Contact-dependent aggregation of functional Ca\textsuperscript{2+} channels, synaptic vesicles and postsynaptic receptors in active zones of a neuromuscular junction

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Abstract

To examine whether Ca\textsuperscript{2+} channels aggregate in a contact-dependent manner, we characterized the distribution of synaptic vesicles and postsynaptic receptors, and compared it to the location of Ca\textsuperscript{2+} entry sites, in a \textit{Xenopus laevis} nerve-muscle coculture preparation using a localized Ca\textsuperscript{2+} detection method. The majority (75%) of Ca\textsuperscript{2+} entry sites at spontaneously formed nerve–muscle contacts were associated with enhanced immunofluorescence to the synaptic vesicle protein, SV2. In contrast, only 11% of recorded sites without Ca\textsuperscript{2+} transients exhibited significant SV2 immunofluorescence. When comparing the spatial distribution of synaptic markers with that of Ca\textsuperscript{2+} entry sites, we found that the majority of Ca\textsuperscript{2+} entry sites (61%) were associated with both enhanced SV2 immunofluorescence and R-BTX fluorescence, thereby identifying putative neurotransmitter release sites where Ca\textsuperscript{2+} channels, synaptic vesicles and postsynaptic receptors are colocalized. Using polystyrene beads coated with a heparin binding protein known to mediate \textit{in vitro} postsynaptic receptor clustering, we show that the location of Ca\textsuperscript{2+} domains associated with enhanced SV2 immunofluorescence at neurite-to-bead contacts. We conclude that the localization of functional Ca\textsuperscript{2+} channels to putative active zones follows a contact-dependent signalling mechanism similar to that known to mediate vesicle aggregation and AChR clustering.

Introduction

Action potential (AP) invasion of a nerve terminal mediates fast synaptic transmission by enabling the entry of Ca\textsuperscript{2+} ions into the nerve terminal through voltage-gated Ca\textsuperscript{2+} channels (Katz & Miledi, 1965; Augustine et al., 1987). It has been proposed that the rapid time course of neurotransmitter release is related to the diffusional dissipation of [Ca\textsuperscript{2+}] gradients and the topological distribution of synaptic vesicles at the nerve terminal (Barrett & Stevens, 1972; Zucker & Stockbridge, 1983). However, the quantitative assessment of the temporal and spatial properties of [Ca\textsuperscript{2+}] gradients generated at Ca\textsuperscript{2+} entry sites, and the distribution of the latter with respect to sites of neurotransmitter release in the presynaptic terminal, remain unclear. We have recently demonstrated that the opening of channels clustered at a Ca\textsuperscript{2+} entry site produce a local [Ca\textsuperscript{2+}] change (Ca\textsuperscript{2+} domain) that, within a millisecond of AP invasion, is constrained to within <200 nm of its site of entry (DiGregorio et al., 1999b). The purpose of this article is to establish if there is a tight spatial coupling between these Ca\textsuperscript{2+}-domains, synaptic vesicles and postsynaptic receptors that may support rapid synaptic transmission in a 'point-to-point' manner (Hille, 1992; Burns & Augustine, 1995; Neher, 1998).

Electron microscopy has been an important tool for the characterization of the ultrastructural features of synapses. The classic images of the vertebrate neuromuscular junction (NMJ) illustrate that presynaptic vesicles tend to cluster opposite to postsynaptic structures thought to contain neurotransmitter receptors at specialized regions termed active zones (AZs, Birks et al. 1960; Couteaux & Pecot-Dechavassine, 1970). However, the evidence for the localization of Ca\textsuperscript{2+} channels within AZs has been restricted to the nonspecific observation of small membrane particles near release sites (Heuser & Reese, 1981; Pumplin et al., 1981; Cooper, 1996). Light microscopy studies of fluorescently-labelled α-conotoxin (Robitaille et al., 1990; Cohen et al., 1991) and antibodies (Boudier et al., 1996) and atomic force microscopy (Haydon et al., 1994) have suggested the presence of Ca\textsuperscript{2+} channel proteins at sites of neurotransmitter release. However, the direct colocalization of functional Ca\textsuperscript{2+} channels, responsible for the Ca\textsuperscript{2+} entry that triggers neurotransmitter release, and synaptic vesicle clusters at the presynaptic terminal has not yet been demonstrated. This is an important measurement as it should demonstrate whether, or not, synaptic vesicles are located within the range of action of AP-elicted Ca\textsuperscript{2+} domains. Moreover, recent evidence (Ahmari et al. 2000) suggests that Ca\textsuperscript{2+} channels and associated synaptic vesicle proteins may be transported to the terminal in aggregates. In addition, it is also thought that the functionality of Ca\textsuperscript{2+} channels can be augmented by interaction with SNARE proteins (soluble N-ethylmaleimide sensitive fusion attachment protein receptors; Umbach et al., 1998; Seagar et al., 1999; Wu et al., 1999). Thus, immunocytochemical techniques alone, which are unable to distinguish between functional and nonfunctional Ca\textsuperscript{2+} channels, may yield equivocal information about the relevance of the colocalization of synaptic proteins and channels.
The Xenopus cultured NMJ has been used extensively to study both the functional and ultrastructural changes underlying synaptogenesis. In this preparation, neurite–muscle contacts (Weldon & Cohen, 1979; Cohen et al., 1987; Buchanan et al., 1989) and latex beads coated with specific extracellular matrix proteins (Dai & Peng, 1995; Peng et al., 1995) have been shown to induce synaptic vesicle and postsynaptic acetycholine receptor (AChR) aggregation similar to that observed at the mature NMJ. In this regard, the heparin-binding associated molecule (HB-GAM) seems to be a key determinant of vesicle aggregation as it is enriched in the extracellular matrix of Xenopus myotomal muscle cells and its local presentation via latex beads induces synaptic vesicle (SV) clustering at bead–neurite contacts (Peng et al., 1995; Daggett et al., 1996). Moreover, this contact-dependent synaptic vesicle aggregation is dependent on local increases in the resting [Ca2+] (Dai & Peng, 1998). As we have recently shown that presynaptic Ca2+ channels aggregate in clusters (DiGregorio et al., 1999a, b) similar in size to clusters of AZs at nerve–muscle contacts (Weldon & Cohen, 1979; Cohen et al., 1987; Buchanan et al., 1989), it was necessary to examine if these channels are spatially associated with synaptic vesicles, and can be induced to aggregate in a contact-dependent manner by similar mechanisms as synaptic vesicles and postsynaptic receptor aggregation.

