Integrins and Modulation of Transmitter Release from Motor Nerve Terminals by Stretch

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The stretch of a frog muscle within the physiological range can more than double the spontaneous and evoked release of neurotransmitter from its motor nerve terminals. Here, stretch enhancement of release was suppressed by peptides containing the sequence arginine-glycine–aspartic acid (RGD), which blocks integrin binding. Integrin antibodies also inhibited the enhancement obtained by stretching. Stretch enhancement depended on intraterminal calcium derived both from external calcium and from internal stores. Muscle stretch thus might enhance the release of neurotransmitters either by elevating internal calcium concentrations or by increasing the sensitivity of transmitter release to calcium in the nerve terminal.

Changes in muscle length regulate both spontaneous and evoked transmitter release from frog motor nerve terminals (*1–3*). The enhancement of evoked release develops fully within 10 to 20 ms of a change in muscle length (*4*), is sustained indefinitely at the new length, and is reversible (*3, 4*). Mean miniature endplate potential (mEPP) amplitude is unchanged, which indicates that the enhancement is pre-synaptic, reflecting an increase in probability of quantal release (*3*). In some frog muscles, where many EPPs are subthreshold for muscle excitation at rest length (*5*), this stretch enhancement constitutes a strong peripheral amplifier of the spinal stretch reflex. Unlike the increase in mEPP frequency produced by K^+ depolarization of the terminal, the stretch-induced increase in release is not blocked in a Ringer solution containing 10 mM Mg^{2+} and 0.1 mM Ca^{2+} (*3*).

"Floating" microelectrodes (*6*) were used to record EPPs and mEPPs from sartorius nerve muscle preparations. Stretches of 1 and 2 mm (approximately 3 and 6%) from rest length (*7*) caused mean increases in mEPP frequency of 32.6 ± 1.6% and 68.9 ± 2.9%, respectively (Fig. 1A). The amplitudes of EPPs recorded in Ringer solution containing 0.6 mM Ca^{2+} and 4 mM Mg^{2+} increased by approximately the same amount (Fig. 1B). The increase in release probability with stretch was approximately linear over the 6% range of stretch tested (Figs. 3 and 4).

In Ringer solution containing no Ca^{2+}, 2 mM Mg^{2+}, and 1 mM EGTA, (Ca^{2+}-free Ringer), the mEPP frequency at rest length fell from a mean of 1.47 ± 0.06 s^{-1} in normal frog Ringer solution (NFR) to 0.59 ± 0.07 s^{-1} (*8*). However, after 1 hour or more in Ca^{2+}-free Ringer, a stretch of 1 or 2 mm still increased the mEPP frequency by 17.9 ± 2.4% and 28.4 ± 3.7%, respectively (Fig. 1A). Thus, the stretch-induced enhancement of mEPP frequency can be obtained without Ca^{2+} influx. When nerve terminals were loaded with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*-*N*-*N*′,*N*′-tetraacetic acid (BAPTA) to buffer internal Ca^{2+} levels (*9*) and then tested in Ca^{2+}-free Ringer, the enhancement of mEPP frequency by stretch was blocked (1.7 ± 4.8% increase at 1 mm of stretch, 7.7 ± 5.8% at 2 mm) (Fig. 1A). Thus, internal stores of Ca^{2+} were required for stretch enhancement of release in Ca^{2+}-free Ringer.

The observation that mEPP frequency is sensitive to the external calcium concentration ($[Ca^{2+}]_o$) suggests that there is a significant resting influx of Ca^{2+} (*10*). Here, the resting mEPP frequency increased by approximately 50% with a change in concentration from 0.3 mM to 5 mM Ca^{2+} in the Ringer solution (Fig. 2). At each concentration, the effect of stretch was an approximately equal proportional increase in mEPP frequency. Thus, the stretch enhancement also depended on $[Ca^{2+}]_o$, presumably reflecting Ca^{2+} influx or loading of internal Ca^{2+} stores. Loading of terminals with BAPTA reduced the stretch enhancement of mEPP frequency in NFR by about 50% and the EPP amplitude in a 0.6 mM Ca^{2+} Ringer solution by about 60% (Fig. 1).

