Presynaptic Differentiation Induced in Cultured Neurons by Local Application of Basic Fibroblast Growth Factor

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Recent studies have suggested a role for molecules residing at the muscle surface in signaling presynaptic development at the neuromuscular junction (NMJ). Since heparan sulfate-proteoglycan is a major component of the extracellular matrix of skeletal muscle, factors that are bound to this proteoglycan, such as basic fibroblast growth factor (bFGF), are in a strategic position for neuronal signaling. To test this idea, we applied bFGF to cultured *Xenopus* spinal cord neurons and monitored the change in intracellular Ca^{2+} level with fura-2 ratio imaging. In one-third of the neurons, local application of bFGF elicited a 30–140% increase in intracellular Ca^{2+} level. Ca^{2+} -free medium or suramin abolished this change. Latex beads coated with bFGF induced clustering of synaptic vesicles at the bead-neurite contacts as evidenced by anti-synaptotagmin antibody labeling and electron microscopy. This response was also blocked by Ca^{2+} -free medium and by suramin. Uncoated beads or beads coated with PDGF were ineffective. This induction was also inhibited by a tyrosine kinase inhibitor, tyrphostin RG-50864, suggesting the role of receptor tyrosine kinase activation in this process. In addition, bFGF-coated beads also induced the localization of depolarization-dependent Ca^{2+} influx to the bead-neurite contacts. In contrast, depolarization caused a distributed Ca^{2+} elevation in untreated neurites. These results suggest that local presentation of bFGF can mimic the muscle target in signaling the development of both a cytoplasmic and a membranous specialization for excitation–secretion coupling observed at the NMJ.

[Key words: neuromuscular junction, basic fibroblast growth factor (bFGF), calcium, synaptic vesicles, *Xenopus*]

During the formation of the neuromuscular junction (NMJ), the contact between nerve and muscle induces the development of synaptic specializations. This is manifested by the clustering of ACh receptors (AChRs) on the postsynaptic side and the clustering of synaptic vesicles (SVs) and voltage-gated calcium channels on the presynaptic side (Hall and Sanes, 1993). Thus,

the nerve–muscle interaction leads to the establishment of a spatial coupling between the transmitter release and detection mechanisms, which is essential for the neurotransmission at this fast synapse. Extensive studies have provided a detailed understanding of the process of postsynaptic development (reviewed in Hall and Sanes, 1993). In contrast, our understanding of the cellular and molecular mechanisms of presynaptic development is rather limited.

Previous studies have suggested that the inductive signal for presynaptic differentiation originates, at least in part, from the target (Dan and Poo, 1994). When a muscle cell is manipulated into contact with a spinal cord neurite, a rapid onset of transmitter release can be elicited (Xie and Poo, 1986). However, inappropriate contact, such as that formed between two motoneurons or between a neuron and a glass bead, does not elicit such response. We have recently shown that this manipulated nerve–muscle contact also induces an elevation in the presynaptic Ca^{2+} level and this increase is causal to the onset of the transmitter release (Dai and Peng, 1993). Neuron–neuron contact, on the other hand, does not elicit this Ca^{2+} signal, thus suggesting the specificity of muscle-derived signals in triggering presynaptic development. In addition to manipulated nerve–muscle contacts, an elevation in presynaptic Ca^{2+} has also been observed at spontaneous nerve–muscle contacts (Dai and Peng, 1993; Zoran et al., 1993).