Materials and methods

Cell culture and electrophysiology

Xenopus nerve–muscle cocultures were prepared from dissociated neural tube tissue obtained from embryos at developmental stages 20–22 (Yazejian et al., 1997). The dissociated cells were plated onto glass cover slips and allowed to grow for 18–36 h (at 22±24 °C) prior to experimental use. For some experiments, beads coated with heparin-binding growth-associated molecule (HB-GAM) were added to the cultures after ~6 h of incubation and experiments were performed after another 12–24 h. HB-GAM coated beads were prepared as described previously (Peng et al., 1995). Neuronal cell bodies were patch-clamped in order to elicit some APs and to dialyse the presynaptic terminal with a pipette solution of the following composition (in mM): K-Aspartate, 85; KCl, 20; MOPS, 40; MgCl₂, 0.5; EGTA, 0.05; ATP-Mg, 2; GTP-Na₂, 0.5; OGB-5 N, 50 μM (Molecular Probes, Eugene, OR, USA), pH 7.0 (5–10 MΩ resistance). Voltage and fluorescence recordings were performed in a similar manner to that described previously (DiGregorio et al., 1999b). Neuronal APs were recorded using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA), filtered at 5 kHz and acquired at 17–55 kHz with a Digidata 1200 A data acquisition system operating under software control (pClamp 7.0, Axon Instruments). All experiments were performed at room temperature (19–21 °C). Normal frog Ringer (NFR), consisting of (in mM): NaCl, 114; KCl, 2.5; MOPS, 10; CaCl₂, 1.8 and d-glucose, 10; pH 7.0, was used to bathe the cultures during recording periods. To eliminate movement artifacts, 20 μM T-tubocurarine (Sigma, St Louis, MO) was added to the NFR to block synthetically evoked myocyte contraction. Extracellular stimulation was performed using borosilicate glass patch pipettes back-filled with NFR (5–10 MΩ resistance) to apply voltage pulses (0.8 ms) elicited by an electronic stimulator (Model SEN 7103, Nihon Kohden, Tokyo, Japan) in series with a stimulus isolation unit (Model 305B, WPI, New Haven, CT).

Ca²⁺-detection

AP-induced fluorescence transients were recorded from nerve terminals using the confocal spot detection method as described previously (DiGregorio et al., 1999b). The diode current was amplified using the capacitor-feedback mode of an Axopatch 200B amplifier (Axon Instruments), low-pass filtered at 1–1.5 kHz and digitized at 18–20 kHz using the Digidata 1200 A. The microscopy stage was equipped with a high-resolution (100 nm) stepper motor (UTS20PP.1, Newport, Irvine, CA) to allow high precision confocal spot displacement within nerve terminals. Fluorescence and phase contrast images were used to document recording sites along the nerve terminal, and were acquired with a cooled CCD camera (Spectra Source, Agora Hills, CA).

Fluorescence transient analysis

AP-induced OGB-5N fluorescence transients were analysed as described previously (DiGregorio et al., 1999b). Peak ΔF/ΔF-values were determined by averaging 3–10 consecutive transients recorded at a single presynaptic site, or by averaging 3–6 transients recorded from sites in the immediate neighbourhood to where the largest transient in a particular scan was obtained. Recording sites were denoted as positive for Ca²⁺ transients if the transients exhibited a rapid time-to-peak (≤ 1.5 ms) and a rapid decay phase (t < 10 ms). Model simulations suggest that the presence of these two features is characteristic of the existence of a Ca²⁺-entry site within the spot detection volume (DiGregorio et al., 1999b). In contrast, recording sites were considered negative for the presence of Ca²⁺-entry sites if they exhibited a time-to-peak greater than 1.5 ms, or lacked the fast decay component, or had a peak ΔF/ΔF of <5%. In cases where no transient was observed, recording sites were only considered negative if the SD in the amplitude of the baseline noise was <5% ΔF/ΔF otherwise the data was rejected.

Immunocytochemistry

Nerve–muscle cultures were washed three times in phosphate buffer saline (PBS) pH 7.4 with an osmolality adjusted to 250 mOsmol/kg and then incubated for at least 15 min in the same solution prior to fixation. The cultures were then fixed for 30 min in 3% (w/v) paraformaldehyde in PBS and permeabilized for 30 min with 0.05% (v/v) Triton X-100 and 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) at either room temperature or 4 °C. In the cases where electrophysiology was performed first, the NFR was replaced at the end of the experiment with the 3% paraformaldehyde solution and subsequently permeabilized as described above. Primary antibodies were diluted 1 : 500 in the permeabilization solution and were incubated overnight at 4 °C or for 1 h at room temperature. The cultures were rinsed thoroughly (3 × 5 min) with PBS and incubated with secondary antibodies diluted 1 : 800 in situ imaging to minimize photobleaching. The monoclonal anti-mouse SV2 antibody was a gift from Dr Kathleen Buckley (Harvard Medical School) and the polyclonal anti-frog cysteine string protein (CSP) antibody was a gift from Dr Cameron Gundersen (University of California, Los Angeles). Secondary antibodies included: Alexa 488 conjugated goat anti-mouse IgG (Molecular Probes), Cy3 conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa 488 conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR).

Tetramethylrhodamine-conjugated α-bungarotoxin (R-BTX, Molecular Probes) was used to visualize clusters of postsynaptic AChRs. For in situ experiments, cells were incubated for 45 min with 0.01 ng/mL of R-BTX in NFR. For the staining of fixed cultures, the

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cells were incubated for 45 min with 0.01 ng/mL of R-BTX in PBS prior to permeabilization.

Only those cells in which the cell outline did not change significantly after fixation were used for analysis. We cannot rule out the possibility that fixation changed slightly the distribution of SV2 or CSP immunofluorescence. However, as a control, our fixation and permeabilization methods did not change significantly the distribution of alpha-bungarotoxin staining (data not shown).

**Fixed cell imaging**

Immunofluorescence images were acquired using a cooled CCD camera (Sensys, Photometrics) coupled to an inverted Olympus microscope. Transmitted light images were formed using a 40 × phase contrast objective or a 100 × (1.3 NA) Plan Fluor objective in conjunction with differential interference contrast optics. For fluorescence imaging, stained cells were illuminated with a 100-W mercury lamp. CCD camera exposure times varied from 0.2 to 3 s in order to maximize the dynamic range of fluorescence intensities. All fluorophores were visualized using a multiband beamsplitter (Model 51004v2BS, Chroma Technology, Brattleboro, VT) in combination with excitation/emission filters using computer controlled filter wheels (Sutter Instruments, Novato, CA). For Alexa 488 immunofluorescence a 480-nm bandpass excitation filter (HQ480/40, Chroma) and a 535-nm band pass filter (HQ535/50, Chroma) were used. For CY3 and rhodamine-based immunofluorescence, a 550-nm excitation filter (XF39, Omega Optical, Brattleboro, VT) and a 570-nm long pass emission filter (380254, Omega) were used. Using these optical filter combinations, we estimated the rhodamine fluorescence wavelength contamination observed in the green image to be < 10% (n = 6) for images taken with identical exposure times. The Alexa 488 fluorescence wavelength contamination in the red image was negligible (< 1%, n = 6). Metamorph imaging software (Universal Imaging Corp., West Chester, PA) was used to operate filter wheels and acquire images.

**Image analysis**

Sixteen-bit images were analysed with either Metamorph or ImageTool (University of Texas Health Science, San Antonio, TX) software packages. For figures, the 16-bit images were exported as 8-bit images and processed using Adobe Photoshop 5.0 (Adobe Systems Inc., San Jose, CA).

**Ca\(^{2+}\) transient colocalization with SV2**

Phase contrast, DIC and fluorescent images were used to define the location of the Ca\(^{2+}\) entry site in relation to immunocytochemical features at the contact region. The mean pixel intensity and its SD were calculated from line intensity profiles (5–10 μm in length) traversing Ca\(^{2+}\) transient recording sites. Relative values for anti-SV2 fluorescence were calculated by normalizing pixel intensities according to the number of SD above the mean pixel intensity. The portions of the line scan that did not traverse a recording site were made to bisect the nerve terminal along its long axis. The line profile was five pixels thick (0.067 μm/pixel). The maximum relative pixel intensity within < 1 μm of a Ca\(^{2+}\) transient recording site was assumed to represent the SV2 immunofluorescence for that location. This method allowed for subtle changes in morphology after fixation due to shrinkage or swelling, and was used for subsequent localization of all markers. Cells in which the morphology after fixation could not be mapped to the original phase contrast image were excluded from the data set.

