# Kinetics, Ca$^{2+}$ Dependence, and Biophysical Properties of Integrin-Mediated Mechanical Modulation of Transmitter Release from Frog Motor Nerve Terminals

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Neurotransmitter release from frog motor nerve terminals is strongly modulated by change in muscle length. Over the physiological range, there is an ~10% increase in spontaneous and evoked release per 1% muscle stretch. Because many muscle fibers do not receive suprathreshold synaptic inputs at rest length, this stretch-induced enhancement of release constitutes a strong peripheral amplifier of the spinal stretch reflex. The stretch modulation of release is inhibited by peptides that block integrin binding of natural ligands. The modulation varies linearly with length, with a delay of no more than ~1-2 msec and is maintained constant at the new length. Moreover, the stretch modulation persists in a zero Ca$^{2+}$ Ringer and, hence, is not dependent on Ca$^{2+}$ influx through stretch activated channels. Eliminating transmembrane Ca$^{2+}$ gradients and buffering intraterminal Ca$^{2+}$ to approximately normal resting levels does not eliminate the modulation, suggesting that it is not the result of release of Ca$^{2+}$ from internal stores. Finally, changes in temperature have no detectable effect on the kinetics of stretch-induced changes in endplate potential (EPP) amplitude or miniature EPP (mEPP) frequency. We conclude, therefore, that stretch does not act via second messenger pathways or a chemical modification of molecules involved in the release pathway. Instead, there is direct mechanical modulation of release. We postulate that tension on integrins in the presynaptic membrane is transduced mechanically into changes in the position or conformation of one or more molecules involved in neurotransmitter release, altering sensitivity to Ca$^{2+}$ or the equilibrium for a critical reaction leading to vesicle fusion.

*Key words: synaptic plasticity; EPPs; mEPPs; muscle length; neuromuscular junction; mechanotransduction; RGD; muscle stretch*

Transmitter release from frog motor nerve terminals is powerfully regulated by muscle length (Fatt and Katz, 1952; Kuffler, 1952). A muscle stretch of 5–10% can cause more than a doubling in endplate potential (EPP) amplitude or miniature EPP (mEPP) frequency, with no change in mEPP amplitude (Hutter and Trautwein, 1956; Turkanis, 1973). Over the physiological range (up to 10–15% stretch), the enhancement is approximately linear with length, is maintained constant at a new length, and is readily reversible (Hutter and Trautwein, 1956; Turkanis, 1973). In contrast to the enhancement of mEPP frequency as a result of K$^+$-induced depolarization, the stretch enhancement is not affected by high Mg$^{2+}$ in the Ringer (Hutter and Trautwein, 1956); hence, it is not a result of a stretch-induced depolarization of the terminal. Moreover, the increase in mEPP frequency is not suppressed in a Ringer containing 10 mM Mg$^{2+}$ and 0.1 mM Ca$^{2+}$, suggesting to Turkanis (1973) that it is not dependent on Ca$^{2+}$ influx. However, this conclusion predated the discovery of stretch-activated channels (SACs) (Yang and Sachs, 1990; French, 1992; Martinac, 1993; Sackin, 1995), some of which might be Ca$^{2+}$-permeable and not blocked by Mg$^{2+}$.

We have reported recently that the effects of stretch on neuro-transmitter release can be suppressed by exposure to peptides containing the tripeptide arginine–glycine–aspartic acid (RGD), which interferes with binding of integrins to their native ligands (Chen and Grinnell, 1995). Changes in external binding of ligands by integrins can trigger profound changes within cells, such as activation of phospholipase C; production of DAG and IP$_3$; stimulation of Ca$^{2+}$ influx and Ca$^{2+}$ release from internal stores; cytoplasmic alkylization; activation of protein kinase C; induction of NO synthase; induction of immediate early gene expression; and initiation of tyrosine phosphorylation cascades that have strong influences on cell shape, motility, and cytoskeletal organization (see, for example, Schwartz et al., 1991; Fujimoto et al., 1991; Damsky and Werb, 1992; Hynes, 1992; Kornberg and Juliano, 1992; Cybulsky et al., 1993; Schwartz, 1993; Miyamoto et al., 1995; Schaller et al., 1995). By their connections both to the ECM and to the semirigid cytoskeleton, integrins are also capable of transducing mechanical signals in both directions across the cell membrane (Ingber, 1991; Wang et al., 1993), potentially causing important biochemical changes, e.g., by bringing kinases closer to their substrates or changing reaction equilibria by altering protein conformation or stress on noncovalent bonds (Damsky and Werb, 1992; Miyamoto et al., 1995; Ingber, 1996).

In this paper, we present additional evidence implicating integrins in the enhancement, and describe experiments designed to determine, in a generic sense, the mechanism(s) of enhancement. Our data suggest that interference with integrin bonds eliminates much or all of the phenomenon, that the enhancement does not require Ca$^{2+}$ influx or release of Ca$^{2+}$ from internal stores (although some basal level of intraterminal Ca$^{2+}$ is necessary), and

that the modulation does not depend on stretch-mediated biochemical processes such as phosphorylation/dephosphorylation reactions. Instead, this seems to be an entirely mechanical form of modulation, altering the probability of a diffusion-limited process that strongly influences release. To the best of our knowledge, this represents the fastest, most powerful form of integrin-mediated mechanical modulation of cellular physiology reported to date.

## MATERIALS AND METHODS

### Preparations and solutions

*Rana pipiens.* *Rana pipiens* (2.5–3 inches, nose to anus) were anesthetized by immersion in 0.1% tricaine methanesulfonate (Sigma), double pithed, and the sartorius nerve-muscle preparation was dissected out. At rest length (with the hind limbs extended backward at a 45° angle, the knees bent, and the distal part of the leg pointed directly backward), the sarcomere spacing is ~2.25 $\mu$m, which was used as the criterion for rest length.

Muscles were held in a bathing chamber accessible to both dissecting and compound microscope examination, fixed at either end to moveable arms (see below). The nerve was pulled into a suction electrode for stimulation at different lengths or phases of a lengthening–shortening cycle. The bath temperature was controlled by a Peltier device affixed to the metal plate, into which the preparation chamber fitted. The temperature could be varied in a controllable way between ~10° and 23°.

Normal frog Ringer (NFR) contained (in mM): 116 NaCl, 1 NaHC03, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 Na-HEPES, and 5 HEPES, pH 7.3. "Zero Ca$^{2+}$ Ringer" contained (in mM): 2 MgCl$_2$, 1 EGTA, 116 NaCl, 2 KCl, 1 NaHCO$_3$, 5 Na-HEPES, and 5 HEPES, pH 7.3. When recording mEPPs, usually 1 $\mu$g/ml neostigmine is added to the Ringer. Recordings at full quantal content were made with 3–6 $\mu$M D-tubocurarine chloride (curare) in the NFR, or after 0.5 hr treatment with $\mu$-conotoxin, which blocks Na$^+$ channels in muscle but not in nerve (Robitaille and Charlton, 1992; Cruz et al., 1985). Reduced quantal content EPPs were obtained by lowering the Ca$^{2+}$:Mg$^{2+}$ ratio in the Ringer, usually to 0.56 mM Ca$^{2+}$, 4 mM Mg$^{2+}$ ("low Ca$^{2+}$ Ringer").

*Terminal loading with Ca$^{2+}$ buffers.* In several experiments, nerve terminals were loaded with BAPTA or dimethyl (DM)-BAPTA. With techniques developed by Robitaille and Charlton (1992), preparations were incubated for 60–90 min at room temperature with 10–25 $\mu$M of the −AM form of the buffer in NFR, made up from a stock solution of 1–5 mM in DMSO, with 0.02% w/w pluronic acid (Molecular Probes) to facilitate loading of the buffer. Twenty micromolar TPEN [tetrakis(2-pyridylmethyl)ethylenediamine] (Molecular Probes) was added to chelate heavy metals. Controls were treated with the same final concentrations of DMSO, pluronic acid, and TPEN as experimentals. The −AM buffer enters the terminals, where it becomes trapped and concentrated, typically into the mM range, as cytoplasmic esterases convert it into the active (Ca$^{2+}$-binding) membrane impermeant form.

### Recording and data analysis

Intracellular recordings were made with microelectrodes of 20–60 M$\Omega$ resistance, filled either with 3 M KCl or with 0.6 M K$_2$SO$_4$ + 5 mM KCl (D'Alonzo and Grinnell, 1985). For almost all recordings, to maintain penetrations during changes in muscle length, "floating" electrodes were used. These are made by breaking off the shank and tip of a standard sharp microelectrode containing an internal filament (Garner Glass Company, KG-33), allowing it to be filled by capillarity and backfilling. This reduced electrode is then affixed on the end of a long (7 cm), partly coiled, flexible 100 $\mu$m diameter silver electrode wire connected to the input stage of an AM-2 Physiological Amplifier (Biodyne, Mission Viejo, CA). The analog output of the amplifier was digitized with a Labmaster DMA analog/digital 12 bit interface (Scientific Solutions, Solon, OH) and stored on the hard disk of a Dell 386 PC.

Stimulation of the preparation and recording of data, as well as subsequent analysis, was carried out using pClamp version 5.5 software (Axon Instruments, Foster City, CA). EPPs were stored and analyzed in blocks of 25 (for each different stretch state of the muscle) using the pClamp modules "Clampex" and "Clampan." MEPPs were detected using an Axon Instruments A2020A Event Detector and recorded and analyzed for frequency in blocks of 25–100 (depending on mEPP frequency) using the modules "Fetchex" and "Fetchan." Data were also recorded by FM tape recorder (Store 4D; Lockheed Electronics Co., Inc., Plainfield, NJ), and by chart recorder (Lineacorder Mark VII WR3101,

Western Graphtec, Irvine, CA). We found that using paired muscles from the same preparation as controls reduced variability in the results. Data are given as mean ± SE, and significance values were determined by Student's *t* test.