The immediate onset of transmitter release and presynaptic Ca^{2+} rise induced by target contact suggests that presynaptic development is triggered by molecules residing on the surface of the muscle cell. This is supported by the observation that muscle membrane extract can also cause a significant increase in neuritic Ca^{2+} in cultured *Helisoma* neurons (Zoran et al., 1993). Furthermore, regenerating motoneurons can form presynaptic specializations on synaptic basal lamina in the absence of muscle (Sanes et al., 1978). These studies indicate that the cue for presynaptic development probably resides within the extracellular matrix (ECM) of the skeletal muscle. Heparan-sulfate proteoglycan (HSPG) is a prominent ECM molecule of skeletal muscle (Anderson and Fambrough, 1983; Anderson et al., 1984; Swenarchuk et al., 1990). Recent studies have shown that one of the major functions of HSPG is to serve as a storage site for heparin-binding growth factors (HBGFs) (Ruoslahti, 1989; Klagsbrun and Baird, 1991; Ruoslahti and Yamaguchi, 1991). The role of these peptide growth factors in synaptogenesis is suggested by our recent results that local application of basic fibroblast growth factor (bFGF), a HBGF that is synthesized by a variety of mesoderm-derived cell types, induces the formation of acetylcholine receptor (AChR) clustering in cultured muscle

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cells (Peng et al., 1991a). Since these HSPG-bound factors can conceivably be presented to the motoneuron growth cone as it makes initial contact with the muscle cell, they seem to be ideal candidates for target-derived molecules that can effect presynaptic development.

In this study, we tested this hypothesis by presenting bFGF-coated beads to cultured *Xenopus* spinal cord neurons and examining the changes at bead–neurite contacts with regard to presynaptic differentiation. We report here that these beads induce the clustering of SVs and an accumulation of a Ca^{2+} influx machinery at their contacts with neurites.

Materials and Methods

Materials. Recombinant human bFGF was a kind gift of Synergen (Boulder, CO). PDGF was from Upstate Biotechnology, Inc. (Lake Placid, NY). Fura-2/AM was from Molecular Probes (Eugene, OR). Monoclonal antibody to synaptotagmin was a kind gift of Dr. Richard Burry (Ohio State University, Columbus, OH). FITC-conjugated goat-anti-mouse antibody was from Organo Technika (Treyburn, NC). Suramin was from FBA Pharmaceuticals (New York, NY). Tyrophostin RG50864 was a kind gift of Rhone-Poulenc Rorer (Horsham, PA). Polystyrene latex beads were from Polysciences (Warrington, PA).

Cell culture. Neurons were isolated from the neural tubes of stage 20–21 *Xenopus* embryos according to published methods (Peng et al., 1991b). They were plated on clean cover glass and cultured in Steinberg medium (60 mM NaCl, 0.7 mM KCl, 0.4 mM $\text{Ca}(\text{NO}_3)_2$, 0.8 mM MgSO_4 , 10 mM HEPES, pH 7.4, supplemented with 10% L-15 medium, 1% fetal bovine serum and 0.1 mg/ml gentamicin). The cultures were first maintained at 22°C overnight and then transferred into a 15°C incubator. They were used within 3 d after plating.

Induction of presynaptic specializations by beads. Polystyrene 10 μm beads were washed with 95% ethanol for 30 min and then rinsed with phosphate buffered saline (PBS) by centrifugation with a microfuge and resuspension. They were then incubated with a solution containing 100 $\mu\text{g}/\text{ml}$ recombinant human bFGF for 2 hr, followed by two rinses. Labeling these coated beads with an antibody against bFGF followed by a fluorescent secondary antibody showed clearly the presence of bFGF on the bead surface. Beads were then applied to cultured neurons which already exhibited neuritic outgrowth. Control uncoated beads were also used in this study. For these beads, care was taken to prevent contaminating their surface with proteinaceous substance after the ethanol wash. Uncoated beads also adhered tightly to cultured neurons. In addition, beads coated with PDGF were also used as controls. These beads were coated with PDGF at concentrations similar to bFGF.

Immunocytochemistry. Cultures on cover glass were fixed with 4% paraformaldehyde in PBS, permeabilized by 0.25% saponin and blocked with PBS containing 1.5% bovine serum albumin. They were labeled with the antibody against synaptotagmin for 1 hr, washed, and then labeled with FITC-conjugated goat anti-mouse secondary antibody for 30 min. They were mounted in a polyvinyl alcohol-based mounting medium containing *n*-propyl-gallate as an anti-bleaching agent.

