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Biphasic modulation of synaptic transmission by hypertonicity at the embryonic *Drosophila* neuromuscular junction

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Puff-application of hypertonic saline (sucrose added to external saline) causes a transient increase in the frequency of spontaneous miniature synaptic currents (mSCs) at the neuromuscular junctions of *Drosophila* embryos. The frequency gradually returns to pre-application levels. External Ca^{2+} is not needed for this response, but it may modify it. At 50 mM added sucrose, for example, enhanced spontaneous release was observed only in the presence of external Ca^{2+}, suggesting that Ca^{2+} augments the response. In a high-K^+ solution, in which the basal mSC frequency was elevated, higher sucrose concentrations produced an increase in mSC frequency that was followed (during and after the hypertonic exposure) by depression, with the magnitude of both effects increasing with hypertonicity between 100 and 500 mM. Evoked release by nerve stimulation showed only depression in response to hypertonicity. We do not believe that the depression of spontaneous or evoked release can be explained by the depletion of releasable quanta, however, since the frequency of quantal release did not reach levels compatible with this explanation and the enhancement and depression could be obtained independent of one another. In a mutant lacking neuronal synaptobrevin, only the depression of mSC frequency was induced by hypertonicity. Conversely, only the enhancing effect was observed in wild-type embryos when the mSC frequency was elevated with forskolin in Ca^{2+}-free saline. In cultured embryonic *Drosophila* neurons, Ca^{2+} signals that were induced by high K^+ and detected by Fura-2, were reduced by hypertonicity, suggesting that the depressing response is due to a direct effect of hypertonicity on Ca^{2+} influx.

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Quantal neurotransmitter release occurs when synaptic vesicles fuse with the presynaptic membrane at specialized release sites. This process is currently the subject of intense investigation in several systems. The embryonic *Drosophila* neuromuscular junction is uniquely valuable for such investigation because of the existence of a number of mutants with defects in key molecules involved in vesicle docking and/or fusion. Although these are lethal mutations, embryos or larvae are viable and can be studied electrophysiologically (Broadie & Bate, 1993*a*,*b*; Broadie *et al.* 1994; Deitcher *et al.* 1998; Aravamudan *et al.* 1999; Yoshihara *et al.* 1999, 2000). In many of these mutants, nerve stimulation does not evoke synaptic currents, so it is desirable to have other tools with which to trigger vesicle fusion. In this preparation, as in many others, vesicle fusion can be greatly enhanced by application of hypertonic solution in the absence of external Ca^{2+} (hypertonicity response).

In neuronal synapses, a comparable hypertonicity response can be induced by high concentrations of sucrose, even when the internal Ca^{2+} concentration ($[Ca^{2+}]_i$) was strongly buffered with BAPTA (Rosenmund & Stevens, 1996; Mochida *et al.* 1998). On the other hand, it has been shown that cleaving of neuronal synaptobrevin (n-syb), SNAP-25 or syntaxin by clostridial neurotoxins blocks the response to hypertonicity (Capogna *et al.* 1997). Similarly, at embryonic *Drosophila* neuromuscular junctions, the hypertonicity response was not evoked or heavily reduced in the absence of n-syb, syntaxin or Unc-13 (Aravamudan *et al.* 1999). Thus, hypertonicity seems to bypass the Ca^{2+}-sensing step, but shares major elements of the basic Ca^{2+}-triggered vesicle fusion mechanism.

To characterize this Ca^{2+}-independent vesicle fusion process, in a previous study we examined the quantal transmitter release induced by hypertonicity at embryonic *Drosophila* neuromuscular junctions in the absence of external Ca^{2+} (Suzuki *et al.* 2002). The frequency of spontaneous quantal transmitter release increased upon puff-application of hypertonic sucrose solutions. Measurements of the total number of quanta released in response to various concentrations of sucrose showed a dose–response curve that reached a peak at 420 mM sucrose (420 mM sucrose added to Ca^{2+}-free external saline) and was lower at higher osmolarities. Thus, the total number of quantal synaptic

events that can be induced by hypertonicity seems to have a ceiling. This ceiling has been interpreted as the size of a readily releasable vesicle pool (Stevens & Tsujimoto, 1995; Rosenmund & Stevens, 1996). However, this interpretation is inconsistent with the observation that forskolin, an activator of adenylyl cyclase, increased and maintained a higher background frequency of spontaneous quantal release in Ca^{2+}-free saline, even after application of 420 mM sucrose. The hypertonicity response to this treatment was as large as in the absence of forskolin, yet had no apparent effect on the subsequent occurrence of spontaneous quantal events. If the pool had been emptied by the 420 mM sucrose, quantal release should have either decreased or ceased transiently after the response. Furthermore, although the dose–response curve apparently hit a ceiling at 420 mM, the response at 600 mM was significantly smaller than at 420 mM, suggesting that at very high concentrations of sucrose another effect of hypertonicity reveals itself and suppresses vesicle release. Thus, the hypothesis that the maximum hypertonicity response represents the size of the readily releasable pool does not fit the data obtained at the embryonic *Drosophila* neuromuscular junction. Hypertonicity does not seem to deplete the readily releasable pool (Suzuki *et al.* 2002).

We have proposed an alternative hypothesis: that hypertonicity facilitates vesicle fusion as well as recruitment of vesicles for release, independent of Ca^{2+}. The total number of quantal events that occur during the hypertonicity response may be determined principally by factors such as the extent and time course of enhancement of vesicle fusion by hypertonicity and the recruitment rate of vesicles for release (Suzuki *et al.* 2002). The second of these factors, the recruitment of vesicles for release, includes the translocation of vesicles to the release site and docking/priming. In other systems, the steps involved in the recruitment of synaptic vesicles are strongly influenced by Ca^{2+}. In adrenal chromaffin cells, for example, the recovery of secretory responsiveness was accelerated by a moderate increase of internal Ca^{2+} (von Ruden & Neher, 1993), and at the calyx of Held in the mouse brainstem, Ca^{2+} accelerates replenishment of the releasable pool (Wang & Kaczmarek, 1998). An acceleration by Ca^{2+} of vesicle replenishment at the retinal bipolar-cell synapse of goldfish has also been suggested (von Gersdorff & Matthews, 1997). Thus one might expect the hypertonicity response to be sensitive to Ca^{2+}. This prediction seemingly contradicts the previous reports that the hypertonicity response is independent of Ca^{2+} (Rosenmund & Stevens, 1996; Mochida, *et al.* 1998).

To resolve this issue we have examined the hypertonicity response at the embryonic *Drosophila* neuromuscular junction in the presence of external Ca^{2+}. We found that the hypertonicity response induced by a relatively low concentration of sucrose is enhanced by external Ca^{2+}. Unexpectedly, we also observed that hypertonic solutions depressed nerve-evoked neurotransmitter release with a prolonged time course, and that the elevation in frequency of miniature synaptic currents (mSCs) induced by high K^+ in the presence of Ca^{2+} was also depressed by hypertonic solutions. Thus, release triggered by Ca^{2+} influx at active zones was depressed at the same time that spontaneous quantal release was enhanced. We further found that the Ca^{2+} signals induced by high K^+ and detected by Fura-2 in cultured embryonic *Drosophila* neurons were reduced by hypertonicity. Taken together, we suggest that the depressing response to hypertonicity is the result of inhibition of Ca^{2+} influx through voltage-gated Ca^{2+} channels by hypertonicity.

METHODS

Fly stocks

The following mutants were used in this study: a null allele of myosin heavy chain gene, Mhc^l (*Mhc*, Mogami *et al.* 1986, balanced with *CyO,* [*yellow*$^+$]) and a double mutant Mhc^l *n-syb*^{F33B} (*Mhc n-syb*). *n-syb*^{F33B} is a null allele of the *n-syb* gene isolated by Deitcher *et al.* (1998) and balanced with T*M6,* [*yellow*$^+$]. For cell cultures embryos of a wild-type strain, Canton S, was used.

