

Neurotransmitter Secretion along Growing Nerve Processes: Comparison with Synaptic Vesicle Exocytosis

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Abstract. In mature neurons, synaptic vesicles continuously recycle within the presynaptic nerve terminal. In developing axons which are free of contact with a postsynaptic target, constitutive membrane recycling is not localized to the nerve terminal; instead, plasma membrane components undergo cycles of exoendocytosis throughout the whole axonal surface (Matteoli et al., 1992; Kraszewski et al., 1995). Moreover, in growing *Xenopus* spinal cord neurons in culture, acetylcholine (ACh) is spontaneously secreted in the quantal fashion along the axonal shaft (Evers et al., 1989; Antonov et al., 1998). Here we demonstrate that in *Xenopus* neurons ACh secretion is mediated by vesicles which recycle locally within the axon. Similar to neurotransmitter release at the presynaptic nerve terminal, ACh secretion along the axon could be elicited by the action potential or by hypertonic solutions. We found that the

parameters of neurotransmitter secretion at the nerve terminal and at the middle axon were strikingly similar. These results lead us to conclude that, as in the case of the presynaptic nerve terminal, synaptic vesicles involved in neurotransmitter release along the axon contain a complement of proteins for vesicle docking and Ca^{2+} -dependent fusion. Taken together, our results support the idea that, in developing axons, the rudimentary machinery for quantal neurotransmitter secretion is distributed throughout the whole axonal surface. Maturation of this machinery in the process of synaptic development would improve the fidelity of synaptic transmission during high-frequency stimulation of the presynaptic cell.

Key words: secretion • exocytosis • synaptic vesicle • acetylcholine • dynamin

NEUROTRANSMITTER secretion from the nerve terminal plays an important role in synaptic competition and plasticity (Thoenen, 1995; Bonhoeffer, 1996; Rao and Craig, 1997). In the developing nervous system, neurotransmitters present in the extracellular medium may participate in axonal pathfinding and navigation by modulating the rate and direction of axonal growth (Kater et al., 1988; Lipton et al., 1988; Zheng et al., 1994; Buzhnikov et al., 1996). In mature neurons, neurotransmitter secretion depends on the exocytosis of neurotransmitter-containing synaptic vesicles (Hanson et al., 1997). These synaptic vesicles are clustered at the active zones specialized for neurotransmitter release and local recycling of synaptic vesicles (Burns and Augustine, 1995). Although the fusion of synaptic vesicles is tightly regulated by the influx of Ca^{2+} during action potential propagation (Bennett, 1997), synaptic vesicles may fuse with the plasma membrane spontaneously. These spontaneous exocytotic

events result in the release of neurotransmitter packets (quanta) and a transient change in the membrane potential in the postsynaptic cell (Del Castillo and Katz, 1954; Xie and Poo, 1986).

Spontaneous neurotransmitter secretion can also be detected in developing axons where it is believed to be localized largely to the growth cone region (Hume et al., 1983; Young and Poo, 1983). In the majority of cases, the insertion of newly synthesized membrane material and the endocytosis of plasma membrane components are also restricted to the distal axon (Craig et al., 1995; Dai and Sheetz, 1995; Futerman and Banker, 1996; Vogt et al., 1996; Zakharenko and Popov, 1998). The confinement of endoexocytic activity to the growth cone region may reflect a unique molecular composition of the distal axon, such as the localization of target (t)-SNAREs (Rothman, 1994) to the growth cone area. However, in hippocampal neurons (Galli et al., 1995; Garcia et al., 1995) and *Xenopus* embryo neurons (Antonov et al., 1998), t-SNAREs were found to have a widespread distribution throughout the axon and were not restricted to the nerve terminal. Moreover, constitutive membrane recycling (Matteoli et al., 1992; Dai and Peng, 1996a), insertion of newly synthesized plasma membrane components (Popov et al., 1993; de

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Chaves et al., 1995; Harel and Futerman, 1996), and quantal acetylcholine (ACh)¹ secretion (Evers et al., 1989) have been observed along the axonal shaft in naive (free of contact with other cells) neurons in culture.

The relationship between the vesicles involved in membrane recycling along the axon and the genuine synaptic vesicles in the nerve terminal remains to be established. The vesicles associated with constitutive membrane recycling along the axon contain some of synaptic vesicle markers (Matteoli et al., 1992; Dai and Peng, 1996a), and their exocytosis can be elicited by membrane depolarization with high KCl (Kraszewski et al., 1995; Dai and Peng, 1996a). Therefore, it has been assumed that these vesicles are similar, if not identical to, the synaptic vesicles (Dai and Peng, 1996b). Alternatively, the constitutive recycling of vesicles along the axon may be a manifestation of the housekeeping recycling pathway which takes place throughout the cell surface (Matteoli et al., 1992).

Previously it has been demonstrated that in *Xenopus* embryo neurons spontaneous secretion of ACh is not limited to the presynaptic nerve terminal. Instead, quantal ACh release can be detected throughout the cell surface, as demonstrated by the whole-cell patch clamp recordings from myocytes brought into contact with neurons (Sun and Poo, 1987; Evers et al., 1989; Antonov et al., 1998). In this study we demonstrate that the properties of neurotransmitter secretion along the axon and at the preformed neuromuscular synapses are strikingly similar. Our results suggest that in developing neurons the assembly of the functional apparatus for neurotransmitter secretion does not require a contact with postsynaptic target. We hypothesize that spontaneous neurotransmitter secretion from growing axons may participate in interneuronal signaling and in the development of neuronal networks.

Materials and Methods

Cell Culture

Cultured *Xenopus* spinal cord neurons were prepared according to previously reported methods (Spitzer and Lamborghini, 1976; Anderson et al., 1977). The cells were plated on acid-washed coverslips and grown in the culture medium consisting of (vol/vol) 50% Leibovitz L-15 medium (GIBCO BRL), 49% Ringer's solution (115 mM NaCl, 2 mM CaCl₂, 2.5 mM KCl, 10 mM Hepes, pH 7.6) and 1% fetal bovine serum (GIBCO BRL). The cultures were used for experiments after 1 d incubation at 20°C. *Xenopus* myocytes were plated separately on Petri dishes, grown in a culture medium supplemented with 3% fetal bovine serum, and then used for experiments after a 24–48 h incubation at 20°C.

Micromanipulation

Manipulation of *Xenopus* myocytes followed the procedures described previously (Girod et al., 1995; Morimoto et al., 1995). In brief, coverslips with plated neurons were transferred to the Petri dish containing *Xenopus* myocytes. Myocytes were gently detached from the surface of the Petri dish by heat-polished micropipettes attached to a hydraulic micromanipulator (Newport). The cells were transferred into the vicinity of the axon, allowed to reattach to the glass surface, and then manipulated into the contact with axon. In the majority of patch clamp recording, the myocyte was firmly attached to the surface of the coverslip and was in tight contact

with the axon. We found that attachment of the myocyte to the coverslip greatly improved the stability of whole-cell patch clamp recordings.

Electrophysiology

Gigaohm-seal whole-cell recording methods followed those described previously (Hamill et al., 1981). Patch pipettes were fabricated from glass micropipets (VWR) and pulled with a two-step puller (Narishigi). After heat polishing, the pipette tip diameter was 1.5–2 μm and the resistance was 2–5 MΩ. The intrapipette solution for the whole-cell recording from myocytes contained 140 mM KCl, 1 mM NaCl, 1 mM MgCl₂, and 10 mM Hepes, pH 7.4. Electrical stimulation of the presynaptic neuron was made by a patch electrode filled with Ringer's solution at the cell body under loose seal conditions. All recordings were done at room temperature. The membrane currents were monitored by a patch clamp amplifier (Warner PC501-A). The data were digitized and stored on a videotape recorder for later playback onto a storage oscilloscope (model 5113; Tektronix) or a chart recorder (model RS3200; Gould). The data were analyzed with the SCAN program, kindly provided by J. Dempster (University of Strathclyde at Glasgow, Glasgow, UK). The threshold for detection of current events was typically set at the level of 20–25 pA. All data reported are mean ± SEM. To determine significant differences between averages, unpaired *t* tests assuming equal variance or analysis of variance (ANOVA) tests were performed.

