
Varicosities of single sympathetic nerve terminals possess syntaxin zones and different synaptotagmin N-terminus labelling following stimulation

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Summary

A study has been made of the probability of exocytosis of synaptic vesicles at different varicosities in single sympathetic terminal axons in the mouse *vas deferens*. An antibody (SV2Ab) against SV2, a proteoglycan in synaptic vesicles, labelled an area of individual sympathetic varicosities that was slightly less than that occupied by dextran-rhodamine, previously orthogradely transported into the varicosities. In contrast plasma membrane bound protein syntaxin, found at active zones of motor nerve terminals, occupied an area of the varicosity that was approximately one-third that of SV2. This suggests that sympathetic varicosities possess specialized zones for exocytosis on their plasma membranes. Antibodies against the N-terminal sequence of synaptotagmin 1 (SNAb), a sequence exposed within synaptic vesicles, were used to determine the probability of exocytosis at different varicosities of single terminal branches. The area of SNAb labelling was not significantly different from that of the SV2 labelling, which implies vesicles that have undergone exocytosis may eventually return to the main pool of vesicles. Varicosities belonging to the same terminal axon, and identified with SV2Ab, showed different extents of labelling with SNAb when secretion was evoked with high potassium concentrations (80 mM) for 30 min in the presence of SNAb. There was up to an order of magnitude difference in the average intensity of SNAb labelling between different varicosities of the same terminal axon whereas there was little difference in the average intensity of SV2Ab labelling. These observations suggest that there is considerable variability in the probability of exocytosis at the specialized zones in different varicosities.

Introduction

The probability for secretion of a quantum of transmitter at somatic neuromuscular junctions on arrival of a nerve impulse varies from active zone to active zone when measured electrophysiologically (Bennett *et al.*, 1986; Zefirov *et al.*, 1995) or when measured by the styryl dye labelling of synaptic vesicles during exocytosis (Betz & Wu, 1995; Bennett, 1996a). Recently it has been possible to record the electrical signs of transmission at visualized varicosities of sympathetic neuromuscular junctions with loose-patch electrodes to show that adjacent varicosities on the same terminal axon have different probabilities for secretion of transmitter (Lavidis & Bennett, 1992, 1993). There is now evidence that the same lack of uniformity in the probability for secretion occurs at boutons in the CNS, although it is still uncertain in this case as to whether this lack of uniformity occurs only between boutons of different nerve terminals or for boutons of the same

terminal branch (Redman & Walmsley, 1983; Ryan *et al.*, 1993; Reuter, 1995).

The question arises as to the identity of the factors which determine these differences in the probability of evoked secretion at different release sites of a nerve terminal. Synaptic vesicles undergo exocytosis in a manner that is regulated by a set of vesicle-associated proteins (Rothman, 1994), at least one of which undergoes a calcium triggered conformational change leading to exocytosis (Dodge & Rahamimoff, 1967; Augustine *et al.*, 1985). It is most likely that this protein is synaptotagmin, which contains five domains (Südhof, 1995): a single transmembrane domain at the N-terminus that spans the membrane of the synaptic vesicle; a linking sequence; two copies of an internal repeat that is homologous to the regulatory region of protein kinase C, with a region termed C2a closest to the transmembrane region and the C2b region furthest

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away; a carboxyl-terminal extension following these two repeats (Perin *et al.*, 1991). The C-terminus end of synaptotagmin is in close association with the presynaptic membrane bound protein syntaxin which in turn, in many terminals, forms a complex with the N-type voltage dependent calcium channel at active zones which use this calcium to mediate secretion (Bennett *et al.*, 1992; Betz *et al.*, 1993; El Far *et al.*, 1995; Sheng *et al.*, 1996) or the P/Q-type voltage-dependent calcium channels at other active zones, which use these channels to mediate secretion (Martin-Moutot *et al.*, 1996). These proteins, together with the synaptic vesicle, constitute a secretory unit which has been termed the 'secretosome' (Bennett, 1996a). After fixation, antibodies to syntaxin can be used to delineate the active zones (Boudier *et al.*, 1996) and those to a proteoglycan in synaptic vesicles (SV2Ab) can be used to determine the distribution of vesicles in the terminal (Bajjalieh *et al.*, 1992). This procedure has been used in the present work to see if there are regions of syntaxin labelling in individual sympathetic varicosities that indicate active zones, a possibility that has as yet only been conjectured upon (Bennett, 1996b).

Antibodies to an N-terminal sequence of synaptotagmin (SNAb) can be used to determine the extent of release from the secretosomes without prior fixation. When a vesicle opens in the process of exocytosis to the extracellular space that contains SNAb, the N-terminus in the lumen of the vesicle is exposed to the extracellular space, where it may be permanently bound by the SNAb (Matteoli *et al.*, 1992). This technique has been developed to determine the extent of exocytosis in tissue cultured hippocampal neurons using fluorescently labelled SNAb (Malgaroli *et al.*, 1995). It has been used in the present work to determine the extent of exocytosis at different varicosities of single sympathetic terminal axons in the mouse vas deferens during stimulation.

Materials and methods

Preparation of the vas deferens

Vasa deferentia were dissected from 6-week-old Balb/c mice. The mice were killed by cervical fracture. Animal ethics approval has been granted by the Animal Care Ethics Committee, University of Sydney. The left and right vas deferens were removed.