Preparations

Embryos of mutants (17–19 h after fertilization) were prepared for electrophysiological experiments as described previously (Kidokoro & Nishikawa, 1994; Nishikawa & Kidokoro, 1995). The egg case was removed by treatment with 30 % hypochlorous acid for 3 min. After washing in saline we selected homozygous mutant embryos with the aid of a stereomicroscope, based upon the marker of [*yellow*$^+$] and dissected them in Ca^{2+}-free saline (see below for ionic composition). The dissected preparation was treated with collagenase (1 mg ml^{-1}) for 30 s to 1 min, and electrical recordings of synaptic currents were carried out with the patch-clamp technique in the whole-cell configuration. The membrane potential was always held at −60 mV. The internal solution contained Cs^+ (see below for ionic composition), and the junction potential of the electrode filled with the Cs^+ internal solution was −5 mV. Thus, the true holding potential was −65 mV.

Hypertonic solutions were prepared by adding sucrose to the external solution and were applied by the puff method. Basically, when 340 mM sucrose was added to external saline, we refer to it as a 340 mM sucrose solution. The puff pipette had a tip diameter of 2–5 μm, and the tip was placed within ~20 μm of the junctional area. The gas pressure used to puff hypertonic solutions was 0.5 kg cm^{-2}. The quantal synaptic events were counted individually within every 1 s interval.

For nerve stimulation, the tip of a microelectrode, with a resistance of 10–20 MΩ after filling with 4 M potassium acetate was placed in the ventral nerve cord, and rectangular pulses of 2 ms duration and about 2 μA intensity were delivered.

For application of RGD peptides, the preparation was kept for 15 min in a low-Ca^{2+} (50 μM), low-Mg^{2+} (50 μM) solution containing the peptide at 0.2 mM. The low divalent solution destabilizes integrin bonds to native ligands, and RGD binds in their place (Gailit & Rouslahti, 1988; Kirchhofer *et al.* 1991; Chen & Grinnell, 1995, 1997). The recording bath solution and the

hypertonic solution in the puff pipette also contained the same concentration of the peptide. To test the effect of forskolin (200 μM) on the hypertonicity response, the preparation was kept for at least 20 min in external saline containing forskolin, and the physiological experiment was carried out in the same medium.

All experiments were carried out at room temperature (18–27 °C).

Solutions

Electrophysiological experiments were carried out either in the Ca^{2+}-containing solution or in Ca^{2+}-free solution. The ionic composition of Ca^{2+}-free saline was (mM): NaCl 140, KCl 2, $MgCl_2$ 6, Hepes-NaOH 5 (pH 7.1). For nerve stimulation to evoke synaptic currents, 0.1 mM Ca^{2+} or 0.3 mM Ca^{2+} was added to Ca^{2+}-free saline, replacing the same amount of Mg^{2+}. The ionic composition of the high-K^+ saline was (mM): NaCl 80, KCl 62, $MgCl_2$ 5.9, $CaCl_2$ 0.1, Hepes-NaOH 5 (pH 7.1). The internal solution in the patch pipette contained (mM): CsCl 158, EGTA 5, Hepes-NaOH 10, ATP 2 (pH 7.1).

For Ca^{2+}-imaging experiments with cultured embryonic neurons, HL3 medium was used (Stewart *et al.* 1994). This medium contained the following ingredients (mM): NaCl 70, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 20, $NaHCO_3$ 10, trehalose 5, sucrose 115, Hepes-NaOH 5 (pH 7.2). A high-K^+ HL3 solution was prepared by replacing all NaCl with KCl. Thus, the total K^+ concentration was 75 mM. A hypertonic, high-K^+ HL3 solution was prepared by adding 420 mM sucrose to the high-K^+ HL3 solution.

Cell cultures

Wild-type embryos were collected on agar plates for 1 h after egg laying and incubated for 3.5 h at 25 °C. Embryos were at the early gastrula stage (stage 7–8). To remove the chorionic membrane, embryos were treated with a mixture of antiformin (about 12 % sodium hypochlorite solution) and 90 % ethanol (1:1 by volume) for 1 min and washed with 70 % ethanol. Embryos were subsequently washed with sterile water, and egg shells were ruptured with a pair of forceps in modified Schneider medium supplemented with fetal bovine serum (20 %), insulin (200 ng ml^{-1}), penicillin (50 U ml^{-1}), streptomycin (50 μg ml^{-1}) and cytochalasin B (2 μg ml^{-1}). The treatment with cytochalasin B prevents cell division and produces giant neurons with multiple nuclei (Wu *et al.* 1990; Saito & Wu, 1991). To remove debris, dissociated cells were washed in the same medium and were plated on 22 mm diameter glass coverslips coated with poly-L-lysine. Cultures were kept in humidified chambers at 25 °C for 5–7 days before experiments.

Fura-2 loading

Free $[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive dye, Fura-2/AM. Fura-2/AM was stored as a stock solution (1 mM) in dimethylsulphoxide at −20 °C and was diluted in the HL3 solution to 2 μM prior to use. Before Fura-2/AM loading, cells were washed with the HL3 medium three times to remove fetal bovine serum and then incubated in the Fura-2/AM solution for 30 min at 25 °C. After loading, the cells were washed twice with the HL3 medium.

Measurement of $[Ca^{2+}]_i$

Using silicon vacuum grease, a plastic frame was pasted on the cover slip on which cells were growing, to form a 200 μl chamber. The Fura-2 fluorescence at 510 nm elicited by excitation at 340 or 380 nm was measured using an inverted Diaphot microscope (Nikon, Tokyo, Japan) equipped with a cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). The excitation wavelength was switched between 340 and 380 nm by a computer-controlled filter changer. Images were recorded at 5 s intervals and were analysed with the Aquacosmos Ca^{2+}-imaging processing system (Hamamatsu Photonics). The F_{340}/F_{380} ratio was measured to estimate $[Ca^{2+}]_i$. The area of measurement was approximately 5 μm × 5 μm. The bath was perfused continuously at a rate of 1 ml min^{-1} during the experiment. Cells were stimulated with a high-K^+ solution for 1 min and subsequently washed with the HL3 medium for 5 min. Only 30–50 % of cultured cells responded well with high-K^+ stimulation. In those responding cells, high-K^+ stimulation was repeated three times with an interval of 5 min. The first stimulation was done with the high-K^+ solution alone for control conditions, the second was with the high-K^+ solution plus Cd^{2+} (200 μM) or PLTXII (0.2 μM) or sucrose (420 mM), and the third was again with the high-K^+ (control) solution to observe the recovery from the treatment.

Calibration of the Fura-2 signal

An *in vitro* calibration was performed using Ca^{2+} solutions of known concentration based on the following equation: $[Ca^{2+}]_i = K_d\beta(R - R_{min})/(R_{max} - R)$, where R is the ratio of fluorescence emission intensities at 340 nm and 380 nm excitations, R_{min} is the same ratio under a minimal Ca^{2+} condition, R_{max} is the ratio at a maximal Ca^{2+} concentration, K_d is the dissociation constant of the Ca^{2+}–Fura-2 complex (K_d = 135 nM at 20 °C) and β is the ratio of fluorescence intensity of the free dye to that of the bound dye measured at a 380 nm excitation wavelength. The value of R_{min} was measured using a 200 μl HL3 solution containing zero Ca^{2+}, 10 mM EGTA and 2 μM Fura-2. The value of R_{max} was measured using a 200 μl HL3 solution containing 2 mM Ca^{2+}, zero EGTA and 2 μM Fura-2. We used the following values: R_{min} = 0.5667, R_{max} = 4.0151 and β = 2.915 (Grynkiewicz *et al.* 1985).