The identities of the pathways of Ca^{2+} influx at rest and during stretch are not known. Gd^{3+}, an effective blocker of voltage-sensitive Ca^{2+} channels and many forms of stretch-activated channels (*11*), totally blocked EPPs at 100 mM concentration but did not significantly affect the enhancement by stretch of mEPP frequency (*12*). Voltage-sensitive N-type Ca^{2+} channels appear to be responsible for evoked quantal release (*13*), but the N-channel blocker ω-conotoxin (GVIA) at a concentration of 1 to 5 mM had no significant effect on the results of muscle stretch. The dihydropyridine agonist Bay K 8644 (1 mM), which prolongs the open time of L-type channels (*14*), sharply increased the stretch enhancement of mEPP frequency, whereas the L-channel blocking agent nitrendipene (10 mM) reduced it (Fig. 3). Thus, at least part of the stretch enhancement may be a result of Ca^{2+} influx through L-type channels.

The rapidity with which stretch increases evoked release probability suggests that the effective tension-mediating connections act on the terminal near the active zones, which are the sites of vesicular fusion and transmitter release (*15*). The most widespread and versatile of known transmembrane proteins implicated in plasma membrane–extracellular membrane (ECM)

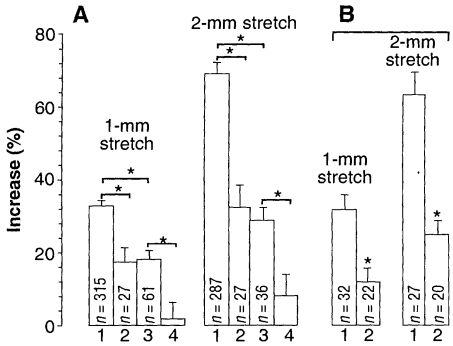

Fig. 1. Percentage increase in mEPP frequency (**A**) and EPP amplitude (±SEM) (**B**) caused by 1- and 2-mm stretch under different conditions. In (A), bars 1 represent treatment with NFR only; bars 2, treatment with NFR and BAPTA; bars 3, no Ca^{2+}; bars 4, no Ca^{2+} with BAPTA. In (B), bars 1 represent treatment with 0.6 mM Ca^{2+}; bars 2, treatment with 0.6 Ca^{2+} and BAPTA. The extracellular Ringer solution was NFR and Ca^{2+}-free Ringer or 0.6 mM Ca^{2+} and 4 mM Mg^{2+} Ringer. BAPTA-AM was used to load terminals. Resting mEPP frequencies in the BAPTA experiments were NFR + BAPTA, 3.23 ± 0.11 s^{-1} and 0 Ca^{2+} + BAPTA, 1.4 ± 0.35 s^{-1}. The number of junctions studied is indicated within each bar; in (A), bar 4, 1-mm stretch, $n = 28$; bar 4, 2-mm stretch, $n = 22$. The asterisk indicates $P < 0.007$ by Student's t test.

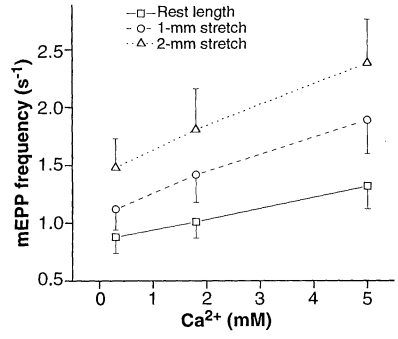

Fig. 2. Relation between $[Ca^{2+}]_o$ in the Ringer solution and mEPP frequency (mean ± SEM) at rest length and at 1- and 2-mm stretch ($n = 20$). Recording commenced a minimum of 10 min after each change in $[Ca^{2+}]_o$.

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interaction are the integrins. Integrin-ligand binding requires divalent cations and in many cases can be blocked by exposure to a short peptide containing the RGD (arginine, glycine, aspartic acid) sequence that mimics the main integrin binding site in several ECM molecules, such as fibronectin, vitronectin, tenascin, and thrombospondin (*16–18*).

