Development of new peptide-based tools for studying synaptic ribbon function

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Francis AA, Mehta B, Zenisek D. Development of new peptide-based tools for studying synaptic ribbon function. J Neurophysiol 106: 1028–1037, 2011. First published June 8, 2011; doi:10.1152/jn.00255.2011.—Synaptic ribbons are proteinaceous specialized electron-dense presynaptic structures found in nonspiking sensory cells of the vertebrate nervous system. Understanding the function of these structures is an active area of research (reviewed in Matthews G, Fuchs P. Nat Rev Neurosci 11: 812–822, 2010). Previous work has shown that ribbons could be effectively labeled and visualized using peptides that bind to the synaptic ribbon protein RIBEYE via a PXDLS motif (Zenisek D, Horst NK, Merrifield C, Sterling P, Matthews G. J Neurosci 24: 9752–9759, 2004). Here, we expand on the previous work to develop new tools and strategies for 1) better visualizing synaptic ribbons, and 2) monitoring and manipulating calcium on the synaptic ribbon. Specifically, we developed a new higher-affinity peptide-based label for visualizing ribbons in live cells and two strategies for localizing calcium indicators to the synaptic ribbon.

exocytosis; hair cell; retina; synapse

THE SYNAPTIC RIBBON IS A PRESYNAPTIC feature found in tonically releasing nonspiking cells of the retina, inner ear, and pineal gland. These proteinaceous structures localize nearby to clusters of voltage-gated calcium channels (Issa and Hudspeth 1996; Midorikawa et al. 2007; Morgans et al. 2005; Zenisek et al. 2004) and tether synaptic vesicles near sites of neurotransmitter release (Matthews and Fuchs 2010). Vesicles tethered to the ribbon itself likely make up most of the vesicles that undergo exocytosis in response to membrane potential changes (LoGiudice et al. 2008; Zenisek 2008). The most abundant protein of the synaptic ribbon is RIBEYE, a splice isoform of the transcriptional corepressor COOH-terminal binding protein 2 (CtBP2) (Schmitz et al. 2000). We previously showed that peptides containing a short sequence, known to bind to the closely homologous CtBP1 (Mollov et al. 1998), can be used as an effective label of synaptic ribbons in living cells when introduced into cells via a patch pipette (Zenisek et al. 2004). This peptide label has proven useful in a number of ribbon-introduced into cells via a patch pipette (Zenisek et al. 2004). Here, we report new variants of this peptide label that offer the potential to extend the utility of the peptide for experimental use. Specifically, we report 1) two strategies for attaching calcium indicators to the synaptic ribbon, and 2) the synthesis of a ribbon label with higher-affinity and better ribbon detection capabilities.

MATERIALS AND METHODS

Peptide Synthesis and Storage

The peptides used for covalent conjugation of fluo-4 were synthesized, purified, and purchased from New England Peptide (≥75% purity); X-rhod-binding peptides were synthesized, purified (75% purity), and purchased from GenScript (Piscataway, NJ). New England Peptide and GenScript were chosen for synthesis because they offered the lowest prices for the projects. The tandem peptide dimers (TPD) were synthesized, purified (≥90% purity), and purchased from AnaSpec (Fremont, CA). AnaSpec was chosen because: 1) the HiLyte Fluor 488 dye is much more photostable and brighter than fluorescein, more cost effective than other photostable dyes, and is only available through AnaSpec; and 2) AnaSpec agreed to take on the project.

Peptides were dissolved in concentrated stock solutions and stored up to 3 mo as frozen stocks. Stock solution concentrations were 5 mM for the X-rhod-binding peptide and 250 μM for the TPD. All other peptides were stored as 10 mM stock solution. The TPD were somewhat difficult to dissolve at this concentration. Solubility was improved slightly by decreasing the pH of the solution to 6.9.

Conjugation of Fluo-4 to Peptides

Direct conjugation to cysteine-containing peptides with fluo-4 maleimide (purchased from Invitrogen). Peptides were synthesized containing the ribbon binding sequence along with an NH2-terminal cysteine (CEQTVPLDLSKRDR). First, 1 mM peptide was incubated in 100 mM HEPES solution buffered to pH 7.2 containing 1 mM tri(2-carboxyethyl)phosphine (TCEP; to reduce cysteines) and 1 mM fluo-4 maleimide (1st prepared as 20 mM stock solution in DMSO) for 1 h at room temperature followed by overnight at 4°C. For some experiments, labeled peptides were purified by Sephadex G-25 gel filtration column in a solution of 120 mM cesium gluconate.

