

# Modulation of Protein Kinase A Activation by Fibronectin Matrix Proteins at Developing Neuromuscular Synapses in *Xenopus laevis* Cell Cultures

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## ABSTRACT

Extracellular matrix proteins, such as fibronectin, laminin, and collagen, have been implicated in a wide variety of cellular properties, which include cell adhesion, migration, differentiation, and proliferation. In this study, we investigated the modulation of protein kinase A (PKA) activity by matrix proteins at developing motoneurons. The cultures of spinal neurons and myotomal cells were prepared from 1-day-old *Xenopus laevis* embryos. Spontaneous synaptic currents (SSC) were recorded from innervated myocytes of natural synapses by whole-cell voltage-clamped recordings ( $V_h = -60 \sim -65$  mV). Bath application of agents, which directly or indirectly activate PKA, such as forskolin (20  $\mu$ M), dibutyryl cAMP (DBcAMP) (1 mM), isoproterenol (10  $\mu$ M), or albuterol (10  $\mu$ M), significantly increased SSC frequency in cultures grown on fibronectin (FN)-coated substratum, but not on laminin- or collagen-coated glasses. The evoked synaptic currents increased in response to forsko-

lin in neurons grown on FN substratum. Trifluavin, an Arg-Gly-Asp-dependent disintegrin, inhibited potentiating action of isoproterenol in neurons grown on FN substratum, suggesting that integrin is involved in the potentiation of the PKA pathway in the regulation of acetylcholine (ACh) release. There is collaboration of neurotrophic factors and the FN matrix in regulating synaptic transmission in response to DBcAMP. Chronic treatment with neurotrophic factors, such as ciliary neurotrophic factor (150 ng/ml), glial cell line-derived neurotrophic factor (30 ng/ml), or neurotrophin-3 (50 ng/ml), enhanced the SSC-increasing action of DBcAMP in neurons grown on FN-coated glasses. These results suggest that the FN matrix potentiates synaptic transmission in response to PKA activation. Neurotrophic factors may collaborate with FN to regulate spontaneous ACh secretion at developing motoneurons, which may play an important role in the maturation of embryonic neuromuscular synapses.

The extracellular matrix (ECM) provides positional and environmental information that is essential for tissue function. ECM proteins, such as fibronectin (FN), laminins, or collagens, form distinct protein networks that show tissue-specific variation in composition and architecture. Cell responses to contact with these networks depend on the type of matrix and on the cell's repertoire of ECM receptors (Schwarzbauer and Sechler, 1999). Connections from the matrix through these receptors determine the organization of cytoskeletal structure and the localization and activation of signaling molecules, leading to unique tissue-specific cell functions. Many of the receptors that integrate matrix information belong to the integrin superfamily of transmembrane proteins. Integrins are heterodimers that mediate adhesion of cells to ECM proteins. Both the  $\alpha$  and  $\beta$  subunits contain large extracellular ligand-binding domains and short cyto-

plasmic domains that bind cytoskeletal and signaling proteins. Binding of integrins to ligands within the ECM or other cells triggers an increase in lateral clustering and occupancy of integrin ligand-binding sites. Integrin occupancy and clustering not only initiate adhesion and cytoskeletal organization via direct physical associations of integrins with other proteins but also activate many intracellular-signaling pathways that regulate cell migration, polarity, survival, growth, differentiation, and gene expression (Giancotti and Ruoslahti, 1999).

Synaptic transmission can be modulated through a number of pre- and postsynaptic mechanisms. Neurotransmitter stored in synaptic vesicles within the nerve terminal are released by exocytosis and modulated by many regulatory processes. Protein phosphorylation plays an important role in the regulation of secretion. Activation of the protein kinase A (PKA) pathway has been shown to facilitate synaptic transmission (Dixon and Atwood, 1989; Chavez-Noriega and Stevens, 1994; Capogna et al., 1995; Trudeau et al., 1996). In

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**ABBREVIATIONS:** ECM, extracellular matrix; FN, fibronectin; PKA, protein kinase A; ACh, acetylcholine; PKC, protein kinase C; CGRP, calcitonin gene-related peptide; DBcAMP, dibutyryl cAMP; ISO, isoproterenol; CNTF, ciliary neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; NT-3, neurotrophin-3; SSC, spontaneous synaptic current; ESC, evoked synaptic current; RGD, Arg-Gly-Asp.

mammalian neuromuscular synapses, it has been demonstrated that cAMP and its derivatives facilitate acetylcholine (ACh) release, apparently by a presynaptic mechanism (Wilson, 1974; Dretchen et al., 1976). PKA may phosphorylate proteins associated with the exocytosis process of synaptic vesicles (Scheller, 1995; Sudhof, 1995). For example, synapsin I, rabphilin-3A, or the 25-kDa synaptosome-associated protein is the substrate for PKA (Jahn and Sudhof, 1994; Risinger and Bennett, 1999). It has also been reported that integrin is involved in the stretch enhancement of ACh release from motor nerve terminals (Chen and Grinnell, 1995). In addition, hippocampal slice experiments have shown that peptides that block ligand binding by a major subclass of integrins prevent the stabilization of long-term potentiation (Xiao et al., 1991; Bahr et al., 1997). We have previously shown that FN and laminin markedly potentiated the action of protein kinase C (PKC) in increasing spontaneous ACh release (Fu et al., 2001b). However, the relationship between matrix proteins and PKA in the regulation of synaptic transmission at neuromuscular junctions remains unclear. The present study further examines the modulation of PKA activity by ECMs at developing motoneurons. Our results suggest that FN may enhance the release of ACh in response to PKA activation and there is interaction between FN-matrix protein and neurotrophic factors in the regulation of transmitter release.