*Imposition of stretch.* For measurements of the kinetics of stretch modulation of release, we used an analog servomotor-drive apparatus in which the two ends of the sartorius were attached to the two writing arms of an MFE 1200 chart recorder that had been removed from the writing assembly with their motors and mounted next to the bath. Mirror image electrical signals from the computer moved the two arms by equal amounts in opposite directions. This greatly reduced the longitudinal displacement of the floating electrode, making it easier to maintain good penetrations. These writing arms are strong enough to impose stretch controlled accurately in magnitude (up to 4 mm) and rate (up to 1 mm/5 msec).

Direct calibration of muscle length was done with an optical technique. For these measurements, muscles were pinned at one end and attached to one of the moving arms at the other. The bottom of the bath was covered with an opaque layer of aluminum foil, into which was cut a uniform narrow slit, immediately to one side of the muscle, through which light was passed. A small infrared phototransistor was located above the slit to measure the amount of light passing through it. A small piece of aluminum foil was affixed at the mid-region of the muscle, projecting over the slit. As the muscle was stretched, an increasing fraction of the light was occluded. This procedure was not done in every muscle, but measurements in several muscles showed almost no variability and established that the muscle length closely paralleled the movement of the stretching arms. Multiple records were averaged to reduce noise in the phototransistor circuit, resulting in the traces shown in Figures 4 and 5. Stimuli to the nerve could be applied at any desired time before, during, or after a stretch. The sartorius muscles in the frogs we used were all ~30 mm long. In most experiments, stretches of 1 and/or 2 mm were imposed, corresponding to increases in length of 3% and 6%.

*Integrin-blocking reagents.* The hexapeptide GRGDSP (RGD, Peninsula Labs) was used as an agent that is known in many other systems to block integrin binding to natural ligands (Pierschbacher and Ruoslahti,
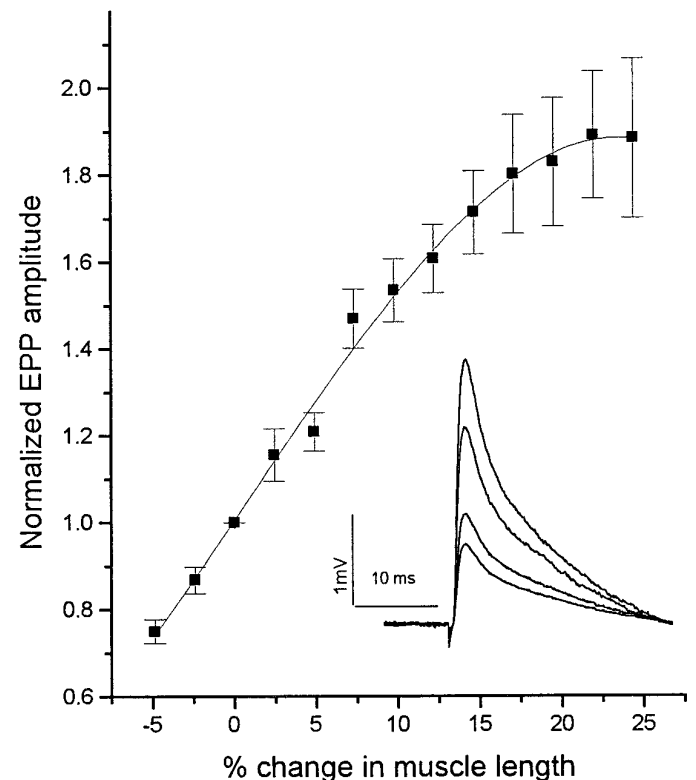


*Figure 1.* Average change in EPP amplitude as a function of stretch of the sartorius muscle. Mean ± SE for six junctions. *Inset,* EPPs obtained from a sensitive preparation at 95, 100, 110, and 120% of rest length. All in low Ca$^{2+}$ Ringer containing 0.56 mM Ca$^{2+}$ and 4 mM Mg$^{2+}$.
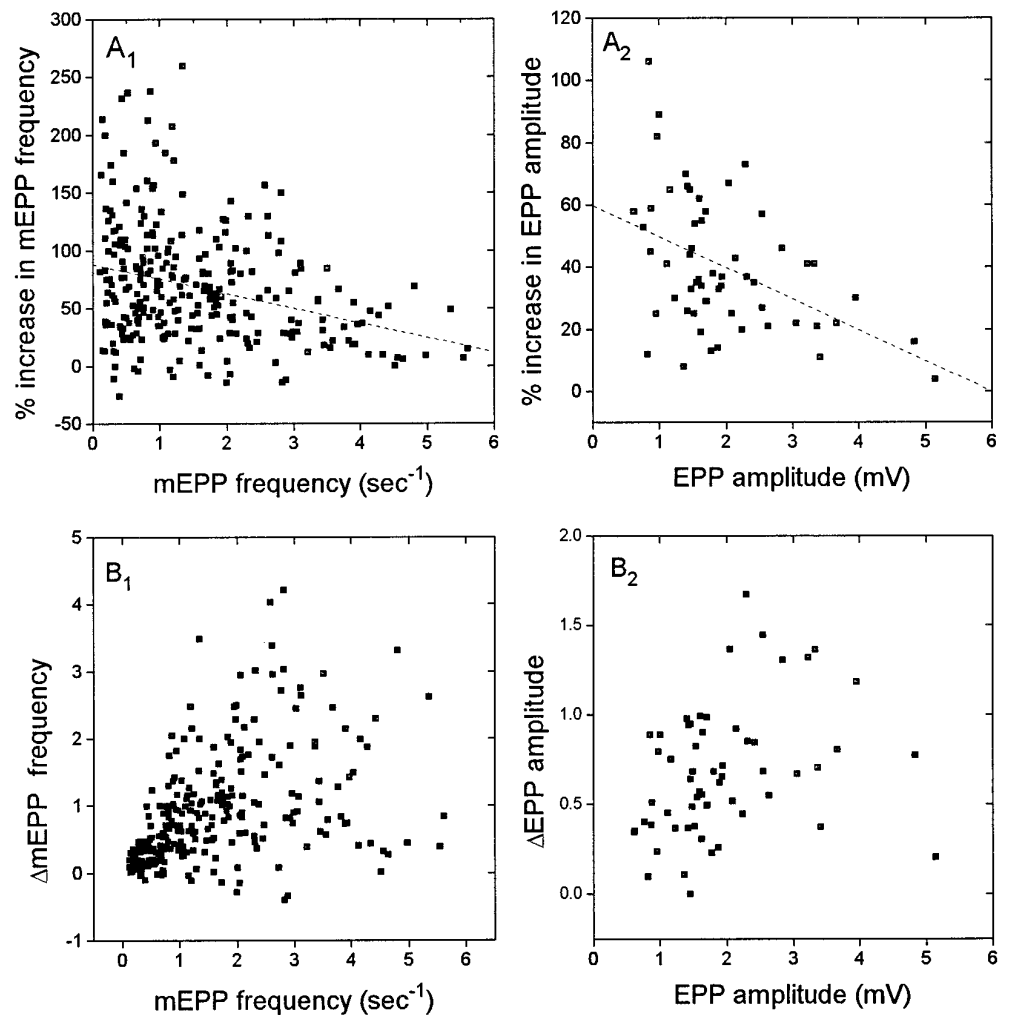
*Figure 2.* Variability and sensitivity of different junctions to 6% muscle stretch. $A_1$ and $A_2$, scattergraphs showing percentage increase in mEPP frequency and EPP amplitude for different junctions, plotted against their release at rest length. The vast majority of junctions showed an increase in release with stretch, but it varied between no change and a two- to threefold increase. The *dashed lines* represent linear best fits. $B_1$ and $B_2$, absolute values of change in mEPP frequency and EPP amplitude in the same junctions. Note that there was little or no correlation between degree of enhancement and resting release levels in the stronger junctions. EPP recordings were in 3 $\mu$M curare.

1987, Albeda and Buck, 1990, Hynes, 1992), whereas GRGESP (RGE) was used as the inactive control.

## RESULTS

### Magnitude and linearity of enhancement of release by stretch

For the population of sartorius junctions as a whole, a 3% stretch caused a 32.6 ± 1.6% (*n* = 315) increase in mEPP frequency; a 6% stretch caused a 68.9 ± 2.9 (*n* = 287) increase (see Figs. 7 and 8). EPP amplitudes are also sharply enhanced by stretch. In a Ringer containing 0.56 mM $Ca^{2+}$ and 4 mM $Mg^{2+}$, in which EPP quantal contents were reduced below threshold for action potential generation (below ~10), the EPP amplitude changed by an average 31 ± 3.9% (*n* = 32) at 3% stretch and 63 ± 5.9% (*n* = 27) at 6% stretch. The inset in Figure 1 shows EPPs recorded from a sensitive preparation at 95, 100, 110, and 120% of rest length. The enhancement was 76% at 10% stretch and 243% at 20% stretch. As Figure 1 shows, the enhancement is nearly linear in magnitude from ~5-10% below rest length to 15–20% stretch. The enhancement tended to plateau above 15–20% stretch. Most of our experiments used stretches of 6% or less.

There is considerable variability in the sensitivity of different junctions to stretch. The basis for this variability is not known. Figure 2A shows plots of the percentage increase in mEPP frequency and EPP amplitude as a function of the values at rest length. There is clearly tremendous variability in the degree of

enhancement for junctions of any given resting mEPP frequency or EPP amplitude. For the population as a whole, there was a mean inverse relationship between the percent enhancement and the synaptic efficacy at rest length. Because EPP amplitude is primarily a function of $Ca^{2+}$ influx, and mEPP frequency also is significantly dependent on external $Ca^{2+}$ (Grinnell and Pawson, 1989), this inverse relationship might be interpreted as indicating that stretch adds a certain amount of free $Ca^{2+}$ within the terminal and that this increment is decreasingly important as resting synaptic strength increases. However, as Figure 2B shows, there was a positive relationship between the absolute stretch enhancement and the rest length synaptic efficacy, at least for the weaker junctions (mEPP frequency up to ~2 Hz and EPP amplitudes up to ~2 mV). For stronger junctions this positive relationship disappeared, and there was no apparent correlation between resting mEPP frequency or EPP amplitude and the degree of stretch enhancement.