Peptide containing cysteine were cross-linked to fluo-4 cadaverine using the heterobifunctional cross-linker sulfo-SMCC (Pierce Biotechnology). To do so, 5 mM sulfo-SMCC, 5 mM peptide, and 5 mM fluo-4 cadaverine were incubated at room temperature for 30 min in a solution containing 120 mM cesium gluconate, 20 mM HEPES, pH 7.2. At this pH, both the amines and cysteines are reactive with the sulfo-SMCC compound. For some experiments, labeled peptides were purified by Sephadex G-25 filtration columns, however, we found that the unpurified mixture also gave satisfactory results.

Dissociated Goldfish and Zebrafish Bipolar Cell Preparation

All procedures for animal care were carried out according to Yale Animal Care and Use Committee (YACUC). Bipolar neurons were prepared as previously described (Coggins and Zenisek 2009). Briefly, adult goldfish (4- to 5-in. length) were placed in low-light conditions for 30 min and then killed by decapitation. Eyes were removed, and the sclera cut off, and lenses removed from the eye cup using forceps. Eyecups were incubated for 20 min in hyaluronidase (Sigma type V; 1,100 U/ml; Sigma-Aldrich) in a saline solution containing (in mM):

- KCl: 120
- NaCl: 30
- CaCl2: 1.8
- MgCl2: 1.0
- Glucose: 10
- HEPES: 10
- pH: 7.4 at 5°C

Dissociated cells from eyecups were collected by gentle aspiration with a Pasteur pipette. Cells were plated at 10,000 cells per 35-mm glass-bottom dish (MatTek Corporation) in the above medium with the addition of 15% fetal bovine serum (FBS; Hyclone, Logan, UT) and 0.2% glucose (Sigma). Cells were incubated at 35°C in a humidified atmosphere containing 5% CO2 for 18–24 h.
120 NaCl, 0.5 CaCl₂, 2.5 KCl, 1.0 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). After incubation, retinas were removed from eye cups, cut into four to eight pieces each, and incubated for 30–35 min in 5 ml of a solution of 30–35 U/ml papain (lyophilized powder; Sigma), 0.5 mg/ml cysteine, and, in mM, 120 NaCl, 0.5 CaCl₂, 2.5 KCl, 1.0 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). After enzymatic treatment, tissue was washed in oxygenated enzyme-free solution and stored at 12°C for up to 5 h. The pieces of retina were dissociated into single cells by mechanical trituration through a fire-polished Pasteur pipette.

Zebrafish-dissociated cell preparations were carried out in a similar manner, with the following differences. After decapitation, dissection of the retina was carried out in a solution containing hyaluronidase (Sigma type V; 1,100 U/ml) and, in mM, 120 NaCl, 0.5 CaCl₂, 2.5 KCl, 1.0 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). After removal from the eye, retinas were incubated in a solution containing hyaluronidase (Sigma type V; 1,100 U/ml) and, in mM, 120 NaCl, 0.5 CaCl₂, 2.5 KCl, 1.0 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4 with NaOH) for 5 min for photoreceptor-enriched preparations and 35 min for bipolar cell preparations.

**Dissociated Cell Electrophysiology**

Bipolar cells and photoreceptors were recognized by their distinct morphologies. To record whole cell currents and introduce labeled peptides into cells, a high-resistance seal was formed onto bipolar cells using a glass pipette, and the patch of membrane beneath the pipette was ruptured using gentle suction. Cells were voltage-clamped using an EPC 10 amplifier (HEKA) controlled by Pulse (Instrutech) acquisition and recording software. The pipette solution contained, in mM, 120 cesium glutamate, 4 Na₂ATP, 0.5 GTP, 4 MgCl₂, 10 tetraethylammonium (TEA)-Cl, 0.5 EGTA (pH 7.2 with NaOH) with added peptides and/or calcium indicators.

