Distribution of the presynaptic calcium sensors, synaptotagmin I/II and synaptotagmin III, in the goldfish and rodent retinas

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Synaptic vesicle exocytosis is triggered by rises in calcium up to 100 µM at the site of vesicle fusion. The synaptic vesicle proteins synaptotagmin 1 and 2 (Syt I and Syt II) bind calcium at similarly high concentrations and have been proposed as the calcium sensors for fast neurotransmitter release. However, 1 µM calcium produces tonic transmitter release at photoreceptor and bipolar cell synapses in the goldfish retina, suggesting that these synapses use a higher affinity calcium sensor. Immunofluorescent staining with a panel of Syt I/II antibodies detected Syt I/II in both photoreceptor and bipolar cell terminals of the rodent retina. By contrast, no staining of either photoreceptor or protein kinase C (PKC)-labeled bipolar cell terminals was detected in the goldfish retina with any of the Syt I/II antibodies. The high affinity calcium sensor synaptotagmin 3 (Syt III) was localized to the synaptic layers of both goldfish and rodent retinas; however, while Syt III was associated with PKC-labeled bipolar cell terminals in the goldfish retina, it did not co-localize with PKC in the mouse retina. These results suggest that, unlike in their mammalian counterparts, synaptic vesicle exocytosis in goldfish photoreceptor and bipolar cell terminals utilizes a calcium sensor other than Syt I/II, possibly Syt III.

Keywords: retina, photoreceptors, bipolar cells

Introduction

Synaptotagmins 1 and 2 (Syt I and Syt II) are integral membrane proteins of synaptic vesicles and have been proposed to be the calcium sensors controlling fast synaptic transmission in the central nervous system (CNS). The evidence in favor of this is compelling. In genetically altered C. elegans, Drosophila and mice lacking Syt I, synchronous neurotransmitter release is drastically reduced (Broadie, Bellen, DiAntonio, Littleton, & Schwarz, 1994; Geppert, Goda, Hammer, Li, Rosahl, Stevens, & Stödhorf, 1994; Nonet, Grundahl, Meyer, & Rand, 1993). Recombinant Syt I and II exhibit calcium-dependent binding to syntaxin 1, a component of the synaptic vesicle fusion complex (SNARE complex), with half-maximal binding occurring at ~200 µM calcium in agreement with the high, local calcium concentrations estimated to be required for transmitter release at many synapses (Li et al., 1995). Yet transmitter release at some central synapses occurs at calcium concentrations lower than that measured for the syntaxin-Syt I/II interaction in vitro. At the calyx of Held, for example, a brief rise of intra-terminal calcium to only 10 µM mimics physiological release (Bollmann, Sakmann, & Borst, 2000; Schneggenburger and Neher, 2000). Likewise, transmitter release by mouse inner hair cells is measurable at 8 µM calcium and maximal at 30 µM (Beutner, Voets, Neher, & Moser, 2001).

Similar to hair cells in the inner ear, photoreceptor and bipolar cells in the retina form ribbon-type synapses and modulate the rate of continuous release of the neurotransmitter, glutamate, in response to graded changes in the membrane potential (DeVries and Baylor, 1993). Capacitance measurements of goldfish bipolar cell terminals show that an increase in intracellular calcium to >100 µM triggers a massive bout of phasic exocytosis (Heidelberger, Heinemann, Neher, & Matthews, 1994). However, small rises in calcium to only 1-2 µM support a lower but continuous rate of exocytosis from both goldfish photoreceptors and bipolar cells (Lagnado, Gomis, & Job, 1996; Rieke & Schwartz, 1996). This high sensitivity to calcium is inconsistent with the reported calcium dependence of Syt I/II binding to syntaxin 1, suggesting that goldfish retinal ribbon synapses may use an alternative calcium sensor. Here we show that ribbon synaptic terminals of photoreceptors and Mb1 bipolar cells in the goldfish retina lack the synaptic vesicle calcium binding proteins, Syt I and Syt II, thought to impart the calcium sensitivity of transmitter release at other synapses.
Methods

