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Hypertonic enhancement of transmitter release from frog motor nerve terminals: Ca^{2+} independence and role of integrins

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1. Hyperosmotic solutions cause markedly enhanced spontaneous quantal release of neurotransmitter from many nerve terminals. The mechanism of this enhancement is unknown. We have investigated this phenomenon at the frog neuromuscular junction with the aim of determining the degree to which it resembles the modulation of release by stretch, which has been shown to be mediated by mechanical tension on integrins.
2. The hypertonicity enhancement, like the stretch effect, does not require Ca^{2+} influx or release from internal stores, although internal release may contribute to the effect.
3. The hypertonicity effect is sharply reduced (but not eliminated) by peptides containing the RGD sequence, which compete with native ligands for integrin bonds.
4. There is co-variance in the magnitude of the stretch and osmotic effects; that is, individual terminals exhibiting a large stretch effect also show strong enhancement by hypertonicity, and vice versa. The stretch and osmotic enhancements also can partially occlude each other.
5. There remain some clear-cut differences between osmotic and stretch forms of modulation: the larger range of enhancement by hypertonic solutions, the relative lack of effect of osmolarity on evoked release, and the reported higher temperature sensitivity of osmotic enhancement. Nevertheless, our data strongly implicate integrins in a significant fraction of the osmotic enhancement, possibly acting via the same mechanism as stretch modulation.

Hypertonic solutions cause a marked increase in spontaneous quantal neurotransmitter release at many synapses (see for example, Fatt & Katz, 1952; Hubbard *et al.* 1968; Kita & Van der Kloot, 1977; Niles & Smith, 1982; Bourque & Renaud, 1984; Doherty *et al.* 1986; Brosius *et al.* 1992; Yu & Miller, 1995; Rosenmund & Stevens, 1996; Mochida *et al.* 1998). At frog neuromuscular junctions, 25–100 mosmol l^{-1} hypertonicity produces a reversible, many-fold increase in miniature endplate potential (mEPP) frequency that develops in 5–10 min to reach a broad peak, and then declines approximately exponentially over a period of 10–20 min to settle at an elevated plateau (Kita & Van der Kloot, 1977; Tanabe & Kijima, 1988; Van der Kloot & Molgó, 1994). Effects on evoked release are smaller and less consistent. Furshpan (1956) and Hubbard *et al.* (1968) reported no effect of moderate changes in osmolarity, while Barton *et al.* (1983) reported an initial increase in EPP quantal content that was proportional to the change in mEPP frequency, but of smaller magnitude. Large increases in osmolarity suppress evoked release at frog and rat neuromuscular junctions (Thesleff, 1959; Hubbard *et al.* 1968; Kita & Van der Kloot, 1977), possibly due to depletion of releasable quanta. Similarly, at the autapses formed on hippocampal cells in culture, Rosenmund & Stevens (1996) showed a transient potentiation of evoked release followed by depression, paralleling changes in spontaneous release. The depression was interpreted as a reflection of depletion, although the Ca^{2+} currents associated with evoked release also were reduced, as has also been reported for Ca^{2+} currents associated with hormone secretion from anterior pituitary cells (Matzner *et al.* 1996).

The mechanism of enhancement of spontaneous quantal release is not known. It is not dependent on Ca^{2+} entry (Furshpan, 1956; Hubbard *et al.* 1968; Blioch *et al.* 1968; Quastel *et al.* 1971; Kita & Van der Kloot, 1977), but there is support from work on the frog neuromuscular junction (Shimoni *et al.* 1977) and avian ciliary ganglion (Brosius *et al.* 1992) for the hypothesis that hyperosmolarity causes release of Ca^{2+} from internal stores. Conversely, in other systems there is convincing evidence that the hypertonicity effect is not dependent on elevated $[Ca^{2+}]_i$. In both hippocampal (Rosenmund & Stevens, 1996) and superior cervical ganglion cell cultures (Mochida *et al.*

1998), loading the cell with BAPTA and/or interfering with internal Ca^{2+} sequestering mechanisms, in a zero-Ca^{2+} Ringer solution, did not suppress the hypertonicity effect. The total increment in quantal release due to application of highly hypertonic solution has been used to determine the size of the 'readily releasable pool' of quanta (Stevens & Tsujimoto, 1995; Rosenmund & Stevens, 1996), which appears to correspond to the similarly named pool in the *Drosophila* neuromuscular junction, where use of the *shibire* mutant led to the identification of the 'immediately releasable', 'readily releasable' and 'reserve' pools (Delgado *et al.* 2000).

We became interested in the enhancement of release by hyperosmotic solutions while studying another form of modulation of release from frog motor nerve terminals, also first noted by Fatt & Katz (1952) – the enhancement of release by muscle stretch. This is a robust modulation, amounting to approximately a 10% increase in both spontaneous and evoked release for each 1% stretch within the physiological range (Hutter & Trautwein, 1956; Turkanis, 1973; Chen & Grinnell, 1995, 1997). This modulation, which is independent of changes in intraterminal $[Ca^{2+}]_i$, is mediated by mechanical stress on integrins which somehow link ligands in the extracellular matrix (ECM) to active zone structures in the terminal, regulating the probability of release (Chen & Grinnell, 1995, 1997). Since a change in osmolarity would be expected to cause shrinking or swelling of the terminal, and potentially a change in stress on molecules connecting the presynaptic and postsynaptic membranes (Robbins & Polak, 1989), this form of mechanical stress could explain at least part of the hypertonicity-stimulated release. In this paper, we present evidence that this is the case.