Image Acquisition and Data Analysis

An Olympus IX 50 inverted microscope equipped with differential interference contrast optics and a 100-W mercury arc lamp was used for fluorescence microscopy. Images were acquired with a charge-coupled device camera (ImagePoint or Sensys, Photometrics) driven by IPLab (Signal Analytics) imaging software, and background subtracted. Images were processed with IPLab and Photoshop (Adobe Systems). Quantitation of data was performed using IPLab software. The distribution of fluorescence intensity along the axon was obtained by measuring the average intensity within circular sampling areas 1 μm in diameter. The sampling was started at the growth cone with regular spacing of ~2.5 μm along the axon.

Application of Hypertonic Solution

For the fast-flow application of hypertonic solution, a micropipette with tip diameter 7–10 μm was positioned within 100–200 μm of the site of recording. A pulse of positive pressure was applied with a Picospritzer. The hypertonic solution contained 300 mM sucrose in culture medium. Application of the hypertonic solution visibly distorted the plasma membranes of both the axon and the myocyte by inducing shrinkage. Withdrawal of the pipette resulted in a rapid (within a few seconds) recovery of both axonal and myocyte membranes to original shape.

FM1-43 Staining

Cells were labeled by superfusion into the chamber with FM1-43 (Molecular Probes) at a concentration of 2 μM in a 60 mM KCl solution for 3–5 min followed by washing in normal culture medium for 30–50 min. Destaining was induced by superfusion with 60 mM KCl in Ringer's solution. In a series of control experiments neuronal cultures were stained with FM1-43, washed in the culture medium supplemented with 5 mM EGTA (Ca²⁺-free medium), and superfused with Ringer's solution containing 60 mM KCl and 5 mM EGTA. The average fluorescence intensity of FM1-43-stained vesicle clusters was measured within rectangular sampling areas (0.22 × 0.22 μm²).

Staining of Mitochondria

To view mitochondria in living neurons, we added 1 μg/ml Rhodamine 123 (Molecular Probes) to the culture medium for 15–20 min, and then extensively washed the cells with fresh culture medium.

Microinjection of Cy3-Tubulin into *Xenopus* Embryos

Cy3-tubulin was a generous gift of G.G. Borisy (University of Wisconsin, Madison, WI). Details of Cy3-tubulin preparation can be obtained from <http://borisy.bocklabs.wisc.edu>. Before microinjection, a 10-μl aliquot of Cy-3 tubulin was centrifuged at 15,000 *g* for 60 min at 4°C to remove particulate material and to reduce pipette clogging and was stored on ice until the time of injection. *Xenopus* embryos were injected with 10–25 nl of 10

1. *Abbreviations used in this paper:* ACh, acetylcholine; ANOVA, analysis of variance; BFA, Brefeldin A; ESC, evoked synaptic current; PPF, paired pulse facilitation; SSC, spontaneous synaptic current.

mg/ml Cy3-tubulin as described before (Chang et al., 1998). The eggs were allowed to develop to stages 19–24 and were then used for the preparation of neuronal cultures.

Detergent Extraction

Neurons labeled with Cy3-tubulin were extracted in a microtubule-stabilizing buffer (60 mM Pipes, 1 mM MgCl₂, 5 mM EGTA, 0.1% Triton X-100, 10 μM taxol, pH 6.8) for 5 min and examined under a fluorescent microscope.

Immunocytochemistry

Monoclonal antibodies to dynamin-1 were purchased from Upstate Biotechnology. Polyclonal anti- α -adaptin antibodies were from Transduction Laboratories. Polyclonal anti-ARF1 antibodies and polyclonal antibodies that recognize σ 3 subunit of AP-3 complex were generously provided by V. Faundez and R.B. Kelly (University of California, San Francisco, CA). The secondary antibody was FITC-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch). Cell cultures were prepared on concanavalin A (1 μg/cm²)-coated coverslips. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, washed three times with PBS, and then permeabilized with 0.1% Triton X-100 in PBS. Cells were incubated with a primary (1:100) and then with a secondary antibody (1:200) for 1 h at room temperature. All antibody solutions were prepared in PBS containing 2 mg/ml bovine serum albumin. Cells were mounted in Vectashield mounting medium (Vector Labs) to resist bleaching.

Results

Spontaneous Secretion of ACh along the Axon Is Due to the Local Recycling of ACh-containing Vesicles

Experiments were performed on 1-d-old nerve muscle cultures prepared from *Xenopus* embryos. Neurons and myocytes in culture formed contacts spontaneously. We will refer to these developing neuromuscular synapses as preformed synapses. Quantal release of ACh at the preformed synapses can be detected by the whole-cell voltage clamp recordings from the postsynaptic myocyte (Hamill et al., 1981). Individual spontaneous synaptic currents (SSCs) in recordings from myocytes reflect spontaneous exocytosis of ACh-containing synaptic vesicles and release of ACh quanta (Chow and Poo, 1985; Evers et al., 1989). To detect the release of ACh in growing axons we chose axon-bearing neurons that were free of contact with other cells. An isolated *Xenopus* myocyte was detached from the substrate, voltage clamped at the resting membrane potential (−70 mV) using whole-cell patch clamp technique, and then manipulated into contact with the axonal shaft (Fig. 1 A). Recordings of the membrane currents in the myocyte using a whole-cell voltage clamp technique revealed fast inward currents (Fig. 1 A). These currents could be detected immediately after manipulation of the myocyte into contact with the neurite, and the SSC frequency did not change during the 30-min period of recordings (Fig. 1 B). Hence, myocytes appear to serve as passive detectors, rather than inducers, of exocytic events (Girod et al., 1995; Ninomiya et al., 1997; Antonov et al., 1998). In our previous study (Antonov et al., 1998) we showed that release of ACh packets can be detected throughout the whole neuronal surface. We characterized the distribution of ACh secretion along the growing axons and demonstrated that the frequency of SSCs displayed a proximodistal gradient with a higher level of activity at the distal axonal region. Moreover, the parameters of individual SSCs (rise time,

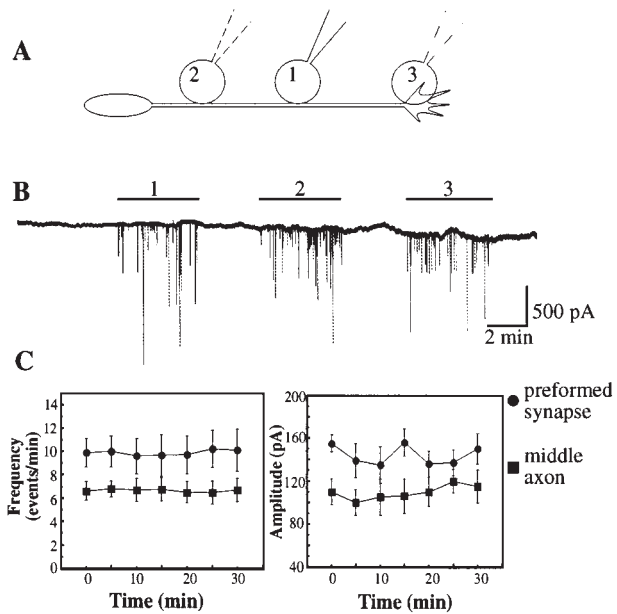


Figure 1. Spontaneous release of ACh from *Xenopus* neurons. (A) Schematic diagram of recording configuration. An isolated *Xenopus* myocyte was detached from the substrate, clamped at the resting membrane potential (−70 mV) using whole-cell patch clamp technique, and then sequentially manipulated into contact with three different sites along the axon. At each site the whole-cell patch clamp recording was performed for ~5 min. (B) A representative example of membrane current recorded from a voltage-clamped myocyte. Downward deflections represent SSCs. SSCs could be detected immediately after establishment of contact between the myocyte and the axon (horizontal black bars). (C) Changes in the frequency and amplitude of the current events with time after the onset of recording, normalized to the values at the beginning of the recording. Data from eight recordings at preformed synapses (circles) and from 10 recordings at the middle axonal segment (squares). Membrane current was continuously recorded for a period of 35 min. In recordings from the middle axon the data were collected immediately after establishment of contact between the myocyte and the axon. Notice that the SSC frequency and SSC amplitude do not significantly change during the period of recording.