Chemicals

The peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) was obtained from Peninsula Laboratories (Belmont, CA, USA). Forskolin, tetrodotoxin (TTX) and collagenase were purchased from Sigma (St Louis, MO, USA). For stock solutions, forskolin was dissolved in ethanol at 10 mM. Ethanol does not have any effect on synaptic transmission at the concentration used in this experiment (Yoshihara *et al.* 2000). A synthetic spider toxin, PLTXII (Body *et al.* 1995), was purchased from the Peptide Institute (Osaka, Japan). Schneider's *Drosophila* culture medium and bovine serum were purchased from Gibco (Grand Island, NY, USA), antiformin and streptomycin were from Wako (Osaka, Japan) and Fura-2/AM from Dojindo (Kumamoto, Japan). Penicillin and insulin were purchased from Sigma.

Statistical analyses

For comparison among multiple groups, ANOVA was used with Scheffé's test. Student's t test was used for comparison of two groups. All data are expressed as means ± S.D., unless indicated otherwise.

RESULTS

Hypertonicity caused a reduction in nerve-evoked synaptic transmission, while spontaneous quantal transmitter release was enhanced

Nerve stimulation in wild-type embryos caused muscle contraction that often stretched nerves and adversely affected terminals. Under these conditions, asynchronous quantal release was increased, and nerve-evoked synaptic transmission was often enhanced. To avoid this instability, throughout this study we used a non-contracting muscle

mutant, *Mhc* (Mogami *et al.* 1986). The basic characteristics of synaptic transmission at the neuromuscular junction in *Mhc* embryos are indistinguishable from those in wild-type embryos (Yoshihara *et al.* 2000).

When the nerve was stimulated at 1 Hz in saline containing 0.3 mM Ca^{2+}, the time course and amplitude of synaptic currents were variable (Fig. 1*A*, before). Because of the variability in the time course, the amount of transmitter release was quantified by integrating the synaptic current to determine the total charge transfer. The average charge transfer before puff-application of hypertonic saline was 1.7 ± 1.4 pC stimulus^{-1} ($n = 5$). Upon application of the 340 mM sucrose solution, the charge transfer evoked by each stimulus declined immediately, and synaptic transmission was depressed for up to ~30 s after the end of the puff pulse (Fig. 1*A*, during; Fig. 1*C*). This hypertonicity-induced depression of nerve-evoked synaptic transmission could be due to an effect on the postsynaptic receptors. However, the failure rate during the 30 s period after the end of the hypertonic puff pulse was 0.86 ± 0.14 ($n = 8$), which is significantly higher than the rate before application of the hypertonic solution (0.50 ± 0.20, $n = 8$, $P < 0.01$, Fig. 1*B*). This indicates that the depression is at least partially due to a reduction in transmitter release from the presynaptic terminal.

It is also possible that failures of synaptic transmission during the hypertonicity-induced depression were due to failure of presynaptic nerve conduction rather than in synaptic transmission. However, when the charge transfer during successful transmission, excluding failures, was compared before and after application of hypertonicity, the ratio was 0.49 ± 0.20 ($n = 5$), which is significantly smaller than 1 ($P < 0.01$). Thus the depression is not likely to be due to failures of nerve conduction, because transmission was less even when nerve conduction occurred. We conclude, therefore, that this depression is due to an effect of hypertonicity on synaptic transmission. That this depression is due to the effect of hypertonicity on the presynaptic terminal is in accord with our previous observation that the mean amplitude of quantal synaptic currents was only slightly smaller during the enhancing hypertonicity response in the absence of external Ca^{2+},

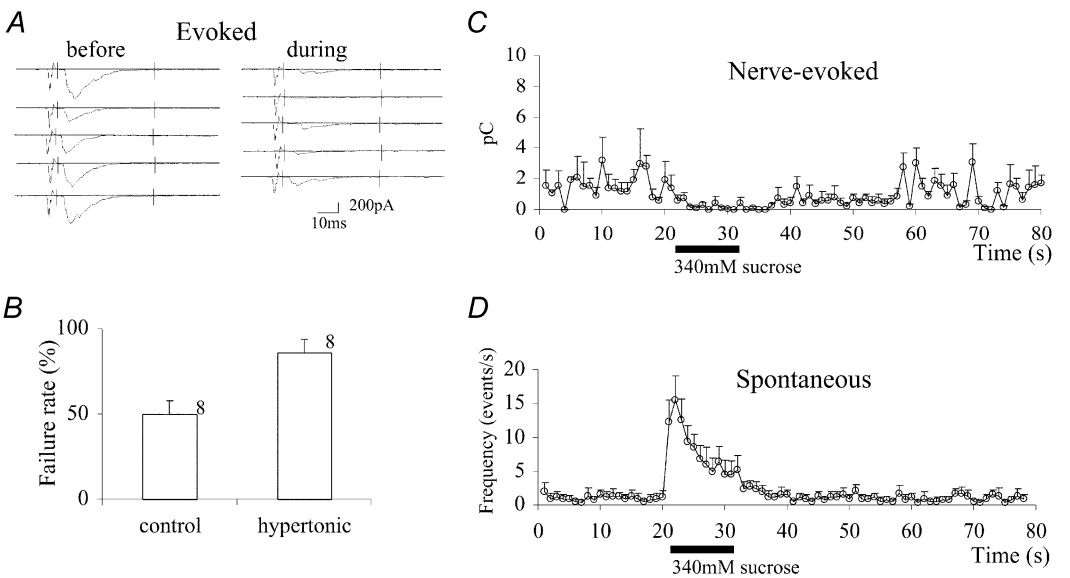

Figure 1. The effects of hypertonicity on nerve-evoked synaptic currents and spontaneous synaptic currents

A, embryonic muscle cells from a non-contracting mutant, *Mhc*, were voltage clamped at −65 mV. Synaptic currents were elicited by stimulation at 1 Hz with a microelectrode placed in the ventral nerve cord. Sample records of nerve-evoked synaptic currents before (left) and during (right) the hypertonicity response are shown. A solution containing 340 mM sucrose (added to the external saline) was puff-applied to the neuromuscular junction while the postsynaptic muscle cell was voltage clamped with a patch electrode in the whole-cell configuration. The external solution contained 0.3 mM Ca^{2+}. *B*, bar graph showing the failure rate before (left, $n = 8$) and during (right, $n = 8$) the hypertonicity response. The vertical bar is the standard error of the mean and numbers are the number of cells examined. *C*, charge transfer during synaptic currents plotted against time. The synaptic current was integrated between the two vertical bars shown in *A* and plotted against time. The puff pulse was applied during the time indicated by the horizontal bar below the abscissa. Data from different cells were superimposed by aligning the onsets of the puff pulse. The bars attached to each data point are the standard error of the mean. *D*, the frequency of spontaneous synaptic events was counted every 1 s and is expressed on the ordinate as the number of events per second ($n = 4$).

suggesting that the postsynaptic effect of hypertonicity is minimal (Suzuki *et al.* 2002). The time course of changes in charge transfer during evoked synaptic currents is plotted against time in Fig. 1*C*. Synaptic depression was more prolonged than enhancement of the frequency of mSCs (see below and Fig. 1*D*) and started immediately after the onset of the pulse of hypertonic solution.