Synaptotagmin Antibodies

The distribution of Syt I/II in the goldfish retina was assessed by immunofluorescent staining of retina sections with a panel of four antibodies recognizing different amino acid sequences (aa) of rat Syt I: anti-Syt\textsubscript{TL} (aa 1-19; Alomone Labs, Jerusalem, Israel), anti-Syt\textsubscript{SS} (aa 120-131; Synaptic Systems, Gottingen, Germany), anti-Syt\textsubscript{TL} (aa 72-223; Transduction Laboratories, Lexington, KY), and 1D12 (gift of Dr. M. Takahashi, Mitsubishi Kasei Institute of Life Sciences, Japan). Two of the antibodies, 1D12 and anti-Syt\textsubscript{SS}, have been demonstrated to cross-react with Syt II. The synaptotagmin 3 (Syt III) polyclonal antibody was raised against a GST-synaptotagmin 3-fusion protein and was the generous gift of Dr. M. Takahashi.

Immunofluorescence

Freshly dissected goldfish, rat, and mouse retinas were fixed for 30-60 min by immersion in 4% paraformaldehyde, cryoprotected in 30% (w/v) sucrose, and cut into 12-µm vertical sections on a cryostat. Dissociated rat retinal bipolar cells were prepared and stained as described for mouse bipolar cells (Berntson, Taylor, & Morgans, 2002). Retina sections were stained by immunofluorescence as previously described (Morgans, El Far, Berntson, Wässle, & Taylor, 1998). For the single labeling experiments, the primary antibodies were used at the following concentrations: anti-Syt\textsubscript{TL}, 1:200; anti-Syt\textsubscript{SS}, 1:10; anti-Syt\textsubscript{SS}, 1:100; 1D12, 1:1000; anti-munc-18 (Transduction Laboratories), 1:1000; and anti-Syt III, 1:1000. The appropriate secondary antibodies coupled to CY3 (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at dilutions of 1:1000. For the double labeling experiment, the procedure followed was essentially the same as for the single labeling experiments, except that goldfish retina sections were incubated overnight at room temperature (RT) with a mixture of the primary antibodies: anti-Syt I/II (1D12) or anti-Syt III diluted 1:1000 plus anti-protein kinase C α (PKC) (Sigma, Saint Louis, MO) diluted 1:20,000; and anti-Syt III diluted 1:1000 plus anti-calbindin (Sigma) diluted 1:1000. The sections were then incubated for 1 hr at RT with a mixture of secondary antibodies: anti-mouse IgG-CY3 (Jackson ImmunoResearch Laboratories) diluted 1:1000 and anti-rabbit IgG-FITC (Jackson ImmunoResearch Laboratories) diluted 1:50. The sections were analyzed with a Leica TCS 4D confocal laser-scanning microscope using a 40X/1.4 N.A. oil immersion objective (Leica, Germany). All confocal images shown are single optical sections of approximately 0.5 µm thickness. Images were collected and imported into Adobe Photoshop for editing. Image enhancement was limited to minor adjustments to image brightness and contrast applied uniformly over the entire image. Some images (Figures 1B and 2A) are photomicrographs taken with an Axioshot photomicroscope (Zeiss, Germany).

Western Blots

Equal quantities of goldfish and rat retinal membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a precast 4%-12% Bis-Tris gel (Novex, San Diego, CA) using MOPS buffer (Novex). The separated proteins were electrophoretically transferred to nitrocellulose and nonspecific protein binding sites blocked by incubation of the nitrocellulose for 1 hr at RT in TBST (tris-buffered saline, pH 7.4, plus 0.2% (v/v) Tween-20) containing 3% (w/v) nonfat dry milk. The membranes were then incubated with either anti-Syt\textsubscript{SS} or anti-Syt III, each diluted 1:1000 in TBST for 1 hr at RT or overnight at 4°C. After three washes in TBST, the membranes were incubated in alkaline phosphatase-coupled anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 1 hr at RT, washed in TBST, and then incubated in Western Blue stabilized alkaline phosphatase substrate (Promega, Madison, WI) for colorimetric detection of immunoactive bands.