**Total Internal Reflection Fluorescence Imaging**

Imaging was performed as previously described (Coggins and Zerisek 2009). Cells were observed through an inverted microscope (Zeiss Axiovert 135 or Olympus IX70) modified for objective-type evanescent field illumination (Axelrod 2001), referred hereafter as total internal reflection fluorescence microscopy (TIRFM), using a 1.65-numerical aperture objective. Illumination was provided by either a 488- or 561-nm solid-state laser. TIRFM images were captured using an intensified charge-coupled device (CCD) camera (Cascade 512B; Roper Scientific). Ribbon locations were observed as bright spots of peptides using an EPC 10 amplifier (HEKA). Slices were viewed with a Zeiss Axioskop 2FS equipped with a water-immersion ×40 differential interference contrast microscopy (DIC) objective. Rod bipolar cells were identified by their shape and position in the slice, as determined by fluorescent imaging. Illumination for epifluorescence was performed using X-Cite 120Q (EXFO, Ontario, Canada) with a 488-nm band-pass excitation filter set for imaging peptide fluorescence (Chroma Technology) or 590-nm band-pass filter set for imaging Alexa Fluor 594. Images were captured and stored with a CCD camera (Hamamatsu ORCA II-HR) acquired using MetaMorph acquisition software.

**RESULTS**

**Generation of a Higher-Affinity Ribbon-Binding Peptide-Based Probe**

Although the original 14-amino acid peptide has proven useful in a variety of experimental systems, its signal-to-noise is somewhat limited by its low affinity for the RIBEYE protein (Kₐ = 27 µM; Zerisek et al. 2004). Since the ribbon contains a high density of RIBEYE and since CtBP2 is known to dimerize, one potential strategy to enhance binding is to attempt to engage two closely spaced RIBEYE molecules simultaneously with a linked dimer. This strategy is analogous to one previously employed to enhance binding to ligand-gated channels (Kramer and Karpen 1998). If the tether is sufficiently long to engage multiple RIBEYE molecules simultaneously, we expect the affinity to be enhanced by a factor reflecting an increase in the effective concentration of the tethered unbinding motif in the peptide. The effect on unbinding is governed by the function:

\[ K_{\text{eff}} = 2K_{\text{eff}}K_d/(C_{\text{eff}} + K_d) \]  

where \( K_{\text{eff}} \) is the effective concentration explored by the unbound peptide when the other peptide is bound (Kramer and Karpen 1998). \( C_{\text{eff}} \) can be calculated by the function:

\[ C_{\text{eff}} = N^{-1} \left( 4/3 \pi r^3 \right)^{-1} \]

where \( N \) is Avogadro’s number, if one assumes a full 360° of motion. In reality, the range of motion is likely limited by nearby proteins, possibly increasing the \( C_{\text{eff}} \). We tested the utility of this strategy by tethering 2 ribbon-binding peptides by a 12-polyethylene glycol linker (PEG12). The sequence is listed as peptide 1 in Table 1. With this peptide, the RIBEYE-

**Table 1. Sequences of peptides used**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Peptide 1</td>
<td>(fluor)-EQTVPFVDSLARFR-P(D12)-EQTVPFVDSLARPK(12)-EQTVPFVDSLARPK(12)</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>EQTVPFVDSLARGCC</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>KPVQWQTUMFPTGSSGQTVPLDSLARDR</td>
</tr>
<tr>
<td>Peptide 4</td>
<td>SKVILFEGPAGFRWRFPBSEPAGSKVILFEGGPEGVQTVPLDSLARDR</td>
</tr>
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binding PVDSL sites are separated by 8 amino acids and a PEG12. Assuming a separation of ~2.5 Å (Stryer 1995) per residue and 53 Å for the PEG12, Eq. 2 gives us an upper limit for $C_{\text{eff}}$ of 1.0 mM, ~30-fold higher than our previous measures for the $K_d$ for a single peptide. From Eq. 1 above, we expect unbinding to be ~30-fold slower, and if we assume that the binding affinity for either monomer will be unchanged, we expect a 2-fold increase in the binding rate, $K_{\text{on}}$, due to the 2 binding sites. Overall, we hypothesized an ~60-fold decrease in the apparent $K_d$.

We introduced the TPD into goldfish retinal bipolar cell terminals via a whole cell patch pipette and imaged fluorescence using either TIRFM or epifluorescence microscopy. Figure 1A shows a bright field image of a synaptic terminal being loaded with the TPD via a patch pipette. Figure 1B shows a TIRFM image from the same terminal after loading with the peptide. Under these conditions, fluorescent spots, representing synaptic ribbons, were visible at TPD concentrations down to 5 nM, whereas spots were not visible at monomer concentrations of 425 nM or below, consistent with an enhanced affinity for RIBEYE with the TPD. Figure 1B shows an image from a cell loaded with 125 nM TPD and imaged with TIRFM.