**R-BTX staining**

R-BTX staining along nerve–muscle contact was also assessed using line profiles of five pixel width. Due to the image shifts between the fluorescence and DIC images, the line profile was initially drawn along the centre of the neurite path, as determined from the DIC image, then parallel shifted > ± 1 μm to align it with a region containing the greatest amount of fluorescence staining. The staining length was obtained from the length of the fluorescence regions with pixel values > 3 SD above the background pixel intensity of a particular scan. To determine the background pixel intensities, line profiles of pixel intensity were made along nerve–muscle contacts in which electrical stimulation did not produce a twitch. The intensity distributions of these pixels were fitted to Gaussian functions. In seven nonfunctional nerve–muscle contacts, the average ratio of the SD to the mean intensity was determined to be 0.29. This ratio was then used to constrain double Gaussian fits of pixel intensity distributions at functional synaptic contacts. The SD calculated from the Gaussian curve fitted to the lower intensities was assumed to identify pixel intensities arising from background staining and is hereafter referred to as the background SD.

**Ca\(^{2+}\) transient colocalization with R-BTX**

If the R-BTX intensity observed at a Ca\(^{2+}\) transient recording site was greater than three-fold above the mean background SD of the line profile bisecting the recording site, then the site was considered positive for R-BTX.

**SV2 colocalization with R-BTX**

Staining lengths for R-BTX were estimated as described above, except that the profile was centred along the nerve terminal as determined from the anti-SV2 fluorescence image. SV2 intensity profiles were initially drawn along the centre of the nerve and then shifted within ± 1 μm so as to transverse the maximum number of staining sites. SV2 staining lengths were determined from intensity values > 1.5 SD above the mean value of the intensity profile (as described above).

**Results**

**Spatial characterization of Ca\(^{2+}\) entry sites and synaptic vesicle distribution**

We have recently demonstrated that the size of presynaptic Ca\(^{2+}\) entry sites in Xenopus cultured NMJs can be estimated from the full-width at half-maximum (FWHM) of the spatial dependence of Ca\(^{2+}\) indicator fluorescence domains (DiGregorio *et al*., 1999b). To determine whether in this preparation AP-induced Ca\(^{2+}\) domains, and hence Ca\(^{2+}\) channels, are localized to putative sites of neurotransmitter release, we examined the clustering pattern of synaptic vesicles in the region where Ca\(^{2+}\) domains were detected. The synaptic vesicle distribution was assessed by immunofluorescence microscopy using an antibody against the synaptic vesicle protein SV2, which has been shown, by Western blot analysis, to be present in the Xenopus nerve–muscle cultures (Poage *et al*., 1999). Antibodies to this molecule have proven to yield a robust identification of presynaptic boutons (Lissin *et al*., 1999; Serpinskaya *et al*., 1999) and active zones (Macleod *et al*., 1999).

AP-induced Ca\(^{2+}\) transients were recorded using the confocal spot detection method from neurites of 1-day-old cultured Xenopus spinal neurons at locations where the neurites contacted myocytes. The illumination spot was focused within the neurites that had been
previously loaded with 50 μM of the low affinity (K_d ~ 30 μM) calcium indicator OGB-5 N (DiGregorio & Vergara, 1997) through a patch electrode (DiGregorio et al., 1999b). Figure 1(A1 and A2) show phase contrast images that illustrate the region of nerve–muscle contact scanned for the detection of Ca^{2+} transients. Images of this type were obtained in each experiment to document the site of Ca^{2+} transient detection and to compare with immunofluorescence images obtained after fixation (see Methods). The illumination spot was
displaced in 0.2 μm increments along the nerve terminal in the direction indicated by the arrow [Fig. 1(A2)]. At each spot location, fluorescence transients were recorded at the presynaptic terminal when APs were elicited at the cell body as shown in Fig. 1(A3, traces a–e). Trace (a) represents an average of five consecutively recorded transients from five spot locations separated by 200 nm in a region of the nerve terminal where the amplitudes of the individual Ca2+ transients were maximal, thereby representing a spatially averaged fluorescence transient over 1 μm. The remaining traces (b–e) also represent 1 μm spatially averaged transients from progressively more distant regions from the site where the largest transients were recorded. It can be observed that transients close to the site of maximal fluorescence change (traces a and b) exhibit rapid times to peak (< 1.5 ms) followed by a fast decay phase (τ = 5 ms), typical of Ca2+ transients when the site of recording encompasses a Ca2+ entry site (DiGregorio et al., 1999b). In contrast, transients more distal to the site of maximal entry (traces c–e) exhibit a slowing in their time-to-peak and a loss of the fast decay phase. The overall extent of the Ca2+ entry site can be more accurately estimated from the full width at half maximum (FWHM) of the isochronal ΔF/F profile (DiGregorio et al., 1999b). Hence, in Fig. 1(A4) we plot the isochronal magnitude of each fluorescence trace at the time point when the largest transient peaked (1.3 ms, dashed line). Note that for the first 13 μm of the scan, the data points represent average isochronal values from five neighbouring spot locations. From the FWHM of the fitted Gaussian function (dotted line), the entry site size was estimated to be 4.1 μm. Given the location and dimension of the entry site within the nerve terminal traces ‘c’, ‘d’, and ‘e’ correspond to spot locations where there were either a very low density of channels or none at all, and traces ‘a’ and ‘b’ represent regions within the nerve terminal where a Ca2+ entry site was located.

To determine the underlying vesicle distribution in the same nerve terminal where we measured AP-induced Ca2+ domains, we fixed the preparation following in situ recordings and then examined the SV2 protein distribution using fluorescence immunocytochemistry (Fig. 1B). The DIC image [Fig. 1(B1)] shows a similar orientation (after fixation) of the synapse in Fig. 1(A3). Panel B2 is a fluorescence image obtained after incubation with primary antibody against SV2 and a secondary antibody conjugated to the fluorescence molecule Alexa 488, thus illustrating the anti-SV2 staining pattern. The arrow indicates the approximate length and direction of the in situ Ca2+ transient scan. Note that the preparation is slightly skewed with respect to the phase contrast image [Fig. 1(A3)]. It can be observed in Fig. 1(B2) that towards the right end of the double-headed arrow, where the Ca2+ entry site was located, SV2 immunofluorescence is maximal. This is better illustrated with a line intensity profile [Fig. 1(B3)] made between the start and endpoints of the fluorescence scan. Here, the brightest regions (*, panel B4) appear to correspond in space with the Ca2+ entry site of the domain shown in Fig. 1(A4). However, there are other relatively bright regions of immunofluorescence ( panel B3) where smaller and slower Ca2+ transients were measured. These transients were similar in amplitude and time course to traces (d) and (e) illustrated in Fig. 1(A3). Thus, SV2 immunofluorescence is obviously enhanced near regions of the nerve terminal where Ca2+ entry sites are present, but regions with less SV2 immunofluorescence staining are associated with recording sites where slower and smaller transients were measured.

