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Differential expression of active zone proteins in neuromuscular junctions suggests functional diversification

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Abstract

Nerve terminals of the central nervous system (CNS) contain specialized release sites for synaptic vesicles, referred to as active zones. They are characterized by electron-dense structures that are tightly associated with the presynaptic plasma membrane and organize vesicle docking and priming sites. Recently, major protein constituents of active zones have been identified, including the proteins Piccolo, Bassoon, RIM, Munc13, ERCs/ELKs/CASTs and liprins. While it is becoming apparent that each of these proteins is essential for synaptic function in the CNS, it is not known to what extent these proteins are involved in synaptic function of the peripheral nervous system. Somatic neuromuscular junctions contain morphologically and functionally defined active zones with similarities to CNS synapses. In contrast, sympathetic neuromuscular varicosities lack active zone-like morphological specializations. Using immunocytochemistry at the light and electron microscopic level we have now performed a systematic investigation of all five major classes of active zone proteins in peripheral neuromuscular junctions. Our results show that somatic neuromuscular endplates contain a full complement of all active zone proteins. In contrast, varicosities of the vas deferens contain a subset of active zone proteins including Bassoon and ELKS2, with the other four components being absent. We conclude that Bassoon and ELKS2 perform independent and specialized functions in synaptic transmission of autonomic synapses.

Introduction

Exocytotic release of neurotransmitters from nerve terminals occurs at specific sites termed active zones. At the ultrastructural level, active zones consist of electron-dense intracellular material that is intimately associated with the inner side of the presynaptic plasma membrane, facing the synaptic cleft (Peters *et al*., 1991). Active zones are characteristic features of CNS synapses where they are arranged in hexagonal lattices, thus defining vesicle docking and release sites (Akert *et al*., 1971). Outside the CNS, active zones are found in somatic neuromuscular junctions. Three-dimensional reconstruction of the active zone in the frog neuromuscular junction revealed elongated bar-like structures that are attached by short pegs to the presynaptic membrane. At regular intervals, side-branches are emerging that connect to synaptic vesicles (Harlow *et al*., 2001). In contrast, morphologically identifiable active zones, as well as postsynaptic densities, are absent from peripheral synapses of the autonomic nervous system. Examples include sympathetic varicosities consisting of bulb-like thickenings of the axons, which contain synaptic vesicles and large dense-core vesicles. Varicosities are often serially arranged along the axon like pearls on a string. In varicosities exocytosis is not confined to morphologically distinct sites, although preference was suggested for specialized membrane subdomains localized in close proximity to the muscle cell (for review, see Thureson-Klein & Klein, 1990; Bennett *et al*., 1998).

In recent years, major protein constituents have been identified that together form the cytomatrix of the active zone (henceforth referred to as CAZ) including RIMs (Rab3 interacting molecules), Munc13, liprin α3, ERCs/ELKs/CASTs, Bassoon. and Piccolo/Aczonin (for review, see Garner *et al*., 2000; Südhof, 2004; Zhen & Jin, 2004). Most of these proteins are composed of multiple domains specialized for protein–protein interactions, and represent small families with several isoforms or splicing variants. Munc13 proteins constitute a family of evolutionary conserved and primarily brain-specific molecules (Brose *et al*., 1995; Augustin *et al*., 1999a). Munc13-1, the most abundant isoform, is thought to be an essential protein for synaptic vesicle priming for exocytosis at the active zone of CNS synapses. Munc13-1-deficient mice are normal with respect to CNS cytoarchitecture and synapse structure, but their neurons are incapable of neurotransmitter release, leading to early postnatal death

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(Augustin *et al*., 1999b). RIMs represent a family of multidomain proteins that bind to Munc13 and Rab3a, a neuronal GTPase localized to synaptic vesicles (Wang *et al*., 2000) thus providing a direct link between synaptic vesicles and active zone constituents. Studies on RIM1 knockout (KO) animals revealed that this protein is essential for synaptic plasticity and neurotransmitter release regulation. The Rab3–RIM interaction is thought to be involved in synaptic vesicle tethering and docking, whereas Munc13–RIM1 binding is likely to participate in synaptic vesicle priming (Koushika *et al*., 2001; Castillo *et al*., 2002; Lonart, 2002; Schoch *et al*., 2002). Piccolo and Bassoon are unusually large, structurally related multidomain proteins (Garner *et al*., 2000). The proteins are thought to play an important role in assembling the cytomatrix at the neurotransmitter release site (Cases-Langhoff *et al*., 1996; Tom Dieck *et al*., 1998; Fenster *et al*., 2000). Homozygous mutant mice lacking the central region of Bassoon are viable and normal at birth, but half of them die during the first 6 months, affected by epileptic seizures. Hippocampal neuronal cultures obtained from these animals are morphologically normal, but they display a reduced number of fusion-competent synaptic vesicles (Altrock *et al*., 2003). Liprin αs (Liprin α1–α4) are members of the LAR-interacting protein family of transmembrane protein-tyrosine phosphatases (Serra-Pages *et al*., 1998), localized at the presynaptic site and associated with other active zone molecules. Genetic deletion of Syd-2 and Dliprin, the *C. elegans* and *Drosophila* orthologues, respectively, of liprin α, resulted in altered active zone morphology, failure of synapse formation and impaired synaptic transmission (Zhen & Jin, 1999; Kaufmann *et al*., 2002). Finally, ELKS constitute a novel family of active zone proteins (Ohtsuka *et al*., 2002; Ko *et al*., 2003), which specifically interact with RIMs, liprins α, and Bassoon and Piccolo (Ohtsuka *et al*., 2002; Wang *et al*., 2002; Ko *et al*., 2003; Takao-Rikitsu *et al*., 2004).

Isolated active zones are insoluble complexes of all CAZ proteins that cannot be easily dissociated, supporting the view that these complexes form structural and functional units. During biogenesis, active zones are preassembled in the soma of neurons, transported *en bloc* by axonal transport in the form of specific transport vesicles termed Piccolo transport vesicles, and then inserted by exocytosis into the presynaptic plasma membrane (Zhai *et al*., 2001; Shapira *et al*., 2003; for review, see Ziv & Garner, 2004). These findings suggest that the biogenesis and assembly of CAZ proteins occurs in a coordinated manner.