In contrast to the variability observed among different junctions, the stretch enhancement of release at any particular junction was essentially the same through repeated cycles of lengthening and shortening. Moreover, the enhancement was extremely stable, showing no evident adaptation with time for as long as the new length was maintained.

The stretch modulation of release is of obvious functional importance to frogs, serving as an amplifier of the spinal stretch
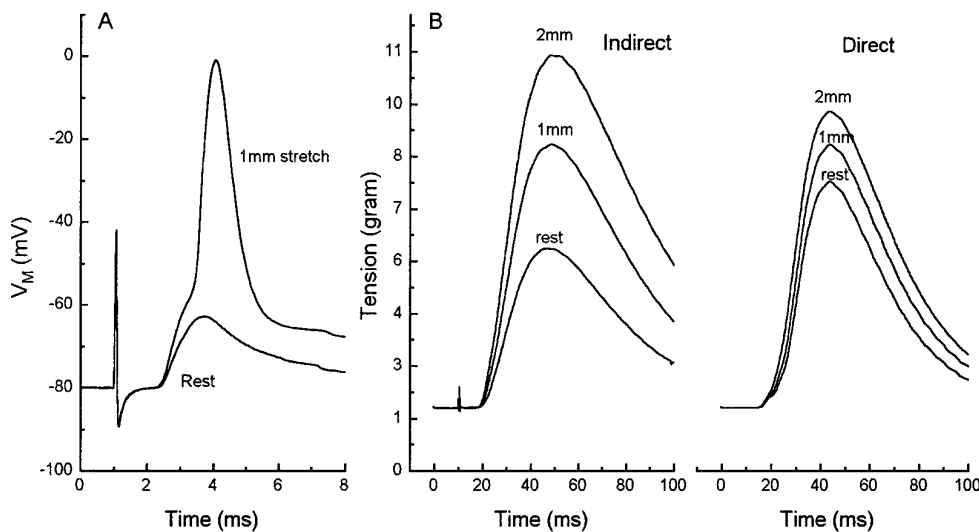
*Figure 3.* Effects of stretch on response in NFR. *A*, intracellular records from an unblocked junction in which stretch of the muscle elicited only an EPP at rest length but a full action potential after 1 mm (3%) muscle stretch. *B*, Records of twitch tension at rest length and after 1 and 2 mm (6%) stretch in a 30 mm sartorius in response to nerve stimulation (*left*) and direct muscle stimulation (*right*). The increase in tension with stretch in the directly stimulated muscle is predicted from the length–tension relationship of individual fibers. The much greater enhancement with indirect stimulation reflects the recruitment of large populations of fibers that were not excited above threshold at rest length. That indirect stimulation produced a larger twitch than direct stimulation at 6% stretch suggests that the direct stimulus did not evoke action potentials in all fibers in the muscle.

reflex. An estimated 30% of the muscle fibers in the frog sartorius do not receive synaptic inputs that are suprathreshold for muscle activation at rest length (Kuffler, 1952; Grinnell and Herrera, 1980; Grinnell and Trussell, 1983). A small stretch, caused by contraction of antagonist muscles, can increase evoked release enough to elevate the EPP above threshold in many of these fibers, increasing the force of contraction when axons to the muscle are excited. As Figure 3 illustrates, in NFR a 1 mm (3%) stretch can elevate an EPP above threshold, and 3 and 6% stretches can greatly enhance the magnitude of a twitch.

## Kinetics of stretch enhancement

A major factor in developing an hypothesis to explain the stretch enhancement is the kinetics of the phenomenon, i.e., the rate of change in release efficacy with change in length. Figure 4 shows the time course of change in EPP amplitude at a single junction when the muscle was stretched by 2 mm (6%) in 50 msec, held at the new length for 45 msec, and then returned to rest length in 50 msec (see Materials and Methods). Low $Ca^{2+}$ Ringer, containing 0.56 mM $Ca^{2+}$ and 4 mM $Mg^{2+}$, was used to reduce quantal content, eliminating muscle contraction. Every point is the average of 50 or more EPPs, each in response to a single stimulus given during a separate cycle of lengthening and shortening. Thus, the data for this figure came from a series of ~1800 separate cycles of lengthening and shortening, presented at a rate of 0.5 Hz. The direct measurement of muscle length shown in Figure 4 was done with an optical technique (see Materials and Methods) after microelectrode recording. As Figure 4 shows, the muscle stretch near the point of physiological recording quite accurately reflected the time course of the command signals to the mechanical arm, but with a delay of ~2-3 msec. The changes in release efficacy paralleled the stretch and shortening with remarkable accuracy and stability, with virtually no delay as the length was changed.

Figure 5 shows the average of six such experiments, normalized for 100% of the full response to 6% stretch, where rest length was taken as the mean of several responses before the onset of stretch and 100% enhancement is the average of all of the EPPs recorded at 6% stretch. Again, there was no significant deviation of the enhancement from a linear, virtually instantaneous dependence on muscle length, with the possible exception of the beginning of the shortening phase, where enhancement slightly lagged the shortening of the muscle, and at the end of the shortening phase,

when slight enhancement persisted for a few milliseconds. We do not know the explanation for these deviations. In general, however, there seemed to be virtually no delay between change in length and change in release efficacy. Clearly the rate of development and decay of the enhancement required no more than 1–2 msec, if that, at a rate of change in length of 2 mm in 50 msec.

Another set of measurements confirmed that there is virtually no delay between stretch and enhancement. Although the requirement that the floating electrode stay in the muscle fiber throughout the experiment precluded the ideal experiment, i.e., abruptly changing length and measuring the rate of change in EPP amplitude, it was possible in some experiments to maintain good penetrations at lengthening rates up to 2 mm/20 msec. Figure 6*A* shows the average of five such experiments, normalized to the amplitude achieved at the end of a 50 msec stretch, with each point the average of five trials per experiment. The amplitude of an EPP elicited immediately at the point of completing the 2 mm stretch command was ~3% less when the stretch was done in 20 msec than when it was done in 50 msec. This might suggest that at the faster rate of lengthening, there is a greater delay in development of enhancement of release. However, as Figure 6*B* shows, this shortfall can easily be explained by a difference in actual muscle length when the terminal was stimulated. At a lengthening rate of 2 mm/20 msec, the stretch of the muscle lagged behind the computer command significantly more than at 2 mm/50 msec. Thus, if the nerve was stimulated at the time of first reaching the peak of the command voltage, causing action potential invasion of the terminal 1–2 msec later, the actual muscle (and terminal) length would have been less in the case of the 20 msec stretch than the 50 msec stretch. Indeed, the degree of stretch of the muscle was ~10% less at 20 msec in the rapid stretch than at 50 msec in the slower stretch, so one might have predicted an even larger difference in enhancement. We conclude that there was no greater delay in development of enhancement at a faster stretch rate and that the modulation of evoked release follows muscle length with essentially no delay. There is no time for complicated chemistry.

## Integrins and mechanical coupling of terminal to ECM/muscle

An early hypothesis to explain the stretch effect was the possibility that stretching the nerve terminal straightened out folds in the presynaptic membrane, exposing more release sites (Hutter and Trautwein, 1956). Subsequent studies of the freeze-fracture ultra-
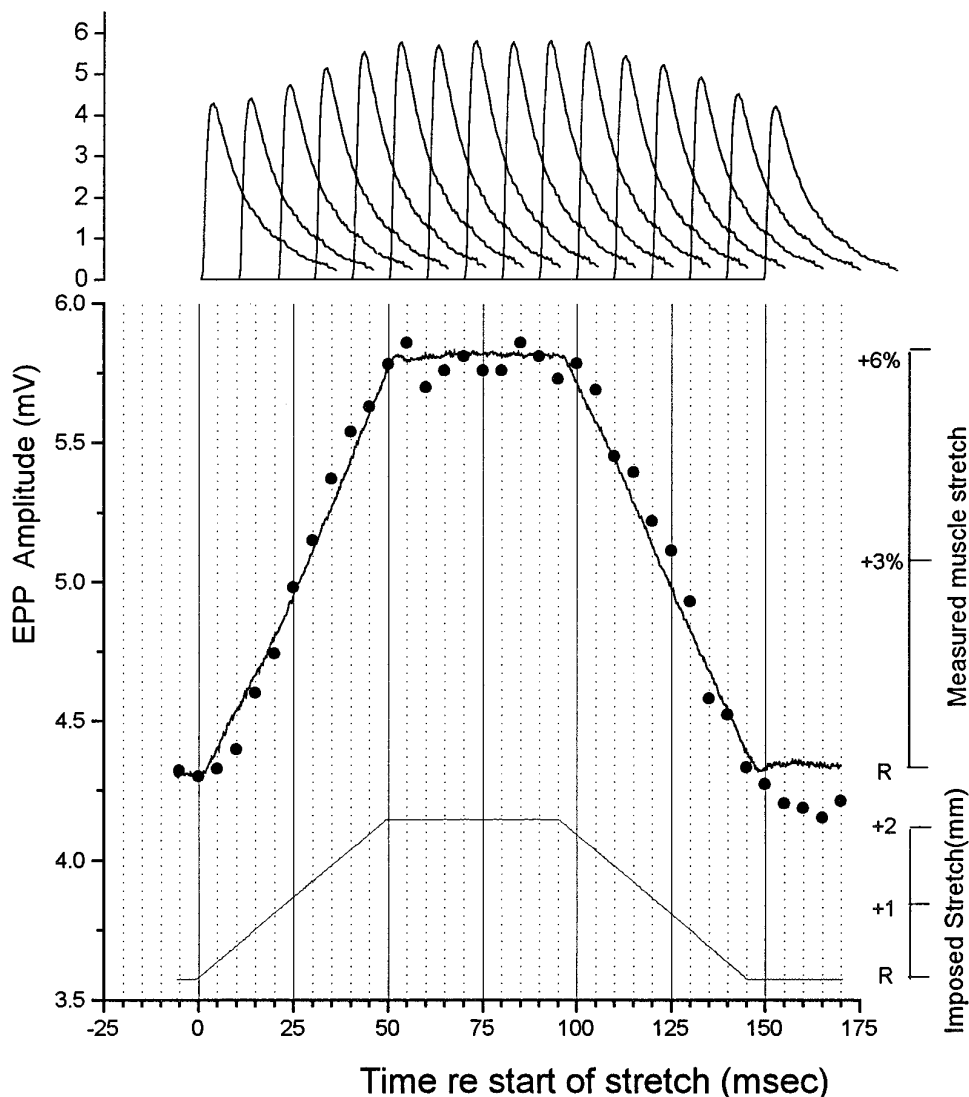
*Figure 4.* Kinetics of stretch enhancement of EPP amplitude in a representative sartorius junction in low $Ca^{2+}$ Ringer. The muscle was attached at either end to a pair of arms that moved simultaneously in opposite directions in response to a command signal from the computer, beginning to lengthen at 0 msec, reaching 2 mm (6%) stretch at 50 msec, holding that length for 45 msec, and shortening in another 50 msec. The actual measured change in length of the muscle (*noisy solid line*) and corresponding EPP amplitudes at 36 time points during the cycle (*filled circles*) are shown in the *middle* records, and sample EPPs taken at different times are shown at the *top*. Each point is the average of 50 or more EPPs, each evoked in a single lengthening–shortening cycle.