To verify that SV2 antibody distribution in this preparation is a good representation of the synaptic vesicle distribution, we compared the staining pattern of SV2 with that of another specific synaptic vesicle associated protein, cysteine string protein (Csp), known to be present in these cultures (Poage et al., 1999). Figure 2A is a DIC image of a fixed neurite–muscle contact that was stained with antibodies against both Csp and SV2. To visualize the Csp protein distribution, we used a secondary antibody against rabbit IgG conjugated to Alexa 488. In Fig. 2B, bright punctuate fluorescence staining is present within the nerve terminal both at muscle contacts (arrows) and at an off-muscle region (*). The SV2 staining was performed using a goat anti-mouse IgG conjugated to CY3, a fluorophore whose different excitation and emission properties allow for the colocalization of two molecules on the same preparation. Figure 2C illustrates a similar pattern of SV2 immunofluorescence that is fully realized with the green and red overlay (Fig. 2D) in which the pixels where red and green are present, are made yellow. This high degree of colocalization was verified in six additional cell pairs. Images of nerve terminals in which the cultures were only incubated in secondary antibody (no primary) exhibited fluorescence intensities similar to the background fluorescence in the myocyte. Attempts to use FM1-43 as an alternative synaptic vesicle marker failed because, as previously reported (Dai & Peng, 1996; Dai & Peng, 1998), this indicator gives a broad distribution of fluorescence at the neurite unlike the localized spotted pattern reported by anti-Csp and anti-SV2 immunofluorescence (data not shown).

To further explore the degree of colocalization between Ca2+ domains and sites of vesicle accumulation, we analysed the relative SV2 immunofluorescence at locations within the nerve terminal where rapid Ca2+ transients were detected. To quantify anti-SV2 fluorescence, the fluorescence intensities in the images were normalized in terms of the number of standard deviations (SD) above the mean fluorescence intensity of a line profile along the nerve and bisecting the site of Ca2+ transient recording (see Methods). The expression of SV2 immunofluorescence in this manner provides a

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Fig. 1. Ca2+-entry site is associated with enhanced anti-SV2 immunofluorescence. (A1) 20 × phase contrast image of a cultured Xenopus nerve (n) and myocyte (m) contact. (A2) 100 × image phase contrast image of the same nerve terminal (nt) corresponding to the dotted rectangle in A1. The white-filled circles denote the exact locations between which the illumination spot was displaced in 0.2 μm steps. The arrow indicates the length and direction of the spot displacement. (A3) Averaged AP-induced Ca2+ transients selected from different locations along the scan illustrated in (A2). Each trace is an average of five consecutively recorded transients obtained from five spot locations. (A4) Isochronal ΔF/F-values of all the transients recorded in the scan depicted in A3 plotted as a function of spot displacement. Each data point is a time average of each transient within ±220 μs around the time point where the largest transient peaked (fine dotted line in A3). These isochronal values, corresponding to the spot displacements between 0 and 12 μm, were determined after averaging five consecutively recorded transients spanning 1 μm. The remaining points are the isochronal amplitudes of single fluorescence transients, recorded in 0.2 μm increments. Horizontal bars (labelled ‘a’–’c’) and the circled data points (‘d’ and ‘e’) identify the isochronal values calculated from the averaged fluorescence transients, according to their letter designation, plotted in A3. The dotted line is a Gaussian fit to the isochronal ΔF/F profile; the double arrow indicates the FWHM of 4.1 μm of the fitted trace. (B1) A 100 × DIC image of the same nerve terminal as in A following fixation and immunocytochemistry with an antibody against the synaptic vesicle protein SV2. (B2) A fluorescence image of the same fixed terminal. The double-headed arrow denotes the direction and length of the fluorescence scan illustrated in panel A1 that spanned the region between the two end points of the dashed lines. (B3) A line intensity profile of the fluorescence image in B2 along the same line as that made for the Ca2+ transient scan shown in A. The values represent an average over the line width (5 pixels, 0.34 μm). *Bright SV2 immunofluorescence intensities in the vicinity of the measured OGB-5 N transients (see labels ‘a’ and ‘b’ in A3 and A4), bright SV2 immunofluorescence regions without a corresponding rapid fluorescence transient.
relative measure of SV2 staining (vesicle aggregation) at the site of Ca\textsuperscript{2+} transient recording with respect to other regions within the same nerve terminal. We tested this by comparing the results from spot detection sites where no Ca\textsuperscript{2+} transients were measured with those from recording locations where rapid Ca\textsuperscript{2+} transients were recorded. Figure 3A shows a histogram plot of the frequency distribution of relative intensities of SV2 immunofluorescence at recording sites that were negative for Ca\textsuperscript{2+} transients (see Methods for criteria). It can be observed that the distribution is nearly centred on a mean relative fluorescence of zero SD (0.33 ± 0.86, \(n = 51\)), implying that throughout this region of the presynaptic terminal the fluorescence is not significantly different from the mean intensity of line profiles. In contrast, Fig. 3B shows that SV2 immunofluorescence intensities at sites where rapid Ca\textsuperscript{2+} transients were recorded are significantly brighter. For 32 recording sites, the mean relative anti-SV2 fluorescence intensity was 2.4 ± 1.1 SD above the mean intensity of line profiles. The cumulative probability plot (Fig. 3C) further illustrates the enhanced anti-SV2 fluorescence intensity at positive Ca\textsuperscript{2+} transient recording sites. Using a Kolmogorov–Smirnov test, the two distributions were found to be significantly different with a \(P\)-value < 0.0001. It should be noted that the majority (48 of 54; 89\%) of the `Ca\textsuperscript{2+}-negative' recording sites have staining intensities < 1.5 SD above the mean intensity. However, 24 of the 32 recording sites (75\%) where the staining intensity showed SV2 fluorescence > 1.5 SD correspond to sites where rapid Ca\textsuperscript{2+} transients were measured. Thus, quantitative analysis reveals that enhanced SV2 immunofluorescence is significantly associated with the existence of Ca\textsuperscript{2+} entry sites.

**Identification of putative active zones: AChR and functional synaptic contacts**

Having established that Ca\textsuperscript{2+} channel clustering is correlated with the aggregation of synaptic vesicles, we investigated whether sites of vesicle aggregation were associated with postsynaptic AChR aggregation, thus putative AZs. To date, the functional identification of neurotransmitter release sites is extremely difficult (Auger & Marty, 2000). As an alternative, we used quantitative immunocytochemistry. Previous work has shown that postsynaptic receptor aggregation along neurite–muscle contacts is associated with the formation of functional synaptic contacts in 2–6-day-old cultures of the *Xenopus* preparation (Cohen & Weldon, 1980; Cohen et al., 1987). We utilized this correlation to develop an optical method of identifying functional synaptic contacts and hence AZs formation.

As our experiments were performed in 24–36 h old cultures, we re-examined whether functional nerve–muscle contacts could be identified by R-BTX staining along the contact region. At low magnification (40 ×), nerve–muscle pairs were identified on the basis of apparent neurite and muscle contact. Functional synaptic contacts were assessed by observing myocyte twitching in response to extracellular stimulation of the neuronal cell body (see Methods). Results from a nonfunctional cell pair are reported in Fig. 4(A1–2). Figure 4(A1) is a 100 × DIC image of a neurite running along a myocyte. Figure 4(A2) demonstrates very little R-BTX staining along