decay time, frequency, and amplitude) recorded from different axonal segments were found to be very similar (Antonov et al., 1998). In this study we focused on ACh secretion at two neuronal regions: the middle axonal segment in naive (free of contact with other cells) neurons and the nerve terminal in the preformed neuromuscular synapses.

The vesicles involved in spontaneous ACh secretion along the axon may recycle locally within the axon, similar to synaptic vesicles in the nerve terminal. Alternatively, these vesicles may be directly transported from the soma via a constitutive biosynthetic pathway (Nakata et al., 1998). In the latter scenario, the cell body-derived vesicles would be expected to carry the molecules of the ACh transporter to produce a detectable SSC upon their exocytosis (Song et al., 1997). To distinguish between the two possibilities, we treated neuronal cultures with 5 μg/ml nocodazole. This treatment resulted in the loss of axonal microtubules (Fig. 2 C), rapidly arrested axonal growth (Fig.

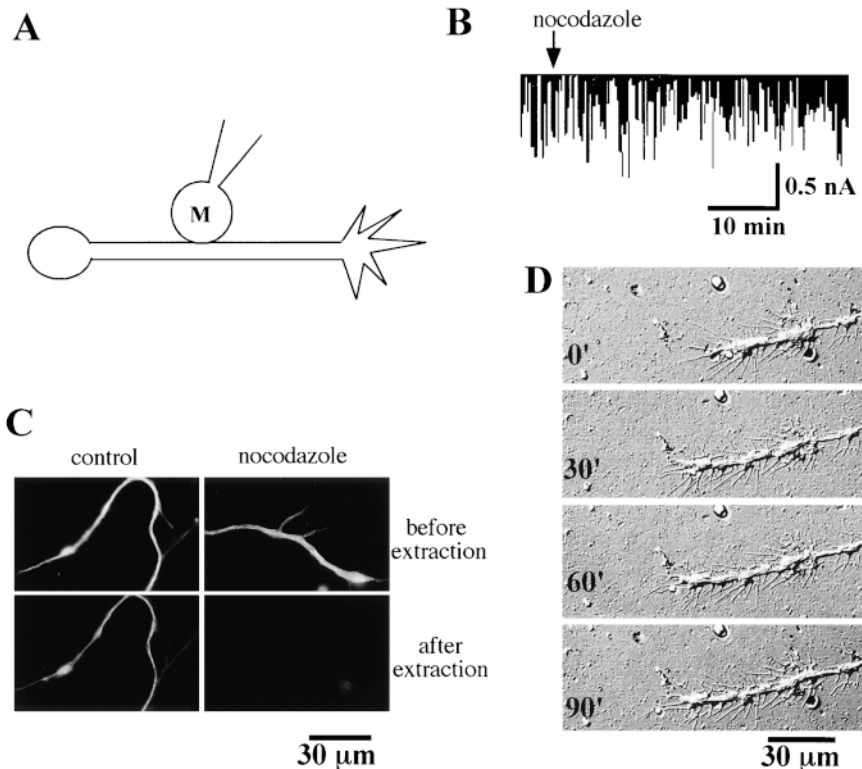


Figure 2. Spontaneous release of ACh in isolated *Xenopus* neurons is mediated by the local recycling of ACh-containing vesicles along the axon. (A) Schematic diagram of recording configuration. Myocyte (M) was manipulated into contact with the middle axonal segment 1 d after cell culture preparation. Whole-cell configuration was established \sim 1–2 min after manipulation. Patch clamp recordings from the myocytes were performed for \sim 30 min. The neurons chosen for experiments were free of contact with other cells and had a single axon \sim 300–500 μ m in length. (B) The trace is a representative example of membrane current recorded from whole-cell voltage-clamped myocyte ($V_h = -70$ mV) manipulated into contact with the middle axonal segment. Downward deflections represent SSCs. SSCs could be detected immediately after establishment of whole-cell configuration (start of the recording). Nocodazole (5 μ g/ml, arrow) was applied to the bath \sim 5 min after the start of recording. (C) Representative fluorescent images of *Xenopus* neurons loaded with Cy3-Rhodamine before (top) and after (bottom) detergent extraction in a microtubule-stabilizing buffer. In control cells (left panels) the bulk of tubulin was re-

tained in the neuron after extraction. In neurons treated for 30 min with nocodazole (5 μ g/ml, right panels), most of the tubulin was removed during the extraction procedure. Similar results were obtained in experiments performed on 10 control neurons and on 10 nocodazole-treated neurons. (D) Nocodazole treatment arrests axonal growth. Representative differential interference contrast (DIC) images of neurite 1 d after cell culture preparation. Numbers indicate time in minutes. Nocodazole (5 μ g/ml) was applied 30 min after the start of the experiment. Within 30 min after nocodazole application, axonal elongation slowed down and the growth cone retracted. Similar results were observed in nine different experiments.

2 D), and inhibited transport of mitochondria along the axon (Fig. 3). Therefore, the treatment with nocodazole is expected to disrupt the delivery of cell body-derived vesicles to the growing axon. However, spontaneous neurotransmitter secretion persisted after nocodazole application both along the axon (Fig. 2 B) and at the preformed synapses (data not shown). Moreover, the disruption of axonal microtubules resulted in a significant increase in the SSC frequency at the middle axonal segment (detailed quantitative analysis of the effects of axonal microtubules on neurotransmitter secretion will be presented elsewhere). These results strongly suggest that constitutive ACh secretion along the axon is not directly related to the exocytosis of cell body-derived vesicles. Instead, ACh secretion is likely to be mediated by a local exoendocytic recycling of ACh-containing vesicles.

To directly demonstrate that ACh secretion along the axon was due to the local recycling of ACh-containing vesicles, we transected the axon from the soma with a microelectrode. Previously it has been shown that this procedure results in the transient increase in SSC frequency at the preformed synapses (Stoop and Poo, 1995) due to the influx of Ca^{2+} (Stoop and Poo, 1995; Ziv and Spira, 1997). Within 15–20 min after transection, both the concentration of cytoplasmic Ca^{2+} and SSC frequency return to control

(before transection) values (Stoop and Poo, 1995). To investigate whether spontaneous ACh secretion persisted along the distal axonal fragments after axotomy, we manipulated the myocyte into contact with the middle axon and recorded SSCs before and for a period of 30 min after

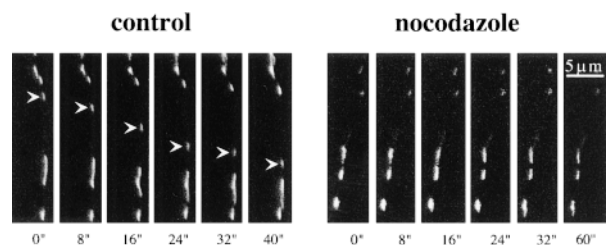


Figure 3. Treatment with nocodazole inhibits transport of mitochondria along *Xenopus* neurites. Representative fluorescent images of mitochondria in a control neuron and in a neuron treated for 30 min with nocodazole (5 μ g/ml). Mitochondria were stained with Rhodamine 123 and visualized with digital fluorescence microscopy. Transport of individual mitochondria (arrows) could be detected in control cells. In neurons treated with nocodazole, none of the \sim 300 observed mitochondria displayed episodes of long-range transport. Numbers indicate the time in seconds after the start of experiment.