Electron microscopy. Neural tubes were sliced into small pieces and plated into polylysine-coated 35 mm Lux Permanox plastic tissue culture dishes (Nunc, Naperville, IL). Within 2 d, neurites could be detected to emanate from these explants. bFGF-coated beads were then applied. Three days after bead addition, the cultures were fixed with 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), postfixed with 1% OsO_4 , stained with 1% uranyl acetate, dehydrated through an ethanol series and embedded in epoxy resin. After removal of the sample block from the culture dish, bead-bearing explants were selected by scoring the desired area of the block with a diamond marker, sawed off the block and thin-sectioned with a diamond knife. The sections were poststained with uranyl acetate and lead citrate and examined with a JEOL 200CX transmission electron microscope.

Calcium measurements. Neurons were incubated in medium containing 10 μM fura-2/AM for 30 min at room temperature. Just prior to optical measurement, the culture medium was replaced with Ringer's solution (120 mM NaCl, 2.5 mM KCl, 10 mM CaCl_2 , 10 mM HEPES, pH 7.4). Fluorescence images were obtained on a Zeiss IM-35 inverted microscope equipped with a Cohu SIT video camera and a Metaltek filter wheel with an electronic shutter. Images were acquired and processed with Image-1 software (Universal Imaging, West Chester, PA). Fura-2 loaded cells were examined through a Nikon 40 $\times$ fluor objective

(N.A. 1.3) at excitation wavelengths of 340 and 380 nm. Images were frame-averaged 16 times, background-subtracted, and stored on optical disk. The fluorescence ratios of the area of interest were calculated on line according to a stored calibration scheme predetermined with Ca^{2+} standards.

To stimulate the neuron, suprathreshold stimuli were delivered to the cell body of neurons via a pipette with a 5 μm heat-polished tip. The stimuli consisted of square voltage pulses, 0.5 msec in duration and 2–6 V in amplitude, generated by a Digitimer isolated stimulator (model D52).

Results

Induction of SV clustering by bFGF-coated beads

To visualize the distribution of SVs, we labeled, fixed and permeabilized cultures with an antibody against synaptotagmin (Matthew et al., 1981; Burry et al., 1986; Peng et al., 1987) followed by a fluorescently conjugated secondary antibody. As shown in Figure 1, *A* and *B*, synaptotagmin was present along the length of the neurite which was not in contact with any target. Using bFGF-coated beads as experimental targets, we examined the redistribution of SVs in response to their stimulation. As shown in Figure 1, *C* and *D* (arrowheads), SVs became more concentrated at bead–neurite contact sites after 1 d. This SV labeling was intensified after 2–3 d of bead contact (Fig. 1*E–H*). From observations made on 45 neurons, we calculated that about 85% of 2–3 d old contact sites were associated with intense synaptotagmin labeling (see Fig. 4).

To confirm that the localization of synaptotagmin indeed represented clustering of SVs at bead–neurite contacts, electron microscopy was conducted to visualize their ultrastructure. As shown in Figure 2, these micrographs revealed that beads established intimate contact with the neuritic membrane and 50–60 nm clear vesicles were clustered at these sites. Large dense-core vesicles (90–100 nm) were also observed in the vicinity of contacts but, unlike small clear vesicles, were not clustered (Fig. 2, *DV*). These features are strikingly similar to the presynaptic nerve terminal at the NMJ (Peng et al., 1987; Matteoli et al., 1988).