structure of frog motor nerve terminals (Heuser et al., 1974; Peper et al., 1974; Heuser and Reese, 1981; Propst and Ko, 1987; Meriney et al., 1996; P. A. Pawson, B. Wolowske, and A. D. Grinnell, unpublished observations) have failed to reveal significant membrane folding, especially near active zones, and it is unlikely that this model could explain the magnitude of enhancement (10% increase for a 1% change in length). Instead, it seems likely that there are mechanical connections between the nerve terminal and the muscle fiber or basal lamina that, in exerting tension on the terminal, somehow modulate transmitter release. There is no paucity of mechanical connections. The terminal is heavily invested in connective tissue and tightly covered (and partially enwrapped) by Schwann cells that are also attached via the matrix to the muscle cell. The rapidity and strength of the modulation suggest, however, that a mechanical stimulus is somehow delivered to the nerve terminal close to the release sites. Integrins, which are widespread, versatile cell membrane adhesion molecules with well established transduction capabilities, clearly play a role in delivering the mechanical stimulus (Chen and Grinnell, 1995).

Integrin–ligand bonds require divalent cations for stabilization and can be blocked by extrinsic RGD-containing peptides. Figure 7 shows the effect of RGD on the stretch enhancement of mEPP

frequency and EPP amplitude in NFR. Preparations were incubated for 1 hr in NFR containing 0.2 mM RGD or the control peptide, RGE, before commencing recording. RGD did not affect mEPP frequency or EPP amplitude at rest length but caused a 33–46% reduction in the enhancement of mEPP frequency and a 50–65% decrease in the stretch enhancement of EPP amplitude, compared with preparations treated with RGE or with no peptide. RGD does not affect the increase in mEPP frequency produced by terminal depolarization. Changing from NFR to Ringer containing 6 mM $K^+$ produced a 55 ± 13% increase in mEPP frequency in the presence of 0.2 mM RGD ($n = 65$), a 49 ± 10% increase in its absence ($n = 45$).

The effectiveness of RGD in suppressing the stretch enhancement is strong evidence for the involvement of integrins in the phenomenon. Moreover, a polyclonal antibody directed against the extracellular domain of *Xenopus* $\beta_1$ integrin subunit, kindly provided by Dr. D. DeSimone (University of Virginia), also partially inhibited the enhancement (Chen and Grinnell, 1995). However, under the conditions outlined above, neither RGD nor the antibody completely suppressed the stretch effect, leaving open the possibility that nonintegrin pathways also contribute. It should be noted, on the other hand, that RGD, like other function-blocking peptides, normally does not cause a complete block of
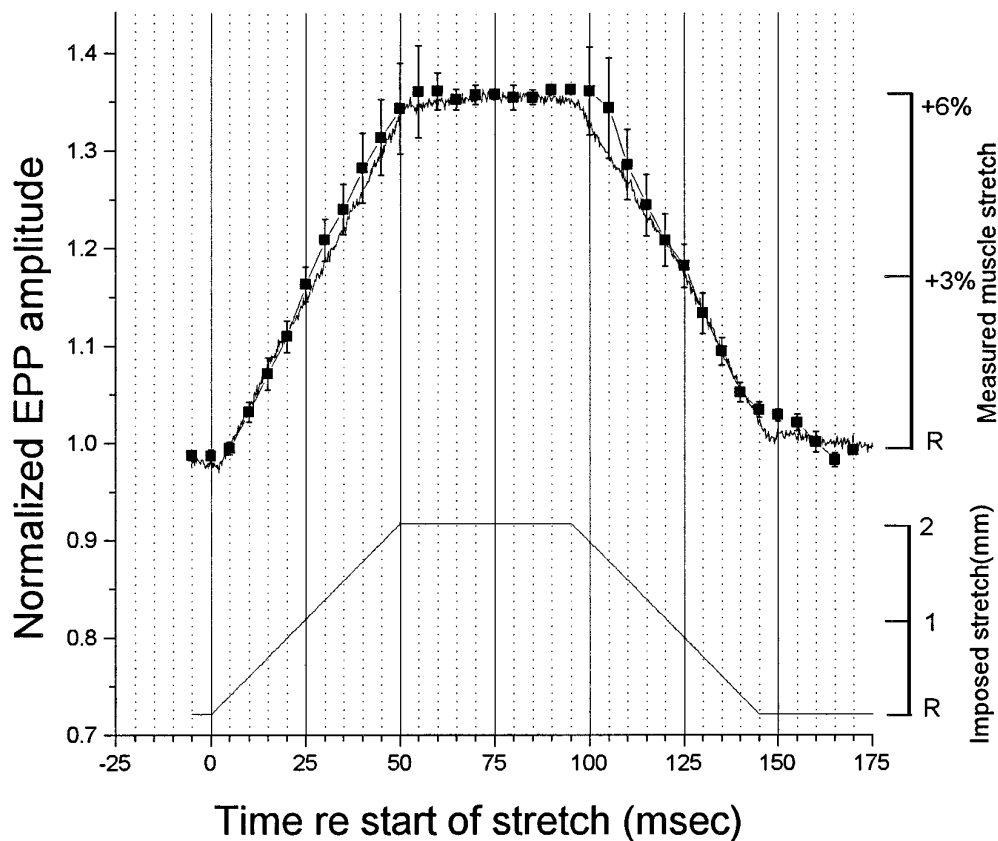
*Figure 5.* Kinetics of stretch enhancement of EPP amplitude. Average of six experiments like that shown in Figure 4. Note the relatively large *arrow bars* at the peak of the lengthening phase and the beginning of shortening. Muscle length is shown by the *noisy line*, where the noise arose primarily in the light source used to measure length (see Materials and Methods). Except at the beginning of the shortening phase, there was virtually no delay between change in muscle length and change in release efficacy.

integrin-binding interactions (Gartner and Bennett, 1985). Especially in a preparation in which integrins are already bound to their native ligands, it should not be expected that extrinsic RGD peptides could entirely displace the natural ligands. It is noteworthy, therefore, that other conditions were found in which RGD could, in fact, totally eliminate the stretch enhancement (see sections D and E in Results).

### D. Lack of dependence of the effect on stretch-induced $Ca^{2+}$ influx

The stretch modulation of release is not blocked by $Mg^{2+}$ (Turkanis, 1973) or by $\omega$-conotoxin GVIA (Chen and Grinnell, 1995), which blocks N-type voltage sensitive $Ca^{2+}$ channels and neuromuscular transmission in frogs (Kerr and Yoshikami, 1984). It is still possible, however, that stretch opens channels (SACs) that are $Ca^{2+}$-permeable and not blocked by $Mg^{2+}$. The most rigorous test of whether $Ca^{2+}$ influx is necessary for stretch enhancement is to eliminate all $Ca^{2+}$ from the external medium. In EGTA-buffered zero $Ca^{2+}$ Ringer, frog muscle fibers tend to twitch spontaneously. This twitching can be prevented, however, by treating the muscle for 25 min with 25 $\mu$M $\mu$-conotoxin, which blocks $Na^+$ channels in muscle but not in nerve (Cruz et al., 1985; Robitaille and Charlton, 1992). After this treatment and 1 hr further incubation in zero $Ca^{2+}$ Ringer, EPPs were gone and resting mEPP frequency was decreased from a mean of 1.47 to 0.69 Hz, but there was still a clear stretch enhancement of mEPP frequency [by 17.9% at 1 mm and 28.4% at 2 mm stretch (see Fig. 8, *column 2*)]. Thus, $Ca^{2+}$ influx, through SACs or any other kind of channel, is not required for the stretch modulation of spontaneous release.