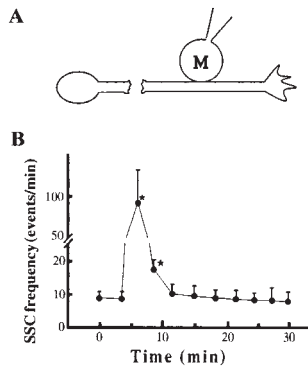


Figure 4. Spontaneous neurotransmitter secretion along the axon can be detected at the distal axonal fragments separated from the soma. (A) ACh release along the axon was measured using patch clamp recordings from the myocyte (M) manipulated into contact with the middle axon. The axon was transected with a sharp microelectrode in the vicinity of the cell body. (B) The time dependence of the SSC frequency in recordings from

myocytes normalized to the control frequency before transection. Typically, the SSC frequency dramatically increased immediately after transection, and returned to the baseline level within 15–20 min after transection. Each data point is a mean \pm SEM of nine different experiments. *, significantly different from control values ($P < 0.05$, ANOVA).

transection (Fig. 4). In agreement with previously reported data (Stoop and Poo, 1995), we observed a dramatic increase in the SSC frequency immediately after transection (Fig. 4 B). With time, the frequency of SSC decreased. For a period of 20–30 min after transection, the average frequency of SSCs, determined for 3-min bins, was not significantly different from that recorded at the middle axonal segment before transection (Fig. 4 B).

Taken together, these results strongly suggest that the majority of ACh secretion events along the developing *Xenopus* axons in culture is mediated by the local recycling of ACh-containing vesicles, rather than by constitutive exocytosis of the cell body-derived vesicles.

ACh Release along the Axon Is Calcium-dependent

Endocytic membrane compartments in the neuron can be stained with fluorescent membrane dye FM1-43. Depolarization-induced destaining of the neurons is believed to reflect the Ca^{2+} -dependent fusion of FM1-43-labeled synaptic vesicles with the plasma membrane and release of the dye into the extracellular medium (Betz and Bewick, 1992; Ryan et al., 1993). In agreement with previously published data (Kraszewski et al., 1995; Dai and Peng, 1996a) we found that after incubation with FM1-43, staining of neurites was not uniform. Occasionally individual fluorescent spots could be resolved (Fig. 5). These spots are likely to represent clusters of synaptic vesicles (Kraszewski et al., 1995). Repeated images were acquired while the neuron was superfused with a 60 mM KCl. The average brightness of the spots rapidly decreased with time during superfusion. No destaining of FM1-43-labeled organelles was observed when superfusion with 60 mM KCl was done in the Ca^{2+} -free medium. This result suggests that, similar to the nerve terminal, exocytosis of synaptic vesicles along the axon is Ca^{2+} -dependent.

As a more direct test for the Ca^{2+} dependence of synaptic vesicle exocytosis, we investigated whether ACh release can be induced by the action potential. Electrical stimulation of the neuronal cell body results in evoked

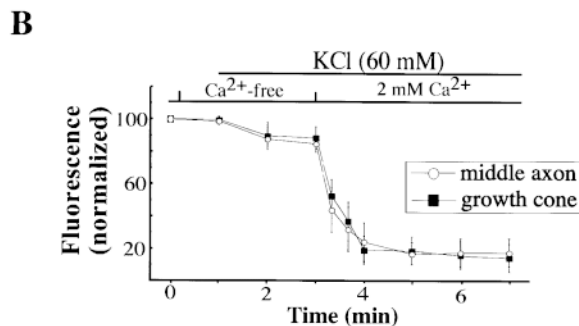
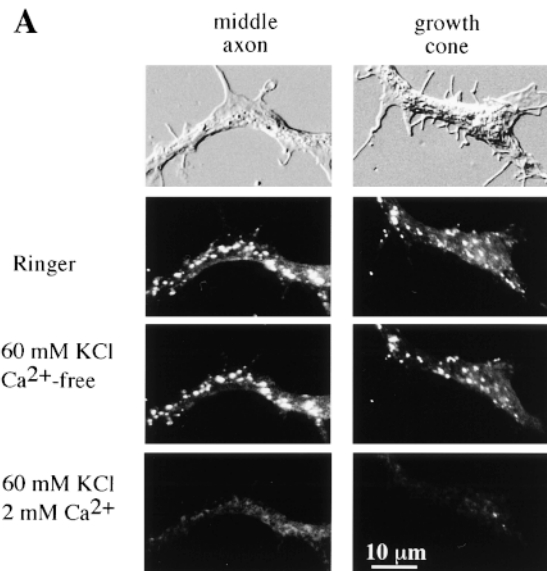


Figure 5. (A) DIC (top) and fluorescence images of *Xenopus* neuron after staining with FM1-43, and after superfusion with 60 mM KCl in Ca^{2+} -free medium followed by superfusion with solution containing 60 mM KCl and 2 mM Ca^{2+} . Staining revealed a characteristic array of fluorescent spots, which are likely to represent clusters of synaptic vesicles. FM1-43-stained organelles were rapidly destained upon KCl-induced depolarization in the medium containing 2 mM Ca^{2+} but not in the Ca^{2+} -free medium. Destaining could be detected both at the middle axonal segment and at the growth cone region. (B) The average brightness of the fluorescent spots during destaining. For each cell the intensity was normalized to that at the onset of depolarization before averaging. Data from 80 fluorescent spots in eight different cells. Destaining at the middle axonal segment (open circles) and at the growth cone region (filled squares) occurred with a similar kinetics. No statistically significant destaining was observed when perfusion medium contained 5 mM EGTA (Ca^{2+} -free medium).

synaptic currents (ESCs) in recordings from the postsynaptic myocyte in *Xenopus* neuromuscular synapses (Fig. 6 A). Action potential-evoked currents reflect simultaneous release of a number of ACh quanta from the nerve terminal. ESCs follow the excitation pulse with a characteristic delay of a few milliseconds (Sun and Poo, 1987). To investigate whether ACh secretion along the axon can be induced by an action potential, we stimulated the neuron at the soma with an extracellular patch electrode. Simultaneously we recorded spontaneous and evoked currents from the myocyte manipulated into contact with the mid-

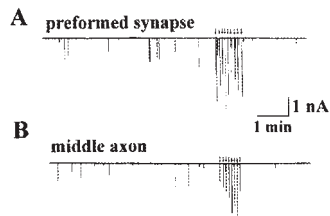


Figure 6. Evoked synaptic currents (ESCs) at different axonal segments. Continuous traces depict the membrane current recorded at the preformed synapse (A) and at the middle axon (B). The neurons were extracellularly stimulated (0.5 ms duration, 0.2 Hz) to generate action potentials. ESCs (arrows) are shown as downward deflections among randomly occurring SSCs.