To test the specificity of bFGF-coated beads in inducing SV clustering, we also treated cultures with uncoated beads. As shown Figure 3, *A* and *B*, no synaptotagmin concentration could be detected at these bead–neurite contacts. To study whether the difference in efficacy between these two kinds of beads was due to adhesive strength, we compared the attachment of beads to neurites under a displacement force. Within 5 min of the establishment of bead–neurite contacts, a stream of culture medium was applied to the contact site from a micropipette (5 μm tip diameter) driven by 3 psi pressure from a pressure ejector. As the pipette was moved slowly toward the bead–neurite contact, most of the beads associated with the neurite could be blown away without the displacement of the neurite. In a smaller number of cases, the beads adhered strongly in such a way that its displacement also resulted in detachment of the neurite from the substratum. The percentage of beads that could not be removed without detachment of the neurite was found to be almost the same for bFGF and uncoated beads (25% out of 63 bFGF beads on seven neurites versus 31% of 58 uncoated beads on seven neurites). This shows that stickiness alone can not account for the SV clustering effect of bFGF beads.

bFGF is a highly basic molecule with pI 9.6. Previous studies have shown that artificial polybasic peptides such as polylysine can induce SV accumulation when they are applied by beads (Burry, 1980; Peng et al., 1987). To test whether this effect is due to its charge alone, we studied the effect of beads

Figure 1. Localization of synaptotagmin within spinal-cord neurites at the contacts with bFGF-coated beads. *Left column*, phase-contrast images; *right column*, fluorescence images of synaptotagmin distribution. *A and B*, Untreated neuron. Synaptotagmin was diffusely distributed along the neurite. *C and D*, 1 d bead–neurite contact. Localization of synaptotagmin at bead contacts (*arrowheads*) was detectable. *E and F*, 2 d bead–neurite contacts; *G and H*, 3 d bead–neurite contacts. Synaptotagmin became highly concentrated at these contacts. In contrast, the bead-free portion of the neurite was less prominent in synaptotagmin staining (*H*).

coated with PDGF, a highly basic peptide growth factor with pI 9.8. As shown in Figure 3, *C* and *D*, PDGF-coated beads were ineffective in inducing synaptotagmin localization. Thus, charge alone cannot explain the clustering effect of bFGF beads.

Quantitation of these results (Fig. 4) showed that SV clusters

were specifically induced by local bead-mediated presentation of bFGF. To test whether this clustering effect was mediated by a ligand–receptor interaction, beads were applied to cultured neurons in the presence of suramin, a polyanion which interferes with the interaction of bFGF and other growth factors with their receptors (Betsholtz et al., 1986; Yaron and Klags-

Figure 2. Clustering of SVs induced by bFGF-coated beads. These electron micrographs show the clustering of 50–60 nm clear vesicles (arrowheads) within the neurite at contact sites with beads (**B**). Large dense-core vesicles (**DV**) were not clustered.

brun, 1990; Corfas et al., 1993). As shown in Figure 3, *E* and *F*, and Figure 4, suramin at 100 μ M completely inhibited the localization of synaptotagmin at neuritic contacts with bFGF-coated beads.

Western blot analyses of spinal cords isolated from *Xenopus* embryos at stage 20–24 (during which the neurons were isolated for culturing) with a monoclonal bFGF receptor antibody showed a band at 110 kDa (data not shown). This shows that these neurons are competent in bFGF-mediated signaling. Since bFGF receptor is a tyrosine kinase (Lee et al., 1989; Johnson et

al., 1990), we tested whether the bead-induced presynaptic development is dependent on the kinase activation. A tyrosine kinase inhibitor, tyrphostin RG-50864 which has been shown to interfere with several receptor tyrosine kinases (Lyll et al., 1989; Levitzki, 1992) was used in this study. We found that at a concentration of 80 μ M, this inhibitor completely blocked the effect of bFGF bead-induced localization of synaptotagmin labeling (Fig. 3*I,J*). However, we could not detect a concentration of bFGF receptors at bead–neurite contacts by immunofluorescence.