Next, we estimated the affinity of the TPD for the ribbon by monitoring the fluorescence of synaptic ribbons as a function of the cytoplasmic fluorescence (Zenisek et al. 2004). The cytoplasmic fluorescence was used as an estimate of concentration within the terminal by assuming that after equilibration the fluorescence within the pipette equaled that of the peptide concentration. The relationship between peptide concentration and fractional ribbon binding was then fit to the function:

$$F_{\text{spot}} / F_{\text{spot, max}} = [\text{Peptide}] / (K_d - [\text{Peptide}])$$

where $F_{\text{spot}}$ is the fluorescence of the ribbon spot at each time point, $F_{\text{spot, max}}$ is the fluorescence of the spot at saturating concentrations, and [Peptide] is the estimated concentration of the peptide (from Zenisek et al. 2004). Figure 1C shows results from these experiments (n = 7 spots in 3 cells). To estimate the $K_d$, we best-fit the data with Eq. 3. Using this approach, we estimated the $K_d$ to be 1.5 μM, somewhat higher than the expected $K_d$ based on the linker size.

To verify that the spots we were imaging were ribbons, we compared the spots labeled with the TPD to those labeled with the monomeric peptide, which we previously verified labels ribbons using retrospective electron microscopy (Zenisek et al. 2004). To do so, we first superfused zebrafish bipolar cells with a solution containing 50 μM monomeric peptide and 20 μg/ml detergent β-escin to permeabilize the cells to allow the peptide to enter, whereas the cells were imaged using epifluorescence microscopy. After washing, prominent spots were visible marking the location of ribbons (Fig. 1D), which disappeared within a minute as the dye was washed away. Following the disappearance of the spots, we then applied a solution containing 2.5 μM TPD and β-escin. The tandem dimer (Fig. 1E) was found to label spots in the same pattern as the monomeric peptide (Fig. 1D). In three cells, we found that each of eight spots imaged with the monomeric peptide also exhibited spots labeled by the TPD. We conclude that the monomeric and dimeric peptides label the same structures.

We next tested whether the TPD was capable of labeling ribbons from other cell types. To do so, we dissociated phot
to receptors from zebrafish retina and introduced the peptide using β-escin permeabilization as above and imaged using epifluorescence microscopy. Figure 1, F and G, shows an example of a photoreceptor ribbon labeled in this way. The structures labeled in photoreceptors appeared to be much larger than those in bipolar cells, consistent with the larger ribbon sizes in these cells.

Use of TPD in Retinal Slice

We next tested whether the TPD were suitable for imaging ribbons in mouse retinal slice recordings of retinal bipolar cells using epifluorescence. The results are shown in Fig. 2. Retinal slice recordings require an upright microscope with sufficiently long working-distance objectives to allow for recording pipettes access to bipolar cells. Such an arrangement places limitations on the numerical aperture of the microscope objective and limits the efficiency of light collection in such experiments. Indeed, we have found that the standard monomeric peptide is not suitable for imaging ribbons in this configuration on our microscope when using epiillumination. This finding was confirmed here. We found that in five cells, no spots were observed in the terminals of voltage-clamped retinal bipolar cells. Figure 2, H–J, shows an example of a bipolar cell terminal loaded with our monomeric ribbon-binding peptide. By contrast, spots were readily visible in epifluorescence when using the TPD. Figure 2, A–G, shows a mouse bipolar cell loaded with both a soluble fluorescent dye (Alexa Fluor 594 hydrazide, in red) and the TPD (in green). Note that the TPD fluorescence exhibits spots specifically in the synaptic terminal. Also of note, we found no obvious effect of the TPD on synaptic transmission. In paired recordings between a rod bipolar cell and an AII amacrine cell, we found that step depolarizations to ~10 mV to a rod bipolar cell loaded with 3 μM TPD evoked synaptic responses of normal size (360 pA) and normal kinetics, similar to previous reports in both rats and mice (Singer and Diamond 2003; Singer et al. 2004; Snellman et al. 2009). We conclude that the ribbon-binding peptide does not block synaptic transmission, although we cannot rule out a more subtle effect of the peptide. Further experiments at higher concentrations will be necessary to determine whether the peptide has any effect on any aspect of synaptic release.