middle axonal segment. Low frequency electrical stimulation of the neuron consistently elicited ESCs in the manipulated myocyte (Fig. 6 B). The average amplitude of ESCs was 2.3 ± 0.2 nA (mean \pm SEM, $n = 16$) and 1.1 ± 0.1 nA ($n = 14$) at the preformed synapses and the middle axon, respectively (Table I). The somewhat higher ESC amplitude at the preformed synapses may reflect a tighter excitation-secretion coupling, or higher density of docked vesicles at the nerve terminal, as compared with that at the middle axon. However, we noticed that the ESC amplitude at the middle axonal segment seemed to depend on the contact area between axonal plasmalemma and the myocyte (see Evers et al., 1989). To take into account the differences in the contact area between the myocyte and the axon, we calculated the ratio of the average ESCs to the average SSC frequency for each recording. Since both of these parameters are expected to be proportional to the area of contact between the myocyte and the neuron, this ratio may serve as an indicator of the efficacy of the excitation-secretion coupling at different axonal segments. The average ratio ESC amplitude/SSC frequency at the preformed synapses and the middle axon showed no significant difference (Table I), suggesting a similar efficiency of evoked neurotransmitter secretion at the middle axon and at the preformed synapses. This conclusion was further supported by the analysis of the delay of ESC onset (as defined by the time between the end of 0.5-ms stimulus and the onset of ESCs), and the fluctuation of the ESC amplitude (as assessed by calculating the coefficient of variation, or SD/mean, of the ESC amplitude observed in each recording). The delay of ESC onset and the fluctuations of the ESC amplitude are believed to reflect the speed and reliability of evoked neurotransmitter secretion (Sun and Poo, 1987; Wang et al., 1995). No statistically significant differences in these parameters were found between preformed synapses and the middle axon (Table I).

The action potential-induced neurotransmitter release is triggered by the rapid elevation of the cytoplasmic Ca^{2+}

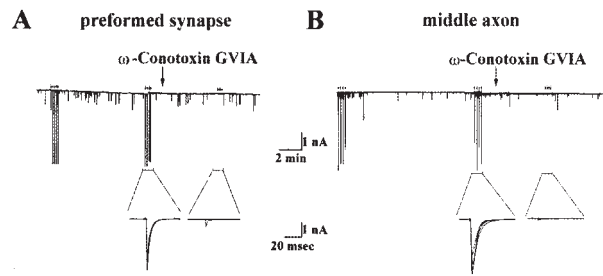


Figure 7. Evoked ACh secretion is inhibited by ω -conotoxin GVIA. Traces are representative examples of the membrane currents recorded from myocytes at the preformed synapses (A) and the middle axonal segment (B). Evoked synaptic currents (small arrows) were elicited by electrical stimulation of the cell body to generate action potentials. Bath application of ω -conotoxin GVIA (1 μ M, large arrow) rapidly inhibited ESCs both at the preformed synapses and at the middle axon. Samples of ESCs before and after ω -conotoxin GVIA application are shown below at a higher resolution.

due to the opening of Ca^{2+} channels (Bennett, 1997). In *Xenopus* spinal cord neurons, evoked neurotransmitter release is mediated primarily by N-type Ca^{2+} channels (Yazefian et al., 1997). Application of a specific blocker of N-type Ca^{2+} channels, ω -conotoxin GVIA, dramatically inhibited evoked neurotransmitter secretion both at the presynaptic nerve terminal and at the middle axonal segment (Fig. 7). Hence, as seen in the presynaptic nerve terminal, the evoked ACh release at the middle axon is mediated largely by N-type Ca^{2+} channels.

The induction of ESCs by electrical stimulation of the neuron suggests that a population of fusion-competent synaptic vesicles is docked at the plasma membrane throughout the axon. To further test this prediction, we applied a pulse of hypertonic solution to neuronal cultures, while continuously recording SSCs at the preformed synapse or at the middle axon (Fig. 8). Application of a hypertonic solution is known to induce an immediate exocytosis of the fusion-competent vesicles at the nerve terminal (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996). The readily releasable pool of quanta defined in this assay appears to be identical to the one drawn upon by action potential-evoked release (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996). We found that a hypertonic solution containing 300 mM sucrose in the culture medium induced a rapid and highly reproducible increase in the frequency of SSCs both at the nerve terminal (Fig. 8 A) and at the middle axon (Fig. 8 B). For a period of 20–60 s

Table I. Characteristics of Spontaneous and Evoked Synaptic Currents at the Preformed Synapses and the Middle Axon

	Average SSC frequency	Average SSC amplitude	Average ESC amplitude	Delay of onset of ESC	Ratio of ESC amplitude/ SSC frequency	Coefficient of variation for ESC
	events/min	pA	pA	ms		
Preformed synapse	$10.4 \pm 0.7^*$	158 ± 12	$2,334 \pm 179^*$	1.96 ± 0.04	266 ± 32	0.52 ± 0.11
Middle axon	$6.2 \pm 0.8^*$	112 ± 9	$1,135 \pm 82^*$	1.89 ± 0.05	286 ± 54	0.68 ± 0.14

Data were obtained by recording membrane currents in the myocytes manipulated into contact with the middle axonal segment of a neuron (Middle axon) and from the spontaneously formed synapses (Preformed synapse). All parameters of SSCs and ESCs were determined for each recording before averaging. In each experiment at least 20 SSCs and five ESCs were analyzed. The data are presented as mean \pm SEM of 15–54 different experiments. The data at the preformed synapses and the middle axonal segments were compared by *t* test. *, $P < 0.05$.

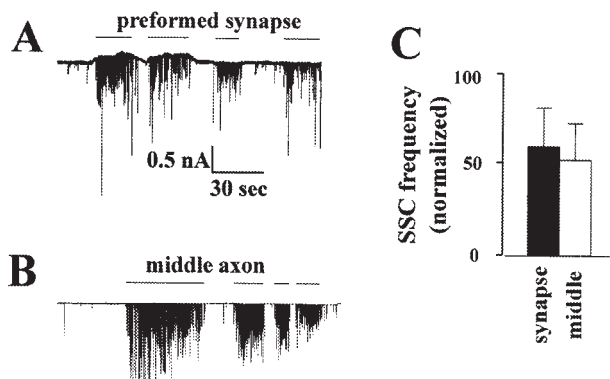


Figure 8. Induction of SSCs by application of hypertonic solution. Current traces are representative examples of whole-cell recordings from myocytes at the preformed synapses (A) and the middle axon (B). Hypertonic solution containing 300 mM sucrose in culture medium was applied to the neurons using a local perfusion system. Superfusion of the neuron with hyperosmotic solution (horizontal black bars) resulted in a rapid increase in the SSC frequency. (C) Normalized frequency of SSCs after application of hypertonic solution to the preformed synapses (solid bar) and the middle axon (open bar). For each recording the average frequency of SSCs during a period of 20–60 s after the onset of sucrose application was normalized to that before the application of hyperosmotic solution. Data are presented as a mean \pm SEM of 10 (preformed synapse) and 14 (middle axon) experiments.

after the onset of sucrose application, the average frequency of SSCs was 526 ± 87 events/min (mean \pm SEM, $n = 10$) and 373 ± 112 events/min ($n = 14$) in recordings from the preformed synapses, and from the middle axon, respectively. These values were ~ 50 -fold higher than that before the application of the hypertonic solution (Fig. 8 C). The increase in the SSC frequency at the preformed synapse induced by the hypertonic solution showed no statistically significant difference compared with the middle axonal segment.

Synaptic Vesicle Recycling and Short-term Plasticity at the Nerve Terminal and along the Axon

Generation of carrier vesicles from the intracellular membrane compartments requires GTP-binding proteins and coats (Rothman and Wieland, 1996; Schekman and Orci, 1996). In many cases, coat assembly is regulated by a small GTP-binding protein ARF1 (Donaldson et al., 1992). To test whether synaptic vesicle recycling at the preformed synapses and at the middle axon is mediated by ARF proteins, we treated neuronal cultures with Brefeldin A (BFA), a specific inhibitor of ARF1-mediated processes. 1 h after the onset of BFA treatment (10 μ g/ml), the frequency of SSCs at the preformed synapses did not change significantly in comparison with control (untreated with BFA) neurons (Fig. 9). Surprisingly, neurotransmitter secretion in the middle segment of neurite was dramatically inhibited. The inhibition of secretory activity by BFA was completely reversible and is unlikely to reflect permanent damage to the neurons.