METHODS

Male and female *Rana pipiens*, ranging from 4 to 7 cm in size, were anaesthetized with 0.1% tricaine methane sulfonate (Sigma), doubly pithed and laid on a bed of ice. Both cutaneous pectoris (CP) muscles were dissected out with approximately 3–5 mm of the nerve attached and immediately bathed in normal frog Ringer (NFR) solution (composition, mM: 116 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $NaHCO_3$, 5 Hepes acid buffer, 1 $MgCl_2$, 3 glucose and NaOH to adjust pH to 7.4 ± 0.1). In experiments testing the effect of elevated K^+, Na^+ was reduced proportionately to maintain the same osmolarity. Once the dissection was completed, the preparation was maintained at 4 °C until use – typically 20–40 min later.

During all electrophysiological recordings, the preparation was pinned down on a thin layer of Sylgard in a Petri dish (1.5–3 ml volume) and maintained at 21 ± 1 °C except where specified otherwise, with Peltier bimetallic elements underlying the metal plate holding the preparation chamber. Resting muscle length was taken to be 2.25 μm per sarcomere, the length at which the muscle was pinned when only osmotic effects were being studied. In most experiments where muscle length was changed, the muscle was stretched or relaxed until sarcomere spacing was the desired value and pinned at that length. Typically the effects of stretch were determined with a stretch of 15–20% above the original muscle length, i.e. to a sarcomere spacing of 2.6–2.7 μm. Since different fibres differed in sarcomere spacing by a few per cent, it was not feasible to specify length more accurately than this. In a few experiments, 'floating' microelectrodes were used to record continuously from individual fibres before, during and after stretch. Muscle stretch was imposed by a computer-controlled mechanical apparatus described in detail elsewhere (Chen & Grinnell, 1997).

Recordings from identified fibres were made with glass microelectrodes (Garner Glass Co., KG-33) pulled on a Narashige horizontal puller. The tips were then bent at about a 45 deg angle to permit penetration at an angle approximately perpendicular to the fibre surface at ×200 using an Olympus ×20 water immersion objective. Electrodes were filled with 0.6 M potassium acetate solution containing 5 mM KCl (D'Alonzo & Grinnell, 1985) and had tip resistances of 60–90 MΩ.

Baseline recordings of mEPPs and/or endplate potentials (EPPs) were obtained in NFR solution from a series of 10–20 identified surface fibres, which could be recognized using structural landmarks and studied repeatedly. Experimental changes in muscle length, external Ca^{2+} concentration, or Ringer solution tonicity were then made and recordings taken again from the same junctions. Changes in bathing solution were done in one of two ways. (1) In cases of change in Ca^{2+} concentration all of the previous solution was removed and exchanged with at least two bath volumes of the new solution. Bath changes took no more than 1–2 min, but were followed by 15–20 min to allow adjustment to the new solution before recordings were made again. (2) Changes in osmolarity were made by perfusion of the preparation with several bath volumes of NFR solution containing 25, 50, 75, or 100 mM sucrose at a flow rate of 1.4 ml min^{-1} (Minipuls 2 pump, Gilson). Most of our experiments involved elevations of only 25–50 mosmol l^{-1}, which caused changes in mEPP frequency similar to those caused by stretch in the physiological range. This is much less elevation in osmolarity than has been used in many other studies, but more likely to be relevant to junctional physiology than elevations of 100–500 mosmol l^{-1}. Preparations were allowed 30–45 min to stabilize after changes in bathing solution before recordings were made. The time course of the hyperosmotic enhancement was obtained by recording from single fibres during the perfusion of the hyperosmotic solution into the bathing chamber. In some cases 25 μM μ-conotoxin (Bachem), a selective blocker of muscle Na^+ channels (Cruz *et al.* 1985), was applied for 10–20 min to prevent twitching (Robitaille & Charlton, 1992). In all experiments where only mEPPs were recorded, 1 μg ml^{-1} of neostigmine (Sigma) was added to enhance mEPP amplitudes. Records were saved on a Pentium processor-based computer using pCLAMP (v. 5.6, Axon Instruments). Means are given ± S.E.M. and significance was judged by Student's *t* test.

'Zero-Ca^{2+} Ringer solution' had the same composition as NFR solution but lacked $CaCl_2$ and contained 2 mM Mg^{2+} and 1 mM EGTA (Sigma). In some experiments, preparations were loaded with 25 μM of the acetoxymethyl ester (AM) form of BAPTA (Molecular Probes) for 1–1.5 h at 4 °C, in the presence of 10 μl ml^{-1} tetrakis(2-pyridylmethyl)ethylenediamine (TPEN, Molecular Probes) before baseline recordings were taken. To disable the native Ca^{2+} buffering by the endoplasmic reticulum, 20 μM thapsigargin (Gibco), prepared by dilution from a stock solution of 5 mM in dimethyl sulphoxide (DMSO), was added to the Ringer solution (Thastrup *et al.* 1989). To test the possible role of integrin binding in the response to hyperosmotic solutions, preparations were pre-treated for 1.5 h in a low divalent (LD) Ringer solution containing 50 μM Ca^{2+} and 50 μM Mg^{2+} (composition, mM: 116 NaCl, 1 $NaHCO_3$, 2 KCl, 10 EDTA, 9.9 $CaCl_2$, 0.175 $MgCl_2$, 5 Hepes sodium salt, 5 Hepes acid) to weaken integrin–ligand bonds, which are divalent dependent in the frog junction (Chen & Grinnell, 1995, 1997), as in many other preparations (Gailit & Ruoslahti, 1988; Kirchhofer *et al.* 1991; Orlando & Cheresh, 1991). Experimental solutions contained 0.2 mM of the hexapeptide

GRGDSP, while controls had NFR solution or the inactive peptide GRGESP. These low divalent solutions were applied for 90 min prior to testing the effects of changes in osmolarity. These were the same conditions used in earlier studies to test the effects of GRGDSP on the stretch enhancement of release (Chen & Grinnell, 1995, 1997).