With 0.3 mM Ca^{2+} in the external solution, mSCs were observed at a mean frequency of $1.1 \pm 1.5\ s^{-1}$ ($n = 5$). These were mostly mSCs, as synaptic events with a similar frequency were also observed in the presence of 3 μM TTX. When a 340 mM sucrose solution was puff-applied for 11 s to the synaptic site, the mSC frequency increased to a peak of $17.2 \pm 6.8\ s^{-1}$ ($n = 5$) and declined quickly during the puff pulse (Fig. 1*D*). This peak frequency was similar to that observed in the absence of external Ca^{2+} ($20.6 \pm 6.7\ s^{-1}$, $n = 5$). The mSC frequency returned to the prepuff level within 20 s after termination of the pulse. The total number of events during the period of 31 s after the onset of the puff pulse was 127 ± 73 ($n = 5$), which was not different from in the absence of external Ca^{2+} (156 ± 61, $n = 5$, $P > 0.05$).

The mSC frequency in a high-K^+ solution containing Ca^{2+} was modulated biphasically by hypertonicity

We have shown here that the depression of charge transfer during nerve-evoked synaptic currents was due to reduced release, while the frequency of mSCs was elevated by hypertonicity. A plausible explanation for this biphasic effect is that the influx of Ca^{2+} during an action potential is reduced, while the fusion rate is enhanced by a mechanism that is independent of the Ca^{2+} level. To test this possibility, we next examined the effect of hypertonicity on mSC frequency already elevated in a high-K^+ solution containing Ca^{2+}. In a high-K^+ saline (62 mM K^+) containing 0.1 mM Ca^{2+} and 3 μM TTX, the mSC frequency was $14.5 \pm 7.6\ s^{-1}$ before application of hypertonic solutions ($n = 31$). The high frequency of mSCs under this experimental condition is most like the result of elevated internal Ca^{2+} concentration due to opening of voltage-gated Ca^{2+} channels by high-K^+-induced depolarization. If hypertonicity inhibits Ca^{2+} influx through voltage-gated Ca^{2+} channels, the frequency of mSCs would be expected to decrease.

When the 340 mM sucrose solution was puff-applied, the frequency increased transiently to a peak of about $35\ s^{-1}$ ($+20\ s^{-1}$ above the prepuff level), followed by a prolonged depression, which lasted more than 30 s after the end of the puff pulse (Fig. 2*A*). At 60 s after the end of the puff pulse, the frequency returned to approximately 60 % of that before the application of hypertonicity (Fig. 2*B*). The peak mSC rate and the extent of depression (the trough amplitude) increased with the sucrose concentration between 200 and 500 mM (Fig. 3*A*), but neither of the effects had saturated at 500 mM sucrose. At 50 mM, there was enhancement of mSC frequency without a subsequent depression (Fig. 3*B*). The enhancing effect of hypertonicity on mSC frequency in the absence of external Ca^{2+} was virtually absent with 50 mM sucrose (Fig. 3*C*). This result indicates that with 50 mM sucrose, the enhancing hypertonicity response is strongly augmented by the presence of external Ca^{2+}. At the higher concentrations of sucrose tested, the effect of external Ca^{2+} on the enhancing hypertonicity response was usually not evident due to the superimposed depressing hypertonicity response.

In high-K^+ saline with 0.1 mM Ca^{2+}, the spontaneous mSC frequency was variable, probably reflecting variable $[Ca^{2+}]_i$ in the presynaptic terminal. When the peak mSC frequency during the enhancing hypertonicity response (induced with the 340 mM sucrose solution) was plotted against the spontaneous frequency before puff application (background rate), a positive correlation was found ($r = 0.67$, significant at $P < 0.02$, Fig. 3*D*). In high-K^+ saline in the absence of Ca^{2+}, the spontaneous mSC frequency was close to zero and the peak rate of the hypertonicity response was smaller than in the presence of Ca^{2+} (Fig. 3*D*, the mean is indicated by a triangle). The peak response in the absence of Ca^{2+} was a mean of $10.8 \pm 6.9\ s^{-1}$ ($n = 8$), which is significantly smaller than that recorded in the presence of Ca^{2+} ($18.3 \pm 6.8\ s^{-1}$, $n = 13$) ($P < 0.05$). These results again suggest that the enhancing effect of hypertonicity is augmented by internal Ca^{2+}.

The mSC frequency in *n-syb*-null embryos was depressed without enhancement by hypertonicity

In the mutant *n-syb*^{F33B} embryos, nerve-evoked synaptic currents are completely lacking, while mSCs are observed at lower frequencies than in wild-type embryos. The frequency of mSCs in *n-syb*^{F33B} was increased when the external Ca^{2+} concentration was raised in high-K^+ saline (Deitcher *et al.* 1998; Yoshihara *et al.* 1999). In these mutant embryos, no hypertonicity response was detected in the absence of external Ca^{2+} (data not shown). Similar results were obtained in the presence of external Ca^{2+} in tetanus-toxin-expressing transgenic *Drosophila* embryos, in which there is no functional n-syb (Aravamudan *et al.* 1999). Thus, n-syb is indispensable for the stimulation of transmitter release by hypertonic solution. If the depressing effect of hypertonicity is independent of the enhancing effect, we might be able to demonstrate the depressing effect in isolation in *n-syb*^{F33B} embryos.

Consistent with these earlier observations, we found that in double-mutant embryos (*Mhc n-syb*) the mSC frequency was higher in high-K^+ saline (62 mM K^+ 0.1 mM Ca^{2+}, 3 μM TTX) than in normal-K^+ saline. The mean frequency was $5.0 \pm 3.3\ s^{-1}$ ($n = 5$) before application of the hypertonic solution, which is about one-third of the frequency reported here in *Mhc*, in accord with the previous observation (Yoshihara *et al.* 1999). With puff application of the 420 mM sucrose solution, the mSC frequency was depressed for a prolonged time without an

initial enhancement (Fig. 4*A*). Thus, the depression induced by hypertonicity is not necessarily coupled to a preceding enhancement of release.

The mSC frequency in the presence of forskolin was enhanced by hypertonicity without depression

The depression of mSC frequency by hypertonicity could be due to an inhibitory effect on Ca^{2+} influx through voltage-gated Ca^{2+} channels, as we postulated above. Alternatively, it could be due to a direct effect of hypertonicity on the vesicle fusion mechanism. It has been demonstrated that forskolin, an activator of adenylyl cyclase, increases the mSC frequency in wild-type and *Mhc* embryos in the absence of external Ca^{2+} (Yoshihara *et al.* 1999, 2000; Zhang *et al.* 1999). If hypertonicity depresses quantal transmitter release by acting directly on the vesicle fusion process, we would expect to observe a depressing effect in the absence of Ca^{2+} after elevating the mSC frequency by forskolin.