Brefeldin A treatment induces a collapse of the Golgi complex into ER (Doms et al., 1989; Lippincott-Schwartz et al., 1989; Dascher and Balch, 1994), and rapidly arrests

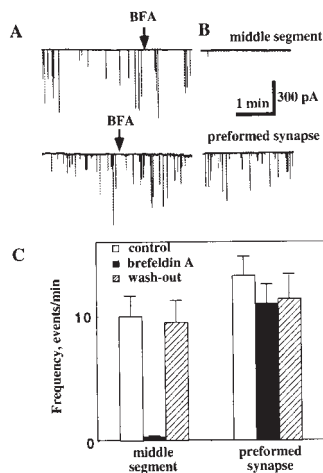


Figure 9. Effect of Brefeldin A (BFA) on quantal ACh release from growing *Xenopus* axons. (A and B) Current traces are examples of whole-cell current recordings from the myocyte manipulated into contact with the middle axonal segment (top traces) or from myocyte at the preformed synapse of *Xenopus* axon (bottom traces) 1 d after cell culture preparation. Arrows indicate the onset of BFA application (10 μ g/ml). After 5 min of recording, the whole-cell pipette was withdrawn

and recording from the same myocyte was performed again 1 h after the onset of BFA application (B). Almost complete inhibition of quantal ACh secretion was observed at the middle axon but not at the nerve terminal after BFA treatment. (C) Quantitative analysis of the effect of BFA on the quantal ACh secretion from the axon. Each bar represents the average of 14–27 series of experiments \pm SEM. About a 30-fold decrease in the SSC frequency was observed at the middle axonal segment 1 h after BFA treatment. The reduction in the SSC frequency found in the spontaneously formed synapses after BFA treatment was not statistically significant ($P > 0.05$, t test). After a 30-min BFA wash out, the frequency of secretion events at various axonal regions was similar to that recorded in control cultures.

axonal growth (Jareb and Banker, 1997; Chang et al., 1998), presumably by blocking the supply of newly synthesized membranes from the trans-Golgi network (Craig et al., 1995). Although we cannot completely exclude the direct contribution of the Golgi-derived vesicles to the ACh secretion along the axon, it appears that SSCs, both at the preformed synapses and along the axon, reflect local recycling of synaptic vesicles. This exoendocytic cycle does not directly depend on the supply of Golgi-derived material (see Figs. 2–4). Additional support for this model is provided by a series of experiments in which we measured the SSC frequency along the distal axonal segments that were transected from the soma. Recordings were started 20 min after the transection and the SSC frequency was constant throughout the period of recording (Fig. 10 and also see Fig. 4). Within 5–10 min after BFA application (10 μ g/ml), the spontaneous neurotransmitter secretion along the axonal fragments was significantly inhibited (Fig. 10). 25 min after the onset of BFA treatment, the frequency of SSCs along the distal axonal fragment dropped to 27% of that at the start of recording (Fig. 10). Since the transected axonal fragments lack the Golgi apparatus, the results strongly suggest an inhibitory action of BFA on local synaptic vesicle recycling along the axon.

To compare the properties of synaptic vesicle recycling along the axon and at the nerve terminal, we used two assays for short-term synaptic plasticity of transmitter release. First, we measured the depression of evoked responses following repetitive high-frequency stimulation of the presynaptic cell. This depression is a characteristic of many synapses and reflects depletion of fusion-competent synaptic vesicles (Zucker, 1996). We compared the rate of

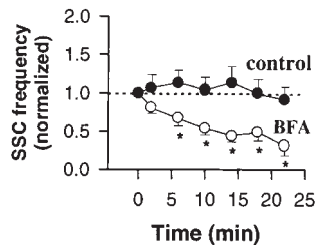


Figure 10. Effect of BFA on ACh does not involve the cell body. The axon was transected with a sharp microelectrode in the vicinity of the cell body as in Fig. 4. Myocytes were manipulated into contact with the middle of axonal fragment 15 min after transection, the time when the SSC frequency returns to control (before transection) values (time 0 on the plot).

Spontaneous neurotransmitter secretion was measured for ~25 min by patch clamp recordings from the myocytes (control). The average SSC frequency was calculated for 3-min intervals and normalized to the SSC frequency at time point 0 (solid circles). Bath application of BFA (10 μ g/ml) 5 min after the start of recording resulted in a decrease in the SSC frequency (open circles). Each bar represents the average of 10 experiments \pm SEM. *, $P < 0.05$, ANOVA.

depression during tetanic stimulation at the preformed synapses to that at the middle axonal segment (Fig. 11). Suprathreshold stimulation applied to the cell body at 5 Hz for 30 s led to an average of $22 \pm 5\%$ ($n = 10$) and $71 \pm 18\%$ ($n = 12$) reduction of the ESC amplitude in the preformed synapse and the middle axon, respectively. Thus, the rate of depression was significantly higher at the middle axon in comparison with the nerve terminal. In the second assay we measured paired pulse facilitation (PPF), the change in the amplitude of ESC when the presynaptic neuron is activated by two successive action potentials. This form of facilitation reflects the enhanced transmitter secretion resulting from the action of residual Ca^{2+} in the presynaptic neuron (Stoop and Poo, 1995; Zucker, 1996). Both at the presynaptic nerve terminal and at the middle axon, a PPF could be observed when the second pulse was applied < 100 ms after the first one. The degree of PPF in recordings from the nerve terminal and the middle axon was similar for all interpulse intervals tested (Fig. 12).

ACh Secretion Can Be Induced by α -Latrotoxin

α -Latrotoxin is a potent stimulator of neurosecretion. Its action is mediated by the binding of the toxin to high-affinity presynaptic receptors (Petrenko et al., 1991; Krasnoperov et al., 1997). An unidentified signaling cascade leads to massive release of neurotransmitter from neurons and neuroendocrine cells (Longenecker et al., 1970; Rosenthal and Meldolesi, 1989). To investigate whether α -latrotoxin elicits ACh release from *Xenopus* neurons,

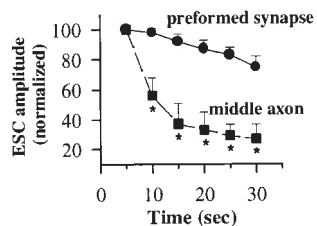


Figure 11. Tetanus-induced depression at the preformed synapses (circles) and along the axonal shaft (squares). Amplitudes of ESCs during a 30-s tetanic stimulation at 5 Hz were averaged in 5-s bins. Each data point (\pm SEM) represents the values averaged for 10 recordings

from preformed synapses and 12 recordings from the middle axon. A significant difference between the two series of experiments was found as soon as 5 s after the onset of stimulation. *, $P < 0.05$, ANOVA.