![Fig. 2. Colocalization of two synaptic vesicle-associated proteins, SV2 and CSP. (A) 100 × DIC image of a fixed preparation in which a nerve (n) was in contact with a myocyte (m). The preparation was incubated with primary antibodies, rabbit anti-CSP and mouse anti-SV2. The secondary antibodies used were Alexa 488-conjugated anti-rabbit IgG and Cy3-conjugate anti-mouse IgG. (B) Fluorescence image using the Alexa 488 filter set (see Methods) of the same nerve terminal. Grey intensity scale of original image was converted to a green intensity scale. (C) Fluorescence image of the same nerve terminal using a Cy3 filter set (see Methods). The image is presented in a red intensity scale. (D) Overlay of the images from B and C, where the yellow colour represents the location of overlap between the two images. The scale bar in panel A is 10 μm and applies to all images.](image-url)
the region of nerve–muscle contact. In contrast, a functional nerve–
muscle contact [Fig. 4(B1)] exhibited a robust enhancement in
staining along the region of nerve–muscle contact [Fig. 4(B2)]. To
quantitatively assess differences in R-BTX staining between func-
tional and nonfunctional contacts, we first analysed line-scans of the
R-BTX fluorescence observed in nonfunctional nerve–muscle con-
tacts in order to establish the background levels of staining (Fig. 4C).
Figure 4(C1) shows the pixel intensity profile of the line-scan along
the non twitching contact illustrated in Fig. 4(A3) and its corre-
spending pixel intensity distribution is shown in Fig. 4(C2). A single
Gaussian distribution of normally dispersed intensities around a mean
background staining can readily fit the data. In contrast, the pixel
intensity distribution [Fig. 4(D3)] obtained from the line-scan
[Fig. 4(D2)] along the twitching nerve–muscle contact indicates the
presence of a significant population of higher pixel intensities. These
intensities correspond to the enhanced R-BTX staining seen in
Fig. 4(B2). Thus, to calculate the contact-length with significant
AChR staining we normalized line-scan intensities according to the
SD of the background fluorescence intensity (see Methods). We set
the threshold for significant staining to be 3 SD above the mean
background intensity [dotted line in Fig. 4(C1) and Fig. 4(D1)]. We
found that this threshold sufficiently isolated the peaks in fluores-
cence from the background. For the two nerve–muscle contacts
presented in Fig. 4, the nonfunctional contact exhibited positive
staining for 0.6% of its contact-length and the functional contact
exhibited positive staining for 33.3% of its contact-length.

When we applied the above normalization and thresholding
method to all the nerve–muscle pairs tested for twitching, we
observed that on average, 33.6 ± 2.8% (mean ± SEM) of the
neurite–muscle contact-length stained positive for R-BTX (n = 9
cell pairs). In contrast, 5.5 ± 1.6% (mean ± SEM) of the neurite–
muscle contact-length of nontwitching contacts stained positive for
R-BTX (n = 7 cell pairs). Hence, the extent of R-BTX staining along
nerve–muscle contacts correlates with the presence of functional
synaptic contacts and the sites of receptor aggregation are thus
potential release sites.

Identification of putative active zones: synaptic vesicles and
acetylcholine receptors

For the development of AZs at nerve muscle contacts, it is necessary
that both Ca2+ entry and synaptic vesicles are localized in close
spatial proximity to postsynaptic receptors. To quantitatively assess
whether all three cellular requirements were fulfilled at developing
Xenopus nerve–muscle contacts, we first examined the location of
enhanced SV2 immunofluorescence domains using the 1.5 SD above
the mean criterion with respect to AChR aggregation. Having
established that R-BTX staining is enhanced at functional nerve–
muscle contacts, it remained to be demonstrated that synaptic vesicle
aggregation also occurred at sites of AChR aggregation. Figure 5
illustrates a particular experiment in which a nerve–muscle contact
(Fig. 5A) was stained with anti-SV2 (Fig. 5B) and R-BTX (Fig. 5C)
in the same preparation. Corresponding regions (see insets) of the
neurite–muscle contact exhibited bright fluorescence staining for both
anti-SV2 and R-BTX. To quantitatively assess the degree of
colocalization between anti-SV2 and R-BTX, we examined the
length of overlap between the two images along line intensity
profiles. Similar to the relative intensity analysis presented in regard
to differences in anti-SV2 staining in the presence and absence of
Ca2+ channels (Fig. 4), we set the threshold for positive anti-SV2
staining to be > 1.5 SD above the mean value of the line profile.
Positive R-BTX staining was set to the more stringent condition of
3 SD above the mean value of the line profile. Figure 5D illustrates
the normalized intensity profiles of both the anti-SV2 staining (thick
trace) and R-BTX (thin trace) for the same cell. The traces were
scaled such that the threshold levels were made identical and the
staining length of each of the two stains could be easily identified.
There is a clear spatial correspondence along the nerve–muscle
contact where anti-SV2 immunofluorescence overlaps with R-BTX

Fig. 3. Ca2+ entry sites are associated with larger relative SV2
immunofluorescence intensities. (A) Histogram plot of relative SV2
immunofluorescence intensities within 1 µm of a confocal spot recording
site in which no rapid Ca2+ transients were detected (see Methods for
criteria). The dashed line is a Gaussian fit to the relative intensity
distribution with a corresponding mean ± SD. (B) Histogram plot of the
relative SV2 immunofluorescence intensities at regions of the nerve–muscle
contact within 1 µm of a confocal spot recording site where rapid Ca2+
transients were detected. The dashed line is a Gaussian fit to the data with
the corresponding mean ± SD. Fluorescence intensities are represented as
the number of SD above the mean value of line scans spanning the
recording site. The two distribution means are statistically different,
P < 0.0001, Kolmogorov–Smirnov test. (C) Cumulative probability plots of
the two anti-SV2 intensity distributions from A (circles) and B (triangles).
fluorescence. It can be observed from this profile that the length of R-BTX positive staining (20.1% of the total contact-length) is longer than that of SV2 immunofluorescence (10.6%). However, the majority (93%) of SV2 positive staining above 1.5 SD is present along the same length as R-BTX.

When pooling results from many nerve–muscle pairs (n = 10), we found that on average, 8.6 ± 0.7% (mean ± SEM) of nerve–muscle contact-lengths exhibited anti-SV2 intensities above threshold. In these same cell pairs, the R-BTX staining was 25.8 ± 3.9% (mean ± SEM) of the total nerve–muscle contact. The contact-length in which anti-SV2 and R-BTX fluorescence were colocalized spanned 7.0 ± 0.9% of the total nerve-muscle contact. Thus, 81% of the SV2 positive contact-length was associated with positive staining of R-BTX, suggesting that most locations of the synaptic vesicle aggregation that correlates with Ca²⁺-entry sites, is also likely to correlate with postsynaptic AChRs. However, the majority of the positive R-BTX staining (73%) was not associated with anti-SV2 staining, indicating that the postsynaptic aggregation of AChRs, though sensitive to the nerve–muscle functionality, is not constrained within the region of presynaptic vesicle aggregation.

Identification of putative active zones: Ca²⁺ channels, synaptic vesicles and acetylcholine receptors

Having verified that, in our cultures, significant vesicle aggregation (anti-SV2 immunofluorescence) localizes with AChR aggregation, we performed experiments to determine whether Ca²⁺ channels