Despite these recent advances, major questions remain. Thus, it is unclear whether all of the CAZ proteins are needed for forming functional active zones. As discussed above, genetic deletions of RIM1, Munc13s and Bassoon resulted in impairments of synaptic transmission, supporting the view that each of the proteins is required for normal synaptic function. On the other hand, somatic neuromuscular junctions were reported to lack both Piccolo and Bassoon (Fenster *et al*., 2000), despite the presence of 'bona-fide' active zones. Conversely, it is not known whether any of the known CAZ proteins is involved in the function of synapses that lack morphologically defined active zones but are capable of fast exocytosis.

In the present study we have used a panel of specific antibodies to systematically investigate whether CAZ proteins are present in peripheral synapses, focusing on somatic neuromuscular junctions and sympathetic neuromuscular varicosities. Our results show that contrary to previous reports somatic neuromuscular junctions contain at least one isoform of all of the examined CAZ proteins. In contrast, sympathetic neuromuscular junctions of the vas deferens contain only a subset of CAZ proteins, including ELKS2 and Bassoon. We conclude that in addition to functioning coordinately in the formation of 'classical' active zones, individual CAZ proteins have specialized roles in peripheral synapses.

Materials and methods

Antibodies

Monoclonal antibodies specific for synaptophysin (clones 7.2 and 7.4), synaptotagmin (clone 41.1), Rab3 (clone 42.1), synapsin I (clone 46.1), SNAP 25 (clone 71.1), syntaxin (HPC1), rabbit sera specific for Bassoon (raised against a recombinant fragment including the C-terminal 330 residues) and Piccolo, and an affinity-purified rabbit antibody raised against recombinant RIM1 were obtained from Synaptic Systems (Göttingen, Germany). Note, however, that the RIM antibody cross-reacts with RIM2 (Fig. 1). Monoclonal and polyclonal antibodies specific for tyrosine hydroxylase were obtained from Chemicon (Temecula, USA). The following antibodies were published previously: rabbit antibodies specific for ELKS2 (Wang *et al*., 2002), liprin α3 (Schoch *et al*., 2002) and monoclonal antibody HPC-1 specific for syntaxin 1 (Barnstable *et al*., 1985). For Munc13-1, a rabbit serum was generated using a recombinant fragment of Munc13-1 (residues 484–1735) as antigen, which was expressed as histidine-tagged protein in SF9 cells according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). To test whether the antibody was specific for Munc13-1, HEK T293 cells were transfected with expression constructs coding for Munc13-1, Munc13-2 (GFP-fusion), Munc13-3 and Munc13-4, followed by immunoblot analysis of cell lysates. The antiserum reacted strongly with the Munc13-1-expressing cells, but not with any of the other transfectants (data not shown). Goat anti-mouse Cy2, goat anti-rabbit Cy2, goat anti-mouseCy3 and goat anti-rabbit Cy3 were obtained from Dianova (Hamburg, Germany). α-Bungarotoxin conjugated with Alexa 488 (1 : 100, green fluorescence) was purchased from Molecular Probes (Invitrogen). All other chemicals used were of analytical grade from Sigma.

Immunohistochemistry

Adult male Wistar rats were anaesthetized with ether and transcardially perfused sequentially with phosphate-buffered saline (PBS) and 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The cervical and thoracic spinal cord, diaphragm and vas deferens were dissected, postfixed in the same fixative for 3–4 h and transferred into cryoprotective 20% sucrose solution in PBS.

Tissues were mounted in freezing medium (Tissue-Tek, Leica, Germany), sectioned longitudinally (vas deferens) or transversally (spinal cord, diaphragm) at a thickness of 8 μm on a cryostat and collected on coated slides (SuperFrost Plus, Menzel Gläser, Braunschweig, Germany). Immunostaining of tissue sections was performed using standard protocols. Briefly, sections were initially washed twice with TBS (10 mM Tris, pH 7.2, 150 mM NaCl) followed by permeabilization in TBST [TBS containing in addition 0.1% (v/v) Triton X-100] and 50 mM NH_4Cl for 15–20 min, afterwards they were shortly rinsed in TBST twice and incubated with blocking solution (TBST containing 10% goat serum, 1% bovine serum albumin and 0.1% sodium azide) for 1 h. Next, the sections were incubated for 12 h with the primary antibody. The concentration of each primary antibody was tested in trial series to optimize for specific labelling, resulting in dilutions ranging from 1 : 50 to 1 : 200. After washing twice in TBST and TBS, respectively (5 min each), the sections were incubated with secondary antibodies for 1 h, briefly rinsed (4 × 3 min) in TBS and mounted. All sections of the same series were processed in parallel.