The role of calcium in bead-induced presynaptic development

To understand whether bFGF can mimic the muscle cell in eliciting an elevation in presynaptic Ca^{2+} level, we measured the neuronal Ca^{2+} concentration in response to bFGF application. Neurons were loaded with fura-2AM and bFGF was locally applied to the growth cone region with a micropipette containing 1 $\mu\text{g}/\text{ml}$ ligand by a pressure ejector. As shown in Figure 5A, the growth cone responded to the bFGF puff by transiently elevating its Ca^{2+} level by 40 nM. In addition, the Ca^{2+} level in the soma also showed a smaller elevation due to the diffusion of bFGF from the pipette to that region (Fig. 5B). Significant Ca^{2+} response ($p < 0.05$), based on measurements before and after bFGF application, was observed in 30% of the spinal cord neurons tested (11 out of 34). In the responsive neurons, a mean Ca^{2+} elevation of 30 nM was observed (see Fig. 8, first bar). These figures are similar to the response of neurons to manipulated muscle contact as previously described (Dai and Peng, 1993). In that study, it was found that 30% of the *Xenopus* neurons responded to muscle contact by an increase in intracellular Ca^{2+} level and transmitter release, probably reflecting the percentage of cholinergic neurons in this culture. When all neurons, including both responsive and nonresponsive ones, were scored together, a significant elevation in intracellular Ca^{2+} level over the resting level was still observed (see Fig. 8, second bar). In contrast, no neurons showed any response to puffs of Ringer's solution without bFGF to the growth cone (see Fig. 8). Thus, this elevation is not due to mechanical stimulation associated with ligand application. This Ca^{2+} transient was abolished in Ca^{2+} -free solution or in solution containing 200 μM suramin (see Fig. 8), suggesting that the activation of bFGF receptor is responsible for this response.

To understand whether this Ca^{2+} signal is essential for the development of the presynaptic development, we conducted bead experiments in Ca^{2+} -free solution. As shown in Figure 3, *G* and *H*, and in Figure 4, the bead-induced synaptotagmin accumulation was abolished under this condition. This shows the Ca^{2+} dependency of the development of the presynaptic specialization.

Calcium elevation induced by bFGF-coated beads

In addition to the clustering of SVs, the contact of muscle target also induces presynaptic localization of depolarization-induced Ca^{2+} influx mechanism at the contact area (Funte and Haydon, 1993). To understand whether bFGF-coated beads can also mimic the muscle target in inducing this kind of membrane specialization, we examined the Ca^{2+} level of neurites before and after bead contacts. In bead-free neurons, the Ca^{2+} level was uniform throughout the soma and the neurite in the resting state (Fig. 6A). When the neuron was electrically stimulated at the soma, the Ca^{2+} level became elevated there as well as along the neurite (Fig. 6B). Results are quantified in Figure 9. Although the Ca^{2+} level was uniform in the resting state (Fig. 9A), the stimulus-induced elevation was more pronounced at sites closest to the soma, where Ca^{2+} rose to 250% of the resting level, than the distal region of the neurite, where a 65% increase was seen (Fig. 9B,C).

Figure 4. Quantitation of synaptotagmin localization at bead-neurite contacts. While bFGF-coated beads were effective in inducing this presynaptic specialization, uncoated beads or PDGF-coated beads were not. The efficacy of bFGF beads was abolished by Ca^{2+} -free solution or by suramin (100 μM). The error bars denote SEs. Number of neurons scored in each case is noted above the bars.

In neurons that were contacted by bFGF-coated beads for 2–3 d, an elevation in resting neuritic Ca^{2+} level was detected at sites of contact in a fraction of the neurons. In neurons that responded to beads, the Ca^{2+} level at the beads reached a mean level that was 60% higher than the bead-free area (Fig. 10). When the soma of these neuron was electrically stimulated, a much higher elevation in Ca^{2+} was detected at bead-neurite contacts than the bead-free area (Fig. 7). As quantified in Figure 10, the bead contacts exhibited a mean elevation of Ca^{2+} about twice as high as the bead-free area of the neurites. This elevation was observed irrespective of the position of the beads along the neurite.