We also tested whether mouse photoreceptor ribbons could be labeled using the TPD. To do so, we locally superfused a Ringer solution containing 2.5 μM TPD along with β-escin (40 μg/ml) using a glass puffer pipette directed at the outer plexiform layer (OPL) in a retinal slice preparation. After application of the TPD solution, we immediately washed the cells and imaged the retina using epifluorescence. Under these conditions, prominent fluorescent structures in the OPL became visible, with many of these structures having the characteristic horseshoe shape of rod ribbons (Fig. 3D). Figure 3 shows an example of these experiments.

Targeting Calcium Indicators to the Synaptic Ribbon

Synaptic vesicle exocytosis in most neurons is triggered by the high local calcium concentration only found near open calcium channels (Heidelberger et al. 1994), and delivery of vesicles to the plasma membrane and/or vesicle priming are also believed to be dependent on calcium microdomains on the ribbon (Babai et al. 2010; Gomis et al. 1999; Mennerick and...
As an assay for utility of our probes, we used whole cell voltage clamp to introduce calcium indicator-ribbon-binding peptide conjugates into goldfish retinal bipolar cells while imaging using TIRFM. Bipolar cells were then imaged during depolarizations from the resting potential of $-60 \text{ mV}$ to more positive potentials, which opens voltage-gated calcium channels and raises cytosolic calcium in the synaptic terminal. A successful calcium indicator conjugate is expected to 1) label punctate regions on the surface of the synaptic terminal, representing ribbon sites, and 2) exhibit an increase in fluorescence in response to depolarizations that activate calcium currents.

**Conjugation of Fluo-4 Maleimide to Cysteine-Containing Ribbon-Binding Peptides**

To target a calcium indicator to the synaptic ribbon, we first decided to use fluo-4 maleimide (Invitrogen) to attach fluo-4 to a cysteine introduced into PXDLS motif-containing peptides. Although fluo-4 has higher affinity than desirable for high-calcium concentrations expected near the calcium channels ($K_d = 345 \text{ nM}$; Invitrogen), we chose to start with this approach because of the availability of fluo-4 maleimide and simple and reliable procedure for coupling maleimides to cysteines. After coupling of the peptide to the calcium indicator, we introduced the peptide into retinal bipolar cells and imaged using TIRFM. Bipolar cells labeled with this conjugated peptide exhibited fluorescent spots, presumably synaptic ribbons, but did not exhibit fluorescence increases associated with membrane depolarization despite robust calcium currents. In our hands, fluo-4 maleimide did not exhibit fluorescent changes with depolarization even in the absence of the peptide. Because of this, we did not pursue this method further.

**Conjugation of Fluo-4 Cadaverine to Ribbon-Binding Peptide**

A second approach centered around the availability of fluo-4 cadaverine (Invitrogen), which contains a free amine. The availability of a free amine allowed us to covalently link the indicator to a cysteine synthesized on to a ribbon-binding peptide using sulfo-SMCC (Pierce Biotechnology), a heterobifunctional cross-linker. Using a patch pipette, we loaded bipolar cell terminals with the ribbon-binding peptides cross-linked to fluo-4 cadaverine and imaged using TIRFM with a 488-nm excitation light. Under these conditions, bipolar cell terminals exhibited dim fluorescent spots, presumably synaptic ribbons, on a background of a dimmer fluorescent terminal footprint, presumably due to calcium indicator-peptide complexes that are not bound to the ribbon (Fig. 4). As expected, on depolarization and opening of calcium channels, the fluorescence increased in both the cytoplasm and at the spots. To analyze the changes in fluorescence elicited by depolarization, we defined areas of interest encompassing both the spots and regions away from the spots and defined regions near the spots to serve as estimates of the local background fluorescence and a region outside of the terminal to serve as the estimate of the background for the nonribbon area. After subtracting the background, we observed specific signals at the ribbon in response to depolarization.
the values were normalized to the average fluorescence of each region at the resting potential for the time preceding the voltage step. An example of the response to a brief depolarization (30 ms) is shown in Fig. 4, A–E. In response to a brief step, the fluorescence increased initially at the ribbon sites (blue and green in Fig. 4E) and then peaked later in regions away from the ribbons, consistent with colocalization of ribbons with calcium channels in goldfish bipolar cells (Midorikawa et al. 2007; Zenisek et al. 2003). Figure 4F shows a response in the same cell to a 500-ms depolarization, and Fig. 4G shows an average response of ribbon spots (black) and nonribbon areas (red) averaged across 3 cells. It is expected that although calcium channels remain open, a standing calcium gradient exists where calcium remains elevated near the open channels relative to the rest of the terminal but that this gradient will dissipate soon after closure of the channels. Of note, the F/F_0 peak, where F_0 is determined as the average fluorescence before the depolarization, is higher and reached faster for the ribbon-bound calcium indicator (Fig. 4G) for the brief depolarizations, but the fluorescence rise at the ribbon is only about threefold higher during the depolarization than it is just after channel closure and at a region several micrometers away from the ribbon. In response to longer depolarizations, the spatial gradient disappears almost altogether (Fig. 4F). One possible explanation for the lack of a large gradient between the apparent cytoplasm fluorescence rise and that of the ribbon may be due to the high affinity of fluo-4, which likely saturates near calcium channels. Therefore, we sought other methods for targeting calcium indicators to ribbons.