The ~50% reduction in stretch enhancement in zero $Ca^{2+}$ Ringer might reflect the loss of a component that is dependent on

$Ca^{2+}$ influx, or it might be the result of destabilization of integrin bonds by the withdrawal of $Ca^{2+}$. We believe that the latter is the important factor because addition of 0.5 mM $Mn^{2+}$, a good stabilizer of integrin bonds as well as an effective $Ca^{2+}$ channel blocker (Gailit and Ruoslahti, 1988; Kirchhofer et al., 1990; Grinnell and Backman, 1991), restored the stretch enhancement to almost its full magnitude (Fig. 8, *column 3*). Moreover, under these conditions, in preparations pretreated with zero $Ca^{2+}$ and RGD to weaken binding to natural ligands before addition of $Mn^{2+}$, RGD totally blocked the stretch enhancement (Fig. 8, *column 4*), suggesting that all of the enhancement is mediated via integrins. The greater effectiveness of RGD in inhibiting the integrin-mediated phenomenon in a $Mn^{2+}$-stabilized preparation is consistent with observations that $Mn^{2+}$ increases the affinity of integrins for RGD at the expense of natural ligands (Gailit and Ruoslahti, 1988; Kirchhofer et al., 1990).

### E. Does stretch act via an elevation in intraterminal $Ca^{2+}$?

Although modulation of mEPP frequency does not require stretch-induced $Ca^{2+}$ influx, $[Ca^{2+}]_i$ is important. If terminals were loaded with BAPTA or DM-BAPTA by immersion in the $-AM$ form of the buffer (see Materials and Methods), the stretch effect in zero $Ca^{2+}$ Ringer was severely reduced. Loading of terminals with DM-BAPTA, a fast, high-affinity $Ca^{2+}$ buffer, reduced resting mEPP frequency to an average 0.46 Hz, and reduced the stretch enhancement to 7.5% at 1 mm ($n = 29$) and 8.8% at 2 mm stretch ($n = 23$) (Fig. 8, *column 5*). It was of interest that 0.5 mM $Mn^{2+}$ added to the zero $Ca^{2+}$ Ringer raised the resting mEPP frequency in the DM-BAPTA-loaded terminals to a mean of 1.64 Hz and increased the stretch enhancement to $22.1 \pm 3.7\%$ at 1 mm ($n = 57$) and $43.4 \pm 6.3\%$ at 2 mm ($n = 49$;
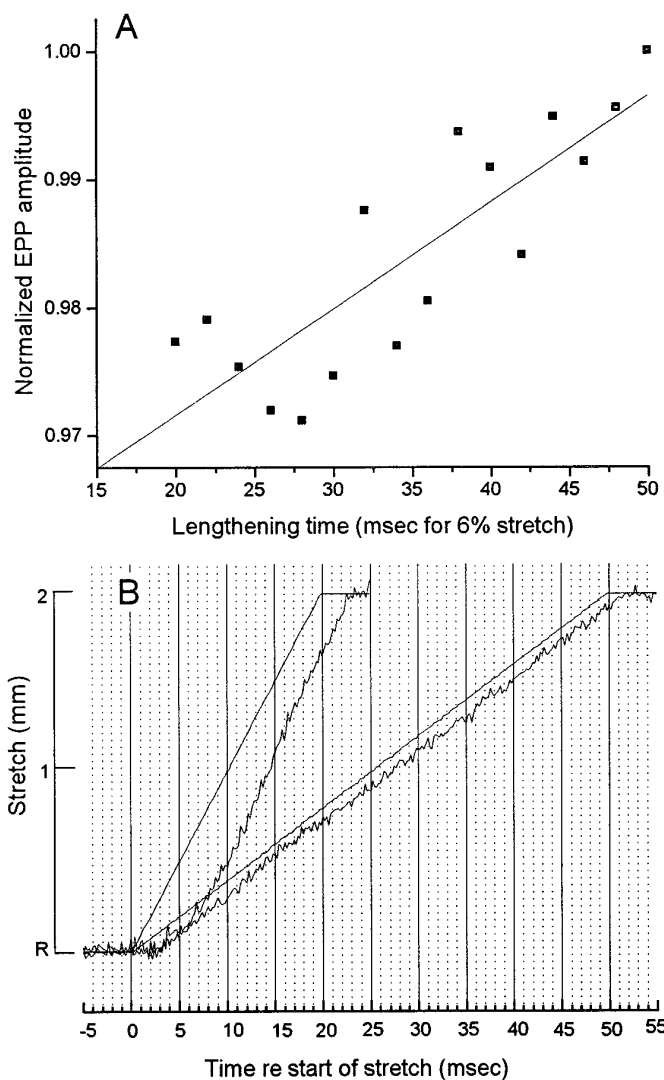
*Figure 6.* *A*, EPP amplitudes in low $Ca^{2+}$ Ringer evoked by stimulation at the moment of reaching the 2 mm stretch command voltage, when the stretch was accomplished at different rates between 2 mm/20 msec and 2 mm/50 msec. The enhancement after the most rapid lengthening (20 msec) was ~3% less than after a lengthening that took 50 msec. *B*, Optical measurement of muscle length during 2 mm stretches in 20 and 50 msec. The greater lag between command voltage and actual muscle stretch at 2 mm/20 msec can explain the slightly reduced enhancement. The muscle length was 30 mm, hence 2 mm represented 6% stretch.



*Figure 7.* Suppression of the stretch enhancement by RGD peptides. The graphs show that enhancement of mEPP frequency and EPP amplitude was approximately linear with length and that 0.2 mM RGD, but not the inactive control RGE, suppressed the effect. EPPs were recorded in 3–6 μM curare.

Fig. 8, *column 6*). Again, after pretreatment with zero $Ca^{2+}$ Ringer and RGD, this $Mn^{2+}$-supported enhancement was totally blocked by 0.2 mM RGD (Fig. 8, *column 7*).

The stretch enhancement of EPP amplitude was also sensitive to internal $Ca^{2+}$ buffering. Loading of terminals by 1 hr incubation in BAPTA-AM did not affect the mean rest amplitude of EPPs in a 0.56 mM $Ca^{2+}$, 4 mM $Mg^{2+}$ Ringer, but did reduce the stretch enhancement by 60–70%, from 31 ± 3.9% ($n = 32$) to 11.3 ± 4.1% ($n = 22$) at 1 mm ($p < .001$) and from 63 ± 5.9% ($n = 27$) to 24.6 ± 3.8% ($n = 28$) at 2 mm stretch ($p < 10^{-5}$).

Binding of integrins can activate second messenger cascades that lead to release of $Ca^{2+}$ from internal stores (see Introduction). It seems unlikely that such a process could act fast enough to explain stretch-induced changes in evoked release, but they could easily explain the increase in mEPP frequency. In an at-
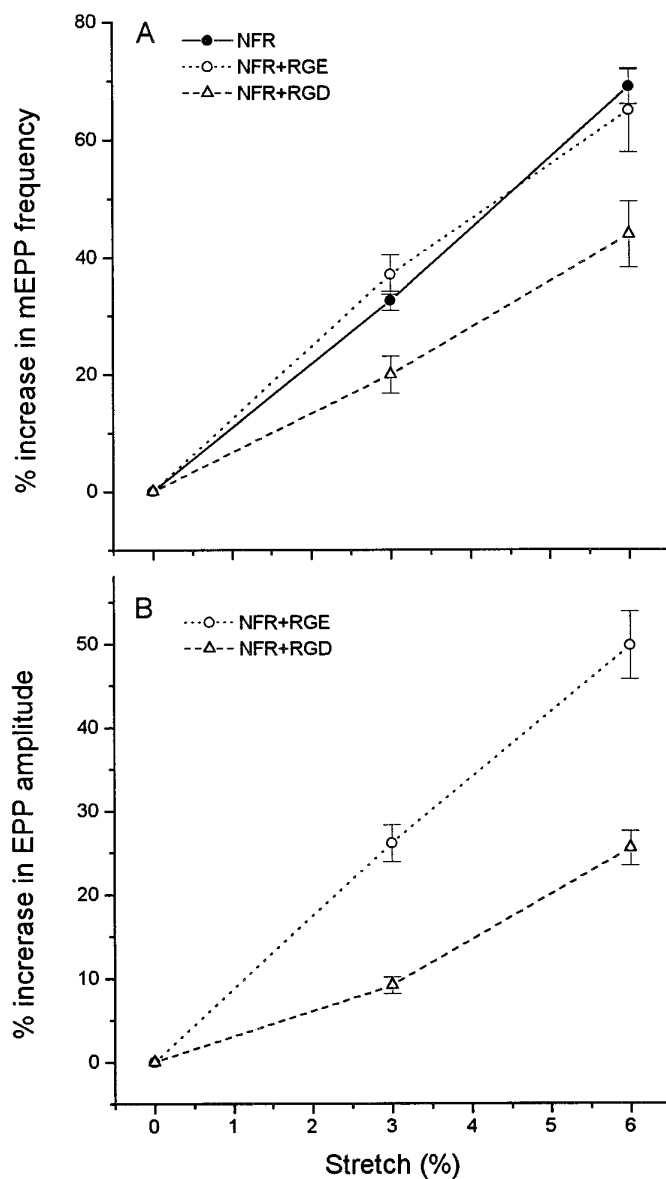
tempt to determine whether stretch acts by causing an increment in $[Ca^{2+}]_i$, we have attempted to "clamp" $[Ca^{2+}]_i$ at a fixed level, while eliminating the transmembrane electrochemical gradient for $Ca^{2+}$. The terminal was loaded with BAPTA by immersion for 60–90 min in 25 μM BAPTA-AM in NFR. Although the effective $K_D$ of $Ca^{2+}$ binding is dependent on ionic strength and the specific intracellular environment, it is ~100 nM (Tsien, 1980), close to the probable normal level. The effectiveness of BAPTA loading can be tested by determining the effect of $K^+$ depolarization on mEPP frequency. Where normally 10 mM $K^+$ increased mEPP frequency by a factor of 5.04 ± 2.02 ($n = 10$), after BAPTA loading the increase was only 1.32 ± 0.63X ($n = 9$).