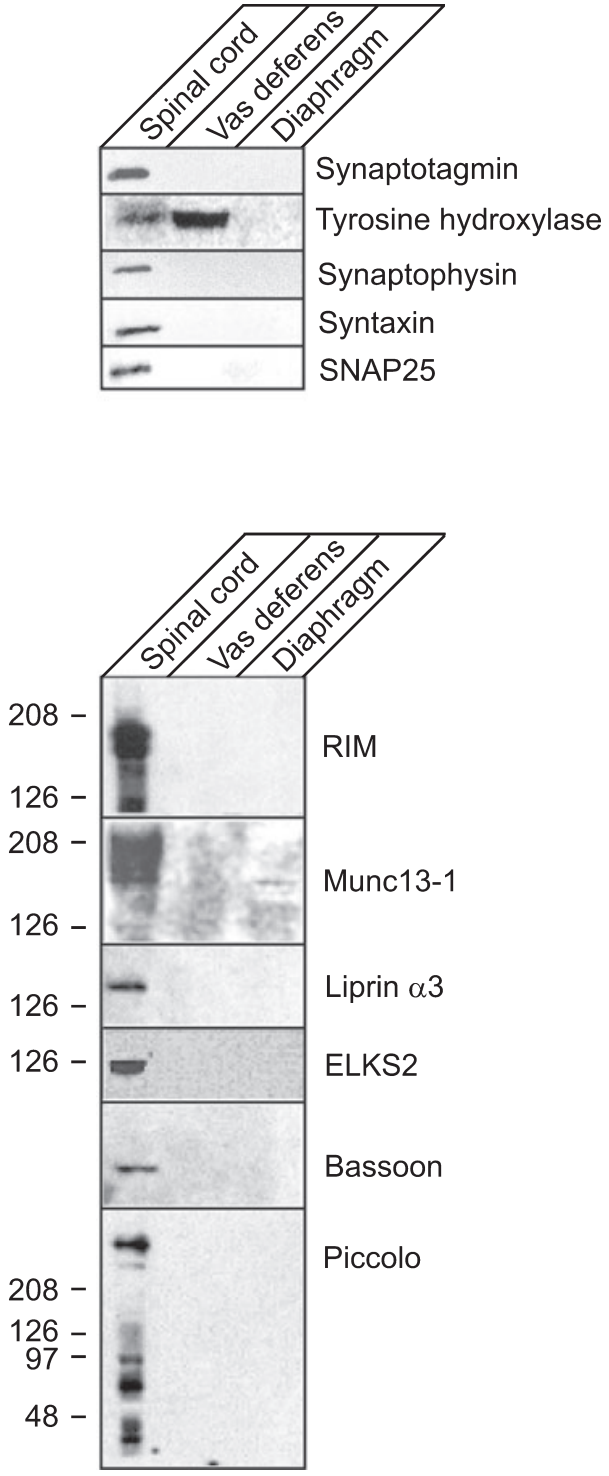


FIG. 1. Analysis of CAZ proteins by immunoblotting using homogenate samples of rat spinal cord, vas deferens and diaphragm. Top: reference blot for the synaptic vesicle proteins synaptotagmin 1 and synaptophysin, the SNAREs syntaxin 1 and SNAP-25, and tyrosine hydroxylase. Note that except for tyrosine hydroxylase, the levels of synaptic proteins are too low for detection in the peripheral tissues.

Double-labelled sections were analysed using a confocal laser scanning microscope with a krypton/argon laser (Leica Microsystems, Wetzlar, Germany) using single- or dual-laser scanning. For co-localization studies, identical scanning sensitivity was used for all sections. Parameters for both channels for each sample were kept similar.

For epifluorescence microscopy, the mounted slides were observed under a Quantix-Axiovert microscope (Zeiss, Germany) using suitable filters. Photographs were taken with a CCD camera (Princeton Instruments, Trenton, NJ, USA) using the program 'Metamorph' for data acquisition (Universal Imaging, West Chester, PA, USA).

Immunoblot analysis of rat tissues

Adult male Wistar rats were killed by CO_2 asphyxiation. The cervical and thoracic parts of the spinal cord, the diaphragm and the vasa deferentia were quickly dissected, and tissues were snap-frozen in liquid nitrogen. Frozen tissue was pulverized in a mortar and pestle and transferred to chilled TBS containing a complete mini-protease inhibitor cocktail tablet (Roche, Mannheim, Germany). A small aliquot was kept for protein estimation, and the rest was immediately boiled in sodium dodecyl sulphate (SDS) sample buffer and stored frozen. SDS–polyacrylamide gel electrophoresis (PAGE) and transfer to nitrocellulose membranes was carried out by standard procedures (Laemmli, 1970; Towbin *et al*., 1979) using 4–12% gradient gels, and 10 μg protein/lane (40 μg/lane for the detection of CAZ proteins) from each tissue was loaded. The blots were developed using appropriate horseradish peroxidase-linked secondary antibodies and super-sensitive enhanced chemoluminescence (Pierce, Rockford, USA).

Electron microscopy

Tissues were fixed as described for immunofluorescence and cryoprotected in 20% sucrose. Twenty-micrometer-thick sections were cut on a cryostat and processed free-floating for electron microscopic immunocytochemistry using previously published methods (Rickmann & Wolff, 1995). In brief, after blocking unspecific antibody binding, anti-Bassoon antibody (diluted 1 : 50), anti ELKS2 antibody (diluted 1 : 25), anti-synaptophysin antibody (diluted 1 : 100) and anti-RIM1 antibody (diluted 1 : 25) were applied for 16 h at room temperature. These were detected with the ABC method (DAKO) followed by a nickel-intensified horseradish peroxidase reaction with 3,3′-diaminobenzidine for 3 min. For negative control we used sections reacted against RIM1, for positive control sections were incubated for synaptophysin. For electron microscopy sections were osmicated and flat embedded. Only well-reacted tissue (close to the surface of the section) was used. All experiments were performed in accordance with the guidelines for animal care of laboratory animals issued by the European Union Commission and the Max Planck Society.

Results

For the characterization of CAZ proteins, a panel of rabbit antibodies was employed, some of which were newly generated for this study (see Materials and methods). Immunoblot analysis of homogenate samples of rat spinal cord revealed major bands of the expected molecular weights for all of the six CAZ proteins analysed, with the two major bands in the RIM blot corresponding to RIM1 and RIM2 (Fig. 1). Furthermore, some breakdown products were detected in the RIM and Piccolo blots. For Piccolo, such breakdown bands were also recognized in mouse brain homogenates but were all absent in brains derived from Piccolo KO mice, confirming that these bands are not due to cross-reactivity with unrelated proteins (data not shown). Despite the use of sensitive detection methods, no cross-reacting bands were observable in homogenates of rat vas deferens and diaphragm (Fig. 1). Furthermore, no cross-reacting bands were observed with any of the pre-immune sera (data not shown). As a

