intensity in cerebellar parallel fiber tracts. For these experiments, we used mag fura-5 AM (Zhao et al. 1997), a low-affinity calcium indicator with high fluorescence levels in the absence of calcium. Parallel fibers were loaded at 22°C and measurements commenced immediately after loading (Fig. 2A). Plots of the fluorescence intensity as a function of either distance from the loading site (Fig. 2B) or time (C and D) show that mag fura-5 rapidly loaded parallel fibers. At distances of 600 and 900 μm from the loading site, peak fluorescence levels were reached 40 and 55 min after loading, respectively \((n = 4)\). After 2 h, bath temperature was then increased to 35°C. This caused a rapid loss of fluorescence along the loaded parallel fiber tract (Fig. 2, A and C). On average fluorescence decreased with a half decay time of 23 ± 2 min \((n = 5)\). Comparable results were obtained even when light exposure was reduced by a factor of 100, indicating that the fluorescence decrease reflected dye extrusion rather than photobleaching \((n = 4, \text{not shown})\). Fluorescence decreases with comparable half decay times were observed using magnesium green AM \((n = 5, \text{not shown})\). In contrast, when the preparation was maintained at 22°C for 4 h there was only a modest loss in fluorescence intensity over time (Fig. 2D).

These results show that AM indicators are slowly extruded from parallel fibers at room temperature but rapidly extruded at physiological temperatures. While such indicator extrusion has been observed previously for both acid and AM indicators (Di Virgilio et al. 1990; Roe et al. 1990; Tombal et al. 1999), it appears to be particularly problematic for parallel fibers, perhaps because like other presynaptic structures the surface-to-volume ratio is large. Notably, we were unable to load AM indicators into parallel fibers at physiological temperatures as rapid extrusion precluded any measurable transport along the parallel fiber band.

Indicators conjugated to high-molecular-weight dextrans are less susceptible to extrusion (Glover et al. 1986; Haugland 1996) and thus may be a viable alternative for measuring \(\text{Ca}_{\text{pre}}\) at physiological temperatures. We therefore performed time-lapse imaging experiments with Texas Red dextran to assess

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Cf. Fig. 2A, right. On average, peaks were 96 ± 3% after 30 min, 49 ± 3% after 60 min, and 13 ± 2% after 90 min compared with the values immediately after the temperature increase. Thus while calcium transients remained stable for ∼30 min in spite of significant dye loss, long-term measurements were not feasible with AM indicators.

We then tested whether dextran-conjugated calcium indicators can be used to obtain stable responses at physiological temperatures. For these measurements, we loaded parallel fi-
bers with calcium green dextran (Fig. 4B). Our time-lapse measurements indicated that the axonal transport for this class of indicator is temperature dependent. Thus to ensure that the indicator concentration was near steady state prior to the imaging, we incubated the slices for ≥3 h at 35°C before performing calcium measurements. To improve our ability to keep dye concentrations relatively constant over the course of the experiment, measurements were performed 500–700 μm from the loading site where relative changes in dye concentrations were minimal after 3 h (cf. Fig. 3, B and C). When measurements were performed at room temperature, both fluorescence intensity (n = 4) and calcium transients remained stable over 120 min (Fig. 4B, left). On average, fluorescence levels after 120 min were 96 ± 1%, and the amplitudes of calcium transients were 104 ± 3% (n = 4), compared with their respective values at the beginning of the measurements. Similarly, when recordings were made with the bath temperature held at 35°C for the entire duration of the experiment (Fig. 4B, right), neither fluorescence intensity nor the amplitudes of the calcium transient changed significantly. On average intensity values were 98 ± 6% and peak fluorescence transients 100 ± 3% (n = 4) after 120 min compared with their respective values at time 0.

Taken together, our results indicate that AM dyes are extruded via a temperature-dependent mechanism and therefore do not permit long-term measurements of Ca_{pre}. In contrast, dextran-conjugated dyes can be used to perform stable measurements of Ca_{pre} provided that the slow speed and temperature-dependence of dye redistribution within the loaded fiber system are taken into account.