In some experiments, vasa deferentia were loaded with the dextran conjugate of lysine fixable rhodamine, molecular weight 10 kDa (Molecular Probes Inc., OR, USA). Dye loading was carried out in a 10 ml bath in which each vas deferens was pinned down and completely immersed. The bath was continually perfused at a high flow rate (20 ml min⁻¹) with a solution containing (mM; modified Krebs solution): 153.1 Na⁻¹, 2.7 K⁺, 1 Ca²⁺, 1 Mg²⁺, 143.6 Cl⁻, 14.9 HCO₃⁻, 1.3 H₂PO₄⁻ and 7.8 glucose. The solution

reservoir was continually bubbled with 95% O₂ and 5% CO₂ at room temperature (20–22°C). The cut prostatic end of the vas deferens was secured in a suction electrode containing a saturated solution of the dye (1 mg of dextran-rhodamine conjugate in 4 µl of 2.5% Triton X-100 in the modified Krebs solution). The dextran-rhodamine conjugate was transported along the sympathetic axons, eventually filling the terminal axons and their varicosities. Each vas deferens was left for 4–5 hours in the dark and at room temperature (20–22°C) to allow time for the dye to load. They were then removed from exposure to the dye solution and perfused at a high flow rate for 1.5 hours at 30°C to remove any non-specific staining within the tissue, presumably caused by diffusion of the dye extracellularly. The low temperature during the loading protocol was used in order to maintain the tissue viability. After this time, the region of the vas deferens from 2 mm beyond the cut prostatic end showed fluorescent labelling of axons and bundles of axons. Some of these axons were varicose. The average distance between these swellings in the axon was about 5 µm, which suggests that these are sympathetic varicosities (Cottee *et al.*, 1996). There was some loading of dye into smooth muscle cells close to the cut end of the vas deferens, but all experiments were carried out beyond this region.

In some experiments, vasa deferentia were exposed to an antibody to an N-terminal sequence of synaptotagmin 1 (anti-CMVSASHPEALA) raised in rabbits (Chiron Mimotopes, Victoria, Australia). Short lengths of vas deferens (about 5 mm) were placed in a modified Krebs solution with a raised K⁺ concentration of 80 mM (with the Na⁺ concentration adjusted to maintain osmolarity and a Ca²⁺ concentration of 2.5 mM) containing the SNAb. The elevated K⁺ concentration depolarises the varicosities while the elevated Ca²⁺ concentration further increases the probability of vesicle exocytosis. After exposure to this solution for 30 min, each vas deferens was washed with phosphate buffered saline (PBS) and fixed.

Immunofluorescence

Each vas deferens was fixed in cold (4°C) 4% paraformaldehyde for 1.5 h, washed in PBS, embedded in OCT embedding medium and frozen using isopentane. Transverse cryostat sections (12 µm thickness) of the vas deferens were mounted on chrome alum coated slides and incubated for 1.5 h with 1% bovine serum albumin (Sigma) in PBS. Sections were then incubated overnight at 4°C with a monoclonal antibody to either SV2 (SV2Ab, courtesy of Dr Kathleen Buckley, Dept. Neurobiology, Harvard Medical School) diluted 1:20 or to syntaxin (Sigma; molecular weight 35 kDa; Smirnova *et al.*, 1995) diluted 1:50. Following three washes in PBS the sections were incubated for 2 h at room temperature with either fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (1:50 dilution, Sigma, for SV2Ab labelling), or FITC-conjugated rabbit anti-mouse IgG1 antibody (1:50 dilution, ICN Biomedicals, for anti-syntaxin labelling). The SNAb labelled sections were also exposed to a rhodamine-conjugated anti-rabbit secondary antibody (1:50 dilution, Sigma). All antibodies were diluted in 1% bovine serum albumin in PBS. The sections were then washed three times with PBS and mounted in p-phenylenediamine dihydrochloride in glycerol. Control sections were incubated with a buffer solution and the

secondary antibody or in a pre-immunised rabbit serum (for the SNAb experiments) to determine the level of non-specific staining. No punctate fluorescent labelling similar to that in the experimental sections was observed in any of the relevant control sections.

Analysis of areas and intensities

Confocal images were transferred to NIH Image 1.60 (available from the user, remote location <http://rsb.info.nih.gov/nih-image/>) and areas were calculated using an automated procedure written with the program's macros. The background signal was calculated by finding the average intensity over a large area which was not deemed to be specifically labelled, and then subtracted from the image. After selecting the centre of a varicosity manually, the local region with pixel values (the fluorescent intensity recorded from the smallest unit of area of the digitised image) greater than 90% of the selected pixel value were averaged in order to estimate the maximum fluorescent intensity in the varicosity. This was done so as to obtain a consistent measurement of the area for small differences in the location of the selected pixel. The area of the continuous region in which the pixel value was greater than 20% or 50% of the maximum was then measured. The 50% threshold was chosen for all experiments involving a comparison between rhodamine-dextran and SV2Ab because a lower threshold did not allow individual varicosities to be discriminated from the inter-varicose axon and adjacent varicosities. The 20% threshold was chosen for all experiments involving a comparison between SV2 and syntaxin, and between SV2 and synaptotagmin labelling in order to yield an area measurement which more fully represented the size of the labelled region. The differences in the protocol used for measuring areas implies that it is not appropriate to compare the measurements made of the rhodamine-dextran areas with the areas measured for syntaxin or synaptotagmin labelling. This measurement protocol is independent of the (linear) intensity scale used, yielding objective area measurements.