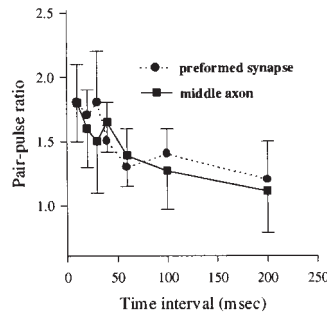


Figure 12. Paired pulse ratio at the preformed synapse (circles) and the middle axon (squares). The ratio of the ESC amplitude induced by the second stimulus to that of the first stimulus is plotted for different interpulse intervals (ranging from 10 to 200 ms). For each neuron the ratio was determined as an average of at least five pairs of ESCs. Data from eight (pre-

formed synapse) and nine (middle axon) experiments were averaged and presented as mean \pm SEM. No statistically significant difference between the PPF was observed between the two series of experiments (ANOVA).

we recorded quantal ACh release from the presynaptic nerve terminal and from the middle axon. Fig. 13 illustrates the result of a typical experiment. At both axonal regions, bath application of α -latrotoxin resulted in a dramatic increase in the SSC frequency (Fig. 13, A and B). 20 min after the onset of α -latrotoxin treatment, the SSC frequency increased ~ 12 fold as compared with the control level of secretion. Potentiation of ACh release followed a similar kinetics at the preformed synapses and at the middle axon (Fig. 13 C).

Distribution of Dynamin, Adaptor Complexes AP-2, AP-3, and ARF in *Xenopus* Neurons

Synaptic vesicle endocytosis at the nerve terminal requires clathrin adaptor complex AP-2 and dynamin (Cremona and De Camilli, 1997). The localization of AP-2 and dynamin in *Xenopus* neurons which were free of contact with other cells was investigated by immunofluorescence using antibodies to these proteins. Immunoreactivity to dynamin and AP-2 was found to have a widespread distribution throughout the axon (Fig. 14). Quantitative analysis of the fluorescence intensity profiles indicated that the intensity of staining was approximately fivefold higher at the growth cone region in comparison with the middle axonal segment.

Inhibition of ACh secretion at the middle axonal segment after Brefeldin A treatment suggests involvement of small GTP-binding protein ARF in synaptic vesicle recycling. Previous reconstitution studies demonstrated that in addition to ARF, synaptic vesicle generation from endosomes requires AP-3 coat complex (Faundez et al., 1998). In *Xenopus* neurites immunoreactivity for ARF and AP-3 was detected both at the growth cone and along the axon (Fig. 14). The intensity of immunofluorescence staining was very close to uniform throughout the whole axonal length.

Discussion

In mature neurons, synaptic vesicle exocytosis is restricted to the active zones of the presynaptic nerve terminals (Burns and Augustine, 1995; Cremona and De Camilli, 1997). However, in developing *Xenopus* neurons, quantal

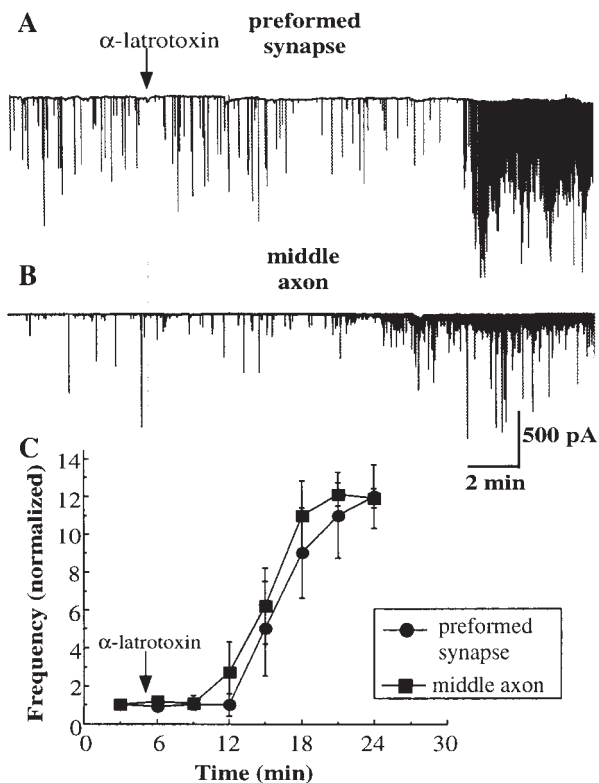


Figure 13. α -Latrotoxin elicits massive neurotransmitter release from *Xenopus* neurons. (A and B). Representative examples of membrane currents recorded from myocytes at a preformed synapse (A) and from the myocyte manipulated into contact with the middle axon (B). α -Latrotoxin (1 nM, arrow) was applied \sim 5 min after the onset of recording. SSC frequency increased with a characteristic delay of \sim 10 min. (C) Quantitative analysis of the effect of α -latrotoxin on neurotransmitter secretion at the preformed synapses (circles) and at the middle axon (squares). Changes in the frequency of the current events with time after the onset of α -latrotoxin application, normalized to the values at the beginning of the recording. Data from five recordings at preformed synapses and five recordings at the middle axon were normalized for each cell before averaging.

ACh secretion can be detected throughout the entire axonal surface. In this study we compared and contrasted the functional properties of neurotransmitter secretion at the nerve terminal and at the middle axonal segment of neurites.

Spontaneous ACh Release along Developing Neurites

Characteristically rapid membrane currents (SSCs) were detected both in recordings from the nerve terminal and from the middle axon. The individual SSCs reflected a simultaneous release of ACh packets from the neuron (Dan and Poo, 1992; Sakmann, 1992). The average number of ACh molecules in the packet can be roughly estimated from the average amplitude of SSCs (110–160 pA, Table I); this number is $\sim 10^3$. Although it is hard to completely exclude the possibility that the quantal ACh release that we observed might be mediated by the activity of a plasma membrane ACh transporter (Falk-Vairant et al., 1996),

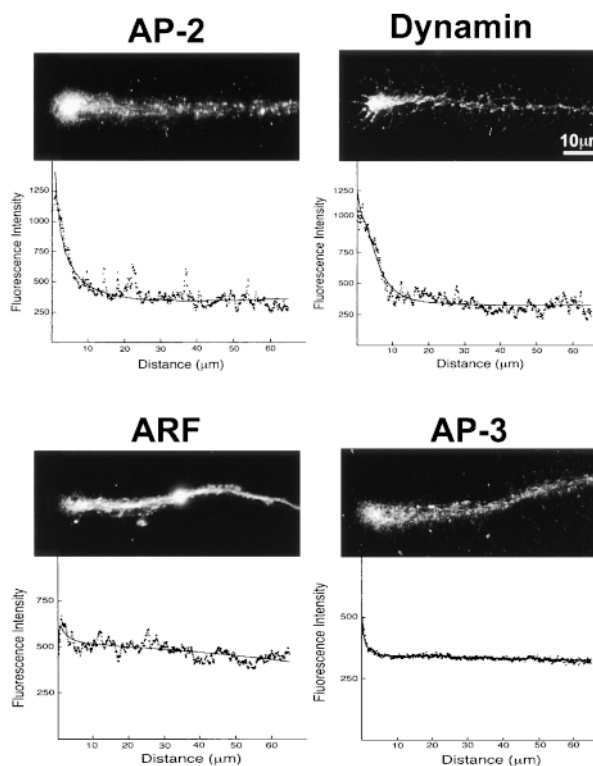


Figure 14. Distribution of dynamin, AP-2, AP-3, and ARF along the neurites. Neurons were fixed, detergent-permeabilized, and then stained with antibodies to dynamin-1, α subunit of AP-2, σ subunit of AP-3, and ARF1. Immunoreactivity recognized by all four antibodies was present throughout the whole axonal surface and had a finely punctate appearance. In the case of dynamin and AP-2, immunoreactivity was concentrated at the growth cone region. Immunoreactivity to AP-3 and ARF was more uniformly distributed throughout the axon. For quantitative analysis of fluorescence intensity distribution, intensity profiles of the axons were created from immunofluorescence images. The sampling areas were chosen along the axon and the average intensity of fluorescence in these areas (arbitrary units) was plotted as a function of distance from the growth cone. For each of the fluorescence profiles, the data from at least 25 different neurons were pooled together.

the overwhelming amount of evidence suggests that in the majority of cases, including developing *Xenopus* neuromuscular synapses (Girod et al., 1995), quantal neurotransmitter release is mediated by the exocytosis of neurotransmitter-filled vesicles.