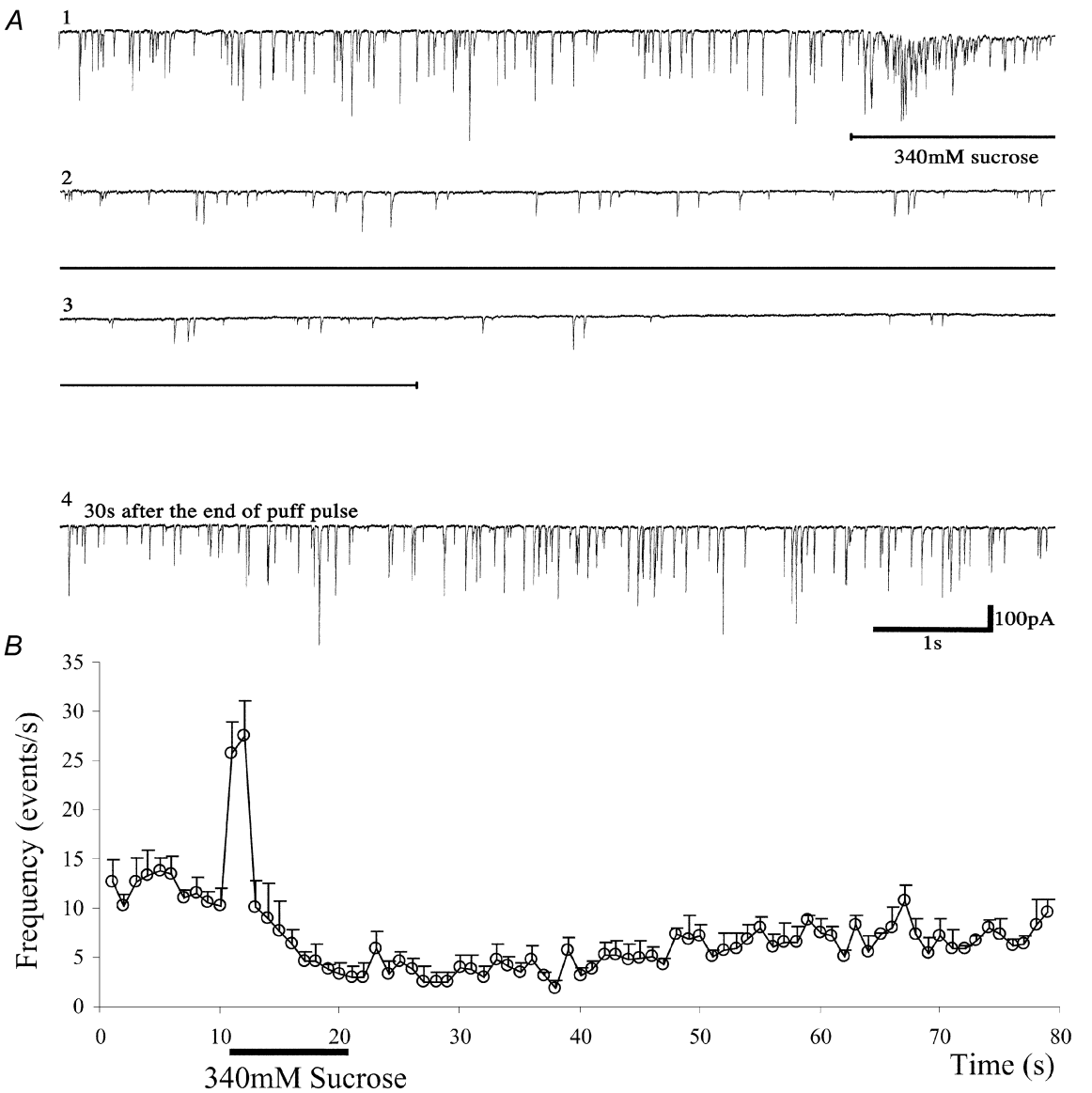

Figure 2. Biphasic effects of hypertonicity on miniature synaptic currents (mSCs) in high-K^+ saline

A, current records before and during the hypertonicity response induced by 340 mM sucrose in an abdominal muscle cell in an *Mhc* embryo. The upper three traces (1–3) are a consecutive record. The fourth trace starts 30 s after the end of the puff pulse, showing the recovery of quantal transmitter release. The hypertonic solution was applied during the horizontal bar shown below the upper three traces. Downward deflections indicate inward currents. The external solution contained 62 mM K^+ and 0.1 mM Ca^{2+}. *B*, the mean frequency of mSCs in five cells is plotted against time. Sucrose (340 mM) was puff-applied at the horizontal bar. Vertical bars attached to each data point are the standard error of the mean. The external solution contained 62 mM K^+ and 0.1 mM Ca^{2+}.

In Ca^{2+}-free saline containing 200 μM forskolin, the mSC frequency in *Mhc* embryos was 3.9 ± 2.6 s^{-1} (n = 6). When a 500 mM sucrose solution was puff-applied, the mSC frequency increased to a peak of 38.7 ± 10.4 s^{-1} (n = 6) and declined to the prepulse level during the puff without exhibiting any depression (Fig. 4*B*). This result is in accord with the result reported previously in wild-type *Drosophila* embryos (Suzuki *et al.* 2002). The absence of a decrease in release suggests that the depression of release observed in high-K^+ saline containing Ca^{2+} is not due to a direct effect of hypertonicity on the vesicle fusion process, but rather to inhibition of voltage-gated Ca^{2+} channels by hypertonicity.

An RGD peptide did not affect the depressing hypertonicity response, but reduced the enhancement

We have shown previously that over 50% of the hypertonicity response in the absence of external Ca^{2+} is blockable with peptides containing the RGD (arginine-glycine-aspartic acid) sequence. Peptides that have this sequence bind to the ligand-binding sites of integrins and block integrin-mediated cell adhesion (Pierschbacher & Ruoslahti, 1987). Since the effects of integrins are mediated by the cAMP–protein kinase A (PKA) cascade (Suzuki *et al.* 2002) and cAMP facilitates synaptic transmission at *Drosophila* neuromuscular junctions (Zhang *et al.* 1999; Yoshihara *et al.* 2000), it is unlikely that position-specific (PS) integrins are involved in the depressing effect. To confirm this prediction we tested the effect of an RGD peptide on the depressing hypertonicity response.

After dissection, *Mhc* embryos were treated with 0.2 mM GRGDSP for 20 min in low-Ca^{2+} and low-Mg^{2+} saline, and the following experiments were carried out in the presence of the peptide. In high-K^+ saline (62 mM K^+, 0.1 mM Ca^{2+}, 3 μM TTX), the 340 mM sucrose solution was puff-applied.

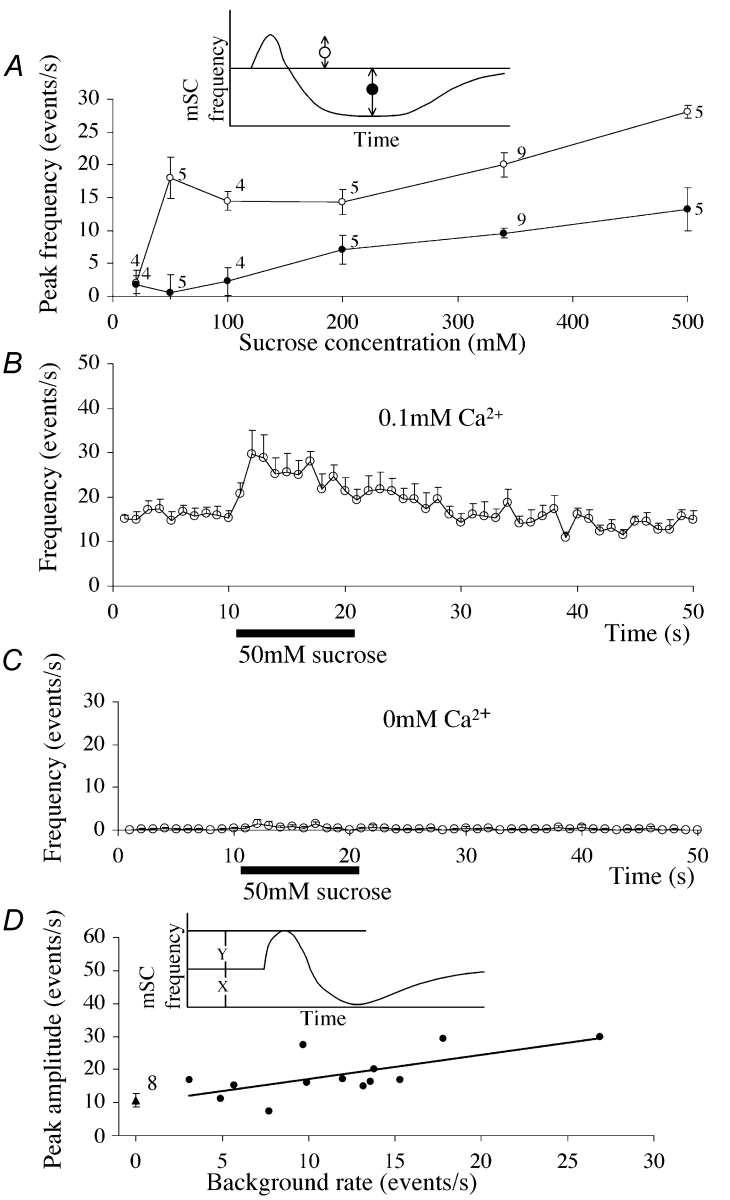

Figure 3. Dose–response curves for the enhancing and depressing effects of hypertonicity and the effect of Ca^{2+} on the enhancing hypertonicity response