RESULTS

Magnitude and kinetics of the hyperosmotic enhancement

Our results were consistent with earlier studies in showing that exposure to hypertonic Ringer solution caused a transient large increase in spontaneous quantal release that declined to a plateau level well above the original release frequency within 20–30 min. The mEPP frequency of control junctions in the present experiments showed a mean sustained increase of 2.45 ± 0.07-fold ($n = 94$) with addition of 25 mM sucrose (Fig. 1, columns 1 and 2) and a 5.74 ± 0.06-fold ($n = 76$) increase with addition of 50 mM sucrose. At these levels of osmolarity, the size distribution of mEPPs is the same as that in NFR solution, and all are effectively blocked by d-tubocurine chloride (data not shown).

The effect of hyperosmolarity on EPP quantal content was much less dramatic. Of 22 junctions studied in three muscles, changing from NFR solution to NFR + 50 mM sucrose caused no significant change (< 5%) in seven junctions, an elevation in EPP amplitude in six (mean $+70 \pm 16$%), and a decrease in nine (mean -23 ± 4%). The mean effect was a 9.6 ± 9.4% ($n = 22$) increase. Because the effect of hyperosmotic solutions on EPP quantal content was not consistent in direction or magnitude, we focused our research on the modulation of spontaneous release (mEPP frequency).

Ca^{2+} independence of hypertonicity enhancement of release

We have confirmed the findings that hyperosmotic solutions enhance mEPP frequency even in the absence of external Ca^{2+} (Blioch *et al.* 1968; Kita & Van der Kloot, 1977; Shimoni *et al.* 1977). After 1.5 h in a zero-Ca^{2+} Ringer solution containing 1 mM EGTA, the addition of 25 mosmol l^{-1} sucrose caused a 3.5 ± 0.20-fold increase in mEPP frequency, a larger effect than the mean 2.45 ± 0.07-fold increase produced by 25 mosmol l^{-1} sucrose in NFR solution (Fig. 1, columns 1–4). To test whether hyperosmotic enhancement was dependent on an elevation in $[Ca^{2+}]_i$ due to release of Ca^{2+} from internal stores, we treated preparations for 1.5 h in an EGTA-buffered zero-Ca^{2+} Ringer solution containing 25 μM

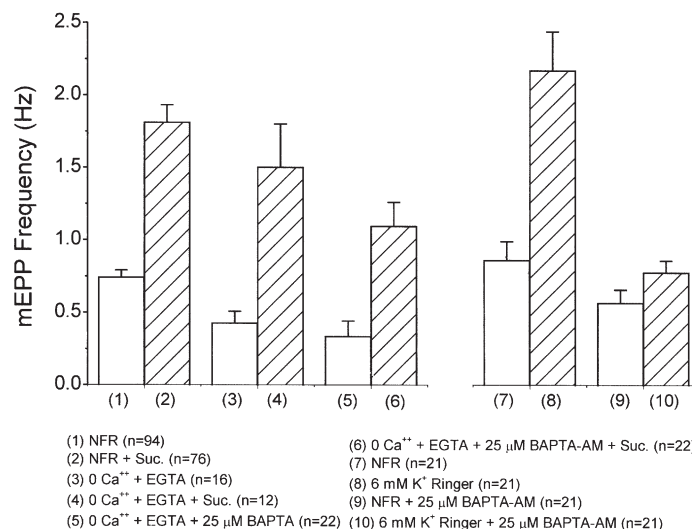

Figure 1. Lack of Ca^{2+} dependence of hypertonicity response

Addition of 25 mM sucrose to NFR solution caused a 2.45 ± 0.07-fold ($n = 76$) increase in the mEPP frequency (bars 1 and 2). Bathing preparations in a zero-Ca^{2+}, EGTA-buffered Ringer solution, which eliminates Ca^{2+} influx, did not reduce the enhancement of mEPP frequency by addition of 25 mM sucrose. MEPP frequency increased by 3.52 ± 0.20 times ($n = 12$) (bars 3 and 4). Intracellular buffering with 25 μM BAPTA AM, coupled with the removal of extracellular Ca^{2+}, also failed to eliminate osmotic enhancement of mEPP frequency. Addition of 25 mM sucrose caused a 3.86 ± 0.12-fold increase ($n = 32$) (bars 5 and 6). Hypertonicity caused a significant effect at the $P < 0.01$ level in all cases. Bars 7–10 show that loading of preparations with 25 μM BAPTA AM was effective in buffering intraterminal Ca^{2+}. Preparations with and without BAPTA loading were slightly depolarized by elevation of K^+ in the Ringer solution to 6 mM, increasing Ca^{2+} influx. Junctions without BAPTA showed an elevation in mEPP frequency of 2.27 ± 0.22-fold ($n = 23$). The same treatment of BAPTA-loaded preparations produced much smaller changes. The resting mEPP frequency after BAPTA loading was slightly reduced relative to the NFR solution condition. Addition of 6 mM K^+ caused a much reduced increase (bars 9 and 10).