## Experimental Procedures

**Chemicals and Solutions.** Albuterol; calcitonin gene-related peptide (CGRP); collagen; dibutyl cAMP (DBcAMP); dobutamine; fibronectin (from bovine plasma, lyophilized from 0.05 M Tris-buffered saline, pH 7.5); forskolin; isoproterenol (ISO); laminin (from basement membrane, Engelbreth-Holm-Swarm mouse sarcoma) (Sigma, St. Louis, MO). Ciliary neurotrophic factor (CNTF); glial cell line-derived neurotrophic factor (GDNF); neurotrophin-3 (NT-3) (Pepro Tech, London, UK). Triflavin was purified from *Trimeresurus flavoviridis* snake venom (Huang et al., 1991).

**Culture Preparation.** *Xenopus laevis* neuromuscular cultures were prepared as reported previously (Tabti and Poo, 1991). Briefly, the neural tube and the associated myotomal tissues of 1-day-old *X. laevis* embryos (stages 20–22) were dissociated in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free Ringer's solution supplemented with EDTA. The cells were plated onto clean glass coverslips and were used for experiments after 24 h at room temperature (20–22°C). The culture medium consisted of 50% (v/v) Ringer's solution (115 mM NaCl, 2 mM  $\text{CaCl}_2$ , 1.5 mM KCl, and 10 mM HEPES, pH 7.6), 49% L-15 Leibovitz medium (Sigma), 1% fetal bovine serum (Invitrogen, Carlsbad, CA), and antibiotics (100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin). In some experiments, the glass coverslips were coated with FN, laminin, or collagen (30  $\mu\text{g}/\text{glass}$ ) and then air-dried. The nerve-muscle cocultures were then plated on these precoated glass coverslips.

**Electrophysiology.** Whole-cell patch-clamp recording methods followed those described by Hamill et al. (1981). Patch pipettes were pulled with a two-stage electrode puller (pp-83; Narishige, Tokyo, Japan), and the tips were polished immediately before the experiment, using a microforge (MF-83; Narishige). Spontaneous synaptic currents (SSC) were recorded from innervated myocytes by whole-cell recording in the voltage-clamp mode. SSC recordings were made at room temperature in the culture medium. For whole-cell recordings, the solution inside the recording pipette contained 150 mM KCl, 1 mM NaCl, 1 mM  $\text{MgCl}_2$ , and 10 mM HEPES, pH 7.2. The extent of the potentiation was measured by the frequency ratio of SSCs, which is defined as the ratio of SSC frequency at peak level observed during the application of drugs to the mean frequency

before drug treatment. Evoked synaptic currents (ESC) were elicited by stimulating presynaptic neurons at the soma with heat-polished glass microelectrodes (tip opening 1–2  $\mu\text{m}$ ) filled with Ringer's solution, and the culture medium was replaced with Ringer's solution. For suprathreshold stimulation of the neuron, a square current pulse (0.3 ms in duration and 2–4  $\mu\text{A}$  in amplitude) was applied through the pipette. Such currents generally induce twitch contraction of the muscle cell when applied to the soma of the innervating neuron. The membrane currents passing through the patch pipette were recorded with a patch-clamp amplifier (Axopatch 200A; Axon Instruments, Burlingame, CA) filtered at 10 kHz. The data were digitized using a Neuro-corder DR 390 (Neuro Data Instruments, New York, NY) and stored on videotape for later playback onto a storage oscilloscope (5113; Tektronix, Beaverton, OR) or an oscillographic recorder (RS3200; Gould, Cleveland, OH). The MacLab (ADInstruments, Mountain View, CA) was used to analyze the frequency of SSCs. The results were expressed as mean  $\pm$  S.E. ( $n$ ). The number of recorded neurons is represented by  $n$ . The statistical significance was evaluated by Student's  $t$  test.

## Results

**Effects of Isoproterenol on Spontaneous Synaptic Currents.** Regulation of transmitter release by PKA activation and ECM proteins at developing motoneurons was studied in *X. laevis* nerve-muscle cocultures. Isolated embryonic spinal neurons in *X. laevis* cultures established functional synaptic transmission with cocultured myocytes soon after nerve-muscle contact, forming natural synapses. SSCs are readily detectable from the innervated muscle cell with the whole-cell recordings. These currents have been shown to be caused by spontaneous ACh secretion from the neuron, because they are abolished by bath application of *d*-tubocurarine and unaffected by tetrodotoxin.  $\beta$ -Adrenergic receptor signaling initiates from ligand binding to G-protein-coupled receptors and leads to the activation of adenylate cyclase and PKA. Bath application of ISO (10  $\mu\text{M}$ ), a nonselective  $\beta$ -adrenergic agonist, did not influence the frequency of SSCs in day-1 natural synapses (Fig. 1A). However, ISO (10  $\mu\text{M}$ ) markedly potentiated SSC frequency in neurons plated on FN-coated glass coverslips (Fig. 1B). The maximal potentiation was reached about 5 min after ISO application, and the potentiation persisted for more than 40 min (Fig. 2A). The SSC frequency ratio for ISO in *X. laevis* cultures grown on FN substratum was  $18.9 \pm 0.7$  ( $n = 6$ ), which was significantly different from control neurons plated on noncoated glasses (Fig. 2B). The effects of the matrix are primarily mediated by integrin (Giancotti and Ruoslahti, 1999). Bath application of triflavin (2.8  $\mu\text{M}$ ), an Arg-Gly-Asp (RGD)-dependent disintegrin (Huang et al., 1991), to 4-h cultures grown on FN substratum inhibited the SSC-increasing action of ISO. The SSC frequency ratio was  $1.2 \pm 0.1$  ( $n = 4$ ), indicating that the potentiating action of the FN matrix was primarily mediated by integrin. The integrin receptor family of vertebrate cells is composed of at least 16 distinct  $\alpha$  subunits and eight or more  $\beta$  subunits that can associate in various combinations. The  $\alpha/\beta$  associations determine the ligand binding specificities of the integrin heterodimers for various ECM proteins, including FN, laminin, vitronectin, and collagens. We thus examined whether other ECM proteins also exert similar potentiation. It was found that ISO did not significantly affect SSC frequency in cultures plated on laminin- or collagen-coated glass coverslips (Fig. 1, C and