As the fluorescently labelled areas are comparable to the area of the point spread function for the microscope (the image obtained from a theoretical point source), the area of the point spread function was measured. Images of 100 nm fluorescent beads were obtained and their areas were measured, using the above technique, to be 0.054 (SEM 0.005) μm^2 ($n = 21$) yielding a radius of the estimated point spread function (ρ) of 0.130 (0.006) μm . These results were obtained using the same objective, filters and confocal pin-hole as that used for the other experiments. The observed fluorescent area (A_o) was used to estimate the true cross-sectional area (A) of the fluorescence by assuming that the true area labelled is circular and that the fluorescent signal then spreads a distance ρ from the surface. With these assumptions,

$$A = A_o - \left(2\rho \sqrt{\frac{A_o}{\pi}} - \rho^2 \right)$$

The correction was carried out on all measured areas, and amounts to a correction of 15–30%. All quoted P values were calculated using a Student's t -test.

Results

Labelling of varicosities with antibodies to SV2 and syntaxin

The spatial extent of contiguous varicosities along individual sympathetic terminal axons was determined by filling them with orthogradely transported rhodamine conjugated to dextran. The assumption here is that the transported dextran fills the whole terminal axon, so delineating the shape of individual varicosities, as shown in Fig. 1A. After fixation, the vas deferens was labelled with the SV2Ab (Fig. 1B), in order to determine the volume in which vesicles are distributed when compared with the extent of the varicosities, as indicated by rhodamine-dextran labelling. The distribution of the area of rhodamine-dextran and SV2Ab labelling of individual varicosities according to this technique, and using the criteria for defining the area given in the methods with a 50% threshold, is given in Fig. 2. The mean area of the rhodamine-dextran labelling was 0.52 (SEM 0.03) μm^2 ($n = 71$), while that of the SV2Ab labelling was significantly smaller ($P = 0.001$) with a mean area of 0.40 (SEM 0.02) μm^2 ($n = 71$).

In other experiments, some sections were labelled with SV2Ab, while other sections from the same vas deferens were labelled with the antibody to syntaxin. Strings of punctate labelling were observed with both

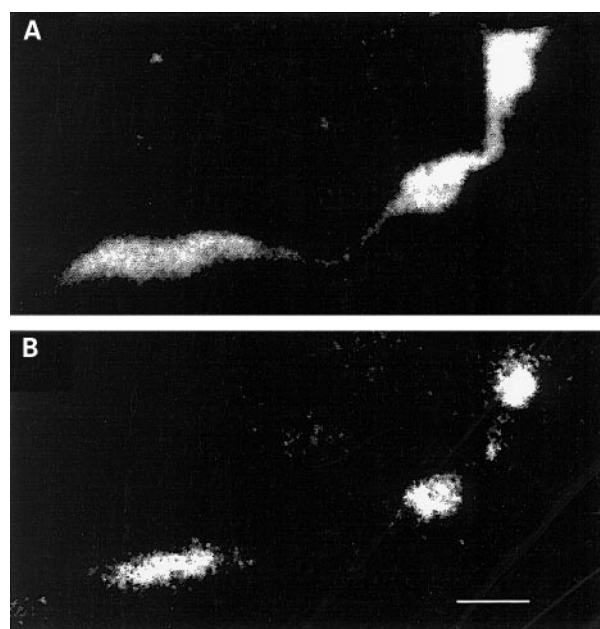


Fig. 1. Comparison of the size of the varicosity with the size of the area of labelled vesicles. (A) shows a varicose terminal axon filled with orthogradely transported rhodamine-dextran. (B) shows the extent of SV2Ab labelling (FITC-conjugated secondary antibody) which indicates the vesicle distribution in the same terminal axon as that in A. Calibration bar is 2 μm .

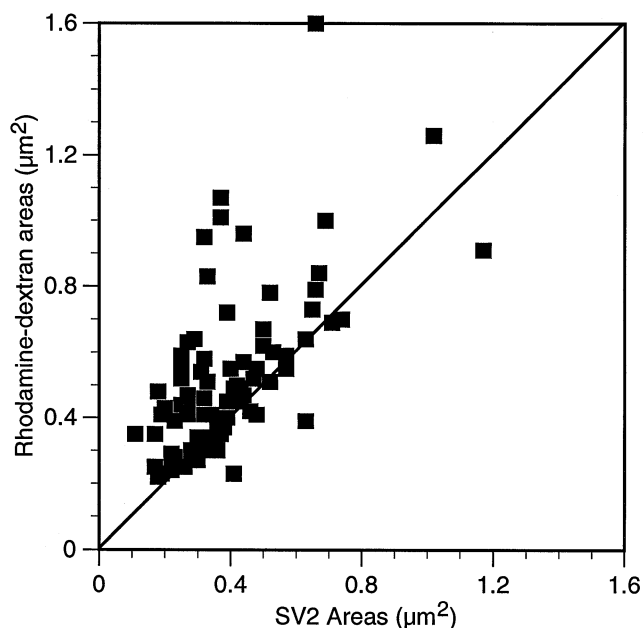


Fig. 2. A comparison of the areas labelled by rhodamine-dextran and SV2Ab in the same varicosities over all experiments. The diagonal line indicates that in most cases the area labelled by rhodamine-dextran is greater than that labelled by the SV2Ab.

labels (Fig. 3A and B). Their average separation was 6.1 (SEM 0.5) μm ($n = 44$ inter-varicose lengths from eight sections; the median was 5.4 μm , implying a positively skewed distribution of distances) in the SV2Ab labelled sections, suggesting that the labelling is situated at or in sympathetic varicosities. Their average measured cross-sectional area was 0.61 (0.04) μm^2 ($n = 63$; Fig. 4A). In the syntaxin labelled sections, punctate regions of fluorescence were also observed. The average fluorescent areas in the syntaxin labelled specimens was 0.22 (0.02) μm^2 ($n = 62$; Fig. 4B), namely about one-third of that occupied by the SV2Ab (compare Fig. 3B with Fig. 3A).