BAPTA AM. The combination of zero-Ca^{2+} external Ringer solution and internal Ca^{2+} buffering reduced the resting mEPP frequency by about 50%, but there was still a strong hyperosmotic enhancement (Fig. 1, columns 5 and 6). Independent experiments showed that the BAPTA loading of terminals was effective, since the same procedures strongly suppressed the increase in mEPP frequency produced by depolarization with 6 mM K^+ in NFR solution (Fig. 1, columns 7–10). Without the extrinsic buffer, depolarization by 6 mM K^+ caused a 127 ± 22% increase. In BAPTA-loaded terminals, the effect of was an increase of only 48 ± 12%.

It is possible that Ca^{2+} could be released from a compartment immediately adjacent to the Ca^{2+}-sensing molecules responsible for triggering release, and that BAPTA is not able to buffer it before it has had its effect. To test this possibility, we treated preparations with 20 μM thapsigargin, an inhibitor of Ca^{2+}-ATPase, which disables the Ca^{2+} buffering capabilities of the endoplasmic reticulum, one of the principal cytoplasmic Ca^{2+} sequestering/releasing compartments (Thastrup *et al.* 1989). Thapsigargin caused a transient increase in mEPP frequency as Ca^{2+} was released from internal compartments; then the frequency dropped back to a baseline level usually somewhat below that in NFR solution. In junctions treated for 1 h or more with thapsigargin, the hypertonicity enhancement was still present. The addition of 50 mM sucrose enhanced mEPP frequency by a factor of 5.74 ± 0.06 without thapsigargin treatment, and by a factor of 7.17 ± 0.08 in thapsigargin-treated junctions (Fig. 2, columns 1–4). Similarly, in a zero-Ca^{2+} Ringer solution and after thapsigargin treatment, 50 mM sucrose induced a 3.2-fold increase in mEPP frequency (Fig. 2, columns 5–6). This increase was less than that without thapsigargin (5.74 ± 0.06-fold increase), suggesting that Ca^{2+} from internal stores can contribute to the response to hypertonicity. There is still a strong response, however, even with $[Ca^{2+}]_i$ release suppressed. We conclude, therefore, that the effect of hypertonicity in enhancing spontaneous release is not dependent on Ca^{2+} influx, and that a large fraction of the modulation persists when $[Ca^{2+}]_i$ release from internal stores close to active zones has been blocked.

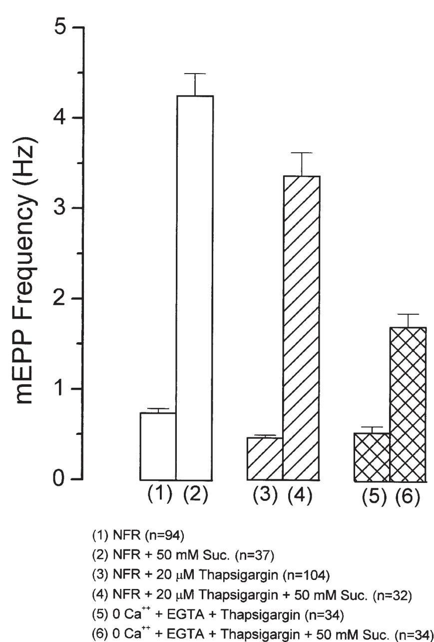

Figure 2. Effects of thapsigargin, an irreversible inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, on osmotic enhancement of release by Ringer solution containing 50 mM sucrose

In NFR solution the baseline frequency (0.74 ± 0.05 Hz, $n = 94$) increased to 4.25 ± 0.25 Hz, ($n = 37$), a 5.74 ± 0.06-fold change (bars 1 and 2). Other preparations were treated for 1 h with 20 μM thapsigargin, during which the mEPP frequency increased sharply but then returned to slightly below the original level. Addition of 50 mM sucrose then increased the frequency from 0.47 ± 0.03 Hz ($n = 104$) to 3.37 ± 0.26 Hz ($n = 32$), a 7.17 ± 0.08-fold increase (bars 3 and 4). Similarly, in 0 Ca^{2+}, EGTA-buffered Ringer solution, thapsigargin-treated terminals still showed a prominent sucrose effect (bars 5 and 6). The effect of hypertonicity was significant at $P < 0.01$ in all cases.

Role of integrins in the hyperosmolarity enhancement of release

Many integrins bind to a specific, characteristic sequence of three amino acids in ligands: arginine, glycine and aspartic acid (RGD) (Albeda & Buck, 1990; Reichardt & Tomaselli, 1991; Hynes, 1992). Addition of an extrinsic peptide containing the RGD sequence can interfere with the native binding. In many preparations, the hexapeptide GRGDSP (hereafter referred to as RGD) has proved particularly potent in interfering with integrin binding to extracellular matrix (ECM) or substrate molecules (Pierschbacher & Ruoslahti, 1987). A closely related hexapeptide, GRGESP (hereafter RGE), is a good inactive control. RGD reduces sharply the enhancement of spontaneous and evoked release by muscle stretch (Chen & Grinnell, 1995, 1997). Because integrin–ligand binding is divalent cation dependent, the interference can be increased if the preparation is pre-treated with a low divalent (LD) Ringer solution containing 0.2 mM RGD. Under these conditions, 0.2 mM RGD inhibits the stretch enhancement by about 60% (Chen & Grinnell, 1995, 1997). RGD does not have a significant effect on resting mEPP frequency (Chen & Grinnell, 1997).