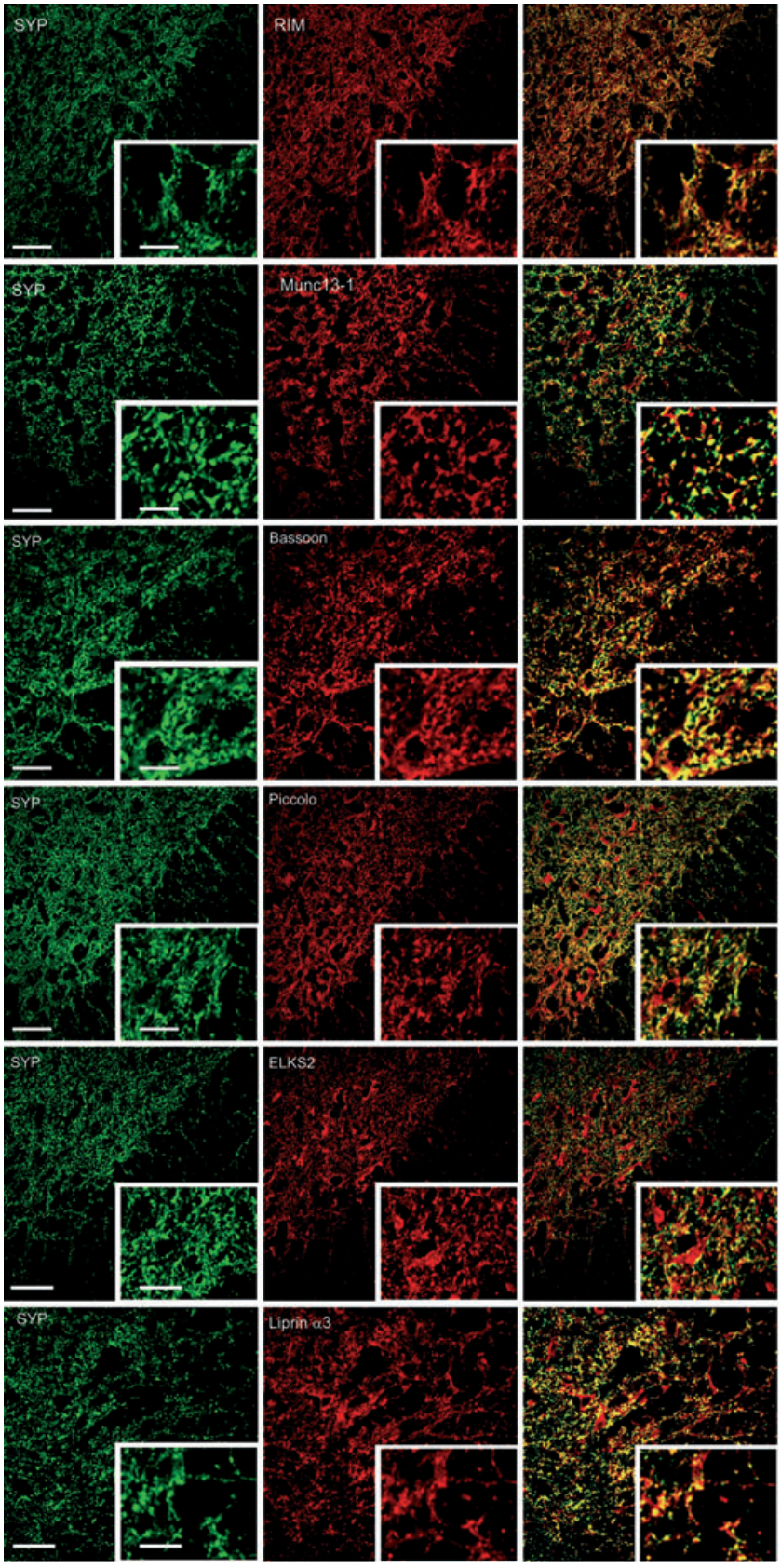


FIG. 2. Localization by immunocytochemistry of CAZ proteins (red channel) in cross-sections of rat spinal cord. Synaptophysin staining (green channel) was used as reference for the identification of presynaptic nerve terminals. Insets show fields at higher magnification. All sections were imaged by scanning confocal microscopy as described in Materials and methods. Scale bar, 40 μm (inset, 20 μm).

further control, the tissues samples were also probed for synaptic markers including synaptotagmin 1, synaptophysin, tyrosine hydroxylase, syntaxin 1 and SNAP25. Again, all proteins were detectable in the spinal cord homogenate but, with the exception of tyrosine hydroxylase, were lacking in the other homogenate samples. Together, these data show that the antibodies for CAZ proteins are specific and do not cross-react with other proteins. Furthermore, they show that the relative contribution of nerve endings to both vas deferens and diaphragm samples is too low to be detectable by immunoblotting of homogenate samples.

Next, immunohistochemical stainings of sections obtained from rat spinal cord were performed for each CAZ protein under study. In order to assess the degree of synaptic localization, all sections were also labelled for synaptophysin, a presynaptic marker. As shown in Fig. 2, stainings for RIM, Bassoon, Piccolo, ELKS2 and Munc13-1 yielded punctate patterns that overlapped with the synaptophysin staining pattern. Furthermore, some staining of the perinuclear cytoplasm was observed in the case of ELKS2 and Piccolo. In contrast, antibodies for liprin α3 resulted in rather uniform labelling of the entire neuron. These data are consistent with fractionation data showing that unlike the other CAZ proteins a sizeable portion of liprin is recovered in the soluble fraction (Wang *et al*., 2002).

Next we examined which of the active zone proteins are present in peripheral neuromuscular junctions. Somatic neuromuscular junctions of the rat diaphragm and the sympathetic neuromuscular varicosities of rat vas deferens were used as models for somatic and autonomic synapses, respectively. The somatic neuromuscular junction is a paradigmatic fast synapse containing small, acetylcholine-containing synaptic vesicles and electron microscopically defined active zones. The smooth muscles of the vas deferens are