Fig. 4. Enhanced R-BTX staining along nerve–muscle contacts in which nerve stimulated muscle twitching was observed. (A₁) A DIC image of a nonfunctional nerve–muscle contact. (A₂) A fluorescence image of the same nerve–muscle contact showing no significant R-BTX staining along the contact site. (B₁) A DIC image of a functional nerve–muscle contact. (B₂) A fluorescence image in which prominent staining of R-BTX fluorescence can be observed on the muscle along the nerve–muscle contact region. (C₁) A fluorescence intensity profile of a line-scan along the region of nerve muscle contact (arrow, A₁). The dashed line indicates a pixel intensity 3 SD above the mean background pixel value (determined from Gaussian fit to distribution in C₂). (C₂) A histogram plot of the distribution of pixel intensities along the linescan illustrated in C₁. The dashed curve is a Gaussian fit (intensity, 312 ± 68.4 arbitrary units; mean ± SD). (D₁) A fluorescence intensity profile of a line-scan along the region (arrow, B₁) of the functional nerve–muscle contact. The dashed line indicates pixel intensities 3 SD above the background fluorescence intensity (determined from the Gaussian fit to the distribution in D₂). (D₂) Histogram plot of the distribution in pixel intensities of the line-scan illustrated in D₁. The dashed line is a double Gaussian fit with the mean : SD ratio of the first Gaussian fixed to 0.28 (see Methods). The resulting mean and SD of the background fluorescence was 249 and 36.5, respectively. The scale bar is 10 μm and applies to all images.
colocalize directly with synaptic vesicles and AChRs. In Fig. 6 we show a synaptic contact in which a rapid Ca\(^{2+}\) transient was measured followed by both R-BTX staining and anti-SV2 staining. In the phase contrast image [Fig. 6(A1)] the arrow indicates the location within the nerve terminal where a rapid Ca\(^{2+}\) transient [Fig. 6(A2)] was measured. The same preparation was then fixed (Fig. 6B) and stained with anti-SV2 antibodies [Fig. 6(B2)] and R-BTX [Fig. 6(B3)]. At the location where the rapid Ca\(^{2+}\) transient was observed (arrows) both bright anti-SV2 staining (2.1 SD above mean) and R-BTX (7.2 SD above mean) were observed. This colocalization between all three markers is, therefore, likely to represent a fully functional synaptic contact. In general, just over 51% (17 of 33) of the recording sites where rapid Ca\(^{2+}\) transients were measured, also stained positively (> 3 SD above background) for BTX. On the other hand, those sites that resulted in a significant transient and stained with > 1.5 SD for anti-SV2, 11 of 18 (~61%), also stained positively with R-BTX. This value compares similarly to the predicted percentage of 61%, calculated from the 81% of SV2 positive contact-length that colocalized with R-BTX (see above), and the percentage of Ca\(^{2+}\) transient recording sites that were greater than 1.5 SD (75%). Thus, a significant proportion of presynaptic Ca\(^{2+}\) channel clusters coaggregate with synaptic vesicles, and postsynaptic AChR at putative release sites along nerve-muscle contacts from 18 to 24 h cultures.

**HB-GAM coated beads induce aggregation of Ca\(^{2+}\) channels and synaptic vesicles**

In the *Xenopus* coculture preparation, presynaptic aggregation of vesicles has been shown to be enhanced in a contact-dependent manner (Cohen *et al.*, 1987; Dai & Peng, 1996). To investigate whether both functional Ca\(^{2+}\) channels and synaptic vesicles are spatially codistributed in artificially induced synaptic contact regions, we performed experiments with HB-GAM coated latex beads. Figure 7A shows an image of a neurite in contact with several 5 \(\mu\)m HB-GAM coated beads (`a'–`e') and these bead-neurite contacts were tested for the presence of rapid Ca\(^{2+}\) transients. The vertical arrows marked with minus signs indicate single recording sites that were negative for Ca\(^{2+}\) transients. They invariably occurred along the neurite at sites without bead contacts. The vertical arrow with a plus sign indicates a single recording site in which rapid calcium transients were detected at a bead–neurite contact (Fig. 7A, bead `c', transients not shown). The horizontal arrows indicate the direction of two high-resolution spot displacement scans along bead–neurite contacts (Fig. 7A, beads `a' and `b') whose isochronal \(\Delta F/F\) profiles are shown in Fig. 7B and C. The insets show transients at the entry site (trace 1) and “off” the entry site (trace 2; see Fig. 2 for description of “on” and “off” entry sites). It should be noted that the bead-induced Ca\(^{2+}\) transients are qualitatively similar to those measured at native junctions. In Fig. 7D and E we show fluorescence images obtained at two focal planes of the same neurite after it was subsequently fixed and stained for the presence of SV2. It can be observed that in regions negative for Ca\(^{2+}\) transients (vertical arrows with negative sign), SV2 immunofluorescence was barely detectable.

**Fig. 5.** SV2 immunofluorescence colocalizes with R-BTX fluorescence. (A) A DIC image of a fixed nerve–muscle contact in which it was colabelled with anti-SV2 antibodies and R-BTX. The small box indicates region viewed in the inset at higher magnification. (B) Fluorescence image of the same neurite–muscle contact using the Alexa 488 optical filter set. The inset is a higher magnification of the same region as in A. (C) A fluorescence image of the same nerve terminal at the same focal plane using a rhodamine optical filter set. The inset is a higher magnification of the boxed region from A. (D) The relative fluorescence intensity profile of line-scans (denoted by dotted line in A) of SV2 (thin trace) immunofluorescence and R-BTX fluorescence (solid trace). The dashed horizontal line represents the threshold (1.5 SD for SV2, 3 SD for R-BTX) for positive staining in order to determine the degree of colocalization. Note that the vertical axis for the R-BTX profile is broken to accommodate for the entire range of intensities.

In addition, at bead–neurite contacts there is a visible enhancement of SV2 immunofluorescence intensity (Fig. 7E, asterisks), particularly at those contacts corresponding to the location of the Ca^{2+} entry sites identified in the two isochronal ΔF/ΔF' plots of Fig. 7B and C, and the rapid Ca^{2+} transient detected at a single site on bead 'c'. Bead 'e' did not appear to induce SV2 immunofluorescence, probably due to the lack of an effective contact with the neurite, which frequently occurs in this type of preparation (Dai & Peng, 1995). In nine other bead–neurite contact experiments, rapid Ca^{2+} transients were observed and exhibited peak ΔF/ΔF'-values of 16.8 ± 1.8% (mean ± SEM, n = 4 neurites). Of these contacts, five out of five tested with anti-SV2 antibodies exhibited significant (> 1.5 SD) SV2 immunofluorescence, demonstrating that beads coated with specific matrix-associated growth factors can mimic, at least in part, the effect of a muscle cell for the induction of both Ca^{2+} channel and synaptic vesicle aggregation.

**Discussion**

By combining the detection of localized AP-induced presynaptic Ca^{2+} transients (DiGregorio & Vergara, 1997; DiGregorio et al., 1999b) with fluorescence-based quantitative immunocytochemical techniques, we demonstrated that Xenopus nerve–muscle implicate a coordinated assembly of Ca^{2+} channels, synaptic vesicles and postsynaptic AChRs to putative AZs similar to that observed at the mature NMJ. Furthermore, we showed that this aggregation can be induced by local presentation of the extracellular matrix molecule HB-GAM, which is also known to aggregate synaptic vesicles and postsynaptic receptors (Peng et al., 1995; Dai & Peng, 1996).

**Using the confocal spot detection method to identify the location of functional Ca^{2+} channels**

Integral to our estimation of where functional Ca^{2+} channels are located is the method of detection of AP-evoked Ca^{2+} transients. One of the advantages of the confocal spot detection method and the use of a low affinity Ca^{2+} indicator is that the spatial profile of the presynaptic [Ca^{2+}] can be assessed within a millisecond of AP invasion. At this time, the diffusional spread of calcium ions is much less than the error set by a finite detection volume (see DiGregorio et al., 1999b) for a full explanation). In short, the kinetic features of the transients allow us to determine whether calcium channels are located within the detection volume (DiGregorio et al., 1999a) in addition to a basic characterization of the free [Ca^{2+}] change associated with a single AP. Given the spot diameter of 0.7 μm (DiGregorio et al., 1999b) Ca^{2+} channels must be located within 0.35 μm of the spot centre in order to detect a Ca^{2+} transient with a rapid rate of rise and a rapid decay rate. If the Ca^{2+} channels are located in these spots, then the spatial resolution of our method is determined primarily by the size of the detection volume.