Figure 3 shows the effect of 0.2 mM RGD on enhancement of spontaneous release measured 30 min or more after addition of 25–100 mM sucrose to LD Ringer solution. The hypertonicity-induced enhancement was reduced by about 40%. The blocking effect of RGD was significant at or exceeding the $P < 0.01$ level at all sucrose concentrations. RGE had no effect. Thus integrins appear

to play a role in the modulation of release by hyperosmolarity.

In earlier studies of modulation of release by muscle stretch (Chen & Grinnell, 1997), it was found that the effect of RGD could be greatly magnified by bathing the preparation in zero-Ca^{2+} Ringer solution containing 0.5 mM Mn^{2+}. Indeed, after such treatment, RGD almost completely blocked stretch enhancement of release. Hence we examined the effect of this treatment on the hypertonicity enhancement of release. Zero-Ca^{2+} Ringer solution slightly reduced the resting mEPP frequency (Fig. 1, column 3) and destabilized integrin bonds. However, addition of 0.5 mM Mn^{2+}, itself a Ca^{2+} channel blocker, restored the resting mEPP frequency to approximately normal levels and allowed a robust enhancement by 25 mM hypertonicity (Fig. 4), probably because it takes the place of Ca^{2+} in stabilizing integrin bonds. However, Mn^{2+}-stabilized integrin bonds are extremely sensitive to RGD, which displaces native ligands (see Discussion). When 0.2 mM RGD was added to muscles treated with zero-Ca^{2+} Ringer solution plus 0.5 mM Mn^{2+}, the effect of an added 25 mM sucrose was largely eliminated (Fig. 4).

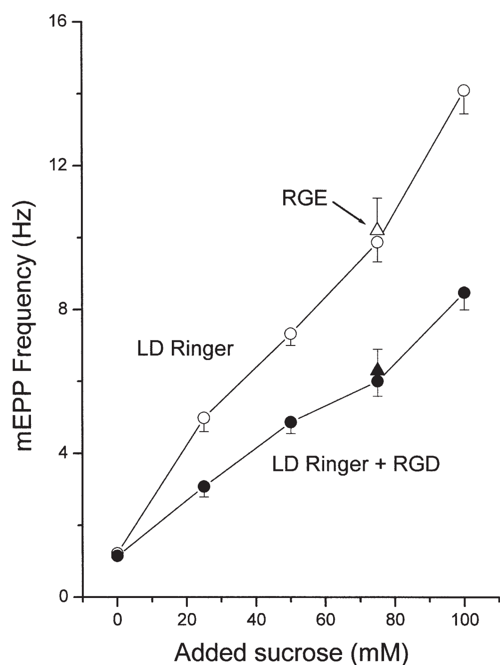

Figure 3. Effect of 0.2 mM RGD on the enhancement of mEPP frequency by addition of 25, 50, 75 and 100 mM sucrose

Control and experimental preparations were pretreated for 90 min in low-divalent (LD) Ringer solution. At all sucrose concentrations there was a significant ($P < 0.01$) inhibition of the hyperosmotic enhancement with the peptide (●) compared to the LD Ringer solution controls (○). Also plotted are control values showing the effect of 75 mM sucrose on mEPP frequency in the presence of the RGE peptide (△, $n = 30$) and paired junctions in RGD (▲, $n = 28$).

Co-variance of stretch and hyperosmolarity effects on spontaneous release

Different junctions exhibit great variability in the magnitude of the hyperosmotic enhancement, just as the magnitude of the stretch effect is highly variable (Chen & Grinnell, 1997). The explanation for this variability is not clear. However, it can be used to test the hypothesis that the mechanisms of hyperosmotic and stretch enhancement overlap to a significant degree. If they do, one would expect that junctions showing a large hyperosmotic enhancement would also be sensitive to stretch, while those that were insensitive to one treatment would be insensitive to the other. Figure 5 shows the data for 59 junctions from five preparations in which the effects of