A, dose–response curves for the enhancing and depressing effects induced by hypertonicity. The magnitudes of the enhancing and depressing effects were measured as indicated in the inset, and the average of those parameters are plotted against the sucrose concentration. The trough level was measured by averaging 10 points during the period including the lowest frequency after the puff pulse. Vertical bars attached to each data point are the standard error of the mean and numbers are the number of cells examined. The bath solution contained 62 mM K^+, 0.1 mM Ca^{2+} and 3 μM TTX. *B*, the enhancing effect at 50 mM sucrose in the presence of Ca^{2+}. The bath solution contained 62 mM K^+, 0.1 mM Ca^{2+} and 3 μM TTX. Sucrose (50 mM) was added to the bath solution. The 50 mM sucrose solution was puffed at the time shown by a horizontal bar. Vertical bars are standard error of the mean. Some bars were smaller than the radius of the symbol (n = 5). *C*, the enhancing effect at 50 mM sucrose in the absence of external Ca^{2+}. The bath solution contained 62 mM K^+ and 3 μM TTX. Sucrose (50 mM) was added to the bath solution. The 50 mM sucrose solution was puffed at the time shown by a horizontal bar. Vertical bars are standard error of the mean. Some bars were smaller than the radius of the symbol (n = 5). *D*, correlation between the peak amplitude of the enhancing hypertonicity response and the background mSC frequency. These data were obtained following puff-application of a 340 mM sucrose solution. The inset indicates the two parameters measured (i.e. the peak magnitude of the enhancing effect, *Y*, and the background mSC frequency, *X*. The regression line has a correlation coefficient of 0.67, which is significant at $P < 0.02$ (two-tail t test). The triangle at the left is the average of the mSC frequency in Ca^{2+}-free saline. The number is the number of cells examined, and the vertical bar attached is the standard error of the mean.

The mSC frequency increased slightly and then decreased for a prolonged period of time (Fig. 4*C*). The peak rate ($+8.2 \pm 2.2$ s^{-1}, $n = 5$) was significantly smaller ($P < 0.05$) than that observed without the RGD treatment ($+17.7 \pm 6.0$ s^{-1}, $n = 13$, $P < 0.05$; Fig. 3*A*). This effect of the peptide on the enhancing hypertonicity response is in accord with our previous results in the absence of external Ca^{2+} (Suzuki *et al.* 2002). In contrast, the depressing effect (-7.8 ± 5.1 s^{-1}, $n = 5$) was not different from without the peptide treatment (-8.0 ± 3.3 s^{-1}, $n = 13$), suggesting that PS integrins are not involved in the depressing hypertonicity response. These results also provide further evidence that an increase in transmitter release is not required for the depression of release by hypertonic solutions.

Hypertonicity depresses the elevation of $[Ca^{2+}]_i$ induced by a high-K^+ solution in cultured embryonic neurons

Voltage-gated Ca^{2+} currents measured at the cell body are reduced by hypertonicity in cultured hippocampal neurons (Rosenmund & Stevens, 1996) and in anterior pituitary cells (Matzner *et al.* 1996). Hence, it is possible that the depressing hypertonicity response we observed is due to a reduction of Ca^{2+} currents in the presynaptic terminal. Since it is technically difficult to measure $[Ca^{2+}]_i$ in the presynaptic nerve terminal of *Drosophila* embryos, we used cultured embryonic neurons to investigate this possibility.

Cultured neurons were loaded with Fura-2 and stimulated with a high-K^+ solution (75 mM K^+ HL3 solution). In the

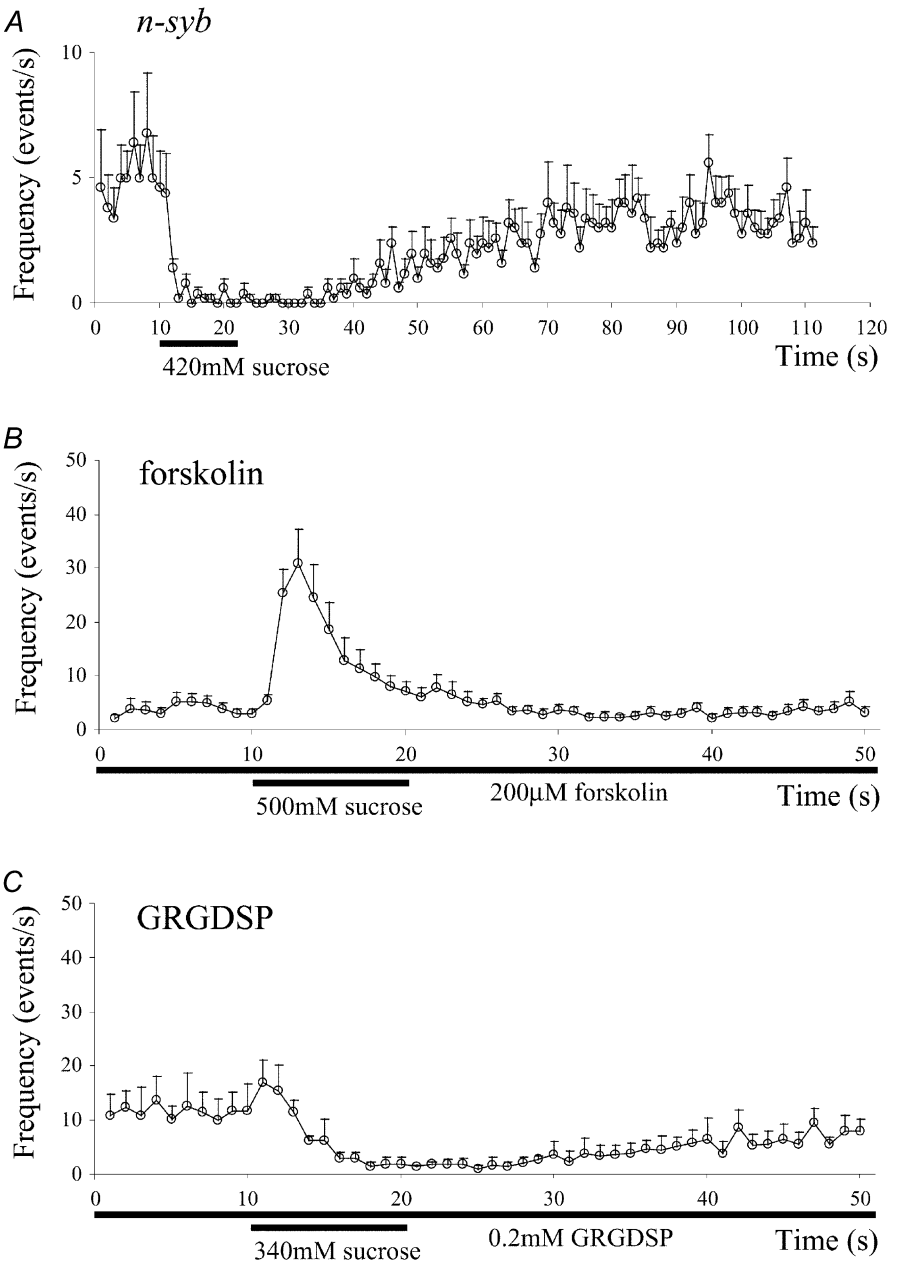

Figure 4. The effects of *neural synaptobrevin* gene (*n-syb*) mutation (*A*), forskolin (*B*) and an RGD peptide (*C*) on the hypertonicity response