To test the hypothesis that RGD-dependent integrin bonds are involved in the stretch effect, we treated preparations with a peptide (GRGDSP, where S is Ser and P is Pro; Peninsula Labs) containing the RGD sequence (*19*). At a concentration of 0.2 mM in NFR, this peptide (hereafter referred to as RGD) reduced the enhancement of both mEPP frequency (Fig. 4A) and EPP amplitude (*12*) by 35 to 40%. This is a large effect, considering that the peptide might not disrupt existing integrin-ECM bonds but may only interfere with the formation of new bonds with natural ligands. Although integrin-ligand bonds differ in the extent to which they are stabilized by Mg^{2+} and Ca^{2+}, a low divalent (LD) Ringer solution containing 50 μM Ca^{2+} and 50 μM Mg^{2+} would be expected to destabilize the bonds (*20*). Indeed, pretreatment of preparations for 1.5 hours in an LD Ringer solution containing 0.2 mM RGD, followed by testing after 1 hour in NFR containing 0.2 mM RGD, resulted in greater suppression of stretch enhancement than was seen without pretreatment (Fig. 4A). Controls pretreated with LD Ringer solution and returned to NFR (both without RGD) showed normal stretch enhancement. The peptide GRGESP (RGE, where E is Glu), which does not interfere with integrin binding to RGD sites in most instances (*17, 19*), did not decrease the stretch enhancement of mEPP frequency (Fig. 4A) or EPP amplitude at concentrations as high as 2 mM.

The RGD peptide appeared to act selectively on stretch enhancement of transmitter release. It did not decrease the mEPP frequency or EPP amplitude at rest length or affect the enhancement of mEPP frequency by K^+ depolarization. An increase from 2 to 6 mM K^+ in the Ringer solution caused equivalent changes in mEPP frequency in the presence or absence of RGD [from 1.34 ± 0.12 (n = 65) to 1.99 ± 0.21 s^{-1} (n = 65) in Ringer solution without RGD and from 1.25 ± 0.19 (n = 39) to 1.94 ± 0.26 s^{-1} (n = 45) in Ringer solution + 0.2 mM RGD]. The enhancement of mEPP frequency by stretch in Ca^{2+}-free Ringer also depended on RGD-sensitive bonds: 0.2 mM RGD decreased the effect of a 2-mm stretch in Ca^{2+}-free Ringer by about 50%, from an increase of 28.4 ± 3.7% (n = 36) to 15.2 ± 5.3% (n = 25) in the presence of RGD ($P < 0.04$). Independent evidence for the involvement of integrins was obtained with a rabbit polyclonal antiserum (P25) to external epitopes of a *Xenopus* β_1 integrin. The β_1 antiserum suppressed the stretch effect, whereas the preimmune serum had no effect (Fig. 4B) (*21*).

The stretch of a muscle might modulate release by elevating the internal calcium concentration ($[Ca^{2+}]_i$) However, this would require two distinct mechanisms: (i) mobilizing Ca^{2+} from internal stores in the case of stretch-enhanced release in Ca^{2+}-free Ringer and (ii) increasing influx of Ca^{2+} in the case of the larger stretch enhancement seen in a Ca^{2+}-containing Ringer solution (Fig. 2). A reduction in the rate of Ca^{2+} efflux—for example, by decreasing Na^+-Ca^{2+} exchanger activity—could elevate $[Ca^{2+}]_i$. However, lowering $[Na^+]_o$ in the Ringer solution by 50%, which strongly reduces exchanger activity (*22*) and increases resting mEPP frequency by about 50% (to 2.51 ± 0.28 s^{-1}), had no effect on the stretch enhancement. In a reduced Na^+ Ringer solution, the mean enhancement was 31.7 ± 4.1% (n = 52) with 1-mm stretch and 71.3 ± 7.6% (n = 50) with 2-mm stretch, compared with 28.4 ± 5.4% (n = 47) and 72.4 ± 9.8% (n = 47) in paired NFR controls. Thus, stretch did not appear to act by decreasing Ca^{2+} efflux.

An alternative explanation would be that strain on integrin bonds modulates the sensitivity of the intraterminal release apparatus to any given Ca^{2+} level and that Ca^{2+} influx and internal Ca^{2+} buffering do not change with muscle length. A change in sensitivity to Ca^{2+} concentration might be attributable to phosphorylation or dephosphorylation of molecules involved in Ca^{2+} sensing or vesicle docking and fusion. Integrin-ligand binding is capable of initiating many different kinds of intracellular events, including activation of tyrosine kinases, second messenger–modulated Ca^{2+} release from internal stores and increased Ca^{2+} influx, changes in gene expression, protein secretion and ion transport, and changes in interaction with cytoskeletal elements (*17, 18, 23, 24*). However, most of the known physiological changes associated with integrin binding develop over seconds to minutes and survive long after removal of the signal, unlike the stretch-induced enhancement (*4*).