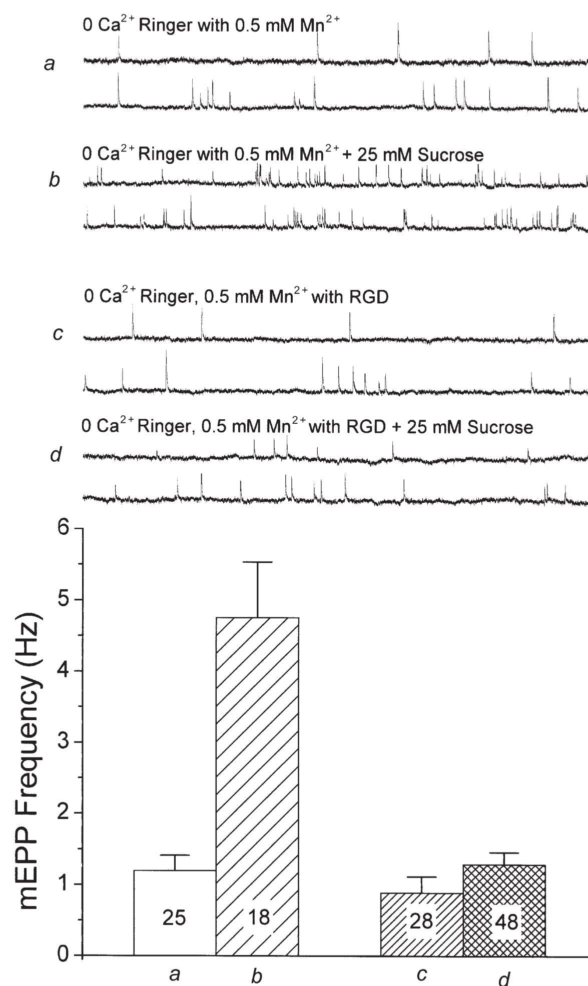

Figure 4. Effects of RGD on Mn^{2+}-treated junctions

In muscles treated with zero-Ca^{2+} Ringer and 0.5 mM Mn^{2+}, the resting mEPP frequency was close to normal (single traces and summary bar graph *a*), and addition of 25 mM sucrose caused an approximately 4-fold increase in frequency (*b*). Preparations treated similarly but in the presence of 0.2 mM RGD showed a slightly depressed resting mEPP frequency (*c*) and a severely reduced enhancement of release by 25 mM sucrose (*d*).

15% stretch in NFR solution were compared with the effect of adding 25 mM sucrose to the Ringer solution at rest length in the same junctions. These experiments were done at 12–14°C to maintain the preparation in good condition throughout more than twice the usual experimental period. Cooling reduced the resting mEPP frequency, but did not affect the response to stretch or hypertonicity. Each treatment, by itself, caused a comparable increase in spontaneous release. Although there was a good deal of scatter in the relative effectiveness of stretch and hypertonicity, this figure shows a clear tendency for a large stretch effect to be correlated with a large hypertonicity effect.

Occlusion of stretch and hyperosmolarity enhancements

If stretch and hypertonicity enhance release by the same or related mechanisms, they might be expected to be additive at low levels of modulation and at least partially occlude each other at more extreme levels. If, for example, hyperosmotic solutions cause a shrinkage of terminals, enlarging the synaptic cleft (Robbins & Polak, 1989), this might exert stress on the same integrin bonds that mediate the stretch effect, partially occluding the effect of stretch itself. To test this hypothesis, parallel sets of experiments were done. In one of these, after recording baseline mEPP frequency at rest length in NFR solution, mEPP frequency was measured in the same identified fibres after an approximately 15% stretch, and again after a further treatment with 25 mM hypertonic Ringer solution. In the second set of experiments, the muscle was treated first with the hypertonic solution and then stretched. Only identified junctions in fibres that maintained a good resting potential throughout were used for data analysis. In the first set of experiments (Fig. 6, *a*), the resting mEPP frequency was a mean of 1.45 ± 0.25 Hz, which increased by approximately 2.7 times to 3.95 ± 0.46 Hz with stretch, and to 4.39 ± 0.4 Hz with subsequent hypertonicity ($n = 26$ fibres in two muscles). The hypertonic solution added a relatively small increment, a mean increase of < 0.5 Hz. This is a much smaller increase both absolutely as a percentage of the baseline frequency than is normally seen with 25 mM hypertonicity. Indeed, several junctions that had experienced a large increase in mEPP frequency with stretch actually showed a decrease with addition of sucrose.

In the second series of measurements (Fig. 6, *b*), the same hypertonicity caused the mEPP frequency to increase from a baseline of 0.9 ± 0.07 to 2.81 ± 0.2, an increase of > 3-fold. In this case, subsequent stretch increased the frequency to 4.98 ± 0.32 ($n = 25$ junctions in two muscles), a substantial addition to the effect of hypertonicity, but a smaller effect, as a percentage of the frequency before stretch, than was usually seen with stretch alone. In a larger set of seven experiments, comparing only junctions with rest frequencies between 0.25 and 1.5 Hz (where the populations overlapped extensively), the effect of stretch was a 4.13 ± 0.58-fold ($n = 26$) increase in NFR, and a 2.79 ± 0.24-fold ($n = 17$) increase in NFR solution plus 25 mM sucrose ($P < 0.05$). Thus both stretch and hyperosmolarity are capable of partially occluding the other.

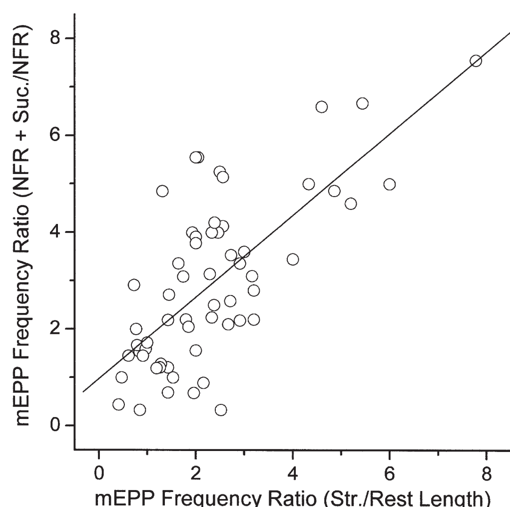

Figure 5. Covariance of effects of 15–20% stretch and 25 mM hypertonicity on mEPP frequency

Data are shown for 59 identified junctions in 5 experiments with preparations held at 12–14° C to help keep fibres healthy through both manipulations. The correlation coefficient was 0.71.