![Image](https://via.placeholder.com/150)

**FIG. 6.** Colocalization of Ca^{2+} transients, anti-SV2 staining, and R-BTX staining. (A1) A phase contrast image of a nerve terminal (nt) to myocyte (m) contact from which a confocal spot detected presynaptic Ca^{2+} transient was recorded. (A2) Averaged AP-induced Ca^{2+} transient (n = 6) recorded from a region of the nerve terminal where it contacted the myocyte (A, arrow). (B1) A DIC image of the same nerve–muscle pair as in A following fixation and staining with anti-SV2 and R-BTX. (B2) Fluorescence image of the same nerve terminal using the Alexa 488 optical filter set. (B3) Fluorescence image of the same nerve terminal using the rhodamine filter set. All arrows point to the location where the rapid Ca^{2+} transient was measured. The scale bar is 5 μm for all.
outside the detection volume, the diffusional delay and amplitude decrement leads to a distinct change in the shape of the transient [see Fig. 1(A3) and insets in Fig. 7B and C]. Therefore, the theoretical accuracy with which we should be able to localize Ca\textsuperscript{2+} channels, presuming there are enough channels to produce a transient larger than the background noise, should be \(< 0.4 \mu m\). In practice, this accuracy is reduced due to our inability to mark the site of recording such that the precise location can be assayed with immunocytochemical methods. We therefore relaxed the accuracy arbitrarily to 1 \mu m and hence for the comparison of anti-SV2 intensity at various recording sites, we always chose the maximum value of anti-SV2 intensity within 1 \mu m of the recording sites.

Interestingly, the confocal detection technique also provides a unique method to characterize the aggregation of Ca\textsuperscript{2+} channels as our attempts to obtain supplementary immunocytochemical evidence using antibodies raised against Ca\textsuperscript{2+} channel subunits produced unsuitable images for high-resolution analysis (data not shown).

Estimation of the spatial distribution of synaptic molecules using quantitative immunofluorescence

The use of a cooled CCD camera for fluorescence imaging allowed us to perform quantitative intensity analysis for the study of synaptic molecule localization. To identify AChR locations we were able to identify easily a background staining intensity and then set the threshold for protein localization above 3 SD of the mean background intensity. When doing this, it provided a consistent method for analysing the length of AChR positive staining along nerve–muscle contacts. Our findings on vesicle aggregation along neurites with a significant correspondence sites of AChR staining are similar to previous results in older \textit{Xenopus} cultures (Cohen et al., 1987) which report a percentage of R-BTX staining per contact-length of 22%, slightly smaller than the ~34% found in functional contacts in our preparation. This difference could be due to the quantitative limitations of photographic film. However it is also possible that the vesicle and AChR distribution is different between 18 and 24 h and 2–6-day-old cultures. On the other hand, the vesicle-dependent staining length reported by Cohen et al. (1987) is ~4-fold greater than the contact-length estimated by us using SV2 immunofluorescence. In the coculture preparation, there are vesicles throughout the nerve terminal (Dai & Peng, 1996), thus our criterion for positive staining, of setting a threshold 1.5 SD above the background, is likely to identify regions of enhanced aggregation rather than the simple presence or absence of vesicles. To further strengthen this proposal, we thought it important to verify that the observed staining patterns with anti-SV2 are likely to represent higher densities of vesicle

![Fig. 7. HB-GAM coated beads induce colocalized aggregation of functional Ca\textsuperscript{2+} channels and synaptic vesicles. (A) A 100\times phase image of a neurite in contact with several HB-GAM coated beads (‘a’–‘e’). Vertical arrows labelled (–) indicate confocal spot locations where no significant Ca\textsuperscript{2+} transient was measured, whereas, the arrow labelled (+) is a spot location where a rapid Ca\textsuperscript{2+} transient was measured. The horizontal arrows (beads ‘a’ and ‘b’) indicate the direction, length and location of confocal spot scans. (B) Isochronal \( \Delta F/F \) profile of a confocal spot scan across bead ‘a’. Inset, averaged Ca\textsuperscript{2+} transients (‘1’ and ‘2’) recorded from the regions of the scan identified by the correspondingly labelled horizontal bars shown in the isochronal \( \Delta F/F \) plot. (C) The isochronal \( \Delta F/F \) profile from the scan across bead ‘b’. Inset, Ca\textsuperscript{2+} transients (‘1’ and ‘2’) recorded from the regions of the scan identified by the correspondingly labelled horizontal bars shown in the isochronal \( \Delta F/F \) plot. (D) Fluorescence image of the same preparation as in A, but fixed and stained with anti-SV2. The confocal spot-recording site are identified as in A, except that the scan start and end locations are indicated by the end of the dashed lines. (E) Fluorescence image taken from a plane 2 \mu m above the image in B. The asterisks identify the location of beads ‘a’–‘d’ for reference. The vertical and horizontal scale bars in the insets represent 0.05 \( \Delta F/F \) and 5 ms, respectively.](image-url)
aggregation in the neurite, rather than a peculiarity of SV2 staining. Thus, we demonstrated that antibodies against another synaptic vesicle associated protein, CSP (Umbach et al., 1994; Umbach & Gundersen, 1997; Poage et al., 1999; Seagar et al., 1999), produced staining patterns identical to those of anti-SV2.

Our results provide firm support to the notion that Ca\(^{2+}\) entry sites, and hence Ca\(^{2+}\) domains, are localized in regions of nerve-muscle contact where both vesicles and postsynaptic receptors (putative AZs) are located. We arrived at this conclusion either from the simultaneous assessment of R-BTX and anti-SV2 staining at a Ca\(^{2+}\) entry site as shown in Fig. 6, or by paired quantification of anti-SV2 fluorescence along Ca\(^{2+}\) entry sites and of anti-SV2 and R-BTX staining in functional contacts, as shown in Figs 3 and 5, respectively. Thus, the first approach demonstrated that 61% of the calcium entry sites were associated with significant SV2 aggregation (> 1.5 SD above the mean intensity) and (> 3-SD) R-BTX staining. On the other hand, paired comparison of global immunocytochemical and physiological data revealed that 81% of SV2 positive contact-length was colocalized with positive R-BTX staining and that 78% of Ca\(^{2+}\) entry recording sites revealed positive anti-SV2 staining. The calculated probability of the paired results predicts that 62% of the Ca\(^{2+}\) entry sites should colocalize with both vesicle aggregation and postsynaptic receptor clustering, similar to the observed probability. Thus, as synaptic vesicle aggregation has been shown to occur juxtaposed to postsynaptic densities (Birks et al., 1960) that represent sites of neurotransmitter release or 'active zones' (Couteaux & Pecot-Dechavassine, 1970), we submit that our data provides unique evidence in support of the existence of functional Ca\(^{2+}\) channel clustering near active zones.

**Developmental implications of the colocalization**

When comparing the distribution of the two molecules, there is an excess R-BTX staining without colocalized SV2 immunofluorescence (73% of the total R-BTX staining). This may be due to 'sloppiness' in the signalling mechanisms inducing the redistribution of Ca\(^{2+}\) channels and synaptic vesicles with the clustering of AChR. Excess R-BTX staining has been shown to occur beyond neurite contact (Anderson et al., 1977) arguing for an imprecise distribution of AChR. Furthermore, this same group showed that the neurotransmitter release was not necessary for this aggregation. Thus, the physical contact-length likely determines the AChR distribution. Moreover, in the mature neuromuscular junction, AChR distribution is not limited to the junctional fold region, but in fact extends laterally beyond the region of proposed neurotransmitter release (Salpeter et al., 1988).