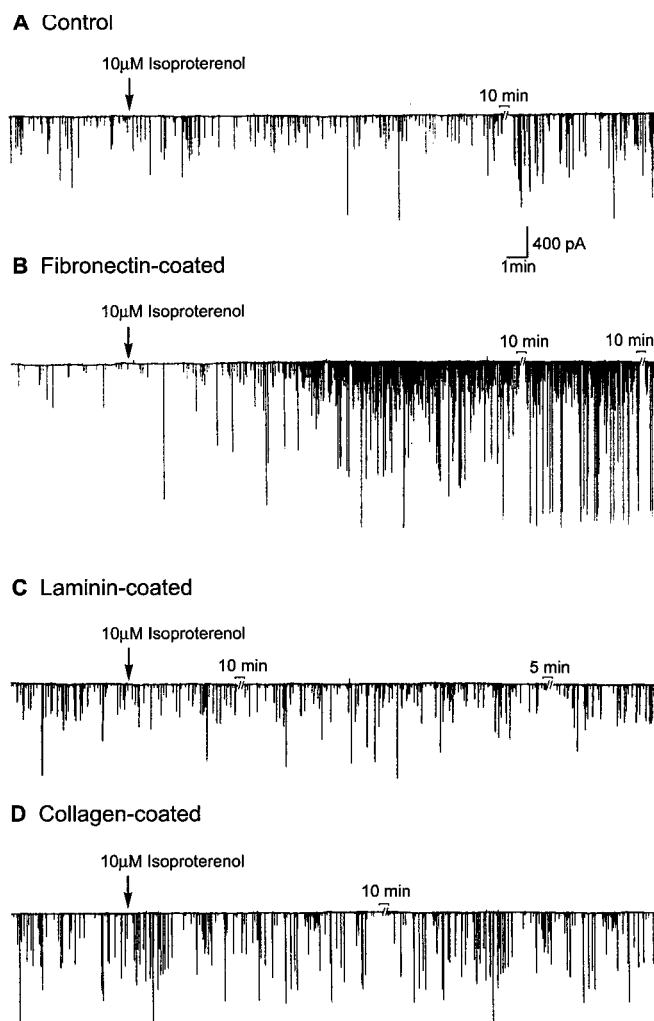
D; Fig. 2B). The SSC frequency ratios were  $1.4 \pm 0.1$  ( $n = 6$ ) and  $1.2 \pm 0.1$  ( $n = 6$ ), respectively.

We further tested the subtype of  $\beta$ -adrenergic receptor responsible for the potentiation effect of ISO. Albuterol (10  $\mu$ M), a selective  $\beta_2$ -adrenergic agonist, also markedly increased SSC frequency in neurons grown on FN substratum, but not on laminin or collagen substratum (Figs. 2B and 3). The SSC frequency ratios were  $11.4 \pm 1.9$ ,  $1.1 \pm 0.1$ , and  $1.2 \pm 0.1$ , respectively ( $n = 5$  for each). On the other hand, dobutamine (10  $\mu$ M), a selective  $\beta_1$ -adrenergic agonist, did not affect SSC frequency in neurons grown on FN substratum (the SSC frequency ratio was  $1.4 \pm 0.2$ ;  $n = 3$ ). Therefore, the SSC-potentiation effect of ISO was mediated by the  $\beta_2$ -adrenergic receptor.

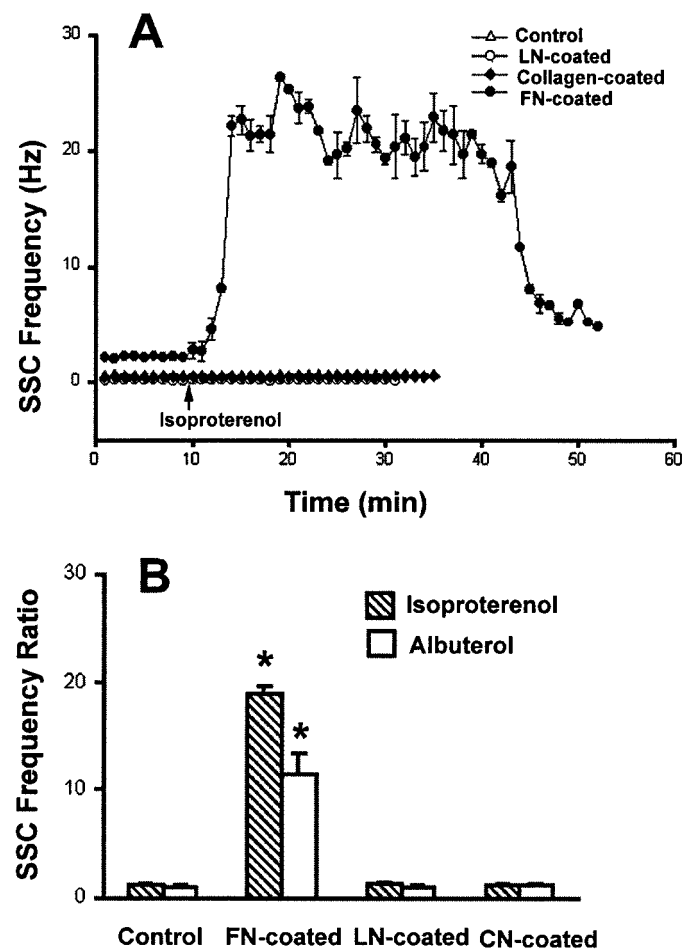
CGRP, a neuropeptide present at presynaptic motor nerve terminals, elevates cAMP levels and stimulates the phosphorylation of ACh channels in myocytes (Laufer and Changeux, 1987; Uchida et al., 1990). We then investigated