**Red dextran-conjugated calcium indicators**

Although dextran-conjugated indicators appear suitable for measuring Ca_{pre}, there are relatively few such indicators available. In particular, few indicators exist that are excited and fluoresce at longer wavelengths. Such indicators would be useful, for several reasons. First, tissue autofluorescence, which can interfere with identification of labeled presynaptic structures and with measurements of Ca_{pre}, is less problematic for long wavelength excitation and emission. Second, it is often useful to have indicators with nonoverlapping emission to label multiple pathways converging onto the same target. Finally, it is desirable to measure calcium in GFP labeled neurons, but GFP fluorescence interferes with the measurements of Ca_{pre} using most available dextran-conjugated calcium indicators.

We therefore synthesized three new dextran-conjugated calcium indicators (Fig. 5, Table 1) based on the fluorophore rhodamine. Both x-rhod dextran and rhod dextran (high affinity) have a relatively high affinity for calcium (K_{D} = 1.4 μM). This was achieved by the separation of the BAPTA phenyl ring and the electron-withdrawing carbonyl group with a methylene group (Fig. 5, A and B, arrows), leaving intact the high calcium ion affinity of a normal BAPTA moiety. By contrast, for rhod dextran (low affinity) the carbonyl group that provides the aminodextran attachment site was directly bound to one of the

![Fig. 2. Temperature-dependent extrusion of acetoxymethyl (AM) indicators. A: fluorescence levels in parallel fiber band are shown after focal application of mag fura-5 AM in the molecular layer for 5 min at room temperature. Times of image acquisition relative to start of loading are indicated. Inverted grayscales were used here and Fig. 3 for visual clarity. B: fluorescence intensities along the parallel fiber band are shown for the indicated times with 0 μm indicating the center of loading site. Traces quantify fluorescence immediately after loading (0.1 h), after 2 h of incubation at room temperature (2 h), and after an additional 2 h at 35-36°C (4 h). C: Fluorescence intensities measured at 5-minute intervals are shown for locations 300, 600, and 900 μm from the loading site. A–C are from same experiment. D: moderate dye extrusion at room temperature: fluorescence intensities measured at distances of 300, 600, and 900 μm from the loading site, in experiment maintained at 22°C.](http://jn.physiology.org/)

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BAPTA phenyl rings, lowering the calcium ion affinity by virtue of the electron withdrawing character of the carbonyl group ($K_D = 3.9 \mu M$). X-rhod dextran and rhod dextran have the same chelator substitution pattern and dextran attachment but differ in the dye portion of the molecule that results in a red-shifted excitation and emission band.

We characterized these indicators by loading them into parallel fibers and measuring evoked fluorescence transients at 35°C (Fig. 6). Parallel fibers were stimulated once or twice (Fig. 6A, left), or with an eight-pulse 20-Hz train (Fig. 6A, right). Previous studies have shown that paired pulses produce very similar incremental increases in calcium, resulting in comparable increases in fluorescence for low-affinity indicators (Reggehr and Atluri 1995). In contrast, for high-affinity indicators, the fluorescence transient evoked by a second stimulus is greatly attenuated due to saturation of the indicator, and there is not a linear relationship between $C_{app}$ and $\Delta F/F$ (Reggehr and Atluri 1995). For x-rhod dextran and rhod dextran (high affinity), the paired-pulse ratios were $0.76 \pm 0.04 (n = 7)$ and $0.82 \pm 0.02 (n = 8)$ respectively, indicating a moderate degree of dye saturation (Fig. 6, A, left, and B, left). By comparison, the previously characterized high-affinity indicators calcium green dextran ($K_D = 0.54 \mu M$) and fura 2 dextran ($K_D = 0.52 \mu M$) display more pronounced saturation (Kretzzer et al. 2000). For rhod dextran (low affinity), the paired-pulse ratio was $0.97 \pm 0.02 (n = 5)$ showing that for this dye there is a near-linear relationship between AP-evoked increases in calcium influx and increases in fluorescence.