That only 81% of SV2 positive regions colocalized with postsynaptic AChRs (see above, 'Estimation of the spatial distribution of synaptic molecules using quantitative immunofluorescence') could be due to random accumulation of vesicles not necessarily at sites of Ca\(^{2+}\)-entry and hence functional release (Dai & Peng, 1996). We cannot rule out the possibility that the fixation process induced an apparent vesicle aggregation. Another, more interesting, possibility is that in some cases vesicle aggregation may occur following nerve-muscle contact yet prior to postsynaptic AChR aggregation. Finally, the 25% of the cases in which significant SV2 fluorescence was not strictly associated with Ca\(^{2+}\) channel domains (Fig. 3C, closed circles) suggests that synaptic vesicle aggregation occurs spontaneously (to a certain extent) without positive Ca\(^{2+}\) aggregation. Interestingly, if we increased the threshold to ~2 SD, then the number of positive SV2 sites which are negative for Ca\(^{2+}\) entry reduces to < 4%. At this threshold, however, only > 50% of the Ca\(^{2+}\) entry sites would be considered positive for SV2, thus suggesting that a significant Ca\(^{2+}\) channel aggregation may occur without vesicle aggregation. Our results seem to be more compatible with this attractive hypothesis, that Ca\(^{2+}\) channel aggregation is a necessary condition for significant vesicle aggregation, also consistent with previous data showing a significant Ca\(^{2+}\) dependence of vesicle aggregation (Dai & Peng, 1998) and a Ca\(^{2+}\) dependent increase in synaptic efficacy (Zoran et al., 1991). Furthermore, the size of clusters of synaptic vesicles may not quantitatively parallel the proximity of docked vesicles to functional Ca\(^{2+}\) channels as combined electrophysiological and ultrastructural evidence, obtained from the Xenopus cultured NMJ preparation, suggests that functional synaptic transmission can occur without significant vesicle aggregation for up to 15 h of synaptogenesis (Buchanan et al., 1989). However, spontaneously forming synaptic contacts from 18 to 24 h cultures do exhibit vesicle clustering opposite to postsynaptic densities. Nonetheless, this ultrastructural feature may represent a later stage of synaptic development as synaptic vesicle aggregation does correlate with enhanced rates of synaptic depression in response to high frequency stimuli at novel neurite-muscle junctions in comparison to spontaneously formed junctions in culture (Zakharenko et al., 1999). Whether calcium channel clustering occurs in naive neurites or novel contacts is unknown, nevertheless, our data are consistent with the timing of calcium channel aggregation at least paralleling that of vesicle aggregation, a known marker of synaptic maturation and indicator of synaptic strength.

**Functional implications of the colocalization of functional Ca channels and synaptic vesicles**

The functional importance of colocalized Ca\(^{2+}\) channel and synaptic vesicle clustering has been proposed to be necessary for efficient neurotransmission. The rapid formation and dissipation of the local [Ca\(^{2+}\)] domains around either single or clustered channels is thought to mediate synaptic transmission (Schweizer et al., 1995; Neher, 1998). Thus, it is not surprising that clusters of vesicles are seen juxtaposed to postsynaptic receptors and possibly interacting directly with Ca\(^{2+}\) channels through the SNARE protein syntaxin (Bennett et al., 1992). Data presented here and previously (DiGregorio et al., 1999b), suggest that the peak of the Ca\(^{2+}\) transient falls to ~10% of its maximum value within <0.5 µm from the edge of the calcium entry site. This steep gradient, which is likely to be greater closer to the membrane (DiGregorio et al., 1999b), requires that releasable vesicles be in the proximity of Ca\(^{2+}\) channels in order to be recruited for release. It is, therefore, not surprising that anti-SV2 staining is enhanced at sites where rapid Ca\(^{2+}\) transients were measured. Our findings are not inconsistent with the suggestion that as many as 95% of releasable vesicles may be directly linked to Ca\(^{2+}\) channels (Rettig et al., 1997), however, they do not provide a demonstration for a nanodomain Ca\(^{2+}\) control mechanism (Adler et al., 1991) due to the resolution limits of the technique (DiGregorio et al., 1999b).

**Candidate signalling mechanisms for contact-dependent aggregation of functional Ca\(^{2+}\) channels**

Although the molecular signalling pathways determining vesicle and channel clustering are unknown, our data supports the suggestion that synaptic differentiation both functionally and ultrastructurally appears to be dependent on cell-to-cell contact (Cohen et al., 1987; Buchanan et al., 1989; Zoran et al., 1991; Funte & Haydon, 1993; Bahls et al., 1998). Various proteins embedded in the basal lamina have been implicated (Dai & Peng, 1995; Dai & Peng, 1996) in addition to the postsynaptically expressed protein agrin (Campagna et al., 1995; Campagna et al., 1997). By studying HB-GAM coated bead-neurite contacts we reinforce the idea that localized application of extracellular proteins could induce both Ca\(^{2+}\) channel and synaptic
vesicle clustering. Although we cannot rule out the possibility that HB-GAM coated beads may preferentially bind to areas with preformed vesicle/channel complexes, the evidence appears to suggest the opposite as muscle cells and coated beads manipulated to come in contact with neurites seem to promote presynaptic differentiation and vesicle aggregation (Buchanan et al., 1989; Dai & Peng, 1995). As a contact-dependent interaction can increase resting [Ca\(^{2+}\)] (Zoran et al., 1993; Dai & Peng, 1995), Ca\(^{2+}\) may be a critical second messenger for presynaptic differentiation. A cAMP-dependent protein kinase has also been implicated in enhanced Ca\(^{2+}\) influx at cell contact-induced synaptic differentiation (Funte & Hayden, 1993). This enhancement in Ca\(^{2+}\) influx is consistent with findings presented here regarding functional Ca\(^{2+}\) channel aggregation at contact sites.

As a counterpart to the above arguments, the association of functional Ca\(^{2+}\) channels with synaptic vesicles may occur in naive (noncontacted) neurites, as suggested by the results with FM-143 by Dai & Peng (1998). However, in those experiments, vesicle turnover was tested in response to nonphysiological depolarization by bath applied KCl, but not by APs. Under these conditions, Ca\(^{2+}\) channels are not required to be colocalized with vesicles to mediate vesicle mobilization. In fact, EGTA was able to block a significant amount of the KCl-induced destaining of FM-143 in naive neurites, but had a minute effect in AP-induced localized Ca\(^{2+}\) domains detected in contact regions (DiGregorio et al., 1999b).

As HB-GAM coated beads can also produce AChR aggregation, it is therefore possible that for contacts at least 12–h-old, the distribution of vesicles and Ca\(^{2+}\) channels, synaptic vesicles, and postsynaptic receptors share a common temporal and or chemical signalling mechanism. Molecules that have been shown to be important in synaptic vesicle aggregation and postsynaptic receptor aggregation are actin cytoskeleton (Dai & Peng, 1996) and tyrosine kinases (Dai & Peng, 1995).

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Abbreviations

AChR, acetylcholine receptor; AP, action potential; AZ(s), active zone(s); CSP, cysteine string protein; FWIM, full-width at half-maximum; NFR, normal frog Ringer; NMJ, neuromuscular junction; PBS, phosphate-buffered saline; R-BTX, tetramethylrhodamine-conjugated α-bungarotoxin.

References