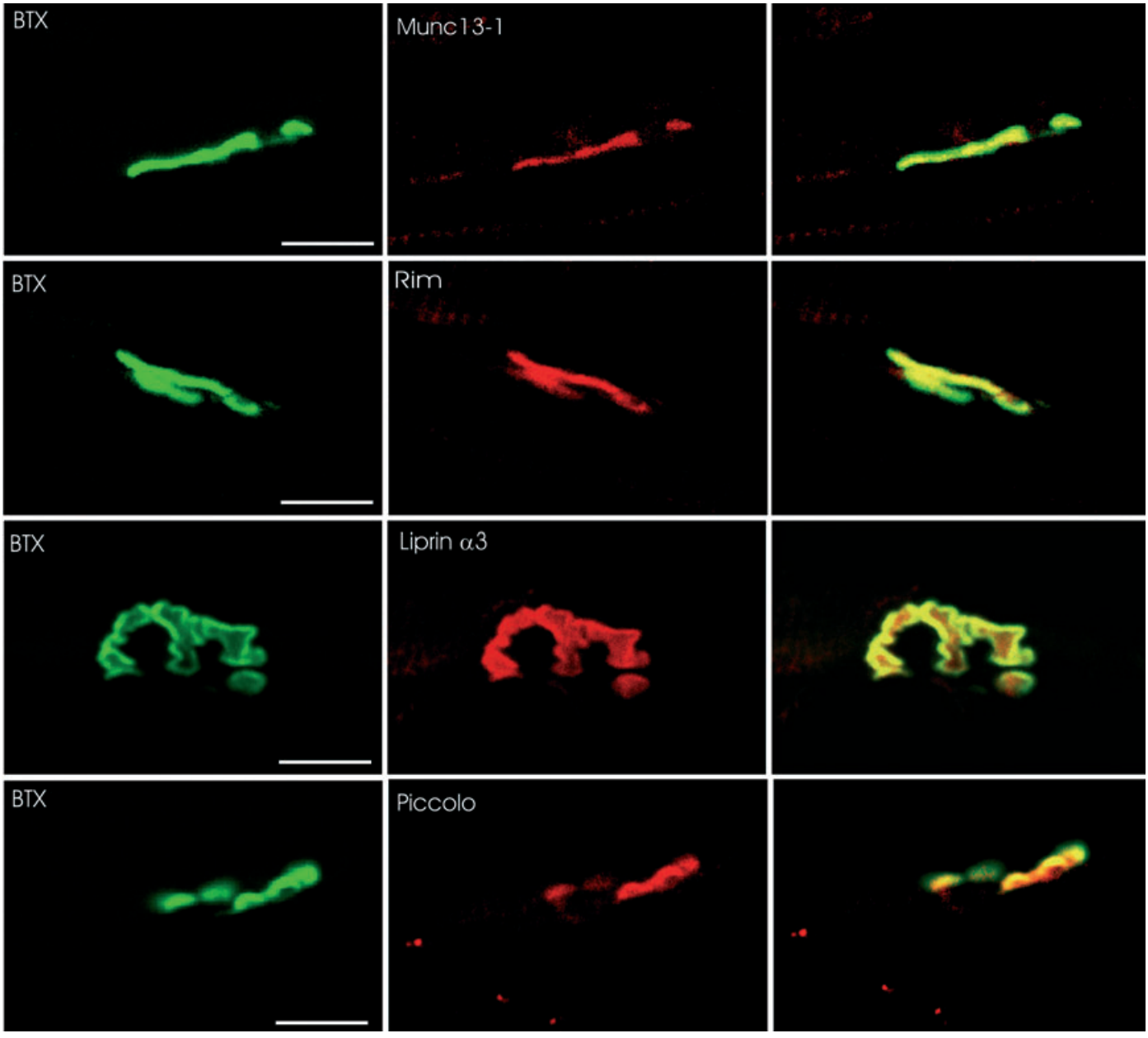


FIG. 3. Presence of CAZ proteins (red channel) in somatic neuromuscular endplates of the diaphragm muscle, analysed by immunocytochemistry of sections of rat diaphragm. Endplates were identified by counterstaining of nicotinic receptors with α-bungarotoxin (BTX) (green channel). Scale bar, 16 μm.