A, the hypertonicity response in *Mhc n-syb*^{F33B} embryos. The bath solution contained 62 mM K^+, 0.1 mM Ca^{2+} and 3 μM TTX. For the puff solution, 420 mM sucrose was added to the bath solution. These data were obtained in five cells and were superimposed after aligning the onsets of the puff pulse. Vertical bars represent the standard error of the mean. *B*, the effect of forskolin on the hypertonicity response in Ca^{2+}-free saline. The bath contained 200 μM forskolin, and the 500 mM sucrose solution was puff-applied at the horizontal bar. The vertical bars indicate the standard error of the mean ($n = 6$). *C*, the effect of an RGD peptide in high-K^+ saline. The experiment was carried out in high-K^+ saline (62 mM K^+, 0.1 mM Ca^{2+} and 3 μM TTX) and a 340 mM sucrose solution was puff-applied during the horizontal bar. These data were obtained in five cells and averaged. Vertical bars represent the standard error of the mean ($n = 5$).

HL3 medium, the resting $[Ca^{2+}]_i$ was 62 ± 10 nM (n = 5) in the neurite and 52 ± 7 nM (n = 8) in the cell body (Fig. 5*A*, Fig. 6*A*, leftmost pair of columns). Upon application of the high-K^+ solution, $[Ca^{2+}]_i$ increased to 453 ± 33 nM (n = 5) in the neurite and 148 ± 9 nM (n = 8) in the cell body (Fig. 5). The difference in the surface-to-volume ratio between the neurite and cell body may be a major factor in the greater elevation of $[Ca^{2+}]_i$ in the neurite (Fig. 6*A*). When application of high K^+ saline was repeated three times at intervals of 5 min, the extent of $[Ca^{2+}]_i$ elevation declined only slightly (Fig. 6*A*). The same data are replotted in Fig. 6*B* as the percentage change compared to the first challenge. The elevation of $[Ca^{2+}]_i$ induced by high-K^+ saline was completely blocked when 0.2 mM Cd^{2+} was included in the high-K^+ saline (Fig. 6*C*, middle columns). This blocking effect of Cd^{2+} was reversible (Fig. 6*C*, right columns). PLTX II (0.2 μM) also blocked the high-K^+-induced elevation of $[Ca^{2+}]_i$, but this blocking effect was irreversible (Fig. 6*D*). These results are in accord with the previous reports that neuromuscular transmission in *Drosophila* larvae was irreversibly blocked (Branton *et al.* 1987), and that voltage-gated inward Ca^{2+} currents in cultured *Drosophila* neurons are suppressed by Plectreurys toxin (Leung *et al.* 1989). Thus, it is most likely that the elevation of $[Ca^{2+}]_i$ observed in these experiments is due to Ca^{2+} influx through voltage-gated Ca^{2+} channels in the surface membrane.

We then tested the effect of hypertonicity on Ca^{2+} influx in the cultured neurons. When 420 mM sucrose was added to the high-K^+ saline, the elevation of $[Ca^{2+}]_i$ was significantly smaller than that induced by high K^+ alone (Fig. 6*E*). With the first application of high K^+ saline, $[Ca^{2+}]_i$ increased to 373 ± 33 nM (n = 7) at the neurite and 190 ± 28 nM (n = 7) at the cell body. With the second application of high K^+ plus sucrose saline, $[Ca^{2+}]_i$ increased to 50 ± 4 %

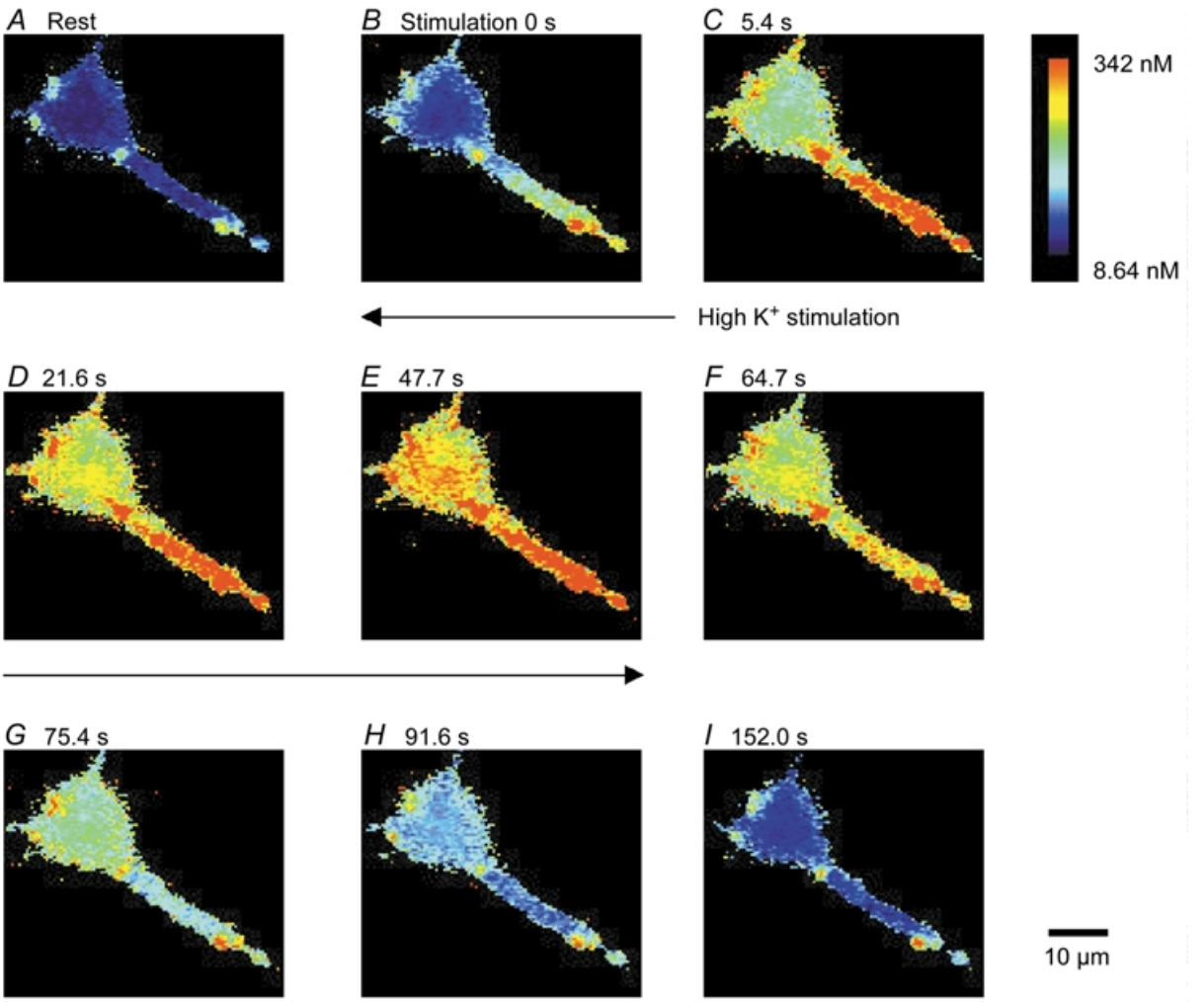

Figure 5. Ca^{2+} signals measured with Fura-2 in cultured embryonic *Drosophila* neurons

Pseudocolour demonstration of $[Ca^{2+}]_i$ before (*A*), during (*B–E*) and after (*F–I*) stimulation with high-K^+ saline (75 mM K^+ HL3 saline). The cultured cell had been loaded with Fura-2/AM. The $[Ca^{2+}]_i$ at rest was 52 ± 8 nM (n = 8) at the cell body and 62 ± 10 nM (n = 5) at the neurite. The $[Ca^{2+}]_i$ increased to 148 ± 9 nM at the cell body and 453 ± 33 nM at the neurite during the first high-K^+ stimulation, and returned to the resting level during a 5 min perfusion with HL3 saline.