Results

Syt I/II Is Absent From Photoreceptor Terminals in the Goldfish Retina

The distribution of Syt I/II was compared in the rat and goldfish retinas by immunofluorescent staining of retina sections with an antibody, 1D12, which has been demonstrated to recognize both Syt I and Syt II in rat (Charvin et al., 1997). The amino acid sequences of Syt I and II are highly conserved between mammals and fish (Wendland et al., 1991), thus cross-reactivity of the antibody in the goldfish is likely. The 1D12 antibody labeled both the outer plexiform layer (OPL) and inner plexiform layer (IPL) in rat retinal sections, but only the IPL in goldfish retinal sections (Figure 1). The strong immunoreactivity in the IPL indicates that the absence of staining in the goldfish OPL is not due to lack of cross-reactivity of the antibody in goldfish. The 1D12 antibody labeled a single band with an apparent molecular weight of 60 kD on a Western blot of goldfish retinal membrane proteins (Figure 1C). The molecular weight is close to that observed for marine ray Syt I and II homologs, o-p65-A and o-p65-B (Wendland et al., 1991).

The absence of Syt I/II in the goldfish OPL was confirmed with a panel of three additional antibodies recognizing different amino acid (aa) epitopes of rat Syt I: anti-Syt\textsubscript{TL} (aa 1-19), anti-Syt\textsubscript{SS} (aa 120-131), and anti-Syt\textsubscript{TL} (aa 72-223). Like antibody 1D12, anti-Syt\textsubscript{SS} also recognizes both Syt I and Syt II. For all three antibodies, strong immunoreactivity was detected in the goldfish IPL and none in the OPL (Figure 2A-C). In contrast, an antibody against another synaptic vesicle protein, munc-18, strongly labeled both plexiform layers (Figure 2D). The OPL is composed almost exclusively of photoreceptor...
ribbon synapses, thus Syt I and II are absent from photoreceptor synaptic terminals in the goldfish retina.

Figure 1. Syt I/II expression in the goldfish and rat retinas. Vertical retina sections from goldfish (A) and rat (B) were stained by immunofluorescence with the Syt I/II antibody, 1D12. C. Goldfish retina proteins were Western blotted with 1D12. is indicates inner segments; onl, outer nuclear layer; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer; and gcl, ganglion cell layer. Scale bars are 35 µm (A) and 40 µm (B).

Syt I/II Are Absent From ON-Bipolar Cell Terminals in the Goldfish Retina

The IPL contains a mixture of conventional synaptic terminals formed by amacrine cells and ribbon terminals formed by bipolar cells. To distinguish between anti-Syt I/II labeling of bipolar and amacrine cell terminals, goldfish retina sections were double labeled with 1D12 and an antibody against PKC α, a marker of Mb1 ON-type bipolar cells in the goldfish retina (Suzuki and Kaneko, 1991). The PKC-labeled bipolar cell terminals (shown in red in Figure 3A) reside in gaps in the synaptotagmin staining (shown in green in Figure 3A). The double labeling demonstrates that, like goldfish photoreceptors, the giant ON bipolar cell terminals of the goldfish retina also lack Syt I/II. In contrast, strong Syt I/II immunoreactivity is detected in the synaptic terminals of retinal bipolar cells in the rat (Figure 3C) and macaque (Koontz and Hendrickson, 1993).