It may be useful, therefore, to consider the possibility that tension on integrin-ECM bonds can in some direct mechanical way change the Michaelis constant of Ca^{2+}-sensing molecules or of molecular interactions involved in vesicle docking and fusion. Such a change could have extremely

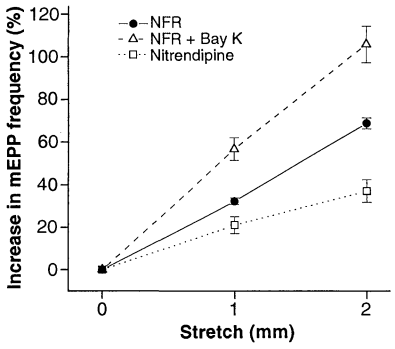

Fig. 3. Effects of L-type Ca^{2+} channel modulators on the stretch enhancement of mEPP frequency. Compared with control records in NFR (n = 319), 1 mM Bay K 8644 (n = 48) increased the stretch enhancement at both 1- and 2-mm stretch ($P < 4 \times 10^{-6}$), whereas the dihydropyridine nitrendipine (10 mM) (n = 46) reduced the stretch enhancement ($P < 0.02$ at 1 mm, < 0.0004 at 2 mm). Resting mEPP frequency in Bay K was 1.41 ± 0.11 s^{-1}. The vehicle for mixed enantiomer Bay K was ethyl alcohol, which at its final concentration (0.3%) had no effect.

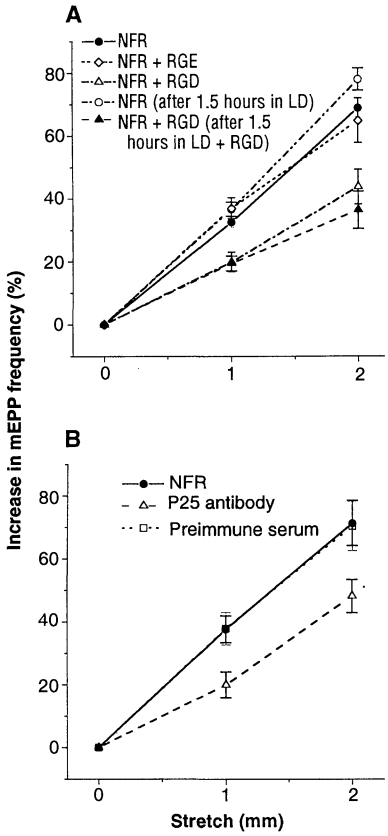

Fig. 4. (**A**) Effects of the peptides RGD and RGE, at a concentration of 0.2 mM, on enhancement of mEPP frequency by 1- and 2-mm stretches (mean ± SEM). RGD and RGE were applied in NFR, with measurements made after 1.5 hours of incubation. Also shown are data obtained after an earlier 1.5-hour incubation in LD Ringer solution to destabilize integrin-ECM bonds. RGE values did not differ significantly from NFR, but RGD suppressed the enhancement by both 1- and 2-mm stretches ($P <$ 0.002 in all cases). (**B**) Effects of polyclonal antiserum (P25) against *Xenopus* β_1 integrin and preimmune serum from the same rabbit. At 1-mm stretch, $P < 0.01$; at 2 mm, $P < 0.02$.

rapid kinetics and would be consistent with all other data. It would be surprising if integrins played a role in synaptic function only at frog motor nerve terminals. However, with the exception of a report that RGD peptides block hippocampal long-term potentiation (*25*), other instances have not been described. Integrins, which can transduce signals in both directions across the cell membrane (*17, 18, 23, 24*), might be involved more generally in cell-cell trophic-inductive interactions, in maintaining synaptic organization, and in regulating synaptic function. Integrins appear also to play a role in the enhancement of transmitter release by hypertonic solutions (*26*) and, if involved in helping regulate transmitter release at central nervous system synapses, might contribute to the excitotoxic response to brain swelling with ischemia.