Because the exogenous buffer has a $K_D$ near or slightly above the normal $[Ca^{2+}]_i$, the free $Ca^{2+}$ level will still be determined in part by the intrinsic buffers that have a higher $Ca^{2+}$ affinity, and
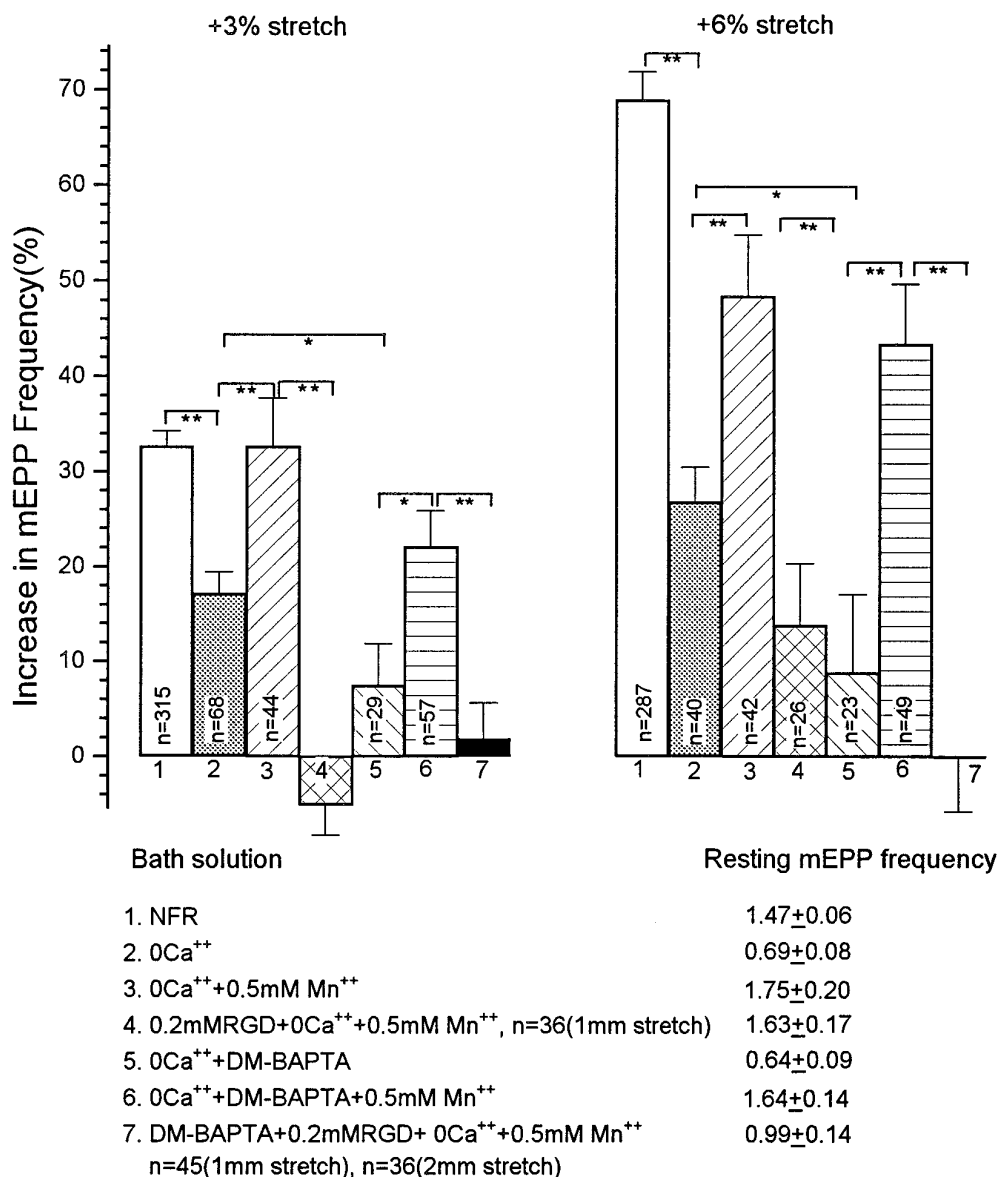
*Figure 8.* Effects of manipulations affecting $Ca^{2+}$ levels on the magnitude of the increase in mEPP frequency with stretch. Zero $Ca^{2+}$ Ringer reduced the enhancement by ~50% (*column 2*), but the enhancement was still highly significant. $Mn^{2+}$ (0.5 mM) restored much of the enhancement (*column 3*), which was then essentially totally blocked by 0.2 mM RGD, applied both before and after addition of $Mn^{2+}$ (*column 4*). Loading of terminals with a strong $Ca^{2+}$ buffer by exposure to DM-BAPTA-AM in zero $Ca^{2+}$ Ringer strongly suppressed the enhancement (*column 5*), but again 0.5 mM $Mn^{2+}$ mostly restored it (*column 6*); 0.2 mM RGD, applied both in the zero $Ca^{2+}$ Ringer and during subsequent addition of $Mn^{2+}$, totally eliminated the enhancement (*column 7*). These data show that $Ca^{2+}$ influx is not necessary for the enhancement and that the enhancement can be totally eliminated by agents that interfere with integrin binding. Absolute mEPP frequencies at rest length are also indicated for each condition, and *asterisks* indicate the probability of the indicated matches (*$p < 0.05$, **$p < 0.005$).

small changes might be quite significant against a low background (hence, capable of modulating release). Therefore, two additional manipulations were done to minimize such changes. Thapsigargin (10 $\mu$M), a membrane-permeant reagent that blocks endoplasmic reticulum $Ca^{2+}$-ATPase, was used to unload the primary internal stores of $Ca^{2+}$ rapidly and irreversibly (Thastrup et al., 1989). Thapsigargin caused a transient increase in mEPP frequency that returned to original levels within 15–20 min. This treatment thus removed a major potential source of release of $Ca^{2+}$ from internal stores. Finally, wishing to fix intraterminal $[Ca^{2+}]_i$ at ~100 nM, we immersed the preparation in an EGTA-buffered Ringer containing 1 nM free $Ca^{2+}$, which would be approximately in electrochemical equilibrium with the assumed intraterminal $[Ca^{2+}]_i$ of ~100 nM at a membrane potential of about −70 mV. To facilitate free exchange of $Ca^{2+}$ across the membrane, the $Ca^{2+}$ ionophore A23187 (2 $\mu$M), diluted from a 10 mM solution in DMSO, was used in several experiments (Duncan and Statham, 1977; Rehder et al., 1992). We saw no evident difference between experiments done with and without A23187. Under these conditions, the mEPP frequency was maintained slightly above its normal level (mean,

1.73 Hz; $n = 32$), and there was still a large stretch enhancement (Fig. 9). Although it remains theoretically possible that stretch causes the release of $Ca^{2+}$ from a thapsigargin-resistant compartment in a sharply confined space near the release sites, it seems much more likely that stretch enhancement does not involve an increment in intraterminal $Ca^{2+}$.

## F. Temperature coefficient of the stretch enhancement

If stretch does not elevate $[Ca^{2+}]_i$, the implication is that the enhancement is a result of either a change in the $Ca^{2+}$ affinity of a $Ca^{2+}$-sensing molecule necessary for vesicle docking or release, or a change in the $K_m$ of a reaction subsequent to $Ca^{2+}$ binding in the release process. We know almost nothing about such reactions, although it is clear that the $Ca^{2+}$-sensing process can be bypassed under certain conditions, e.g., by $\alpha$-latrotoxin, which somehow induces massive release when it binds to its receptor neurexins, which are associated with synaptotagmin, syntaxin, and $Ca^{2+}$ channels in a "synaptosecretosome" (Rosenthal et al., 1990; O'Connor et al., 1993). Two generic hypotheses can be proposed: first, that stretch changes $Ca^{2+}$ binding properties or critical
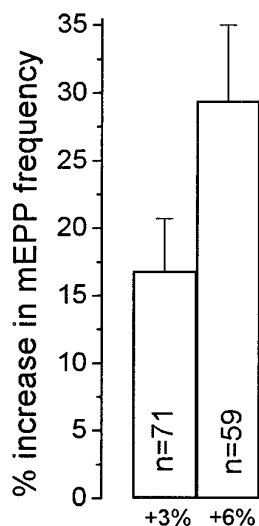
*Figure 9.* Stretch enhancement of release with 3 and 6% stretch in preparations in which $[Ca^{2+}]_i$ was "clamped" at ~100 nM by loading the terminal with BAPTA, disabling the main intrinsic $Ca^{2+}$ buffers with 10 $\mu$M thapsigargin, and reducing or eliminating the electrochemical gradient for $Ca^{2+}$ across the terminal membrane by immersion in a Ringer containing 1 nM $Ca^{2+}$ and 2 $\mu$M of the $Ca^{2+}$ ionophore A23187. Under these conditions, stretch enhancement of release was still about one-half that found in NFR and very close to that obtained in zero $Ca^{2+}$ Ringer (see Fig. 8).

reaction kinetics by a metabolic modification of some molecule(s), e.g., by a phosphorylation or dephosphorylation; and second, that the modulation of release probability is accomplished by a purely mechanical alteration in the position or conformation of one or more molecules, changing the equilibrium for a critical reaction. Any process fitting the first hypothesis would be expected to have a $Q_{10}$ of 2 or higher, whereas a purely mechanical modification of molecular conformation or position, affecting a diffusion-limited process, would be expected to have a $Q_{10}$ closer to 1.

Both the absolute EPP amplitude and mEPP frequency change markedly with temperature. Figure 10*A* shows the time course of change in EPP amplitude with 2 mm stretch and shortening in a representative preparation at 22.2° and after 1 hr equilibration at 13°C. The absolute EPP amplitude was decreased by nearly 50% at the lower temperature, consistent with the finding that quantal size decreases with temperature (Adams, 1989), but the percentage change in release with the change in length was actually somewhat greater. When the change with length is normalized (Fig. 10*B*), it is clear that the time course of the change in amplitude was essentially indistinguishable at the two temperatures during both lengthening and shortening phases (Fig. 10*B*).