These data show that the depolarization-induced Ca^{2+} influx mechanism is uniformly distributed along the neurite in the absence of stimuli for presynaptic development. bFGF-coated beads induce a concentration of this mechanism to the site of bead-neurite contact, where the clustering of SVs also takes place as shown above.

Discussion

In this study, we have shown that bFGF-coated beads are effective stimuli for inducing presynaptic differentiation on cultured spinal cord neurons. They can mimic the muscle target to induce the formation of a cytoplasmic specialization as manifested by the clustering of SVs and a membrane specialization as manifested by the localization of a voltage-sensitive Ca^{2+} influx mechanism. These two types of specializations form the basis for the rapid, depolarization-dependent transmitter release at mature NMJs. Thus, the signal conveyed by these beads is comprehensive enough for the development of a full-range of specializations for presynaptic functions. Except for the stimulus-induced elevation in presynaptic Ca^{2+} level at the bead-neurite contacts, the data presented here did not address the releasability

Figure 3. Lack of synaptotagmin localization under various experimental conditions. *A* and *B*, Uncoated beads; *C* and *D*, PDGF-coated beads; *E* and *F*, bFGF-coated beads in the presence of 100 μM suramin; *G* and *H*, bFGF-coated beads in Ca^{2+} -free medium; *I* and *J*, bFGF-coated beads in the presence of 80 μM tyrphostin RG-50864 (a tyrosine kinase inhibitor). Arrowheads point to bead-neurite contacts.

Figure 5 (left panels). Elevation of intracellular Ca^{2+} level induced by bFGF. bFGF was applied via a micropipette positioned about 10 μm from the growth cone. The ligand was targeted to the growth cone, although diffusion also carried it to the soma area. *A*, Fura-2 ratio images taken at intervals of 30 sec (*a–j*). bFGF was ejected from the pipette immediately before image *c* was taken and the application was stopped after image *f* was taken. The pseudocolor scale shows Ca^{2+} concentration (nM). *B*, Quantitation of Ca^{2+} level in the growth cone and in the soma.

Figure 6. (right upper panel) Ca^{2+} elevation in a bead-free neuron. *A*, Resting state; *B*, after electrical stimulation of the soma. The stimulation-induced elevation in Ca^{2+} level was most pronounced in the proximal portion of the neurite.

Figure 7. (right lower panels) Elevation in neuritic Ca^{2+} at contacts with bFGF-coated beads. A bead-laden neuron is shown in phase contrast in *A*. The area containing the bead–neurite contacts is enlarged and shown in *B* and *C*. In the resting state (*B*), the Ca^{2+} level was slightly elevated

Figure 8. Quantitation of the elevation in growth cone Ca^{2+} level induced by bFGF. The neurons were bathed in Ringer's solution containing 10 mM Ca^{2+} during the experiment. The first bar is the result of bFGF-responsive neurons only. The second bar summarizes results from all 34 neurons measured. Application of Ringer's alone without bFGF did not induce Ca^{2+} elevation (third bar). The effect of bFGF was abolished if neurons were incubated in Ca^{2+} -free Ringer's (fourth bar) or in Ringer's containing 200 μM suramin (fifth bar). Error bars are SEs. Number of neurons tested is indicated above each bar. Significant differences ($p < 0.01$) exist between second and third, second and fourth, or second and fifth bars.

of the SVs clustered at those sites. However, our more recent studies with the fluorescent vesicular probe FM1-43 (Betz and Bewick, 1992) have shown that SVs clustered at contact sites with bFGF-coated beads can indeed undergo depolarization-dependent exocytosis and recycling (Dai and Peng, 1994). Thus, the presynaptic specialization formed on these artificial targets is indeed functional.