Differential Distribution of Syt III in Rodent and Goldfish Retinas

The absence of Syt I/II immunoreactivity suggests that goldfish photoreceptor and bipolar cell terminals use an alternative calcium sensor for synaptic vesicle exocytosis. In the rat retina, the inner and outer plexiform layers have been shown to be enriched in the high affinity calcium sensor, synaptotagmin 3 (Syt III), in addition to Syt I/II (Butz, Fernandez-Chacon, Schmitz, Jahn, & Südhof 1999). We therefore investigated the possibility that photoreceptor and bipolar cell terminals in the goldfish retina contain Syt III. Immunoblotting with a Syt III antibody labeled a single band at 74 kD in both goldfish and rat retina (Figure 4A), consistent with the antibody recognizing Syt III in both species. Syt III staining in the rat retina (Figure 4B) labeled dense puncta in the IPL and sparser puncta in the OPL. In addition, immunoreactivity was also detected around neuronal cell bodies in the INL. In the goldfish retina (Figure 4C), anti-Syt III yielded dense labeling in both the OPL and IPL, as well as labeling of cell membranes in the INL and GCL. In addition, in the goldfish retina but not the rat retina, Syt III labeled processes descending through the ONL to the OPL (Figure 4B and Figure C).
Figure 3. Syt I/II is absent from Mb1 ON-type bipolar cell terminals in the goldfish retina. A. Confocal fluorescence micrograph of a goldfish retina section double labeled for Syt I/II (green) and the ON-bipolar cell marker, PKC α (red). Areas of co-localization are yellow. B. High magnification images of two ON-bipolar cell terminals from A showing the PKC and Syt III staining separately and superimposed. C. Phase contrast image of a dissociated rat bipolar cell. D. The same rat bipolar cell as in C labeled by immunofluorescence for Syt I/II. opl indicates outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer; and gcl, ganglion cell layer. Scale bars are ~15 µm in A and 4 µm in C.

Examination of the OPL in the mouse retina sections double labeled for Syt III and PKC, or Syt III and the horizontal cell marker, calbindin (Haverkamp and Wässle, 2000), revealed no co-localization of Syt III puncta with either horizontal cell or rod bipolar cell processes (Figure 5B and 5C). Thus, Syt III does not appear to be postsynaptic at rod photoreceptor ribbon synapses in the rodent retina. The possibility remains that Syt III in the rodent OPL is localized to domains within the photoreceptor terminals.

Figure 4. A. Western blot of goldfish (lane 1) and rat (lane 2) retinal membrane proteins for Syt III. Migration of 97-kD and 52-kD molecular weight markers is indicated to the left. Syt III immunofluorescence is shown in vertical sections through the rat retina (B) and goldfish retinas (C). opl indicates outer plexiform layer, and ipl, inner plexiform layer. Scale bar is ~25 µm for B and 30 µm for C.

To examine whether Syt III is localized to ON-bipolar cell terminals in the IPL, goldfish and mouse retina sections were double labeled for Syt III and PKC α (Figure 5 and Figure 6). In the mouse retina, the PKC-labeled terminals stratified below the bulk of the Syt III staining. Confocal images of the double labeling in mouse revealed very few Syt III puncta associated with the PKC-labeled bipolar cell terminals, indicating that mouse PKC-labeled cell terminals are devoid of Syt III. In contrast, in the goldfish retina, Syt III staining was localized to PKC-labeled bipolar cell terminals (Figure 6). Unlike synaptic vesicles, which fill the bipolar cell terminals, the Syt III staining in the goldfish bipolar cells appeared as small patches within the terminals. This is consistent with the localization of Syt III to specialized domains within the bipolar cell synaptic terminals, perhaps at the active zones themselves.

Figure 5. Syt III is not localized to ON bipolar cells or horizontal cells in the mouse retina. A. Confocal image of a mouse retina section double labeled for Syt III (green) and PKC (red). B. Confocal image of the OPL of a mouse retina section double labeled for Syt III (green) and the horizontal cell marker calbindin (red). C. High magnification image of the OPL double labeled for Syt III (green) and PKC (red). Areas of co-localization in panels A-C appear yellow. Very little co-localization is observed between Syt III and either bipolar cells or horizontal cells. opl indicates outer plexiform layer, and ipl, inner plexiform layer. Scale bar is ~30 µm for A, 25 µm for B, and 9 µm for C.
Discussion

Continuous synaptic vesicle exocytosis at ribbon synapses of photoreceptors and bipolar cells in the goldfish retina is estimated to occur at calcium concentrations between 1-10 μM (Lagnado et al., 1996; Rieke & Schwartz, 1996). This is much lower than the calcium concentration of approximately 200 μM required for the binding of syntaxin 1 by Syt I/II, the proposed calcium sensor for synaptic vesicle-mediated neurotransmitter release (Geppert & Sudhof, 1998). Here we show by immunohistochemistry that Syt I and II are absent from photoreceptor and bipolar cell ribbon synapses in the goldfish retina, indicating that, contrary to current models of neurotransmitter release, the presence of Syt I or II is not obligatory for calcium-triggered synaptic vesicle exocytosis.