Labelling of different varicosities with antibodies to an N-terminal sequence of synaptotagmin 1 following stimulation

The varicosities were exposed to the SNAb in a high K^+ solution, and the position of varicosities was determined by identifying SV2Ab labelling in the fixed tissue (Fig. 5A and B), as described in the Methods section. Contiguous varicosities of single terminal axons were identified in this way in the smooth muscle layers towards the surface of the vas deferens. This enabled observations to be made of the extent of labelling with SNAb of individual varicosities in a string following stimulation with an elevated potassium concentration. Comparison between Figs 5A and 5B shows that there was considerable non-uniformity in the average intensity of the labelling of different

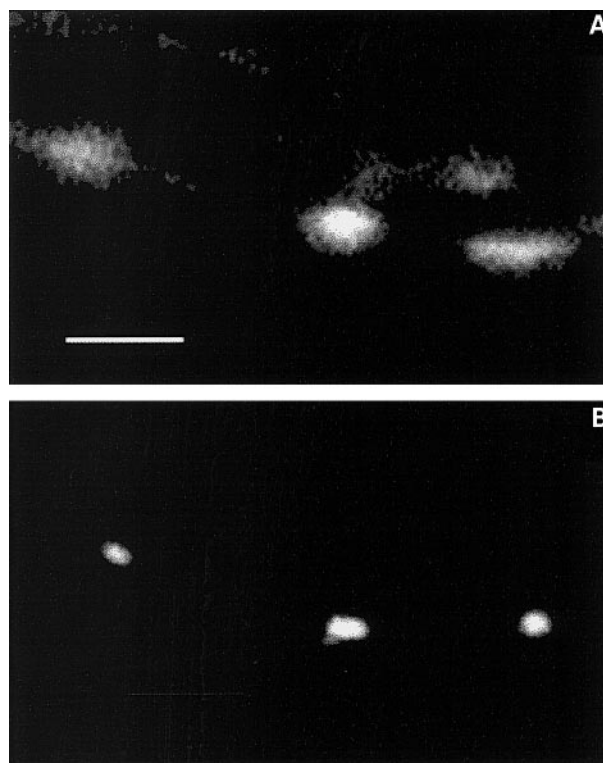


Fig. 3. Comparison of the size of the area of labelled vesicles with the area of syntaxin labelling. (A) shows the extent of SV2Ab labelling in a terminal axon. (B) shows the distribution of the syntaxin antibody labelling in the same terminal axon. Calibration bar is 2 μm .

varicosities with SNAb but not with SV2Ab (quantified in Fig. 5C and D). The results from another terminal axon are shown in Fig. 6, where some of the varicosities show almost no SNAb labelling.

The relative average fluorescence intensity of SV2Ab was measured for the different varicosities of the same terminal axon and then the relative average fluorescence intensity of SNAb measured for the same varicosities. The results of Fig. 5C and D show that the average intensity of SV2Ab fluorescence varies by less than 40% between varicosities, but that of SNAb varies by up to an order of magnitude. If allowance is made for the difference in area of SNAb fluorescence for each varicosity, by multiplying the intensity of fluorescence by the area and then normalizing the result to that of the varicosity with the largest value, then Fig. 5D shows that there is more than a tenfold difference in the SNAb labelling between varicosities. Results similar to these were obtained for 13 varicose terminal axons. Even though there was extensive variation in the intensity of SNAb fluorescence between varicosities, the area of SNAb fluorescence, in any varicosity where there was a significant SNAb signal, was similar to the area of SV2Ab fluorescence over a wide range of varicosity sizes (Fig. 7; $P = 0.16$; $n = 30$