By assaying transmitter release or increase in intracellular Ca^{2+} accompanying manipulated or spontaneous nerve-muscle contact *in vitro*, previous studies have demonstrated a role for muscle in the establishment of the functional NMJ (Xie and Poo, 1986; Dai and Peng, 1993; Funte and Haydon, 1993; Zoran et al., 1993). Furthermore, it was found that molecules associated with the muscle membrane can induce these early presynaptic changes (Zoran et al., 1993). Since bFGF was previously shown to be bound to the surface of skeletal muscle cells (DiMario et al., 1989; Gonzalez et al., 1990), the current work thus further supports the role of target surface-bound molecules in the development of presynaptic specializations (Dan and Poo, 1994).

Since bFGF is a highly basic molecule, it is conceivable that its effect is mediated by the positive charge. Indeed, beads coated with polybasic compounds such as polylysine or polyornithine are inducers of SV clustering in cultured spinal cord and cerebellar neurons as previously shown (Burry, 1980; Peng et al., 1987). There is evidence that these polycations can actually mimic the endogenous ligands, such as bFGF, in activating their cell surface receptors (Dauchel et al., 1989; Kuo et al., 1990). However, not all basic molecules are effective in this induction.

Figure 9. Quantitation of the Ca^{2+} distribution along the neurites without contact with beads. Each symbol represents a single neurite. For the measurement, each neurite was divided into 50 segments of equal length and the mean Ca^{2+} level of each segment was plotted. The neurite-soma junction was designated 0% distance, and the growth cone was designated 100% distance along the neurite. *A*, Before stimulation. Results from four neurites are plotted. *B*, After stimulation. Results from three neurites are plotted. *C*, Sigmoid least-square fit of all data points from three neurites, before stimulation (solid line); after stimulation (dotted line). The stimulus-elicited Ca^{2+} increase is most pronounced in the proximal part of the neurite.

As shown here, PDGF, another highly basic molecule, failed to induce SV clustering when applied on beads. Inhibition by suramin and by a tyrosine kinase inhibitor supports the notion that the SV clustering induced by bFGF beads is mediated by a receptor. It should be noted, however, that the effect of suramin is not specific to bFGF receptor. It has been shown that this drug also blocks ATP receptor (Edwards et al., 1992) which has been shown to be involved in the modulation of transmitter release at developing *Xenopus* NMJs (Fu and Poo, 1991).

The signal-transduction process for presynaptic development activated by bFGF-coated beads is Ca^{2+} -dependent and may involve the activation of tyrosine kinases. Significantly, recent data have shown that the development of the postsynaptic specializa-

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at sites of bead-neurite contact (arrows) in comparison to the bead-free portion of the neurite. After a single electrical stimulus delivered to the soma, the Ca^{2+} level was elevated in the neurite (*C*). The increase at the bead-neurite contacts was much higher than the bead-free area. The image shown in *C* was acquired within 1 sec after somatic stimulation.

Figure 10. Comparison of Ca^{2+} concentration between bead-contact (+) and bead-free (−) area of the neurite. To measure the level under the bead, a 10 μm circle was drawn around the bead and the mean Ca^{2+} concentration within this circle was calculated. In the resting state (R), a small but significant elevation existed between the bead and bead-free areas. Stimulation (S) greatly enhanced this difference.

tion on cultured muscle cells also involves activation of tyrosine kinases (Qu et al., 1990; Peng et al., 1991a; Wallace et al., 1991; Baker and Peng, 1993). The fact that beads can induce SV clustering and localization of Ca^{2+} influx wherever they come into contact with the neurite suggests that the chemosensitivity for presynaptic development is distributed over the entire neuritic membrane and beads can locally induce this development. The mechanism for the localization of SVs at bead–neurite contacts may involve local organization of a cytoskeletal specialization, since previous ultrastructural studies have shown the presence of an actin network within the nerve terminal at NMJs *in vivo* and *in vitro* (Peng, 1983; Hirokawa et al., 1989). This hypothesis is supported by our recent observation that F-actin, as visualized by rhodamine-phalloidin labeling, is concentrated at bead–neurite contacts (Z.D. and H.B.P., unpublished observations). Furthermore, in *Aplysia* growth cones, small polybasic beads induce the rapid assembly of actin networks at the membrane-bead interface (Forscher et al., 1992). These actin-containing structures, termed inductopodia, can propel beads to move on the surface of the growth cone. A local actin network within the neurite may interact with proteins on SVs, such as synapsins, to effect their localization (Hirokawa et al., 1989; Betz and Henkel, 1994). A previous study showed that the expression of synapsin IIb in neuroblastoma cells can promote synaptogenesis (Han et al., 1991). Thus, the contact with the postsynaptic target may first activate a tyrosine-kinase based signal-transduction mechanism, leading to the local assembly of an actin-based cytoskeletal specialization. This specialization then interacts with SVs via their intrinsic proteins to effect their clustering.