The analysis of the secretory activity in growing neurites performed in this study is based on the assumption that myocytes do not directly elicit ACh release from neurons. It is possible that the myocytes manipulated into contact with the axon rapidly stimulate the local assembly of the secretory machinery at the site of contact between the two cells and, therefore, serve as inducers of exocytic activity. A few lines of evidence argue against this model: (a) SSCs in recordings from the middle axon could be detected without any measurable delay after the manipulation of the myocyte into contact with the axon (Fig. 1 B) (see also Evers et al., 1989); (b) the frequency of SSCs remained at

the same level during a period of recording (Fig. 1 C); (c) constitutive membrane recycling along *Xenopus* neurites which are free of contact with other cells can be visualized with FM1-43 dye (Fig. 5) (Dai and Peng, 1996a). Taken together, these data suggest that constitutive ACh release occurs in naive neurites. It should be noted that we cannot completely exclude the possibility that manipulation of a myocyte into the contact with an axon rapidly potentiates neurotransmitter release from the nerve cells. This induction, however, should take place on a time scale of a few seconds, a possibility that seems unlikely.

In conditions where the activity of the axonal transport system is severely impaired by disruption of axonal microtubules (Figs. 2 and 3), quantal ACh secretion along the axon can still be observed. Moreover, ACh secretion persists in axonal fragments which are separated from the cell body (Fig. 4). Therefore, the majority of exocytic events that we detect is mediated by vesicles which recycle locally within the axon. This notion is also indirectly supported by the striking similarities of the electrophysiological parameters of neurotransmitter secretion at various axonal regions (see below).

Properties of Quantal Neurotransmitter Secretion at the Presynaptic Nerve Terminal and along the Axon

Quantitative comparison of the parameters of SSCs and ESCs at the preformed synapses and along the axonal shaft yields insights into the mechanisms of synaptic vesicle recycling at different axonal regions. We measured the frequency of SSCs, the average SSC amplitude, and the parameters of action potential-evoked ACh secretion (Table I). In all parameters examined, ACh secretion from the middle axon showed little or no difference compared with that at the presynaptic nerve terminal in preformed synapses. These results suggest that regardless of their location, synaptic vesicles are likely to contain the basic complement of proteins required for synaptic vesicle docking, fusion, and neurotransmitter accumulation (Sudhof, 1995). It is particularly intriguing that the secretion of ACh along the axon could be triggered by both the action potential and by hypertonic solution, suggesting the existence of a pool of fusion-competent vesicles docked at the plasma membrane throughout the axon. Moreover, similar coefficients of variation of ESCs, a delay in the onset of ESCs, an inhibition of ESCs by ω -conotoxin GVIA, and induction of the massive ACh release by α -latrotoxin all point to the similar properties of excitation-secretion coupling at the nerve terminal and along the axon.

In recordings from myocytes manipulated into contact with the middle axon, individual secretory events were integrated over relatively large region (~ 5 – $10 \mu\text{m}$) along the axon. Previously reported data (Kraszewski et al., 1995; Dai and Peng, 1996a) and our experiments with FM1-43 (Fig. 5) hint to the possibility that synaptic vesicles in naive neurites are grouped in clusters with a characteristic spacing of a few micrometers. The limited spatial resolution of our electrophysiological technique does not allow to determine whether ACh secretion events are restricted to this cluster or, rather, are uniformly distributed along the axon.

An indication of how the secretory apparatus along the

axon might differ from that at the nerve terminal comes from the analysis of the synaptic response to tetanic stimulation. Under high-frequency repetitive stimulation, developing neuromuscular synapses exhibit a reduction in the amplitude of action potential-evoked responses, which reflects the depletion of fusion-competent vesicular pool. This depletion is partially balanced by the mobilization of vesicles from reserve pools to docking sites, and/or by the priming of docked vesicles (Zucker, 1996). Synaptic depression in response to tetanic stimulation was much more pronounced at the middle axon in comparison to the nerve terminal. Therefore, the nerve terminal is likely to develop a mechanism for the rapid replenishment of the fusion-competent vesicles in the nerve terminal, which is essential for the fidelity of synaptic transmission during the high-frequency excitation of the presynaptic cell.

Interestingly, neurotransmitter secretion along the axon (but not at the nerve terminal) was drastically reduced by the specific inhibitor of ARF-mediated processes, BFA. Members of the ARF family may participate in clathrin-coated vesicle formation from the plasma membrane (D'Souza-Schorey et al., 1995). However, in *Xenopus* neurons recycling of synaptic vesicles at the nerve terminal was not affected by BFA (Fig. 9). This result is consistent with the model in which synaptic vesicles at the nerve terminal form from the plasma membrane in a single budding step involving clathrin and dynamin, but not ARF. The vesicles do not communicate with endosomal compartments; instead, they are ready to reenter synaptic vesicle pool immediately after dissociation of clathrin coat (Takei et al., 1996). Our data suggest that in naive neurons recycling of synaptic vesicles along the axon uses ARF, consistent with the model for synaptic vesicle formation proposed by R. Kelly (University of California, San Francisco, CA) (Faundez et al., 1997, 1998). According to this model, ARF1 and AP-3 coat complex are involved in formation of synaptic vesicles from endosomes, whereas formation of synaptic vesicles from the plasma membrane is ARF independent. It remains to be elucidated whether differential mechanisms of synaptic vesicle recycling at the nerve terminal and along the axon are related to the experimentally observed differences in responses to tetanic stimulation.

Implications for the Assembly of the Secretory Apparatus at the Nerve Terminal

Although crucial to our understanding of interneuronal communication, the mechanism of synaptic vesicle assembly is poorly understood. Based on the similarity of molecular components, it has been suggested that the synaptic vesicle recycling pathway is ontogenically related to the constitutive endosomal membrane recycling pathway found in neuronal as well as in nonneuronal cells (Matteoli et al., 1992; Bennett and Scheller, 1993). It has been hypothesized that the vesicular recycling observed along developing axons in culture is a manifestation of the constitutive housekeeping function of endosomal membrane recycling (Matteoli et al., 1992). The exact molecular composition of these vesicles remains unknown. Our data indicate that constitutive membrane recycling along growing neurites is likely to be accompanied by quantal neurotransmitter secretion. The vesicles involved in neurotrans-

mitter secretion along the axon appear to be surprisingly similar to the genuine synaptic vesicles at the nerve terminal. These results are consistent with a scenario in which the whole axon formed by a naive neuron can be considered as a giant precursor of the nerve terminal. Each site is equally competent for the spontaneous and action potential-evoked neurotransmitter secretion and the assembly of the rudimentary secretory apparatus in naive neurons does not require any interaction with a postsynaptic target.

Synaptic development involves the interaction between pre- and postsynaptic cells, and a chain of protein-protein interactions leading to the differentiation of both pre- and postsynaptic membranes (Dai and Peng, 1996b; Sheng and Kim, 1996; Rao and Craig, 1997). Neurotransmitter secretion from the presynaptic neuron is crucial for synaptic maturation and proper development of neuronal circuits (Thoenen, 1995; Bonhoeffer, 1996; Rao and Craig, 1997). Our results corroborate that the capability for neurotransmitter secretion already exist in naive neurites free of contact with other cells. It is tempting to speculate that constitutive neurotransmitter secretion along growing nerve processes may play a role in the establishment of synaptic contacts in the developing nervous system.

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Dr. Chang's name was accidentally misspelled in the first footnote in the paper by Zakharenko et al. The footnote should read as follows:

S. Zakharenko and S. Chang contributed equally to this work.