whether CGRP also exerts presynaptic potentiation. CGRP at 3  $\mu$ M, which is high enough to produce postsynaptic effect, did not affect SSC frequency in cultures plated on FN-coated glasses. The SSC frequency ratio was  $1.6 \pm 0.1$  ( $n = 6$ ), which was not different from control neurons ( $1.2 \pm 0.1$ ;  $n = 6$ ). These results suggest that the action of CGRP may be mainly of postsynaptic origin.

**Effects of Forskolin and DBcAMP on the SSCs.** Our results showed that the FN matrix facilitates the  $\beta_2$ -adrenergic receptor in the regulation of embryonic synaptic transmission. Activation of the  $\beta_2$ -adrenergic receptor affects various physiological functions via the activation of PKA. We thus investigated whether the FN matrix affects the downstream pathway after  $\beta_2$ -receptor activation. The action of forskolin, an adenylate cyclase activator, and DBcAMP, a direct PKA activator, was examined. Bath application of forskolin (20  $\mu$ M) or DBcAMP (1 mM) significantly increased SSC frequency in neurons grown on FN substratum, but not those grown on either laminin or collagen substratum (Fig. 4). The SSC frequency ratios in response to forskolin and



**Fig. 1.** Effect of matrix proteins on the regulation of spontaneous ACh release by isoproterenol. The continuous traces depict the inward membrane currents recorded from the innervated myocyte in day 1 *X. laevis* cocultures. *X. laevis* nerve-muscle cocultures were plated onto glass that was previously coated with FN, laminin, or collagen. The myocyte was whole-cell voltage-clamped at  $-60$  mV. Downward deflections represent SSCs (filtered at 150 Hz). Note that compared with control (A), bath application of isoproterenol resulted in a significant enhancement of SSC frequency on cultures grown on FN (B), but not on laminin (C) or collagen (D) substratum.



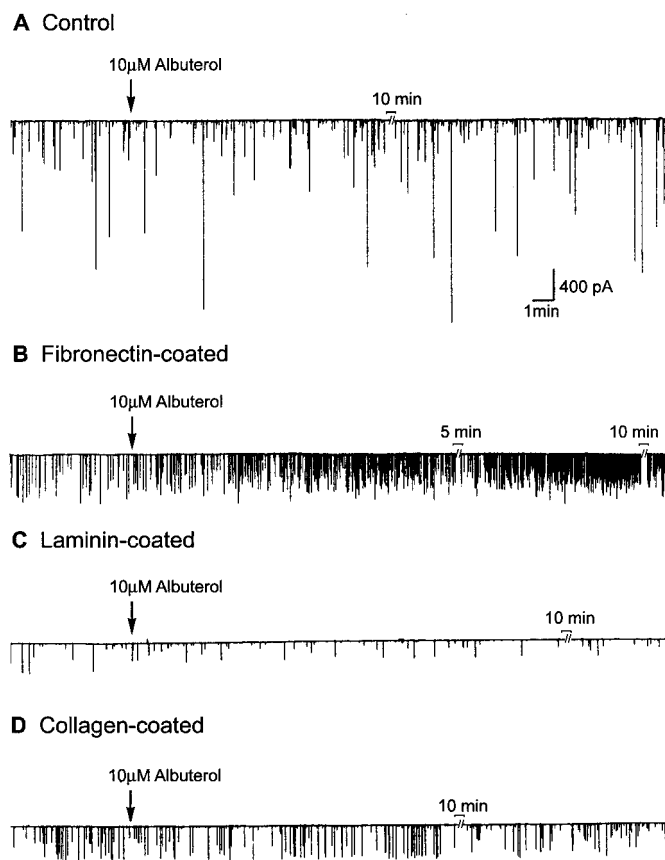
**Fig. 2.** Summary of the effect of ECM proteins on the regulation of spontaneous ACh release by isoproterenol and albuterol. *X. laevis* nerve-muscle cocultures were plated onto glass that was previously coated with fibronectin, laminin, or collagen. The myocyte was whole-cell voltage-clamped at  $-60$  mV. A, time course-response curves of isoproterenol in neurons grown on different matrix substratum ( $n = 5-6$ ). B, potentiation of the spontaneous ACh release in response to isoproterenol and albuterol in neurons grown on FN substratum. The SSC frequency ratio is defined as the ratio of the peak frequency observed during the application of drugs to that of mean frequency before drug treatment ( $n = 5-6$ ). \*  $p < 0.05$  compared with control; LN, laminin; CN, collagen.