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6. Sartorius nerve muscle preparations were dissected from mature (4 to 5 cm body length) *Rana pipiens* and held in NFR (116 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $NHCO_3$, 3 mM dextrose, and 5 mM Hepes, pH 7.4) in an apparatus that permitted elongation or shortening of the muscle at both ends simultaneously. EPPs and mEPPs were recorded by means of "floating" microelectrodes consisting of just the distal shank and tip of a microelectrode, filled with 0.6 M K^+ acetate and 5 mM KCl and held on the end of a coiled flexible electrode wire. A suction electrode was used to stimulate the sartorius nerve.
7. Rest length was defined as the muscle length in situ with the frog on its back with its hind limbs extended at approximately 45° from the body axis and extended directly back from the knee. The sarcomere spacing at rest length was 2.2 to 2.3 mm, and 1-mm stretch represented approximately a 3% increase in muscle length.
8. To avoid spontaneous twitching in Ca^{2+}-free Ringer solution, we pretreated the preparation for 15 min with 25 mM μ-conotoxin, which blocks Na^+ channels in muscle, but much less effectively in nerve (*27, 28*), and has no apparent effect on transmitter release or synaptic potentials.
9. Preparations were immersed for 1.5 hours in Ca^{2+}-free Ringer containing 25 mM BAPTA-AM, made from a stock solution of 5 mM in dimethylsulfoxide (DMSO), with 0.02% pluronic acid (Molecular Probes) to facilitate loading of the dye and 20 mM tetrakis(2-pyridylmethyl) ethylenediamine (TPEN, Molecular Probes) to chelate heavy metals, according to techniques worked out by Robitaille and Charlton (*28*). The -AM form of the buffer is membrane-permeant but becomes trapped and concentrated inside the terminal into the Ca^{2+}-binding form after esterification. Controls were treated with the same final concentration of DMSO, pluronic acid, and TPEN.
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21. Preparations were treated for 1.5 hours in LD Ringer solution, then returned to NFR for 1 hour before testing, at 1:100 serum dilution throughout. The antigen used to generate the P25 immunoglobulin G was a β_1 fusion protein of about 50% of the full sequence.
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A VAMP-Binding Protein from *Aplysia* Required for Neurotransmitter Release

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Before the fusion of synaptic vesicles with the plasma membrane, a protein complex is thought to form between VAMP—an integral membrane protein of the vesicle—and two proteins associated with the plasma membrane, SNAP-25 and syntaxin. The yeast two-hybrid interaction cloning system has now been used to identify additional proteins from *Aplysia* that interact directly with VAMP. A 33-kilodalton membrane protein, termed VAP-33 (VAMP-associated protein of 33 kilodaltons), was identified whose corresponding messenger RNA was detected only in the central nervous system and the gill of *Aplysia*. Presynaptic injection of antibodies specific for VAP-33 inhibited synaptic transmission, which suggests that VAP-33 is required for the exocytosis of neurotransmitter.

A fundamental property of all neurons that interact by means of chemical synaptic transmission is that neurotransmitter is stored in synaptic vesicles in the nerve terminal and released by exocytosis in response to an increase in presynaptic Ca^{2+} (*1*). Transmitter release can be modulated by activity, and such modulation is a common feature of synaptic plasticity that is thought to contribute to memory formation. Significant progress toward a molecular description of transmitter release has been achieved by identifying and analyzing proteins associated with synaptic vesicles (*2, 3*). The purification of vesicle-associated proteins and the isolation of their cognate genes (*4*) have been facilitated by the abundance of synaptic vesicles in the brain. Other proteins have been identified by their ability to interact physically with vesicle-associated proteins in immune complexes (*5, 6*). However, conventional biochemical binding assays and immunoprecipitation methods rely on the formation of relatively stable, high-affinity protein-protein complexes; they do not detect weaker or more transient interactions. To identify proteins important for vesicle exocytosis that bind more weakly to components of previously identified synaptic complexes, we have used the two-hybrid system developed in yeast (*7*). With this approach, we have now identified a protein from *Aplysia californica* that specifically interacts with the synaptic vesicle protein VAMP (also termed synaptobrevin) (*8*).

The partial complementary DNA (cDNA) clones isolated in this screen contained an open reading frame encoding a 19-kD polypeptide. With a polymerase chain reaction (PCR)–based strategy, additional cDNAs were isolated that contained a single long open reading frame encoding a putative 260–amino acid polypeptide with a predicted molecular mass of 30 kD (Fig. 1A). The recombinant protein expressed in bacteria migrated at 33 kD on SDS–polyacrylamide gel electrophoresis; the polypeptide was therefore referred to as VAP-33 (VAMP-associated protein of 33 kD).

When expressed as a bacterial fusion protein with glutathione-S-transferase, VAP-33 bound specifically to in vitro–translated

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