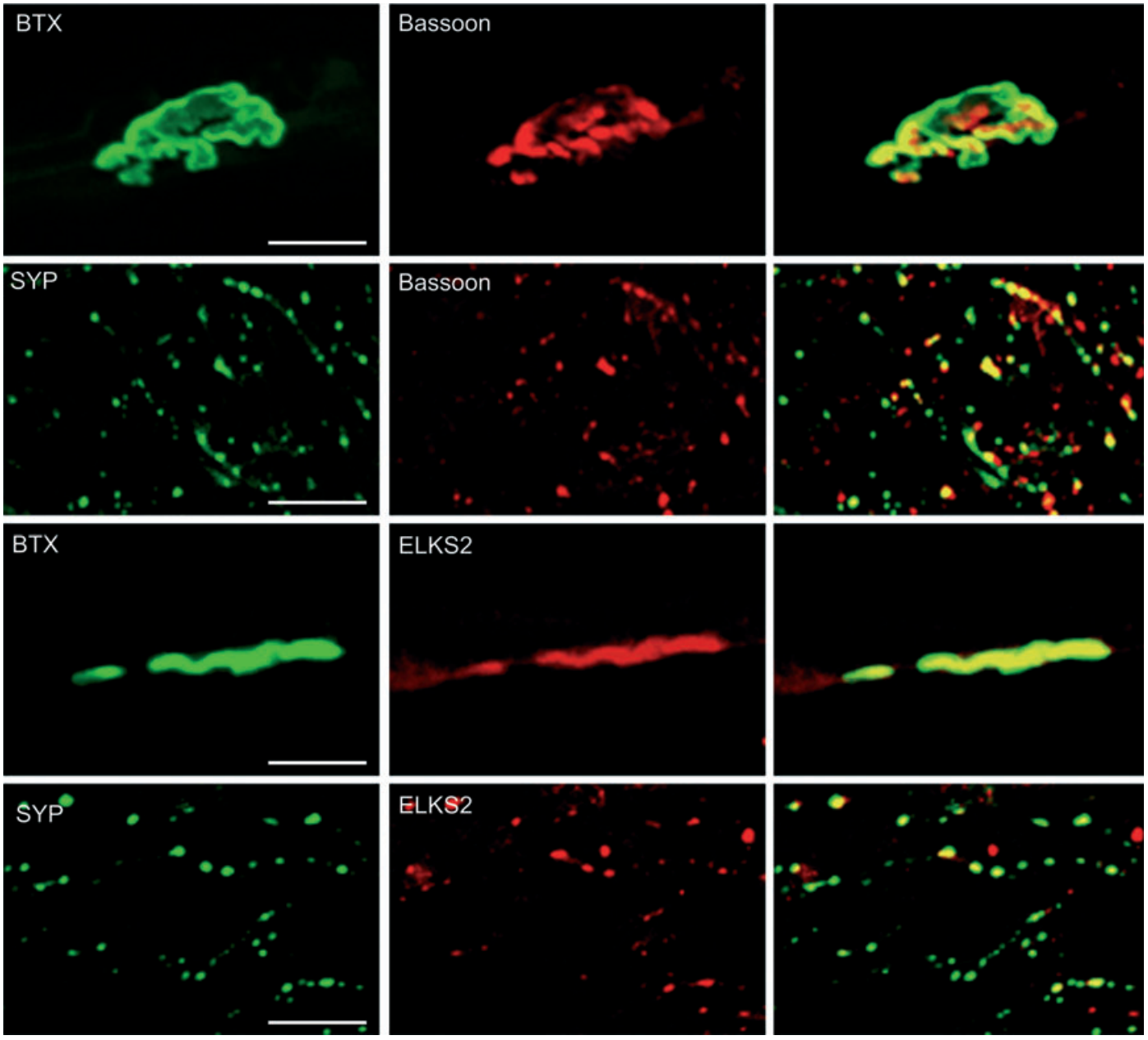


FIG. 4. Bassoon and ELKS2 are present both in neuromuscular endplates and in nerve terminals of the vas deferens. Sections of rat diaphragm (first and third row) and of rat vas deferens (second and fourth row) were double-labelled for Bassoon and ELKS2, respectively, and synaptophysin (green channel) to identify nerve terminals. The panels show individual neuromuscular endplates and sympathetic varicosities, respectively. Scale bar, 16 μm.

innervated with sympathetic varicose axons (Merrillees, 1968). These varicosities contain ATP as a fast-acting transmitter, and noradrenaline that is stored in both small and large dense-core vesicles (Stjarne, 2000). Varicose nerve endings also form junctions with the smooth muscles (Richardson, 1962) but, in contrast to the neuromuscular endplates, the junctional clefts are larger (Bennett *et al.*, 1998) (50–100 nm as compared with 20–50 nm). Furthermore, there are no obvious pre- and postsynaptic specializations (Bennett, 1996). Both types of synapses were previously shown to contain synaptic vesicle proteins and the SNAREs syntaxin and SNAP-25 (Brain *et al.*, 1997).

Labelling of neuromuscular endplates in the rat diaphragm revealed strong signals for RIM, Munc13-1, liprin α3 and Piccolo (Fig. 3). All sections were counterstained either for α-bungarotoxin (Fig. 3) or for synaptophysin (not shown), revealing near-perfect co-localization. It should be noted that our finding disagrees with a recent report stating that Piccolo is absent from neuromuscular endplates (Fenster *et al.*, 2000). However, in this study a different antibody was used that may have yielded lower staining intensities, thus escaping detection in this preparation. In parallel experiments, staining was carried out with sections obtained from rat vas deferens. Whereas the varicosities were strongly positive for synaptophysin, no specific staining was observable for any of the proteins listed above (not shown). Together, these data strongly support the correlation between the presence of these CAZ proteins and the presence of ultrastructurally identifiable active zones.

Next, we stained sections containing both types of neuromuscular junctions for ELKS2 and for Bassoon. As with the other CAZ proteins, both proteins were readily detectable in neuromuscular