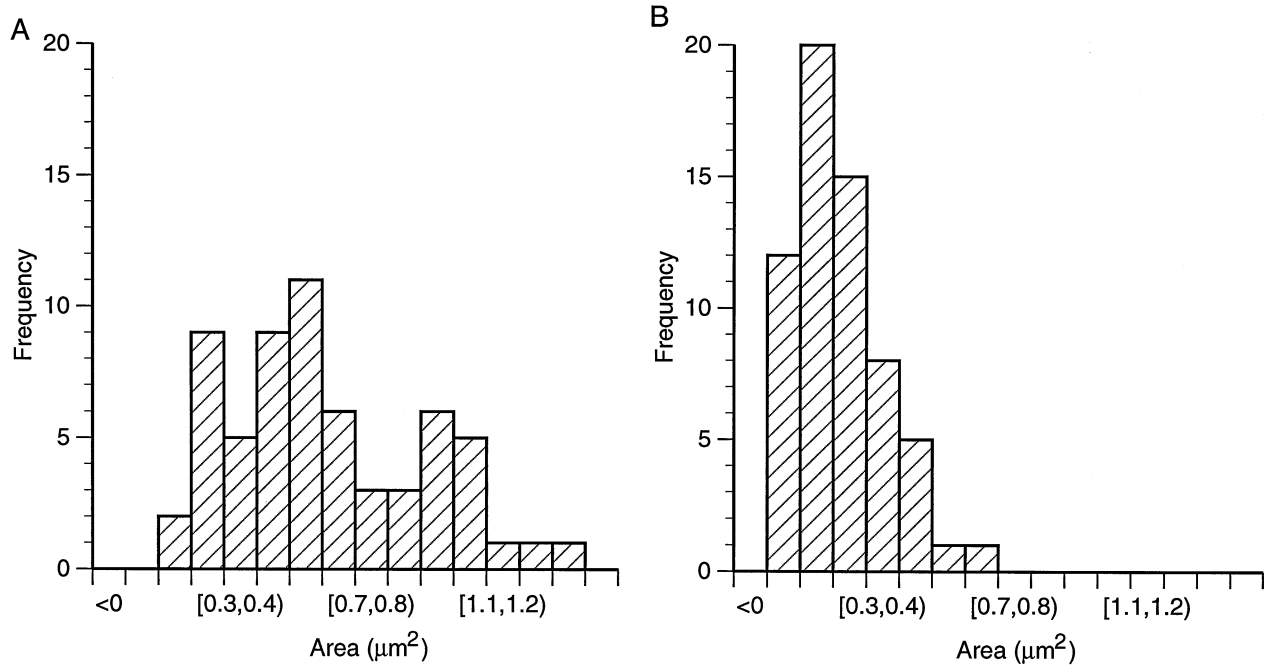


Fig. 4. (A) shows the frequency distribution of the area of individual varicosities labelled with SV2Ab, while (B) shows the frequency distribution of the area for the syntaxin antibody labelling. The area of the syntaxin labelling is characteristically smaller than the area labelled by the SV2Ab.

varicosities). This result was confirmed in an additional set of six varicose terminal axons ($P = 0.54$; $n = 19$ varicosities), the images of which were smoothed (with a Gaussian filter) prior to processing in order to reduce the effect of noise on the measurement protocol.

Discussion

Varicosities were identified in the present work with an antibody (SV2Ab) against a keratin sulphate proteoglycan in the synaptic vesicle membrane (Scranton *et al.*, 1993). This proteoglycan appears to be ubiquitous, occurring in all small vesicles in nerve terminals and in nerve terminals using different transmitters (Bajjalieh *et al.*, 1994). It cannot then be used to distinguish the pool of recycling vesicles at the presynaptic membrane from the large store of vesicles further back in the nerve terminal. The area of the accumulations of SV2Ab labelled pools of vesicles was 0.40 (SEM 0.02) μm^2 which was only slightly less than that measured with rhodamine-dextran of 0.52 (0.03) μm^2 and is similar to the maximum cross-sectional area of varicosities measured from serial section reconstruction at the ultra-structural level of 0.4 (0.1) μm^2 (Cottee *et al.*, 1996; calculation of this area assumes a section parallel to the axis of the terminal axon, yielding an approximately elliptical cross-section). These observations indicate that the store of synaptic vesicles occupies a volume similar to that of the varicosity. This

agrees with the ultra-structural findings which show that although there is a tendency for the highest density of vesicles to occur opposite the site of closest approach of the presynaptic membrane to the underlying smooth muscle, vesicles are found throughout the varicosities (Luff *et al.*, 1995; Cottee *et al.*, 1996). Furthermore, the observation that the varicosities occur in sets of two or more, the measured separation between the varicosities of 6.1 (0.5) μm and the measured cross-section of the varicosities, are all characteristic of sympathetic varicosities (Cottee *et al.*, 1996). It should be noted that the density of sensory varicosities in the monkey vas deferens is less than 0.1% (Leong & Singh, 1990). If this observation is true in the mouse vas deferens, then we are very unlikely to be selecting sensory varicosities in the present experiments.

The plasma membrane protein syntaxin is exclusively found at active zones in motor-nerve terminals (Boudier *et al.*, 1996) and in discrete regions within hippocampal boutons (Bennett *et al.*, 1992), although there is doubt over whether syntaxin is localized to specialised release sites in the rat striatum and frontal cortex (Sesack & Snyder, 1995). The C-terminus of synaptotagmin binds to the cytoplasmic domain of syntaxin, which is in turn bound to the N-type calcium channels in the presynaptic membranes where these occur (for review, see Südhof, 1995). In the present work antibodies to syntaxin were found to be localized in relatively discrete zones on the varicosities, compared with that of the SV2Ab labelling, so that the area