Goldfish photoreceptor and bipolar cell terminals must utilize an alternative calcium sensor to Syt I/II for synaptic vesicle exocytosis, probably a different member of the synaptotagmin gene family. The relatively low calcium concentrations required for continuous vesicle cycling at the goldfish photoreceptor and bipolar cell ribbon synapse hint that the calcium sensor may have a higher affinity for calcium than has been measured for Syt I and II. Of the synaptotagmin family, Syt III exhibits half maximal binding to syntaxin at ~1 μM (Li et al., 1995). Syt III immunoreactivity was detected in both the inner and outer plexiform layers in the goldfish retina. Double labeling for PKC revealed that Syt III was localized to small patches overlying the PKC staining (Figure 6), suggesting that it is unlikely to be a component of bipolar cell synaptic vesicles, which fill the terminals (von Gersdorff, Vardi, Matthews, & Sterling, 1996). This interpretation of the staining is consistent with previous subcellular fractionation experiments showing that Syt III is concentrated in rat brain synaptosomes but does not co-purify with synaptic vesicles (Butz et al., 1999). Thus Syt III may function as a calcium sensor at specialized domains of the plasma membrane of goldfish bipolar cell terminals. It remains unknown which, if any, member of the synaptotagmin family of calcium sensors is found on synaptic vesicles in goldfish photoreceptors and bipolar cells.

Comparison of the staining of Syt I/II and III in goldfish, rat, and mouse retinas revealed differences in the distribution of different synaptotagmins between the fish and rodent species. Unlike the goldfish retina, intense immunoreactivity for Syt I/II is detected in both the OPL and IPL of the rat (Figure 1) and mouse retinas (not shown), and is present in the synaptic terminals of isolated rat bipolar cells (Figure 3B and 3C). The staining in the rat and mouse OPL is consistent with the presence of Syt I/II on synaptic vesicles in rodent photoreceptors as previously reported (Von Kriegstein, Schmitz, Link, & Sudhof, 1999). Furthermore, in contrast to the punctate Syt III immunoreactivity over goldfish bipolar cell terminals, no Syt III staining was associated with mouse bipolar cell terminals (Figure 5). The reason for the difference in Syt I/II and III expression between mammalian and fish retinal ribbon synapses is unclear. It raises the possibility that the calcium sensitivity of transmitter release at ribbon synapses may differ between fish and rodent retina. In the rat and mouse retinas, the recently discovered, retina-specific α1F calcium channel subunit is localized to photoreceptor and bipolar cell active zones (Morgans, 2001; Berntson et al., 2002). It will be interesting to find out whether the calcium channels in goldfish photoreceptors and bipolar cells are also restricted to active zones or have a more diffuse distribution necessitating a higher affinity calcium sensor.
Conclusions

The data presented here show that, whereas Syt I/II immunoreactivity is abundant in both photoreceptor and bipolar cell terminals in the rodent retina, Syt I and II are absent from photoreceptor and ON-bipolar cell terminals in the goldfish retina. Thus, contrary to current models, the presence of Syt I/II is not obligatory for calcium-triggered synaptic vesicle exocytosis. Further differences were found between goldfish and rodent retinas in the expression of Syt III. Punctate Syt III staining localized to ON-bipolar cell terminals in the goldfish retina, but no Syt III staining was found in ON-bipolar cells in the mouse retina. The differences in the distributions of Syt I/II and III between the goldfish and rodent retinas suggests that they may differ in their calcium dependence of neurotransmitter release.

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References