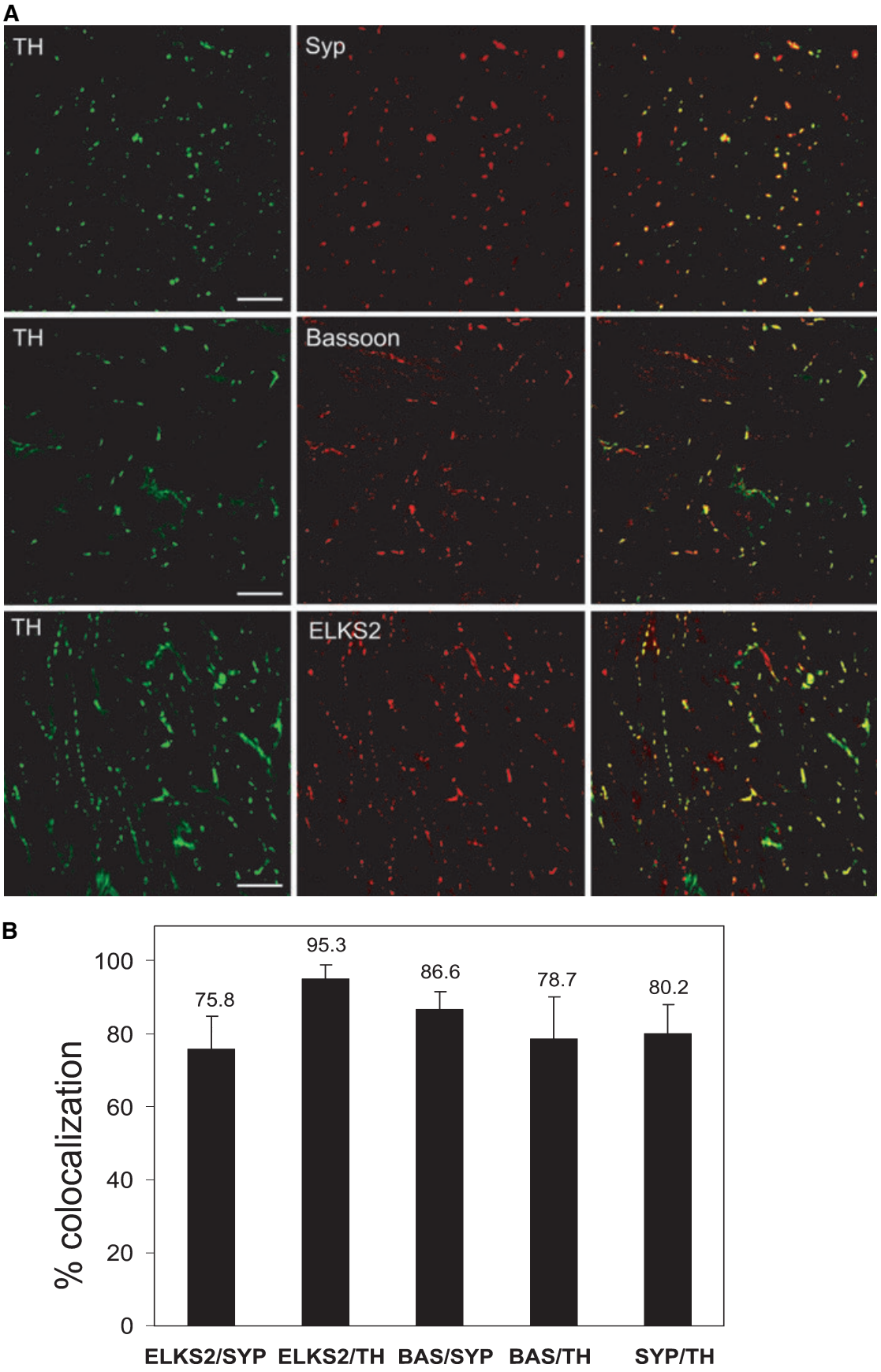
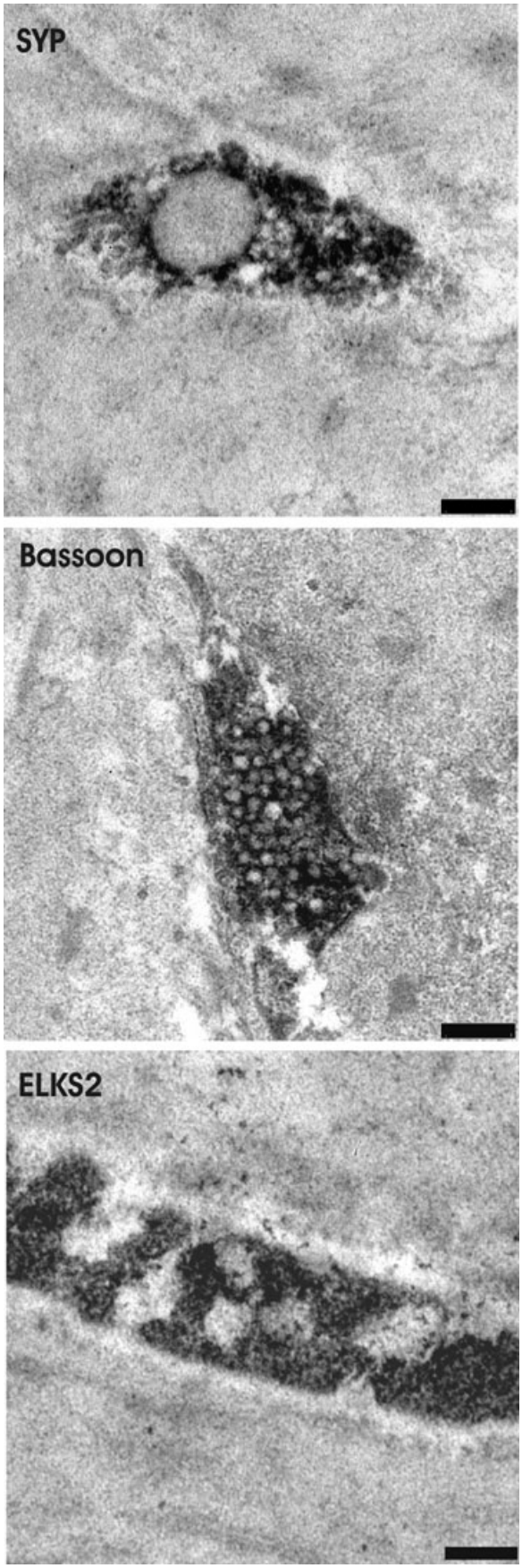


FIG. 5. (A and B) Co-localization of Bassoon and ELKS2 (red channel) with tyrosine hydroxylase (green channel) identifies nerve terminals of the vas deferens as sympathetic varicosities. Sections of rat vas deferens were double-labelled for tyrosine hydroxylase and the respective CAZ protein, and analysed by confocal microscopy. Scale bar, 20 μm.



endplates (Fig. 4). These findings show that neuromuscular endplates contain a full complement of CAZ proteins. Surprisingly, however, strong signals were also detectable in the varicosities of the vas deferens, identified by counterstaining for the vesicle marker synaptophysin (Fig. 4). In order to verify that the punctate staining pattern indeed represents sympathetic varicosities, double-labelling experiments were performed with an antibody specific for tyrosine hydroxylase, an enzyme marker for sympathetic nerve endings (Nagatsu, 1995). A high degree of co-localization between tyrosine hydroxylase and synaptophysin, ELKS2 and Bassoon was observed (Fig. 5A). To obtain more quantitative data, the number of varicosities positive for each of the antigens was related to the number of varicosities positive for synaptophysin and tyrosine hydroxylase. The degree of co-localization varied in each case, ranging from 76% for ELKS2/synaptophysin to 95% for ELKS2/tyrosine hydroxylase (Fig. 5B). To confirm that the labelled structures indeed represent varicose nerve terminals, we performed immunoelectron microscopy on vas deferens sections for synaptophysin, ELKS2 and Bassoon using the immunoperoxidase method. In all cases, strong labelling of varicosities was observed that were readily identifiable by their dense packing with synaptic vesicles (Fig. 6). As a negative control, the sections were also incubated with antibodies specific for RIM. As expected, no reaction product was detectable in these sections.

Discussion

In the present study, we have investigated whether CAZ proteins are localized to peripheral synapses. Using a panel of antibodies specific for representative members of six CAZ protein classes we found that somatic neuromuscular junctions contain all CAZ proteins examined. In contrast, sympathetic neuromuscular varicosities contained ELKS2 and Bassoon, but lacked the other four proteins. We conclude that ELKS2 and Bassoon are probably required for synaptic transmission in these synapses, being involved in functions that do not require interactions with the other major proteins including RIM, liprin α3, Munc13-1 and Piccolo.