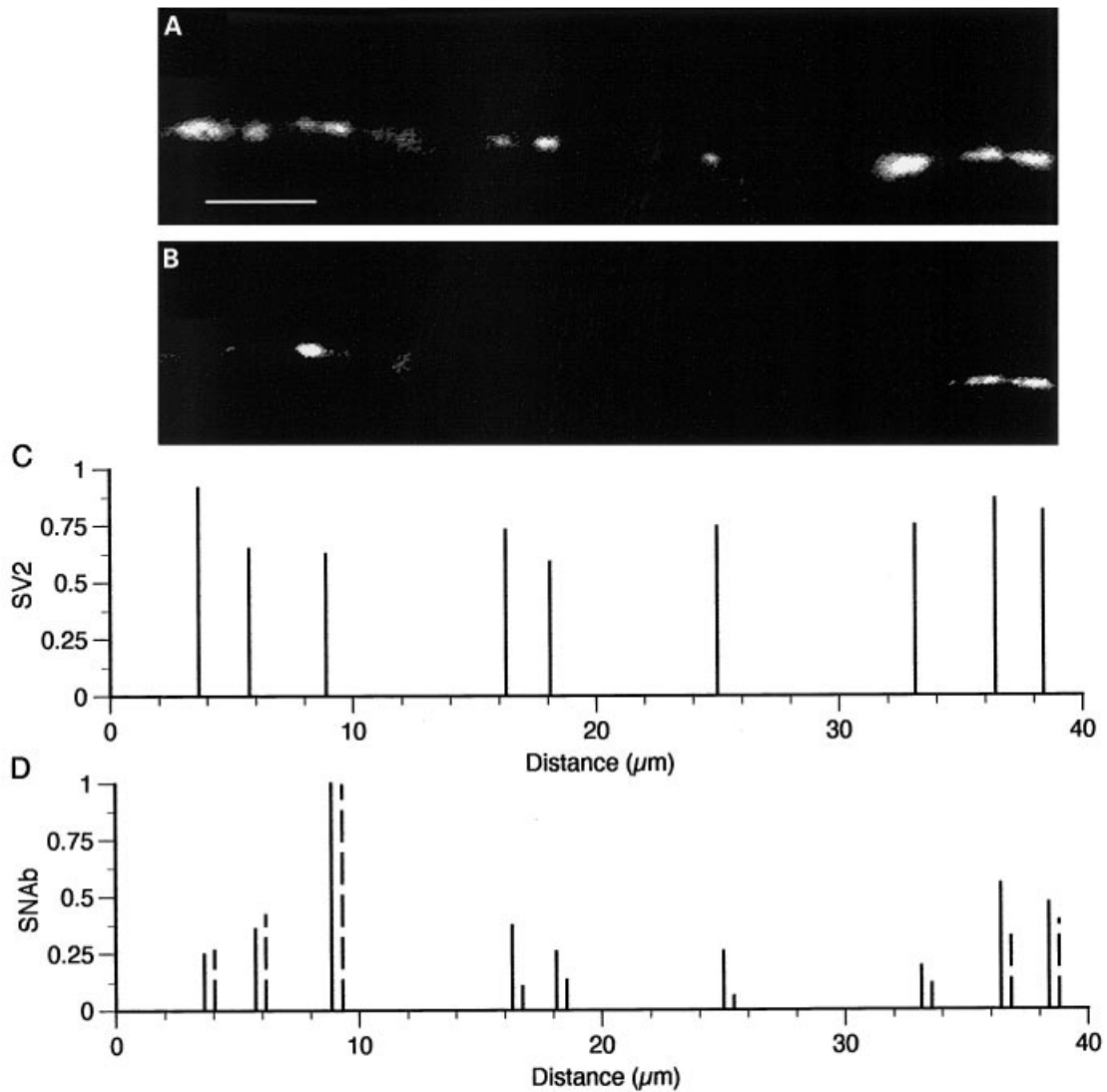


Fig. 5. Expression of SV2Ab and SNAb in varicosities of a sympathetic terminal axon during stimulation. (A) a varicose sympathetic terminal axon identified with the SV2Ab (FITC-conjugated secondary antibody). (B) the same terminal axon labelled with the SNAb in the presence of 80 mM potassium for 30 min before subsequent fixation (rhodamine-conjugated secondary antibody). (C) quantification of the extent of SV2Ab labelling along the length of the set of varicosities shown in (A) and (B); the ordinate is the relative average intensity of fluorescence. (D) quantification of the extent of rhodamine fluorescence (SNAb) along the length of the set of varicosities shown in (A) and (B); the ordinate is relative fluorescence for the solid vertical lines, and relative average intensity of fluorescence multiplied by the area of the fluorescence in the varicosity for the broken vertical lines. The distance scale in (A) and (B) is not quite the same as that in (C) and (D) because the distance in the former pair is measured between the midpoints of the varicosities, rather than recording the horizontal distance. Calibration bar in (A) is also for (B) and is 5 μm .

of the syntaxin labelling was on average approximately one-third of the area of the SV2Ab labelling. It could be argued that the smaller size of syntaxin labelling was due to the peripheral location of syntaxin with a uniform surface density. If this were the case, then the density of syntaxin labelling would be expected to form a peripheral pattern of labelling around the varicosities. This was not observed in any of the varicosities labelled with the syntaxin primary antibody.

The discovery of discrete zones of syntaxin labelling on sympathetic varicosities may allow for the first time an objective evaluation of what constitutes an automatic neuromuscular junction (Bennett, 1996b) by analogy with the definition of the active zone at motor nerve terminals as being delineated by the area containing syntaxin (Boudier *et al.*, 1996). The syntaxin labelling covers a zone with an area of 0.22 (0.02) μm^2 . It should be noted that the comparison of this area (a surface area projected onto the focal plane) with the