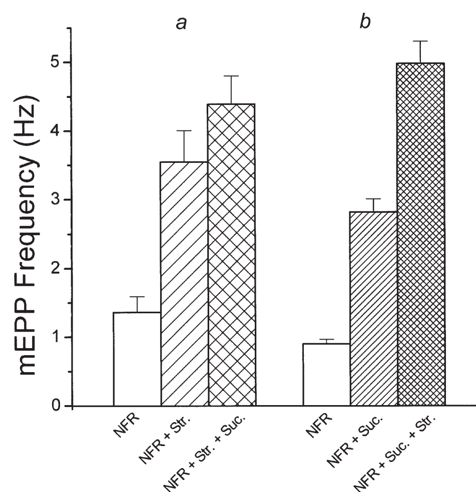

Figure 6. Partial mutual occlusion of effects of stretch and hypertonicity on mEPP frequency

a, data from 23 identified fibres in two muscles studied first in NFR solution, then stretched by approximately 15%, and finally exposed to 25 mM sucrose in NFR solution. *b*, data from 25 identified fibres in the two muscles paired with those above, tested first in NFR solution, then in 25 mM hypertonic solution, and finally with stretch. Occlusion was particularly evident when stretch preceded hypertonicity.

DISCUSSION

Ca^{2+} independence of osmotic enhancement of spontaneous release

Our data show that the hypertonicity-induced increase in mEPP frequency does not depend upon the presence of external Ca^{2+}, nor is it affected by loading of terminals with BAPTA, in agreement with findings in hippocampal (Rosenmund & Stevens, 1996) and superior cervical ganglion neurons (Mochida *et al.* 1998). We conclude, therefore, that it is not dependent on Ca^{2+} influx or on a general elevation of intraterminal Ca^{2+}. We cannot exclude the possibility that part of the hypertonicity response is mediated by internal release of Ca^{2+} from compartments so close to release sites that BAPTA cannot fully block its effect. The response, while still quite large, was significantly reduced in a series of measurements where Ca^{2+} binding by internal compartments had been blocked by thapsigargin. The enhancement of release by stretch has been shown to require a basal level of intraterminal Ca^{2+} but to be independent of an elevation in $[Ca^{2+}]_i$, either from outside or by internal release from local compartments (Chen & Grinnell, 1995, 1997). A partial dependence on Ca^{2+} release from local internal stores might help explain the larger magnitude of the hypertonicity response relative to stretch, especially at high levels of hyperosmolarity. It might also help account for the findings of Shimoni *et al.* (1977) that the response to hypertonicity decreased in a reversed transmembrane Ca^{2+} gradient.

Involvement of integrins in the hyperosmolarity effect

Perhaps the most convincing evidence that a similar mechanism is involved in both forms of modulation is the observation that the peptide GRGDSP, which interferes with integrin binding to native ligands, sharply reduced the effect of hypertonicity in a low divalent Ringer solution (Fig. 3). The reduction in mEPP frequency (approximately 40%) was less than that observed with the stretch effect (approximately 60%), but still highly significant. More dramatically, in zero-Ca^{2+} Ringer solution containing 0.5 mM Mn^{2+}, the osmotic enhancement of release by 25 mosmol l^{-1} sucrose was reduced about 90% by RGD (Fig. 4). This magnification of the effect of RGD by Mn^{2+}, which was also clear in the case of stretch enhancement (Chen & Grinnell, 1997), is consistent with the observation by several groups that Mn^{2+} appears to sharply increase the affinity of integrin for short peptides containing the RGD sequence, which therefore are better able to displace the native ligand (Gailit & Ruoslahti, 1988; Kirchhofer *et al.* 1991; Grinnell & Backman, 1991). It has already been shown that the stretch enhancement is a mechanical modulation dependent on integrin binding, presumably to a molecule in the extracellular matrix (ECM) (Chen & Grinnell, 1997). Although integrins could in principle be operating in different ways, the reduction in effect seen with block of their binding is consistent with the hypothesis that at least part of the osmotic modulation involves tension on the same integrins that are involved in the stretch enhancement of release. It should be emphasized, however, that the integrin-blocking peptides did not totally block osmotic enhancement of release, even in a Mn^{2+}-containing solution.

A role for integrins in modulation of release has not been reported at synapses other than the frog neuromuscular junction. However, similar experiments in larval *Drosophila* neuromuscular junctions reveal that integrins are involved in osmotic enhancement of spontaneous release in that preparation as well (K. Suzuki, A. D. Grinnell and Y. Kidokoro, unpublished observations). Moreover, a *Drosophila* integrin mutant, *volado*, has been shown to have defective olfactory memory (Grotewiel *et al.* 1998). Integrins have been demonstrated both pre- and postsynaptically at rat neuromuscular junctions where they appear to play a number of important developmental roles (Martin *et al.* 1996; Martin & Sanes, 1997; Nishimura *et al.* 1998; Wong *et al.* 1999). Integrins have been implicated in the stabilization of long-term potentiation in the mammalian hippocampus (Bahr *et al.* 1997), and the widespread and regionally differentiated distribution of different integrins subunits in the brain suggests that they may play a variety of roles in CNS function (Pinkstaff *et al.* 1999).

Co-variance and mutual occlusion of the effects of hypertonicity and stretch

Our experiments showed that junctions that were particularly sensitive to stretch tended to be particularly sensitive to hypertonicity as well. This does not mean that stretch and hyperosmolarity enhance spontaneous release via the same mechanism, but is consistent with that possibility. If similar mechanisms are involved, one might also expect the effects of stretch and hyperosmolarity to occlude each other to some extent. This prediction was at least partially borne out (Fig. 6). Although the effects of stretch and hypertonicity were clearly additive, some junctions, especially those that had undergone a large enhancement by one manipulation, showed a reduced or opposite effect of the other. It must be noted, however, that this occlusion is almost certainly not the result of the modulation reaching a saturation level, since high levels of hypertonicity, in particular, can produce much larger changes in release probability than occurred in our experiments.