MEPP frequency typically dropped by 50% or more when a preparation was cooled from 22–23° to 10–13°, but again the effects of stretch were undiminished. As Figure 11 shows for a representative preparation, the percentage changes in mEPP frequency were somewhat larger at the lower temperature, even though the absolute levels were depressed. When the absolute frequency at 10° was increased by depolarizing with 10 mM $K^+$, raising the level approximately to that in NFR at 23°, the absolute changes in frequency with length at 10° and 23° were almost exactly the same. Thus, both the dynamics of the modulation and the percentage change in release effectiveness with stretch are undiminished by cooling within the range tested, and the $Q_{10}$ values of these phenomena are close to 1.
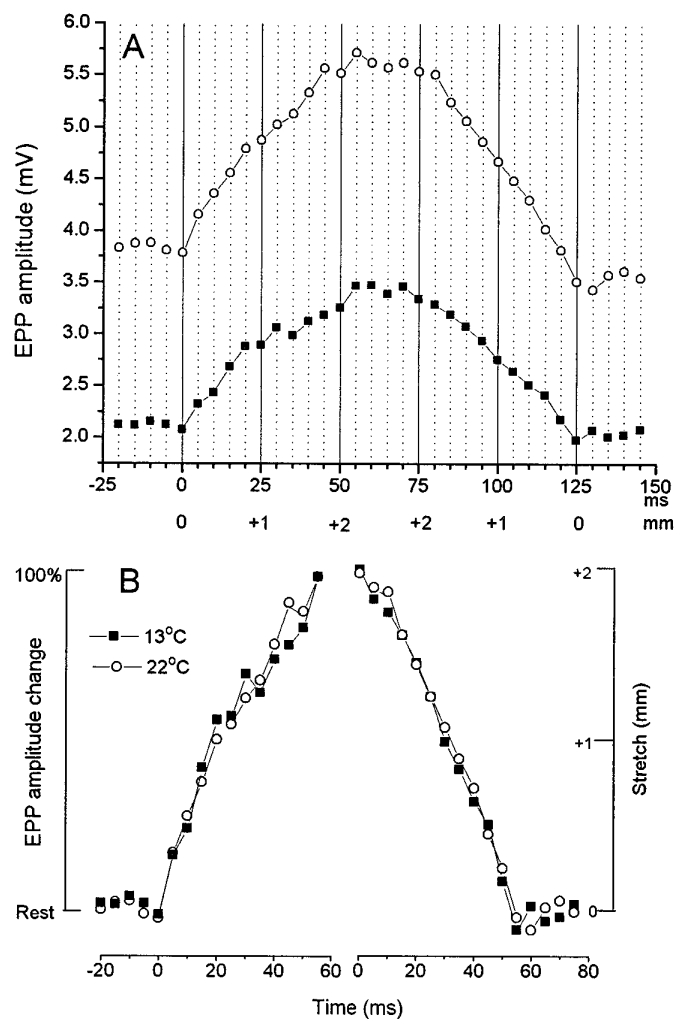


*Figure 10.* Effects of change in temperature on stretch enhancement of the EPP in low $Ca^{2+}$ Ringer. *A,* Average of records from five lengthening–shortening cycles at a single representative junction at 22.2° and at 13°, showing the change in absolute EPP amplitude with stretch and shortening [using a stretching regime similar to that of Figs. 4 and 5 but with stretch maintained at +2 mm (6%) for only 25 msec]. The absolute amplitude was much decreased at 13°, but the percentage change with stretch was actually increased at the lower temperature. *B,* Normalized plots of the lengthening and shortening phases at the two temperatures. The kinetics of the change in amplitude with change in length were essentially unaffected by temperature in this range.

## DISCUSSION

Stretch is a powerful modulator of both evoked and spontaneous transmitter release at the frog neuromuscular junction. *In vivo,* as the hind limb is flexed and the sartorius is stretched by activation of opposing muscle groups, action potentials in sartorius nerve terminals would be expected to elicit larger EPPs and generate action potentials in a higher percentage of muscle fibers, increasing the force of sartorius contraction. A similar enhancement of release obtains in the cutaneous pectoris (A. Kashani and A. D. Grinnell, unpublished observations) and is probably characteristic of most frog muscles.

We consider it probable that enhancement of spontaneous and evoked release are both manifestations of the same change in the release machinery caused by the level of tension on integrins in the membrane. However, important properties of the stretch enhancement can be studied only in one or the other phenome-
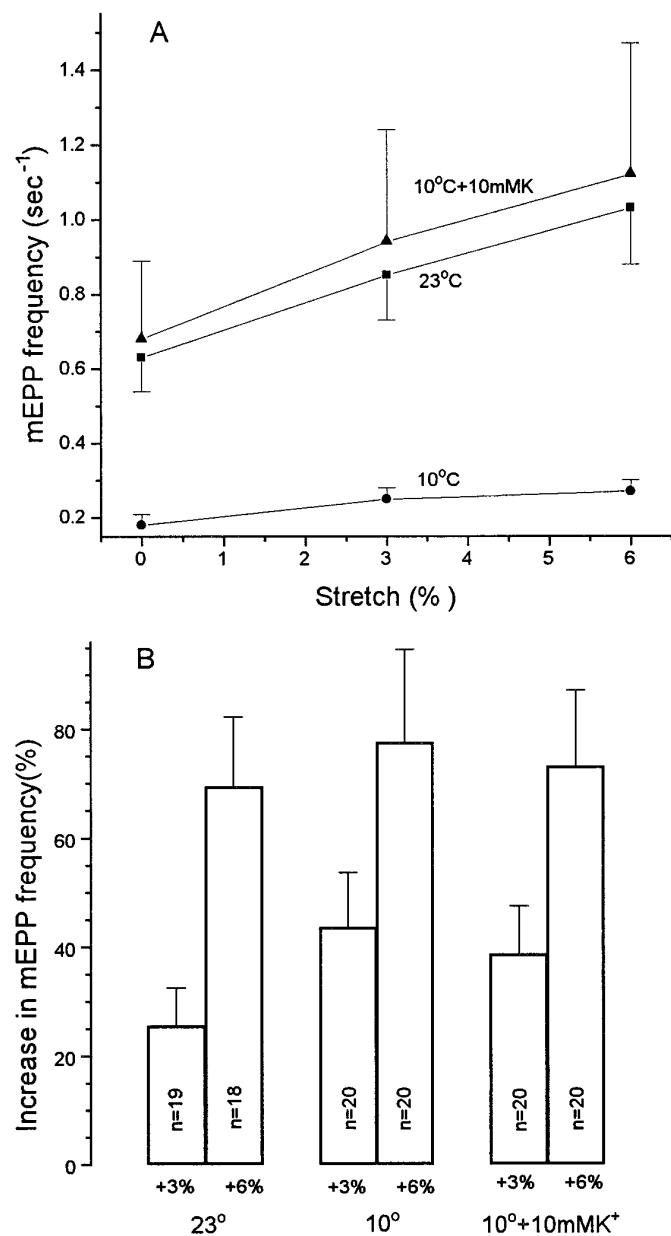
*Figure 11.* Effects of temperature on sensitivity of mEPP frequency to stretch. *A,* mEPP frequency as a function of muscle length at 10° and 23° in NFR, and at 10° in NFR plus 10 mM $K^+$. *B,* Percentage change in mEPP frequency at 3 and 6% mm stretch under each of these conditions. Cooling reduced the resting mEPP frequency but had little effect on the enhancement by stretch. When the resting mEPP frequency was increased by $K^+$ depolarization to approximately the level in NFR at 23°, the effect of stretch was indistinguishable from that at 23°C.

non. The rapid kinetics of the modulation can be tested carefully only with EPPs, for example, and might not apply to mEPPs. Conversely, a lack of dependence of modulation on $Ca^{2+}$ influx can be demonstrated only for mEPPs because EPPs require some $Ca^{2+}$ in the external medium. Stretch-activated channels could still be involved in the modulation of EPP amplitude. On the other hand, the similar nonadapting nature of the change in EPP amplitude and mEPP frequency at a new length, the similar magnitude and linearity of the changes, and the low $Q_{10}$ of both are consistent with the idea that they result from the same mechanism. Both show a similar susceptibility to blockage by RGD

peptides, and hence involve integrins at some step in the process. We have no data incompatible with the conclusion that they are modulated by the same mechanism, but it is essential to keep in mind that this is not necessarily the case, especially given evidence that spontaneous and evoked quantal release can behave differently with different manipulations (Barrett and Magleby, 1976; Andreu and Barrett, 1980; Narita et al., 1990b; Umbach et al., 1990; Zengel and Sosa, 1994).

The modulation of release at frog neuromuscular junctions is extraordinarily rapid, linear with change in length, and nonadapting, all properties that argue against the involvement of second messenger pathways. External $Ca^{2+}$ is not needed for stretch modulation of mEPP frequency, ruling out changes in $Ca^{2+}$ influx through voltage- or stretch-sensitive channels. However, it is more difficult to exclude the possibility that stretch causes a release of $Ca^{2+}$ from internal stores, especially in view of the finding that loading of terminals with a strong $Ca^{2+}$ buffer suppresses the enhancement. Integrin binding is capable of activating phospholipase C, releasing DAG and $IP_3$ (Cybulsky et al., 1990; McNamee et al., 1993; Schwartz, 1993), which can sharply elevate $[Ca^{2+}]_i$. In most cases this is much too slow a process to explain the stretch enhancement of EPP amplitude, but in a tightly confined space this might not be the case. In olfactory receptors, for example, $IP_3$ can rise in concentration within 25 msec after the activation of receptors (Restrepo et al., 1993). Another candidate pathway would be through mobilization of cyclic ADP-ribose, thought to be the endogenous activator of $Ca^{2+}$-induced $Ca^{2+}$ release (Galione, 1994), primarily from the endoplasmic reticulum (Etcheberrigaray et al., 1991). However, even if mobilization of $IP_3$ or cyclic ADP-ribose could elevate $[Ca^{2+}]_i$ quickly enough to explain the change in release, there are so many potent buffering and pumping systems regulating internal $[Ca^{2+}]_i$ that a nonadapting change seems highly improbable.