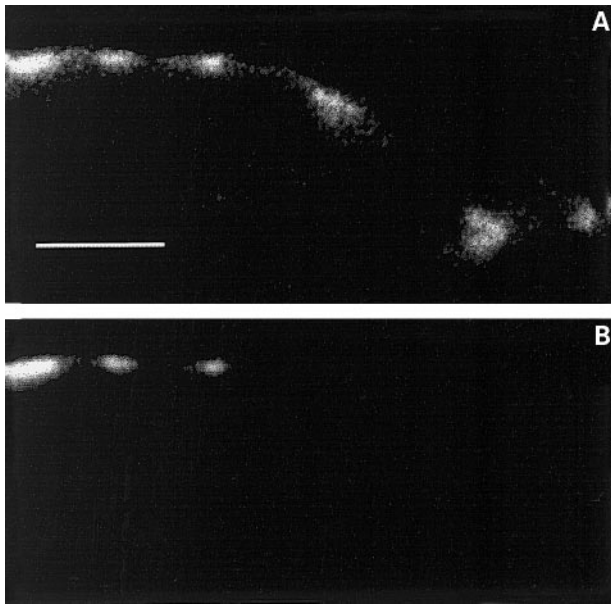


Fig. 6. Expression of SV2Ab and SNAb in varicosities of a sympathetic terminal axon during stimulation. (A) a varicose sympathetic terminal axon identified with the SV2Ab (FITC-conjugated secondary antibody). (B) the same terminal axon stimulated with 80 mM K^+ for 30 min in the presence of SNAb (rhodamine-conjugated secondary antibody). In this terminal axon some of the varicosities (on the right hand side of the image) show almost no detectable SNAb labelling. Calibration bar in (A) is also for (B) and is 5 μm .

SV2 labelled area (a volume projected onto the focal plane) must be interpreted with care. An important conclusion which can be drawn, however, is that the area of syntaxin labelling is much smaller than that which would be expected if syntaxin were distributed over the whole varicosity. Indeed, the area of syntaxin labelling is similar to the total varicosity area within 100 nm of a smooth muscle cell in the mouse vas deferens (Cottee *et al.*, 1996). Hence, all of the syntaxin labelling could be explained by its location at sites within 100 nm of the smooth muscle cell which it immediately apposes. These observations support the hypothesis that there are regions of the varicosity plasma membrane that are specialized for triggering regulated exocytosis and that this region of junctional contact within 100 nm of the post-junctional membrane defines the sympathetic neuromuscular junction in this muscle. It will be of great interest to see if such syntaxin zones are of the same size for different autonomic neuromuscular junctions.

SNAb was developed against a peptide having the N-terminal sequence of synaptotagmin 1 which is the isoform of synaptotagmin in sympathetic neurons (Li *et al.*, 1994). Antibodies of this kind have been used previously to label synaptic vesicles undergoing exocytosis in hippocampal cultures as neurons from this part of the brain also use the synaptotagmin 1

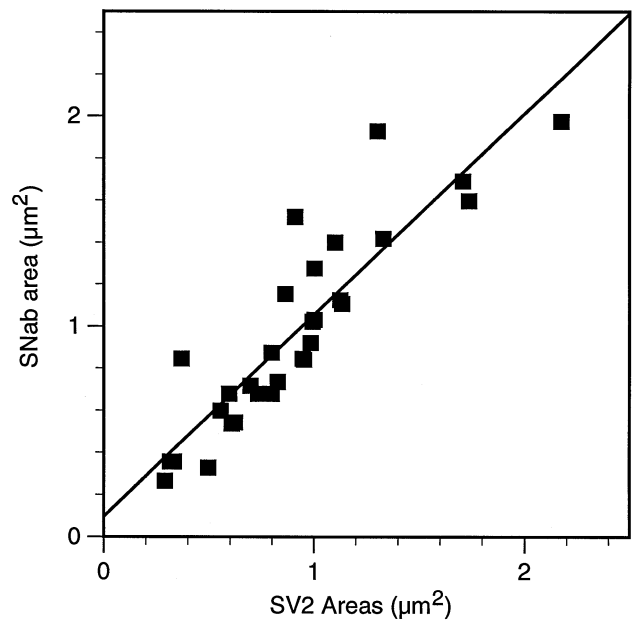


Fig. 7. Correlation between the size of the regions labelled with SNAb and SV2Ab. The pooled results for 30 varicosities from which measurements of both SNAb labelling after potassium stimulation and SV2Ab labelling were determined are shown. Each filled square is for a different varicosity. The line gives the best fit to the data according to a least squares regression analysis for which the correlation coefficient was 0.89.

isoforms (Ullrich *et al.*, 1994). A potential limitation of this technique is that it assumes a uniform access of the primary antibody to the varicosities. Whilst the SV2Ab labelling acts as an internal control for differences in the access of the secondary antibodies, the protocol used for exposing the tissue to the SNAb primary antibody does not have an internal control. The observation that only 3% of the surface area of the varicosity is in close apposition to smooth muscle cells (i.e. within 50 nm; Cottee *et al.*, 1996) suggests that close synaptic apposition presents a relatively minor obstacle for the penetration of the primary antibody. If the concentration of the primary antibody is the rate limiting factor in vesicle labelling, then the time that the vesicles are exposed to the synaptic cleft (i.e. the time between exocytosis and endocytosis), will affect the labelling. The prolonged exposure of the vas deferens to the high K^+ concentration SNAb solution (30 min) reduces the chance of a non-uniform distribution of extracellular SNAb during the stimulus protocol and increases the total number of vesicles exocytosed, which increases the fluorescent signal. In measuring the labelling at individual varicosities, we have also assumed that there is no exchange of vesicles between varicosities over the course of the stimulation protocol. The lateral movement of vesicles within the frog neuromuscular junction has been shown to be

insignificant in the resting state and during intense nerve activity (Henkel *et al.*, 1996), although the rate of vesicle movement can be enhanced with the phosphatase inhibitor okadaic acid. If these results also hold for sympathetic neuromuscular junctions, then the movement of vesicles between varicosities is unlikely to be a significant factor in the current experiments.