DBcAMP in neurons grown on FN substratum were  $3.5 \pm 0.1$  ( $n = 9$ ) and  $13.5 \pm 1.5$  ( $n = 6$ ), respectively. When the forskolin concentration was increased up to  $40 \mu\text{M}$ , forskolin alone was also able to increase spontaneous ACh secretion in neurons grown on noncoated glasses (the SSC frequency ratio was  $3.4 \pm 0.1$ ;  $n = 5$ ). These results suggest that FN, but not laminin or collagen, potentiates SSC-increasing actions of the agents, which directly or indirectly activate PKA.

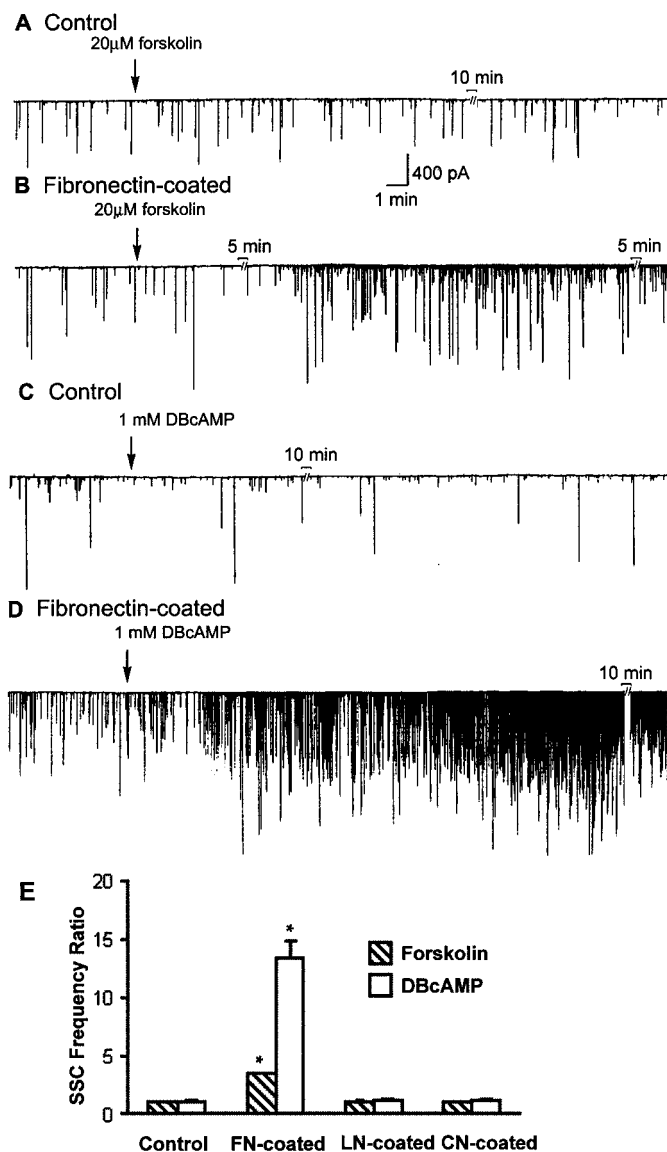
**Effects of Forskolin on the ESCs.** The ESCs were recorded via whole-cell recording at a myocyte that has contacted with the nerve terminal. ESCs were induced by stimulating the soma with suprathreshold currents. As shown in Fig. 5, the soma was electrically stimulated to elicit ESCs after observation of the spontaneous release of ACh for 5 min. Bath application of forskolin ( $20 \mu\text{M}$ ) increased not only SSC frequency but also ESC amplitude in neurons grown on FN substratum, but not those grown on laminin or collagen substratum. The ESC ratio is defined as the ratio of mean ESC amplitude after bath application of forskolin to that of mean ESC amplitude before drug treatment. The ESC ratios were  $1.74 \pm 0.09$  ( $n = 4$ ) and  $1.01 \pm 0.06$  ( $n = 4$ ) for neurons grown on FN-coated and noncoated glasses, respectively.

**Collaboration of Fibronectin with Neurotrophic Factors.** The signals from integrin receptors are integrated



**Fig. 3.** Effect of matrix proteins on the regulation of spontaneous ACh release by albuterol. The continuous traces depict the inward membrane currents recorded from the innervated myocyte in day 1 *X. laevis* cultures. *X. laevis* nerve-muscle cocultures were plated onto glass that was previously coated with FN, laminin, or collagen. The myocyte was whole-cell voltage-clamped at  $-60 \text{ mV}$ . Downward deflections represent SSCs (filtered at  $150 \text{ Hz}$ ). Compared with control (A), bath application of albuterol resulted in a significant enhancement of SSC frequency in neurons grown on FN (B), but not on laminin (C) or collagen (D) substratum.

with those originating from growth factors to stimulate mitogen-activated protein kinase cascades and regulate immediate-early gene expression (Giancotti and Ruoslahti, 1999), which regulate many cellular functions (Schwartz et al., 1995). The engagement and clustering of integrins can affect the efficiency of signaling pathways triggered by growth factors (Miyamoto et al., 1996). Therefore, we examined whether the FN matrix and neurotrophic factors collaborate in the regulation of spontaneous ACh release in response to PKA activation. The neurotrophic factors were bath-applied to 4-h cultures, and SSCs were recorded after further incu-