Differences between the stretch and hyperosmolarity forms of modulation of release

Osmotic and stretch modulation are similar in being reduced by peptides that block integrin–ligand binding, in not being dependent on Ca^{2+} influx or release of Ca^{2+} from internal stores, and in exhibiting co-variance and partial occlusion. It is necessary also, however, to emphasize that there are differences between the two phenomena and to assess the significance of these differences, as follows.

(1) The absolute magnitude of enhancement of mEPP frequency achievable by changes in osmolarity far exceeds the enhancement that can be obtained by stretch. Mean mEPP frequency can be increased by more than 30-fold by large changes in the osmolarity of the Ringer solution (Blioch *et al.* 1968; Doherty *et al.* 1986). Stretch enhancement, in contrast, seldom exceeds a mean of 3- to 4-fold (Hutter & Trautwein, 1956; Turkanis, 1973; Chen & Grinnell, 1995, 1997). Either osmotic enhancement involves additional mechanisms or terminal shrinkage in a hypertonic medium is a much more efficient way of exerting tension on the mechanical pathway modulating release than is muscle stretch. Neither possibility can be ruled out.

(2) The two forms of modulation exhibit very different time courses. The osmotic enhancement develops over minutes, shows a prominent phasic component, and usually decays only slowly to a plateau level. Stretch modulation, in contrast, has a negligible delay and no phasic component and changes linearly with length (Chen & Grinnell, 1997). Nevertheless, shrinkage of a terminal due to osmotic loss of water might be an extremely effective way of exerting tension on bonds between integrins in the presynaptic membrane and their natural ligands in the ECM; and the time course of changes in tension might be very complicated as structures change shape and size due to changes in volume. We do not consider differences in time course to be compelling evidence for different mechanisms.

(3) The Q_{10} of osmotic enhancement has been reported by others to be about 2 in mammals (Hubbard *et al.* 1968) and up to 7 in frogs (Kita *et al.* 1982). The Q_{10} of the stretch enhancement is close to 1 (Chen & Grinnell, 1997). This difference clearly suggests a difference in mechanism, or in factors coupling stretch and osmotic changes to the same mechanism. We did not study the temperature coefficient systematically. It should be noted, however, that in experiments done at 12–14 °C (Fig. 5), the mean enhancement of release by 25 mosmol l^{-1} was 3.04 ± 0.23-fold ($n = 56$), fully equivalent to the enhancement at 21 °C (2.45 ± 0.07-fold, $n = 76$). Hence for moderate increases in osmolarity, the Q_{10} may not be large.

(4) Stretch increases the probability of evoked release in parallel with that of spontaneous release, and to a similar extent (Chen & Grinnell, 1997). In contrast, levels of hyperosmolarity up to 100 mosmol l^{-1} have been found to have no effect (Furshpan, 1956; Hubbard *et al.* 1968) or to increase evoked release only moderately (Barton *et al.* 1983; Tanabe & Kijima, 1988), while larger changes in osmolarity decreased evoked release (Thesleff, 1959; Hubbard *et al.* 1968; Kita & Van der Kloot, 1977; Rosenmund & Stevens, 1996). Although large increases in osmolarity might be depleting the pool of readily releasable vesicles, it seems unlikely that the moderate increase in frequency produced in our experiments could be causing depletion or explain the relative lack of effect on EPP amplitude of adding 50 mM sucrose. These differences in effect on mEPP frequency and evoked release imply, at the very least, an osmotically driven mechanism of suppression of evoked, but not spontaneous, release.

In summary, our data provide evidence that some fraction of the enhancement of spontaneous release by hypertonic solutions involves integrins, possibly acting via a mechanism common to stretch enhancement. However, there remain some major differences, the most interesting of which are the differences in effect on evoked release and the possible differences in Q_{10}. Both stretch and hypertonicity modulation of release by-pass the Ca^{2+}-triggering step in release. What other aspects of the docking and fusion processes are involved is not clear at this point. At mammalian neuromuscular junctions, hypertonicity is no longer effective after cleavage of SNAP-25 or synaptobrevin by clostridial toxins (Dreyer *et al.* 1987; Gansel *et al.* 1987), and at *Drosophila* neuromuscular junctions, the hypertonicity effect is eliminated in the absence of synaptobrevin, syntaxin, or Unc-13 (Aravamudan *et al.* 1999). Similarly, in hippocampal cell cultures, cleavage of syntaxin, synaptobrevin, or SNAP-25 eliminates the response to hypertonicity (Capogna *et al.* 1997). Thus the hypertonicity response, while bypassing Ca^{2+} triggering, does require much of the molecular apparatus thought to be involved in vesicle docking and fusion. An understanding of the mechanism(s) of integrin-mediated modulation of release is likely to be helpful in understanding molecular events involved in vesicle fusion.

The mechanical modulation of release responsible for the stretch and hypertonicity responses might conceivably also play a role in the long unexplained suppression of evoked and spontaneous release from frog neuromuscular junctions (Parmentier *et al.* 1981; Ashford *et al.* 1982) and of secretion from chromaffin cells and mast cells (Heinemann *et al.* 1987) by moderate levels of hydrostatic pressure. If increased hydrostatic pressure can compress some synaptic structures more than others (e.g. moving pre- and postsynaptic structures closer together), it could change the tension on mechanical links that are involved in regulating release.

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