It is of interest that 0.5 mM $Mn^{2+}$ could almost fully restore the stretch enhancement in zero $Ca^{2+}$ Ringer. In addition to blocking $Ca^{2+}$ channels (Hagiwara and Takahashi, 1967), $Mn^{2+}$ increases resting mEPP frequency (see Fig. 8) but does not support evoked release. The mechanism of this enhancement of mEPP frequency is not known, although it is possible that $Mn^{2+}$ permeates through voltage-sensitive $Ca^{2+}$ channels and displaces $Ca^{2+}$ from internal stores (Kita et al., 1981; Misler and Falke, 1987; Narita et al., 1990a). It is noteworthy that $Mn^{2+}$ also increased the resting mEPP frequency and sharply enhanced the stretch effect in preparations that had been loaded with DM-BAPTA (see Fig. 8), which would be expected to buffer any internal changes in free $Ca^{2+}$. However, by analogy with the divalent cation binding properties of the BAPTA derivative fluo-3, which binds $Mn^{2+}$ ~70 times more strongly than $Ca^{2+}$ (Minta et al., 1989), if a significant amount of $Mn^{2+}$ did permeate the membrane, it might displace $Ca^{2+}$ from BAPTA, elevating intraterminal $Ca^{2+}$. $Ca^{2+}$ release from internal stores under these conditions might be largely unaffected by the presence of BAPTA. On the other hand, even in a BAPTA-loaded, $Mn^{2+}$-treated preparation, 0.2 mM RGD completely suppressed the stretch effect and reduced the resting mEPP frequency, arguing that a major function of $Mn^{2+}$ is stabilization of integrin–ECM bonds. This finding suggests several conclusions: that integrin binding is a critical link for essentially 100% of the stretch effect; that stretch does not work primarily by elevating $[Ca^{2+}]_i$ (although a moderate level of internal free $Ca^{2+}$ may be necessary); and that the enhancement of resting mEPP frequency by $Mn^{2+}$ may be a result, at least in part, of effects on
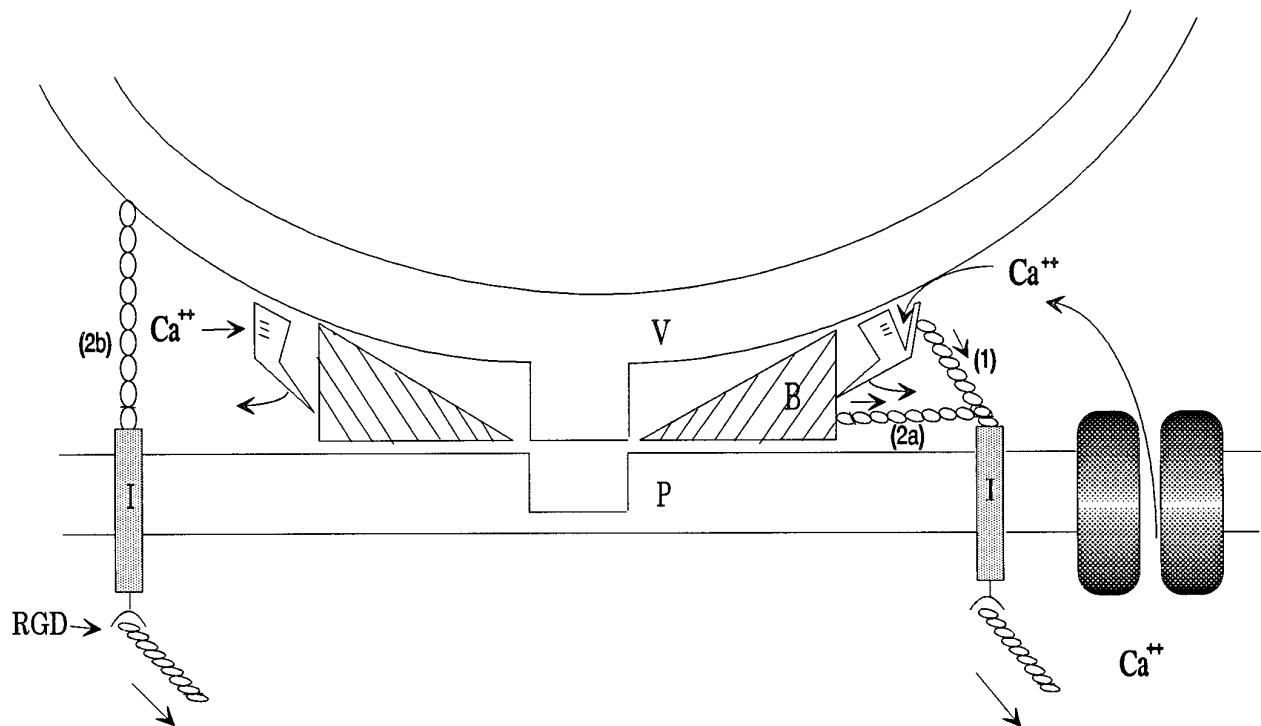
*Figure 12.* A schematic model suggesting, generically, how extracellularly applied tension on integrins might increase the probability of vesicle fusion. The fusion process is pictured as resulting when a docked vesicle (*V*) is allowed to be pulled into contact with the plasma membrane (*P*) at a specific site. This process is normally triggered by $Ca^{2+}$ interaction with a $Ca^{2+}$-sensing molecule that can, through an unknown number of steps, activate the fusion process, perhaps by displacing a molecule (*B*) that physically blocks contact of the two membranes. Tension on integrins (*I*) might alter the $Ca^{2+}$ binding affinity of the $Ca^{2+}$ sensor (*1*) or physically exert force contributing to the removal of the block of fusion (*2a*) or pulling of the vesicle down toward fusion-promoting molecules in the plasma membrane (*2b*). Another interpretation of alternative (*2b*) might be an increased probability of vesicle–plasma membrane fusion, the result somehow of a direct effect of mechanical stress on the conformation of the plasma membrane lipid. RGD inhibits the modulation by blocking integrin binding to ligands in the ECM.

the integrin-mediated pathway that helps regulate mEPP frequency.

A stretch-induced chemical modification of one or more intermediaries in the release pathway, e.g., by phosphorylation or dephosphorylation, also is unlikely. Reactions leading to phosphodiesterase activation, cGMP hydrolysis, and $Na^+$ channel modulation occur within tens of milliseconds in photoreceptors (Detwiler and Gray-Keller, 1992), but this is a rapidly adapting process and it is highly unlikely that the phenomenon could reverse as quickly as it develops. Instead, the development and the decay of the stretch modulation occur with a delay of no more than 1–2 msec, perhaps virtually instantaneously. This rules out stretch modulation of any second messenger or enzymatic pathway.

The very low $Q_{10}$ (close to 1 for both EPP and mEPP enhancement) also militates against any biochemical reaction being the direct result of stretch. Instead, lengthening of muscle seems to cause a change in release probability by mechanically altering the $K_m$ of a diffusion-limited process.

The nature of this mechanical process is still unknown and must be the subject of future research. It is highly unlikely that either the volume or the surface area of the terminal changes significantly during stretch, so the effects would be on shape and perhaps on local membrane conformation near points of attachment to the ECM or other connective tissue. Integrins are important anchoring molecules, connecting the cytoskeleton to the ECM. Because of the semirigidity of the cytoskeleton and its role in organizing arrays of associated molecules (Ingber, 1996), ten-

sion on integrins could transmit mechanical stimuli virtually instantaneously to other regions of the cell, potentially causing a change in the proximity or reactivity of molecules that interact in the process of vesicle docking and fusion. If this is the case, it represents a particularly rapid, powerful form of mechanical modulation of intracellular physiological processes.

It is important, however, to emphasize the specificity of this mechanical pathway and the role of integrins in it. After RGD treatment in a $Mn^{2+}$-treated preparation has eliminated the stretch enhancement, most of the effects of stretch on terminal structure would seem to be unchanged. Muscle stretch still causes terminals to elongate, with whatever changes in cytoskeletal organization are associated with this elongation; mechanical stress on the membrane persists at sites of non-integrin-mediated connective tissue connections to the terminal, and transmitter release is normal, but the stretch enhancement of release is eliminated. We do not yet know the nature of the critical connections between integrins and the ECM, nor do we have information regarding the chain of connections between integrins and the release apparatus inside the terminal. The data indicate that there are such connections, however, with a powerful effect on release. These connections must somehow be fitted into molecular models of vesicle docking and fusion.

We propose that the integrin-mediated pathway is working in one of two general ways, indicated schematically in Figure 12. Tension on integrins might, via an unknown chain of intraterminal connections: (1) change the $Ca^{2+}$-binding affinity of the $Ca^{2+}$ sensing molecules responsible for triggering the molecular ma-

chinery that causes (or disinhibits) vesicle fusion with the plasma membrane; or (2) transduce mechanical force directly onto molecules or structures affecting the probability of vesicle fusion, either (2a) by facilitating the reactions leading to vesicle fusion (or helping displace molecules that block fusion), or (2b) by directly increasing the likelihood of lipid/lipid interactions between the plasma and vesicle membranes, e.g., by pulling vesicles closer to the plasma membrane, or, via localized stress on the plasma membrane lipid, somehow facilitating fusion.

It would be surprising if frog neuromuscular junctions were the only synapses in which integrins helped regulate transmitter release. Given their transduction capabilities and the wide variety of physiological changes that they can trigger in cells, integrins might play different but important roles in central nervous system synapses, e.g., in response to subtle osmotic changes associated with use. It is of interest that interference with integrin binding by RGD peptides inhibits long-term potentiation in the hippocampus (Staubli et al., 1990), and a *Drosophila* mutant, *volado*, which lacks an $\alpha_1$ integrin subunit, suffers an olfactory learning deficit (R. L. Davis, X-R. Zhu, and K. H. Wu, unpublished observations).

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