**Fig. 4.** Potentiation of the FN matrix on the spontaneous ACh release in response to forskolin and DBcAMP. The continuous traces depict the inward membrane currents recorded from the innervated myocyte in day 1 *X. laevis* cultures. *X. laevis* nerve-muscle cocultures were plated onto glass that was previously coated with fibronectin. The myocyte was whole-cell voltage-clamped at  $-60 \text{ mV}$ . Downward deflections represent SSCs (filtered at  $150 \text{ Hz}$ ). Compared with control (A) and (C), bath application of forskolin (B) or DBcAMP (D) resulted in a significant enhancement of SSC frequency in cultures grown on FN substratum. E, the quantitative data are shown. Note that DBcAMP and forskolin increased spontaneous ACh release in neurons grown on FN, but not on laminin or collagen substratum. \* $p < 0.05$  compared with control; LN, laminin; CN, collagen.

bation of 18 to 24 h. As shown in Fig. 6, chronic treatment with neurotrophic factors, such as CNTF (150 ng/ml), GDNF (30 ng/ml), or NT-3 (50 ng/ml), markedly enhanced the SSC-increasing effect of DBcAMP in neurons grown on FN substratum. The SSC frequency ratio increased by 2-fold after chronic treatment with neurotrophic factors. When the cultures were plated on noncoated glasses and chronically treated with these neurotrophic factors, none of these neurotrophic factors alone affected SSC frequency in response to DBcAMP (CNTF,  $1.02 \pm 0.08$ ; GDNF,  $1.04 \pm 0.03$ ; NT-3,  $1.04 \pm 0.03$ ;  $n = 3$  for each). These results indicate that the FN matrix can collaborate with neurotrophic factors to regulate synaptic transmission in response to PKA activation.

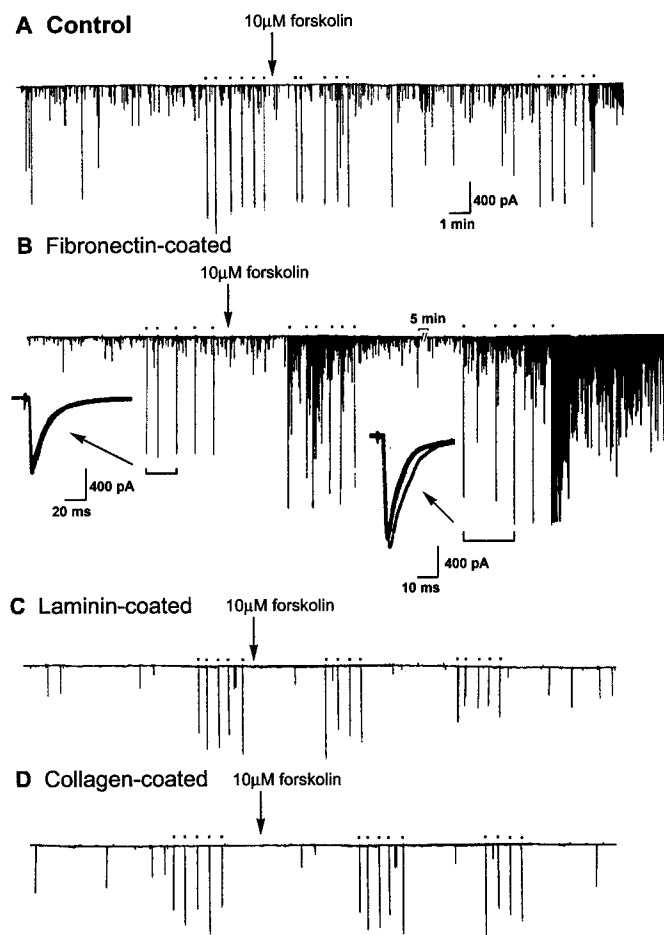
## Discussion

In the present study, we found that isoproterenol and albuterol increased SSC frequency in neurons grown on FN substratum, but not in those grown on laminin or collagen substratum. These results suggest that FN facilitates the signal transduction caused by presynaptic activation of  $\beta_2$ -

adrenergic receptor.  $\beta$ -Receptor activation stimulates the membrane-bound adenylyl cyclase via the bridge Gs-protein. The subsequent rise of cAMP in the cytosol activates the downstream enzyme of PKA. Furthermore, the SSC-increasing action of forskolin and DBcAMP was also significantly potentiated by FN-matrix protein, indicating that FN-matrix protein may potentiate PKA action in the regulation of transmitter secretion.

Protein phosphorylation represents an important mechanism for regulating synaptic activity. PKA, casein kinase II, calmodulin kinase II, and PKC have all been implicated in different aspects of long-term changes in synaptic efficacy (Charriaut-Marlangue et al., 1991; Alberini et al., 1995; Wang and Kelly, 1995; Mayford et al., 1996). Activation of the PKA pathway has been shown to facilitate synaptic transmission in mammalian neuromuscular synapses, although the relevant phosphorylation targets are mostly unknown (Losavio and Muchnik, 2000). Vesicle docking and fusion reactions are potential targets for phosphorylation-mediated regulation of synaptic transmission. Recent results have demonstrated that proteins involved in membrane fusion, such as rabphilin-3A, synaptophysin, synaptotagmin, the 25-kDa synaptosome-associated protein, and syntaxin, can be phosphorylated in vitro (Genoud et al., 1999; Risinger and Bennett, 1999). We found here that activation of the PKA pathway at a low concentration of isoproterenol, albuterol, forskolin, or DBcAMP did not significantly affect ACh release in neurons grown on FN substratum. It is possible that a synergistic effect of the FN matrix and PKA activation on spontaneous ACh release occurs through a direct interaction in the phosphorylation of membrane fusion proteins.