Short Linear Texas Red-Binding Motif-Containing Peptides

The experiments described above demonstrate a method for covalently attaching fluo-4 to a ribbon-binding peptide. However, the utility of such a probe for measuring calcium is limited by its relatively high affinity for calcium, which likely results in the saturation of the calcium indicator on the ribbon itself. Work by Nolan and colleagues (Rozinov and Nolan 1998) used phage display to identify short (13 amino acids) linear sequences that bind to the fluorophore Texas red and chemically similar compounds with moderate affinity (1 μM). We attempted to exploit this finding by synthesizing peptides containing both the RIBEYE-binding and Texas red-binding
membranes (peptide 3 in Table 1) separated by a short flexible linker and introducing the peptide into the cell along with Texas red. Cells loaded with this combination of the peptide and Texas red failed to exhibit fluorescent spots, and this technique was not further pursued.

**High-Affinity Hairpin Texas Red-Binding Motif-Containing Peptides**

The Nolan laboratory also identified short peptide motifs that, when constrained, exhibit high affinity to Texas red and similar fluorophores, including some X-rhod calcium indicators (Marks et al. 2004). Nolan and colleagues used a dimerization motif (Bodenmuller et al. 1986) to constrain the binding site in phage display screens to identify a high-affinity Texas red binding sequence \(K_d = 25 \mu M\). We next attempted to use this longer hairpin-containing motif to bind to calcium indicators. To take advantage of this interaction to deliver calcium indicators to the ribbon peptide, it was necessary to synthesize a 50-amino acid peptide (GenScript) with a ribbon-binding motif along with the constrained high-affinity Texas red-binding site (peptide 4 in Table 1).

We next looked at cells loaded with a combination of peptide 3 (50 μM) and 45 μM X-rhod-5F, a low-affinity calcium indicator \(K_d = 1.6 \mu M\), which contains the Texas red fluorophore. After achieving whole cell configuration, we found that terminals loaded with the calcium indicator and peptide 4 exhibited the expected dim spots when imaged using TIRFM, whereas cells containing rhod-5F alone exhibited no spots \(n = 4\) cells. In response to step depolarizations, fluorescence rose throughout the terminal, but increases were much more pronounced and faster at ribbon sites than at other locations on the footprint of the cell. Figure 5, A–E, shows an example of a single cell depolarized for 100 ms to 0 mV. For each spot, the fluorescence was taken as the fluorescence of the spot with a nearby region subtracted to estimate the contribution of local background to spot fluorescence with the fluorescence normalized to the average fluorescence before the depolarization. For the nonribbon, a region outside of the terminal was subtracted from the fluorescence in the footprint away from the spots. Figure 5F shows an average of 25 spots in 5 cells subjected to 100-ms steps (black) and 5 nonspot regions in the footprints (red) of the same cells. Figure 5G shows the same analysis for 12 spots in 3 cells subjected to 500-ms depolarizations.