With the above qualifications, the average intensity of fluorescent labelling of a sympathetic varicosity with SNAb depends on both the number of vesicles that undergo an exocytotic event during the stimulation period as well as the number of transit times of the vesicle through the recycling process, which at somatic neuromuscular junctions appears to take about 2 min (Betz & Bewick, 1993). Present estimates suggest an abundance of synaptotagmin amounting to 7–8% of the total vesicle protein (Chapman & Jahn, 1994), indicating that any vesicle that undergoes exocytosis in the presence of SNAb will be labelled. It is possible then that some vesicles recycle more than once during potassium stimulation over several minutes, so that the SNAb labelling will give an underestimate of the extent of exocytosis from a varicosity under these circumstances.

The SNAb labelling occupied the same area of the varicosities as did SV2Ab, and this was slightly less than that of the rhodamine-dextran labelling. This result suggests that after a vesicle undergoes an exocytotic–endocytotic cycle(s), and is labelled by SNAb, it eventually ends up in the store of vesicles that occurs throughout the large extent of the varicosity volume and is labelled by SV2Ab. Similar results have recently been obtained for synaptic vesicles at the active zones of motor-nerve terminals, in which vesicles labelled with the styryl dye FM1-43 as they undergo an exocytotic–endocytotic cycle end up in the large store of vesicles to be found in the terminal behind the active zones (Betz & Wu, 1995; Bennett, 1996a). As the present technique specifically labels synaptotagmin-containing vesicles, it is less likely than the FM1-43 technique to be affected by constitutive endocytosis.

Electrophysiological analysis of the extent of secretion from varicosities, visualized with mitochondrial dyes, has shown that even adjacent varicosities have different probabilities for secretion (Lavidis & Bennett, 1993). However it has been difficult to reconstruct varicosities at the ultra-structural level after they have been visualized for the electrophysiological analysis, as the varicosities are only a few micrometres in length. It has not been possible then to verify that the loose-patch electrode is over only a single sympathetic varicosity, although this has been achieved for the giant boutons at crayfish neuromuscular junction (Cooper *et al.*, 1995), which are of about 10 μm extent and contain 10 to 30 release sites. The present results using SNAb to determine the relative probabilities for different varicosities avoids this problem, as the

varicosities are also identified with SV2Ab. Using the extent of SNAb fluorescence as a measure of exocytosis indicates a similar heterogeneity in secretion between different varicosities as is shown with the loose-patch electrode technique. These differences were still apparent when allowance was made for the different size pools of vesicles in different varicosities. In order to obtain a measurement that is proportional to the number of labelled vesicles, rather than their density, the intensity of fluorescence was multiplied by the cross-sectional area over which the vesicles were distributed. Using this measurement technique there was still an order of magnitude difference in the number of SNAb labelled vesicles per varicosity. The differences in labelling cannot, therefore, be explained by equal amounts of exocytosis occurring in varicosities of different sizes.

There is experimental evidence that the opening of a single N-type calcium channel is sufficient to trigger the exocytosis of a synaptic vesicle, as required by the secretosome hypothesis (Yoshikami *et al.*, 1989; Stanley, 1993). However the extent to which all the N-type calcium channels in a nerve terminal form part of a secretosome is not clear at this time. A recent description of the stochastic events that occur between the opening of an N-type calcium channel and exocytosis triggered by a conformational change in synaptotagmin has been given both for the case of all the calcium channels contained in secretosomes as well as for the case in which there is a random distribution both of calcium channels and of synaptic vesicles with their associated proteins (Bennett *et al.*, 1995). The results show that the numbers of vesicles secreted depends upon the spatial distributions of channels and vesicles. Secretosomes may be distributed in a regular array at release sites and incorporate all classes of calcium channels that mediate secretion there, as probably occurs at the active zones of somatic neuromuscular junctions (Heuser & Reese, 1973; Robitaille *et al.*, 1990) and possibly in boutons (Akert, 1973; Scholz & Miller, 1995). In this case the probability for secretion at different release sites within a single nerve terminal will differ according to their area; that is, the number of secretosomes they possess (Bennett, 1996b). There is evidence that this is the case, as larger active zones within a somatic neuromuscular junction possess larger probabilities for the secretion of a quantum (Bennett *et al.*, 1989). Furthermore, motor-nerve terminals with larger cumulative lengths of active zone have a higher quantal release from the whole terminal than do terminals with smaller cumulative lengths of active zones (Herrera *et al.*, 1985). These observations suggest that the probability for secretion at a release site should be directly related to the calcium influx at the release site following an action potential, even given the stochastic nature of channel opening under these circumstances.

The spatial and temporal resolution provided by contemporary imaging techniques does not yet allow the Ca^{2+} concentration changes close to a release site to be recorded during the influx of Ca^{2+} ions (Llinás *et al.*, 1995). Nevertheless, the calcium concentration observed some 100 ms or so after a stimulus in a nerve terminal at a release site ($[\text{Ca}^{2+}]$), at a time when the calcium is at uniform concentration throughout the terminal, is proportional to the calcium entry (see for example, Fig. 3 in Brain & Bennett, 1995). It is interesting in this regard that the change in $[\text{Ca}^{2+}]$ in different sympathetic nerve varicosities following stimulation differs from one varicosity to the next along a single

terminal axon in the mouse vas deferens (Brain & Bennett, 1997). The model of transmission from varicosities that emerges is that the size of the syntaxin site in a varicosity reflects the number of secretosomes that are docked and available for release. This pool of available vesicles is fed from a large store of vesicles which eventually receives recycled vesicles after exocytosis. The probability of secretion of a quantum from a varicosity is then determined by the size of the syntaxin site. Whether any or all of these conjectures are correct awaits confirmation from electron microscope experiments.

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