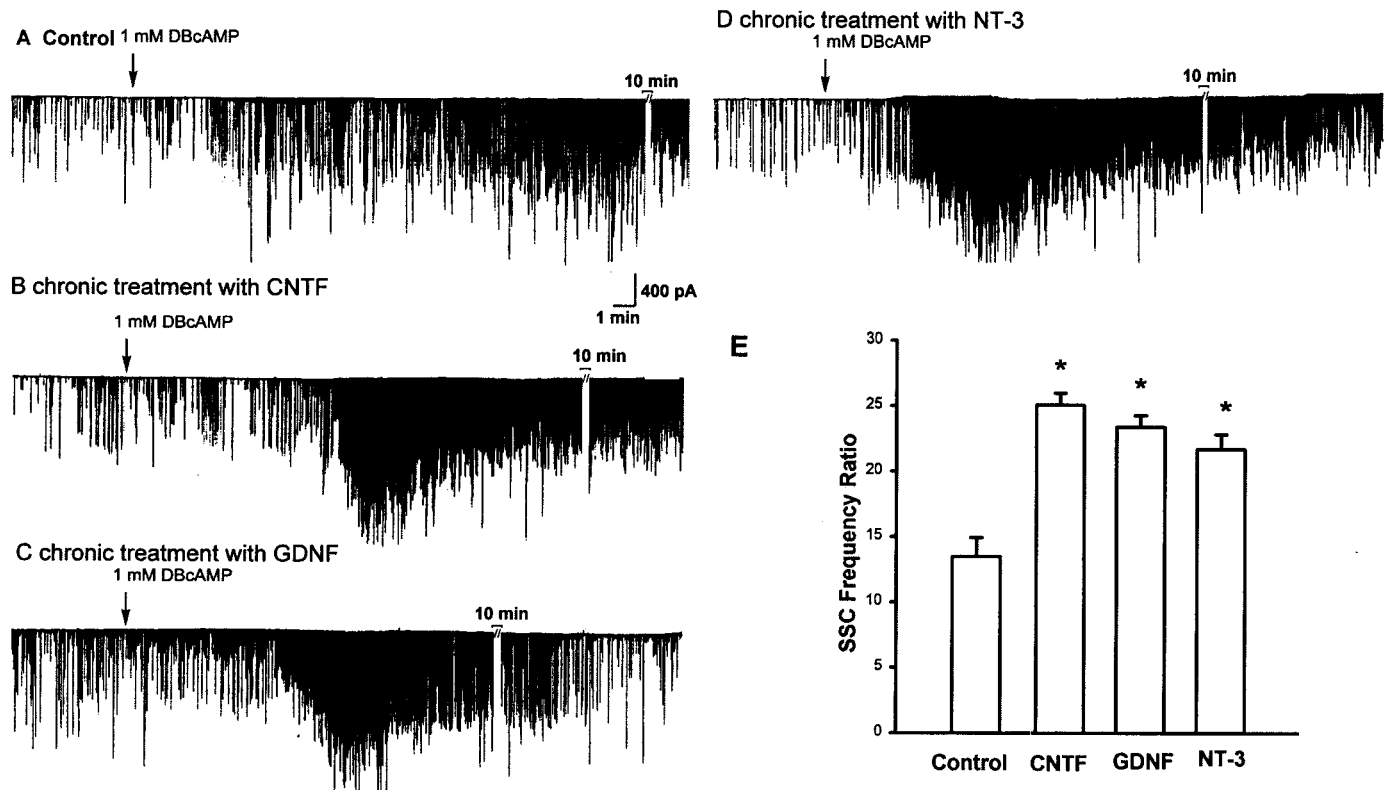
ECM proteins bind to its specific integrins. The integrins that bind to FN are RGD-dependent, whereas the integrins for laminin are RGD-independent. Triflavin, an RGD-dependent disintegrin (Huang et al., 1991), inhibited potentiating action of ISO in neurons grown on FN substratum, suggesting that integrin is indeed involved in the potentiation of the PKA pathway in the regulation of ACh release. Recently, Cohen et al. (2000) demonstrated that  $\alpha 3 \beta 1$  integrin is concentrated at the active zones of *X. laevis* motor nerve terminals, which link ECM to cytoskeletal elements and participate in the formation of signaling complexes.  $\alpha 3 \beta 1$  integrin can interact with many kinds of ECMs, such as FN, laminin, and collagen (Hynes, 1992). Our results showed that FN, but not laminin or collagen, binding to integrin could potentiate PKA action in the regulation of spontaneous and evoked ACh release. Why ECM specificity exists in the regulation of transmitter release in response to PKA activation remains unclear. We found previously that both FN and laminin enhanced spontaneous ACh release in response to PKC activation (Fu et al., 2001b). Such a noteworthy difference in the action of laminin needs further investigation. It may result from the different signaling pathways activated by FN and laminin. In addition, we recently found that laminin, but not fibronectin, increased the axonal growth rate in *X. laevis* embryonic cultures. The axonal length of day-1 naive neurons was  $303.5 \pm 16 \mu\text{m}$  ( $n = 77$ ) for neurons grown on laminin-coated coverslips, which was longer than those grown on FN-coated or noncoated glasses [ $171.1 \pm 15.8 \mu\text{m}$  ( $n = 26$ ) and  $148.0 \pm 5.2 \mu\text{m}$  ( $n = 180$ ), respectively]. Therefore, different ECM proteins may regulate different neuronal