One surprising finding was the occasional observation of flashes of calcium indicator fluorescence independent of the timing of the depolarization. The intensity of the fluorescent flashes varied considerably and at times could be rather large, on occasion exceeding the increases in fluorescent observed in response to depolarization. Such sparks were observed in 6 of 30 cells. Figure 6 shows 2 examples of these oscillations, 1 detected at a ribbon site (Fig. 6A) and 1 away from any detectable ribbons (Fig. 6B). Although the source of these flashes are unknown, these results are reminiscent of events in other cells thought to arise from release of calcium from internal stores (Cleemann et al. 1997; Zenisek et al. 2003). Of note, we also occasionally observed similar events in cells loaded with fluo-5F imaged using TIRFM.

![Fig. 5. Use of X-rhod-5F- and rhodamine-binding peptide as ribbon calcium indicator. Dissociated goldfish bipolar cells were whole cell voltage-clamped using internal solution containing both the RIBEYE and rhodamine-binding peptide (peptide 4 of Table 1) and the calcium indicator X-rhod-5F. A: TIRFM image of retinal bipolar cell before (left) depolarization. Note prominent spots, showing locations of ribbons. B: same image as in A but with circles indicating ROI for analysis in E. Circles define ROI surrounding synaptic ribbons (black, red, cyan, and green) and in a location away from ribbons (blue). Scale bar = 1 μm. C and D: same as in A, showing images of the same cell during (middle) and after (right) 100-ms depolarization to 0 mV at the time points indicated in the graph in E. Timing of image in A denoted by lines connected to trace in B. E: fluorescence intensity as a function of time for regions defined in A (left) normalized to F/F₀, as described for Fig. 3. Responses were normalized to the average fluorescence in the 500 ms preceding the depolarization. Note the rise in fluorescence is larger at ribbon sites as expected for colocalization between ribbons and calcium entry sites. F: average response of ribbon spots \(n = 25\) spots; black) and regions outside the spots \(n = 5\) for 5 cells depolarized for 100 ms to 0 mV. G: same as in F but for 12 spots in 3 cells subjected to 500-ms steps to 0 mV. Error bars represent standard errors. Time scale in D, same as in B and C.](http://jn.physiology.org/)

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Specifically, we sought to modify a ribbon-binding probe to aid in the understanding of sensory synaptic transmission. In this study, we sought to develop new tools to target the synaptic ribbon. To target calcium indicators to the ribbon, we uncovered two successful approaches: 1) covalent binding of a calcium indicator to a ribbon-binding peptide; and 2) linking ribbon-bound calcium indicators.

Since local concentrations of signaling molecules may differ dramatically from global measurements, we sought to determine whether fluorescent calcium indicators could be targeted to the synaptic ribbon. Calcium triggers neurotransmitter release in synapses and has been implicated in the priming, docking, or translocation of vesicles on the ribbon before exocytosis. Despite the importance of this signal, it has been difficult to monitor calcium concentrations on the ribbon itself independent of the cytoplasm except under special circumstances. One previous study took advantage of fortuitous binding of the calcium indicator fluo-3 in bullfrog sacculus hair cells to monitor changes in calcium dynamics at the ribbon (Issa and Hudspeth 1996). Unfortunately, this approach has not proven generalizable to other ribbon synapses. Additionally, previous studies using TIRFM or confocal microscopy have identified hotspots of calcium entry near the ribbon, indicative of calcium entry through clustered calcium channels, but such studies report calcium near channels but not necessarily on the ribbon itself and in some cases required high levels of EGTA to restrict the calcium signal (Beaumont et al. 2005; Frank et al. 2009; Midorikawa et al. 2007; Tucker and Fettiplace 1995; Zenisek et al. 2003). Moreover, the unbound calcium indicators diffuse rapidly, spreading the signal and distorting the spread of calcium itself. In another promising approach, genetically encodable calcium indicators concatenated to synaptic vesicle proteins have been used as a means to introduce relatively immobile calcium buffers near calcium channels in zebrafish (Dreosti et al. 2009). As with our peptide-based approach, such a technique allows one to measure calcium near channels without the spread of calcium signals experienced by the introduction of highly diffusible calcium indicators.

To target calcium indicators to the ribbon, we uncovered two successful approaches: 1) covalent binding of a calcium indicator to a ribbon-binding peptide; and 2) linking ribbon-binding peptides to a single molecule or possibly shortening the linker.
binding peptides to calcium indicators via a high-affinity Texas red-binding sequence. Both approaches offer distinct advantages and disadvantages. Covalent binding generated a smaller probe, which is more easily diffusible into cells. This approach is limited, however, by the commercial availability of reactive analogs of calcium indicators, which at this time is restricted to fluo-4. Fluo-4 has relatively high affinity for calcium ($K_d = 345$ nM) and may be saturated in the local high-calcium environment of the ribbons, which are found near dense collections of calcium channels. For this reason, this compound may be better suited as a tool to compete with endogenous calcium-binding proteins on the ribbon than it is a sensor of calcium concentrations.