We also assessed the ability of these indicators to measure the time course of $C_{app}$ changes (Fig. 6B, right). Previous studies have established that stimulus-evoked $C_{app}$ in parallel fiber boutons have half decay times ($t_{1/2}$) of ~10 ms. High-affinity indicators do not accurately report the time course of $C_{app}$ due to indicator saturation and slow off rates (Sabatini and Regehr 1998). This was the case for rhod dextran (high affinity) and x-rhod dextran ($t_{1/2}$ of 32 ± 2 ms, $n = 8$ for rhod dextran, $t_{1/2}$ of 40 ± 2 ms, $n = 7$ for x-rhod dextran). Fluorescence transients obtained with rhod dextran (low affinity) had the fastest decay rate ($t_{1/2}$ of 21 ± 2 ms, $n = 5$).

The responses of the three indicators to trains of stimuli also illustrate differences in their ability to track $C_{app}$ during ongoing activity (Fig. 6, A, right, and C). For fluorescence signals mediated by rhod dextran (high affinity) and x-rhod dextran, 20-Hz trains slightly accentuated the amount of saturation (Fig. 6C). By contrast, for rhod dextran (low affinity), stimulus-evoked fluorescence increases remained similar throughout the train. These findings demonstrate that although rhod dextran (low affinity) can slightly distort the time course of calcium decay, it is well suited to quantifying calcium entry during moderate frequency trains in a manner that is similar to fluo-4 dextran.

**Measuring calcium influx in single synaptic boutons**

In addition to obtaining aggregate measurements from a population of synapses, as we did here for parallel fibers, it is
often desirable to measure Ca_pre from individual presynaptic boutons. To date most functional imaging studies of single synapses in brain slices require the direct filling of the presynaptic neuron with calcium fluorophores (Helmchen et al. 1997; Koester and Sakmann 2000). However, this approach is not feasible for the study of most synapses.

Alternatively, presynaptic boutons could be labeled by focally loading axons with dextran indicators. This approach could prove particularly promising in systems with GFP expression in distinct subsets of presynaptic axons (Feng et al. 2000). However, focal loading of individual synaptic boutons

**FIG. 4.** Measuring calcium transients with AM indicators and dextran-conjugated indicators at physiological temperatures. Similar experiments are shown for magnesium green AM (A) and calcium green dextran (10,000 MW; B) loaded into parallel fibers. Graphs plot normalized fluorescence intensity (top) and peak ΔF/F of calcium transient (bottom) evoked by single stimuli every 60 s at 22°C (left) and at 35-36°C (right). Insets in each graph compare calcium transients at times 0 and 90 min. In A, imaging commenced 2-3 h after loading at room temperature. In B, slices were loaded for 2 min at room temperature and incubated at 34°C for 2-3 h. Scale bars, 10 ms.

**FIG. 5.** Properties of red calcium indicator conjugated to dextrans. Structures (left) and emission spectra with increasing calcium concentrations (right) are shown for x-rhod dextran (A), rhod dextran (high affinity; B), and rhod dextran (low affinity; C). For excitation wavelengths used to obtain the emission spectra see Table 1. C: the arrows in A and B indicate the methyl group determining the higher affinity of x-rhod dextran and rhod dextran (high affinity). D: calcium dependence of fluorescence for the 3 indicators. Traces indicate fits of the Hill equation to fluorescence values in (A–C), with the Hill coefficient set to 1.
with dextran indicators in brain slices has not been demonstrated. In addition, the excitation and emission spectra of GFP and its variants are broad and overlap with those of currently available dextran-conjugated indicators (Bolsover et al. 2001).