The localization of the depolarization-dependent Ca^{2+} influx mechanism at bead–neurite contacts is likely due to the accumulation of Ca^{2+} channels which are known to be clustered at presynaptic active zones at the NMJ (Robitaille et al., 1990; Cohen et al., 1991). We have attempted to visualize the localization of Ca^{2+} channels at bead–neurite contacts with fluorescent ω -conotoxin GVIA, a specific probe for N-type Ca^{2+} channels. However, no significant concentration of the toxin binding sites at bead–neurite contacts has been observed. This could mean that the density of Ca^{2+} channels is not high enough to be

visualized with this fluorescent method. Alternatively, this toxin may not be a good marker for the Ca^{2+} channel in *Xenopus* embryonic neurons, since our electrophysiological studies have shown that a large portion of the Ca^{2+} current blocked by this toxin is reversible (Z.D., unpublished observation). Further studies are necessary to understand the physiological basis of this membrane specialization induced by beads.

In addition to the possible effect on Ca^{2+} channel clustering when locally applied via beads, bFGF also directly stimulates a transient elevation in neuronal Ca^{2+} level by itself when applied in the bath. The mechanism of this stimulation is not known, although a previous study has documented that bFGF can upregulate voltage-dependent Ca^{2+} channels in retinal glia cells (Puro and Mano, 1991). bFGF beads, on the other hand, elicits a prolonged elevation in Ca^{2+} level at the bead–neurite contacts in the resting state (Fig. 10). This prolongation may be due to the increased density of Ca^{2+} channels clustered at the beads.

Previously, we showed that bFGF-coated beads are also effective in inducing the formation of AChR clusters on cultured muscle cells (Peng et al., 1991a). This raises the possibility that a target-derived molecule can be used as both pre- and postsynaptic inducers. In addition to bFGF, the HSPG on the surface of muscle cells can also harbor other muscle-derived peptide growth factors, including additional members of the FGF family such as FGF-5 and FGF-6 (DeLapeyrière et al., 1993; Han and Martin, 1993; Hughes et al., 1993), and the newly discovered heparin-binding growth-associated molecule (HB-GAM) (Mernies and Rauvala, 1990; Li et al., 1990). All of these factors are potential candidates for signaling presynaptic development. In addition, HSPG can also interact with other components of the ECM on skeletal muscle, such as agrin (Wallace, 1990; Saito et al., 1993). The fact that regenerating axons *in vivo* can form presynaptic specializations on synaptic basal lamina, where agrin is concentrated, suggests its possible involvement (Sanes et al., 1978; McMahan, 1990). There is evidence that neurotrophins may also play a role in presynaptic development, as suggested by their effect on enhancing the transmitter release (Lohof et al., 1993). The skeletal muscle can synthesize several neurotrophins, including brain-derived neurotrophic factor and neurotrophin-3 (Henderson et al., 1993). In addition to their roles in motoneuron survival, it is conceivable that they may also be involved in the regulation of synaptogenesis. Whether the presynaptic induction is mediated by a specific molecule or by a multiplicity of molecules remains to be determined. The bead–neurite culture model used in this study should be useful in assaying the role of these molecules in synaptogenesis.

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