Active zones are generally thought to organize sites of vesicle release at fast synapses. Similar to their postsynaptic counterpart, the postsynaptic densities, active zones are primarily morphologically defined, characterized by their electron-dense appearance in most electron micrograph techniques. Electron density is thought to represent a high concentration of macromolecules forming a supramolecular matrix of interacting proteins. The molecular characterization of major active zone proteins in recent years yielded a molecular explanatory framework for many of the functions active zones are thought to perform. For instance, all CAZ proteins consist of multiple protein–protein interaction domains and specifically bind to each other, thus creating a dense network of proteins that was predicted based on the electron microscopic studies. In particular, Piccolo and Bassoon, by virtue of their enormous size, are almost destined to provide a scaffolding backbone (Garner *et al.*, 2000), although biochemical characterization is hampered by major problems in the handling of these large proteins. Furthermore, the presence of calcium-binding C2-domains in three of the CAZ proteins (Piccolo, RIM and

FIG. 6. Localization by immunoelectron microscopy (peroxidase method) of Bassoon, ELKS2 and synaptophysin (as presynaptic marker) to varicosities of the vas deferens. The images show representative individual varicosities that are easily identifiable by the presence of numerous synaptic vesicles. Scale bar, 200 nm.

Munc13) (Garner *et al*., 2000; Wang *et al*., 2000; Gerber *et al*., 2001) suggests that the active zone may respond to changes in calcium levels and thus may possess the means to adjust to changing requirements in synaptic strength. Moreover, ELKS2 and RIM are effectors of Rab-GTPases (Wang *et al*., 1997; Monier *et al*., 2002), opening the possibility that CAZ–Rab interactions contribute to synaptic vesicle docking. Synaptic vesicles are an integral part of active zones: they are docked to and exocytose at 'slots' defined by the surrounding electron-dense material (reviewed in Südhof, 2004).

Since their initial discovery, most CAZ proteins have been investigated with respect to their function in CNS synapses, including the analysis of animal strains in which individual proteins are deleted (see Introduction). Thus, functional data concerning the involvement of CAZ proteins in peripheral synapses are largely absent. Based on our finding that somatic neuromuscular endplates contain all six CAZ proteins examined, we predict that deletion of any of the proteins is likely to result in an impairment of neuromuscular transmission, and it cannot be excluded that such impairments contribute to motor defects observed in the *C. elegans* mutants unc13 (Richmond *et al*., 1999), syd2 (Zhen & Jin, 1999) and RIM/unc10 (Koushika *et al*., 2001). Furthermore, the correlation between the presence of CAZ proteins and electron densities at presynaptic release sites suggests that both CNS synapses and somatic neuromuscular junctions require the joint action of all interacting CAZ proteins for function. Altogether, these findings make sense as neuromuscular junctions are classical fast synapses that share functional and morphological properties with active zone containing CNS synapses, except that they are larger and contain multiple and elongated active zones.

Apart from conventional synapses, CAZ proteins are found to be present in specialized synapses of mammalian sensory pathways called ribbon synapses. In the retina, they are associated with a ribbon-specific protein termed RIBEYE. RIBEYE is a ribbon major protein component and it has been suggested to play a role in building a unique scaffold for these specialized synapses (Schmitz *et al*., 2000), whereas its homologue CtBP1 (C terminal binding protein 1), present in all types of synapses, has been suggested to be involved in the development and function of CNS synapses (Tom Dieck *et al*., 2005). Recent investigations on wild-type and Bassoon mutant retina have revealed that Bassoon directly interacts with RIBEYE and might be responsible for physical integrity and assembly of functional ribbon synapses. In the Bassoon, functional KO mouse ribbons are still identifiable but most of them lost their contact to the plasma membrane ('floating') (Tom Dieck *et al*., 2005). It has been suggested that Bassoon links ribbons to the plasma membrane by virtue of its interaction with RIBEYE (Dick *et al*., 2003).

The most surprising finding of our analysis was that two CAZ proteins are expressed in sympathetic varicosities, including ELKS2 and the very large protein Bassoon, whereas all others are absent. Sympathetic varicosities lack electron-dense structures characteristic for active zones. However, the presynaptic membrane is not uniform but rather contains areas of close apposition (50 nm) with the muscle cell (Bennett *et al*., 1998). Clusters of postsynaptic purinergic receptors of 1 μm diameter are often seen in apposition to many varicosities, however, such clusters are also sometimes found unrelated to varicosities and some varicosities have no apposing receptor cluster (Bennett *et al*., 1998). Presently it is not known whether exocytosis is confined to such sites in varicosities, or whether exocytosis, as generally assumed, may occur on the entire surface of the varicosity. In this context it needs to be kept in mind that active zones exclusively define release sites for small synaptic vesicles whereas large dense-core vesicles that are abundant in sympathetic varicosities can exocytose anywhere on the presynaptic plasma membrane (Thureson-Klein & Klein, 1990). Thus, it is tempting to speculate that Bassoon and ELKS2 define hotspots for the release of small synaptic vesicles. These sites are probably not quite as highly organized as electron-dense active zones. Support for such a view is provided by a recent study demonstrating that ELKS1a is expressed in pancreatic beta cells. Here, ELKS1a is concentrated in hotspots at the plasma membranes that co-localize with both granule docking sites and syntaxin clusters (Ohara-Imaizumi *et al*., 2005). The molecular interactions in which Bassoon and ELKS2 are involved in sympathetic varicosities need to be further explored. Recently, a direct interaction between them has been reported (Takao-Rikitsu *et al*., 2004). ELKS2 is viewed as a central player in organizing active zones because of its ability to interact not only with Bassoon but with many of the other CAZ proteins, including RIM, liprin, Bassoon and Piccolo (Takao-Rikitsu *et al*., 2004). In the absence of these proteins it is conceivable that ELKS2 binds to other, hitherto unknown, proteins, and that it recruits secretory vesicles by virtue of its binding to Rab proteins.

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Abbreviations

CAZ, cytomatrix of the active zone; KO, knockout; PBS, phosphate-buffered saline; RIMs, Rab3 interacting molecules; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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