We found that we could focally load Texas Red dextran into cerebellar mossy fibers of mice in which a small percentage of cerebellar mossy fibers express GFP (Feng et al. 2000). Texas Red dextran has excitation and emission spectra similar to ones of the red indicators but is easier to visualize as all three calcium dependent indicators emit little fluorescence at resting calcium levels (Fig. 5D). As shown using 2-Photon laser scanning microscopy, loading with dextrans allows for detection of individual boutons against a low-noise background (Fig. 7A). Furthermore, mossy fibers expressing GFP were well separated from dye-loaded boutons (Fig. 7, B and C).

We went on to measure fluorescence transients from individual boutons using standard epifluorescence optics. As in the experiments described in the preceding text, we used slices of GFP transgenic mice. We focally loaded mossy fibers with x-rhod dextran combined with Texas Red dextran for visualization. Labeled fibers could be readily identified without interference from GFP. Mossy fibers were activated with single stimuli and responses from individual terminals were recorded with a photo-

![Image of Figure 6](http://jn.physiology.org/)

**FIG. 6.** Characterization of red dextran-conjugated calcium indicators in the parallel fibers at 35-36°C. Responses are normalized to facilitate comparison. A: fluorescence transients measured in response to 1 or 2 stimuli (40 Hz; left) and trains of 8 stimuli (20 Hz; right). Traces are averages of 30–40 trials. Scale bar, 15% (top), 3% (middle), and 4%. ΔF/F (bottom). B: summary data quantifying the paired pulse ratio (left) and half decay time after a single pulse (right). C: summary graph plots transients in response to a train of 8 stimuli (20 Hz), normalized to the 1st response.

![Image of Figure 7](http://jn.physiology.org/)

**FIG. 7.** Imaging single presynaptic boutons with red dextran-conjugated calcium indicators. A: cerebellar mossy fiber bouton labeled with Texas Red dextran. B: same field, different bouton expressing green fluorescent protein (GFP). C: overlay of the images in A and B. Scale bar, 5 µm. D: calcium transient evoked in single cerebellar mossy fiber bouton labeled with x-rhod dextran. Trace shown is average of 50 trials.
multiplier (Fig. 7D). Thus focal loading of dextran-conjugated indicators enables studies of Ca\(_{\text{pre}}\) at individual synaptic boutons in systems that were previously inaccessible. Red dextran dyes should prove particularly useful for such measurements, due to the low level of autofluorescence at longer wavelengths.

**DISCUSSION**

**Measuring Ca\(_{\text{pre}}\) at physiological temperatures**

A major goal of our study was to determine what method is best suited to measuring Ca\(_{\text{pre}}\) at physiological temperatures. We found that indicator extrusion was a major limitation for AM-based calcium indicators. Despite the relative stability of Ca\(_{\text{pre}}\) measurements with AM indicators at room temperature, at physiological temperatures, these indicators were rapidly extruded from presynaptic fibers. Thus the use of AM indicators requires loading and equilibration at room temperature, before the temperature can be changed to physiological levels. However, in these experiments, indicator extrusion leads to a complete loss of the signal. This places a practical limit on these types of experiments of 20 min to one hour. Additionally, loss of indicator during the experiment can alter the buffer concentration and lead to a perceived alteration in the time course and amplitude of the calcium transient. Moreover, as the indicator concentration decreases, the background correction becomes increasingly important and can affect the determination of \(\Delta F/F\).

Our findings contrast with a recent report of AM indicators being used to measure somatic calcium transients for several hours at physiological temperatures with only a moderate decrease in fluorescence intensity (Stosiek et al. 2003). Although these differences are not well understood, it is likely that the much higher surface-to-volume ratio of parallel fibers relative to cell bodies contributes to the more pronounced extrusion within presynaptic structures. In a variety of neuronal and nonneuronal cell lines, dye extrusion is mediated by organic anion transport systems (Di Virgilio et al. 1990). Differences in the expression of transporters could also contribute to the more pronounced extrusion of AM indicators from parallel fibers.