Our second approach of using high-affinity peptides with intrinsic binding affinity for Texas red offers the advantage of having the flexibility to use any of several X-rhod calcium indicators with different affinities. However, the size of the required peptide sequence (5,348 Da) will slow the delivery of the peptide and may limit the accessibility of such a probe in some systems. Nonetheless, this approach yielded specific staining of ribbons in the synaptic terminal of retinal bipolar cells and exhibited fast responses associated with the onset and offset of depolarization. These results may also provide a framework for delivering other molecules to the synaptic ribbon or perhaps may be generalizable for targeting calcium indicators or other molecules to other peptide-binding motifs.

**Calcium Sparks in Retinal Bipolar Cells**

Although previous studies have reported calcium oscillations, the underlying source of those changes in calcium levels seem to be oscillations in membrane potential (Burrone and Lagnado 1997; Protti et al. 2000; Zenisek and Matthews 1998). To our knowledge, our measurements show the first demonstration of spatially restricted calcium oscillations in voltage-clamped retinal bipolar cell that oscillate independently of membrane potential. The source of calcium for these calcium oscillations remains unknown, although the literature suggests several possibilities. Previous work has demonstrated that under certain conditions the endoplasmic reticulum (Kobayashi et al. 1995) and mitochondria (Zenisek and Matthews 2000) can serve as internal calcium stores in goldfish bipolar cell terminals. Furthermore, IP3 receptors have been localized to retinal bipolar cell terminals in some species (Koulen et al. 2005; Micci and Christensen 1996). Alternatively, calcium may enter through spontaneous openings of voltage-gated calcium channels or calcium-permeable nonvoltage-gated channels on the plasma membrane such as ligand-gated receptors or TRP or CRAC channels. Regardless of the source of the calcium, our results demonstrate that in a subset of cells, the calcium oscillations observed can significantly increase the calcium concentration on the ribbon. The results raise the possibility that calcium stores may influence some aspects of transmitter release under some conditions in these cells.

**Limitations of New Ribbon Probes and Future Directions**

In this study, we describe strategies and tools for a higher-affinity synaptic ribbon-binding probe and for attaching calcium indicators to the synaptic ribbon. Although these new probes will add to the toolbox of researchers, additional improvements would be desirable and may be the subject of future development. First, it may be possible to increase further the affinity of peptide-based probes for the synaptic ribbon. The general strategy here of tethering multiple binding sites to a single molecule could be expanded to engage additional RIBEYE molecules or other proteins resident on the ribbon via multifrmed peg molecules or other linkers. Similarly, improvements to the affinity of the underlying monomer would also increase the affinity of the TPD probe. Moreover, it should be possible to attach different fluorochromes, function blocking peptides, or other molecules to these probes for concentration to the ribbon.

The calcium-indicator probes described here should be able to provide qualitative temporal information about calcium changes on the ribbon. At present, the probes may have limitations in applications where one wishes to measure calcium in a more rigorous quantitative manner. Specifically, the affinity of the probes, coupled with the proximity to calcium sources, may result in partial saturation of calcium indicator binding on the ribbon, which results in a nonlinear relationship between calcium and fluorescence. Moreover, temporal and spatial inhomogeneities in local peptide concentration make direct calculation of calcium concentrations from fluorescence intensity difficult. To get around these problems, it should be possible to perform ratiometric measurements to measure calcium concentration by including in the patch pipette ribbon-binding peptides with the same affinity for the ribbon but labeled with a calcium insensitive fluorophore of a different color. Similarly, there is no obvious technical barrier to synthesizing or labeling the X-rhod-binding peptide with a fluorophore excitable with blue light (e.g., HiLyte Fluor 488) for ratiometric imaging. Using such approaches would allow one to calculate calcium concentrations from the ratio of green-to-red fluorescence, analogous to methods used for other ratiometric calcium indicators (Gryniewicz et al. 1985).

**REFERENCES**


The diverse roles of ribbon synapses in sensory...

**References**