**Fig. 5.** Enhancement of the evoked synaptic currents by the fibronectin matrix in response to forskolin. The continuous traces depict the inward membrane currents recorded from the innervated myocyte in day 1 *X. laevis* cocultures. *X. laevis* nerve-muscle cocultures were plated onto glass that was previously coated with FN, laminin, or collagen. The myocyte was whole-cell voltage-clamped at  $-60$  mV. Downward deflections represent SSCs and ESCs (as shown by filled circles). Compared with control (A), bath application of forskolin resulted in a slight enhancement of ESC amplitude in the neurons grown on FN (B), but not on laminin (C) or collagen (D) substratum.

functions. Focal adhesions are specialized sites of attachment found in cultured cells at locations where the extracellular domains of cell-surface integrins bind to immobilized ECM proteins, such as fibronectin (Schwartz et al., 1995). This interaction results in clustering of the integrins and the association of their intracellular domains with cytoskeletal protein that anchor bundles of polymerized actin filaments (stress fibers) to these sites. A number of signaling proteins are recruited to focal adhesions, including the adapter protein paxillin and the focal adhesion tyrosine kinase (Schwartz et al., 1995; Giancotti and Ruoslahti, 1999). Thus, focal adhesions have both structural and signaling functions. Synaptic vesicles are thought to be clustered as a result of their interactions with cytoskeletal proteins. An actin network has been found close to the presynaptic membrane (Hirokawa et al., 1989), and it is likely that the actin filaments play an important role in the maintenance of the cluster of synaptic vesicles (Doussau and Augustine, 2000). PKA has also been reported to be involved in cytoskeletal rearrangement after integrin binding to FN (Glass and Kreisberg, 1993). FN may increase the pool of readily releasable vesicles or more efficient vesicular-docking mechanisms, which thus potentiates PKA action in the regulation of transmitter release. In addition to potentiating PKA action, FN also enhanced PKC activity in the regulation of spontaneous ACh release (Fu et al., 2001b). These results suggest that FN may affect a common pathway to regulate transmitter release in response to PKC or PKA activation.

In vitro and in vivo studies have shown that motoneurons respond to a variety of neurotrophic factors (Kato and Lindsay, 1994). NT-3, CNTF, and GDNF have been shown to support the survival or differentiation of motoneurons during embryonic development and rescue motoneurons from axotomy-induced cell death after birth (Oppenheim et al., 1991; Li et al., 1994; Yan et al., 1995). The signals from integrin receptors are integrated with those originating from growth factors to stimulate mitogen-activated protein kinase cascades and regulate immediate-early gene expression and many cellular functions (Schwartz et al., 1995; Giancotti and Ruoslahti, 1999). The engagement and clustering of integrins can affect the efficiency of signaling pathways triggered by growth factors (Miyamoto et al., 1996). Here we show that the FN matrix collaborates with NT-3, CNTF, and GDNF to potentiate the SSC-increasing action of DBcAMP. We recently also found that NT-3 greatly potentiated the SSC-increasing action of  $\alpha,\beta$ -methylene ATP, *N*-methyl-D-aspartate, and carbonyl cyanide *m*-chlorophenylhydrazone in neurons grown on FN substratum (Fu et al., 2001a). It seems that ECM and neurotrophic factors may regulate transmitter release downstream after the elevation of cytosolic  $\text{Ca}^{2+}$ . They may affect the neuronal  $\text{Ca}^{2+}$  disposal mechanism or exocytosis in collaboration. Therefore, FN may increase the size of readily releasable vesicular pools and/or up-regulate the  $\text{Ca}^{2+}$  sensitivity of exocytosis. It is unclear how integrin participates in increasing the  $\text{Ca}^{2+}$  sensitivity of exocytosis upon the activation of protein kinases. Spontaneous ACh



**Fig. 6.** Collaboration of neurotrophic factors and the fibronectin matrix in the regulation of SSC in response to DBcAMP. The continuous traces depict the inward membrane currents recorded from the innervated myocyte in day 1 *X. laevis* cultures. The cultures were plated onto noncoated glass coverslips (A) or glasses that were previously coated with FN (B–D). CNTF (150 ng/ml) (B), GDNF (30 ng/ml) (C), or NT-3 (50 ng/ml) (D) was bath applied to 4-h cultures. The myocyte was whole-cell voltage-clamped at  $-60$  mV after further incubation of 18 to 24 h. Downward deflections represent SSCs (filtered at 150 Hz). The quantitative data are shown in E. The SSC frequency ratio is defined as the ratio of the peak frequency observed during the application of drugs to that of mean frequency before drug treatment. Note that chronic treatment with neurotrophic factors enhanced the SSC-potentiating effect of DBcAMP in cultures grown on FN-coated glasses. \* $p < 0.05$  compared with control ( $n = 3-6$ ).



release at the developing neuromuscular synapse may play a trophic function in the synapse maturation. In *X. laevis* nerve-muscle cultures, many of spontaneous synaptic potentials are capable of eliciting action potentials and contractions in the muscle cells (Xie and Poo, 1986). This frequent suprathreshold excitation produces a global influence on the maturation of embryonic neuromuscular synapses (Fitzsimonds and Poo, 1997). The regulation of ACh release by protein kinases, ECMs, and neurotrophic factors is coordinated in a complex manner, and the determination of molecular details of the precise mechanisms of its interaction needs further investigation.

In conclusion, our results suggest that FN potentiates PKA action in the regulation of ACh release, and neurotrophic factors may modulate this PKA potentiation at developing motoneurons, which may play an important role in the regulation of embryonic synaptic transmission.

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