In contrast, dextran-conjugated indicators were retained inside parallel fibers and thus permit long-term measurements of Ca\(_{\text{pre}}\) with only moderate changes in fluorescence intensity. The long-term stability of both calcium transients and fluorescence levels indicate that this class of dyes is not compartmentalized inside organelles. These results further show that calcium indicators in general are not chemically altered at higher temperatures. It is unlikely that dextran-conjugation of an indicator will influence its sensitivity to degradation. Therefore the fluorescence loss observed with AM-based indicators is most likely explained by active extrusion from the cytoplasm.

An important practical consideration in measuring Ca\(_{\text{pre}}\) with dextran indicators is that they equilibrate within fibers very slowly at room temperature. The transport is greatly accelerated at physiological temperatures in a manner that suggests mechanisms other than simple diffusion. However, even at high temperatures it takes several hours for dye levels to distribute uniformly within the presynaptic structures. As a result, the most stable Ca\(_{\text{pre}}\) measurements with dextran involved incubating the slices for 2 h at 34°C before commencing Ca\(_{\text{pre}}\) measurements.

Dextran dyes offer several advantages over AM indicators. Because they are retained inside fiber tracts for up to several days they can be loaded in vivo, thus permitting labeling of even distant targets (Edwards and Cline 1999; Fetcho and O’Malley 1997; Kreitzer et al. 2000; McPherson et al. 1997; O’Donovan et al. 1993; Takahashi et al. 2002; Wachowiak and Cohen, 2001). Unlike AM dyes, dextran dyes can also be loaded into heavily myelinated fiber tracts such as the cerebellar white matter, the optic tract, or the thalamocortical pathway. Furthermore dextran dyes label only a small percentage of fibers (Fig. 7A), making it feasible to isolate individual dye-filled terminals without interference of other labeled structures. Here we have shown that red dextran dyes are particularly suitable for single bouton measurements by labeling mossy fibers in cerebellar slices. This approach could readily be applied to other synapses. In preliminary studies, we were able to load retinogeniculate axons in the optic tract and perform measurements from individual terminals in the lateral geniculate nucleus (LGN).

**Red dextran dyes**

The three red dextran-conjugated indicators we have introduced here promise to be useful in several ways. The existence of two low-affinity dyes with nonoverlapping emission spectra, fluo-4 dextran (Kreitzer et al. 2000) and rhod dextran (low affinity), now enables experiments in which the presynaptic activity patterns from two distinct populations are faithfully monitored. For example, these dyes could be loaded into the optic tracts of both eyes in vivo to measure Ca\(_{\text{pre}}\) in retinogeniculate terminals originating from ipsilateral and contralateral eye converging into the same target area in the LGN. Low-affinity indicators are particularly useful for quantitative studies where the time course of Ca\(_{\text{pre}}\) is to be determined, changes in calcium influx are to be quantified, or Ca\(_{\text{pre}}\) is to be quantified during trains. Higher-affinity indicators such as rhod dextran (high affinity) and x-rhod dextran are more appropriate for more qualitative studies requiring higher sensitivity. These red indicators can be used with the other high-affinity indicators fura-2 dextran and calcium green dextran to allow simultaneous measurement of calcium arising from three populations of presynaptic fibers. For example, in the LGN this could include corticothalamic synapses and retinogeniculate inputs from the ipsi- and contralateral retina.

One attractive feature of the red indicators is in applications where the tissue has high levels of autofluorescence for excitation in the 340–490-nm range. There is often much less tissue fluorescence for excitation of 550–600 nm. Although we have focused on the measurement of Ca\(_{\text{pre}}\), red dextrans will be more generally useful for recording from neurons. The ability to measure calcium transients in cells in a manner that is compatible with GFP is particularly attractive and will become increasingly important with the more widespread use of GFP-labeling to guide measurements from specific types of neurons or synapses.

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