($n = 10$) of the first response at the neurite and 43 ± 4 % ($n = 12$) at the cell body. These values are significantly smaller than the corresponding values shown in Fig. 6*B* for the second high-K^+ challenge (88 ± 3 % at the neurite, $n = 5$, and 100 ± 5 % at the cell body, $n = 8$, $P < 0.01$). At the third stimulation they were 82 ± 13 % at the neurite and 81 ± 3 % at the cell body, values that are not different from the corresponding values shown in Fig. 6*B* (77 ± 1 % at the neurite and 88 ± 11 % at the cell body; $P > 0.05$). Thus, the effect of hypertonicity on high-K^+-induced Ca^{2+} influx was reversible.

We conclude that hypertonicity reduces the Ca^{2+} influx induced by high-K^+ stimulation in cultured embryonic *Drosophila* neurons.

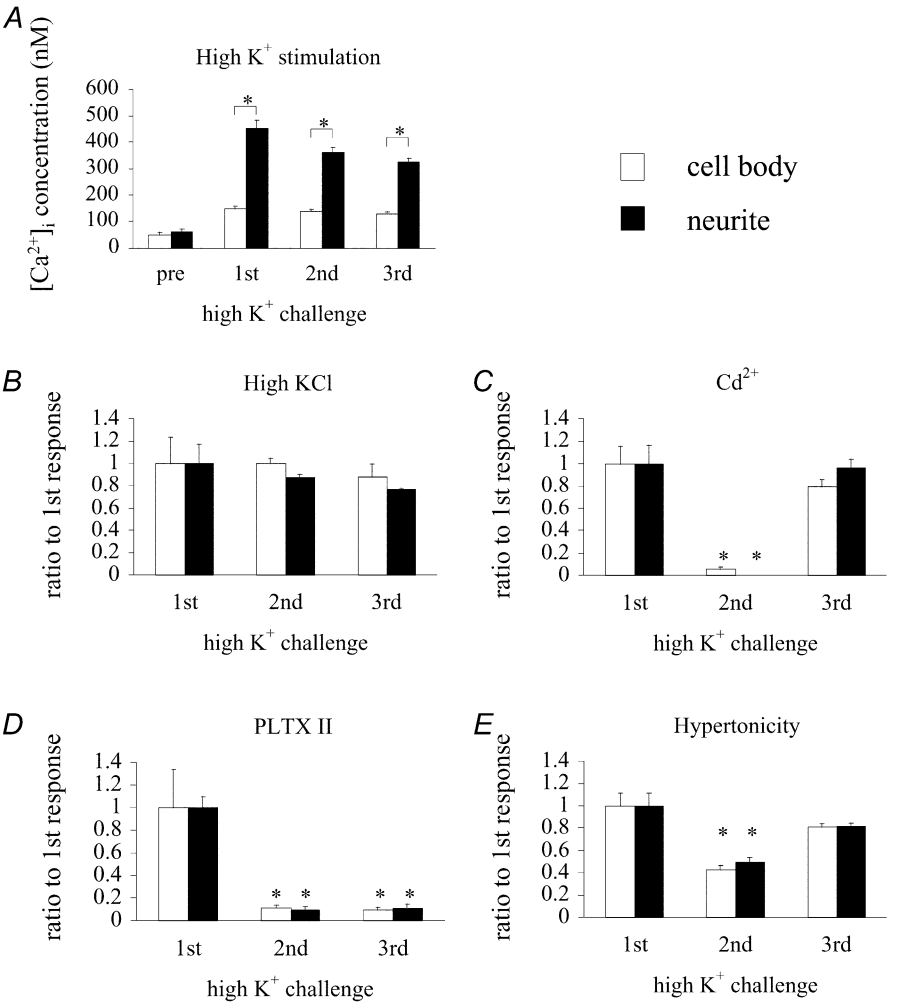

Figure 6. Effects of Cd^{2+}, PLTXII and hypertonicity on the high-K^+-induced elevation of $[Ca^{2+}]_i$

A, control: the cells were stimulated with high-K^+ HL3 saline three times for 1 min at intervals of 5 min, during which the HL3 solution was continuously perfused. The ordinate indicates the Ca^{2+} concentration in nanomolar. Open columns are the data obtained at the cell body and filled columns are those obtained at the neurite. The height of each column represents the mean response in eight cell bodies and in five neurites. Vertical bars at the top of each column are the standard error of the mean. The pair of columns at the left represents the resting state (pre). The three pairs to the right are for three consecutive challenges with high-K^+ HL3 saline. *Significant difference between the cell body and neurite ($P < 0.001$). *B*, the same data as in *A*, but the high-K^+ responses are expressed as a percentage of the first response. *C*, the effect of Cd^{2+}; 200 μM Cd^{2+} was added to the high-K^+ solution at the second challenge. No response was detected. *Significant difference ($P < 0.01$) between the column with the symbol and the corresponding one in *B*. The response had fully recovered at the third challenge. The number of cells examined at the cell body was six, and that for the neurite was also six. *D*, the effect of PLTXII. PLTXII (0.2 μM) was added to the high-K^+ solution at the second challenge. Recovery of the response was not observed at the third challenge. *Significant difference ($P < 0.01$) between the column with the symbol and the corresponding one in *B*. The number of cells examined at the cell body was five, and that at the neurite was also five. *E*, the effect of hypertonicity. Sucrose (420 mM) was added to the high-K^+ solution at the second challenge. *Significant difference ($P < 0.01$) between the column with the symbol and the corresponding one in *B*. The number of cells examined at the cell body was 12, and that at the neurite was 10.

DISCUSSION

Hypertonic solutions increase spontaneous quantal transmitter release at embryonic *Drosophila* neuromuscular junctions in the absence of external Ca^{2+} (Suzuki *et al.* 2002), and we have now shown that this response can be amplified in the presence of external Ca^{2+} (Fig. 3*B*). However, with external Ca^{2+}, where it is possible to test effects on evoked release by nerve stimulation, we found that nerve-evoked synaptic currents were depressed by hypertonicity (Fig. 1*A* and *C*). This is at least in part a presynaptic depression, since the failure rate also increased during this period (Fig. 1*B*). The depression of evoked release was seen at the same time the spontaneous release was elevated (Fig. 1*D*). Hypertonicity had a similar depressing effect on mSC frequency when the baseline frequency was elevated in high K^+ (Figs 2 and 3). These observations might suggest that the elevated spontaneous release depletes the population of readily releasable vesicles, thus suppressing evoked release. However, we feel that this is not the case, since forskolin increased the background mSC frequency in Ca^{2+}-free saline and augmented the response to hypertonicity without evidence of depression (Fig. 4*B*). Furthermore , in *n-syb*^{F33B} mutant embryos there was no enhancement of mSC frequency, but the depression remained (Fig. 4*A*). Thus, the enhancing and depressing effects of hypertonicity are separable. These observations lead us to conclude that the depressing effect is not coupled to the enhancement, and that these two effects are the result of two separate processes induced by hypertonicity.

The effect of hypertonic solutions on nerve-evoked synaptic currents has been studied in several preparations. Low levels of hypertonicity (up to 100 mosmol) increased evoked release either moderately or not at all (Barton *et al.* 1983; Tanabe & Kijima, 1988; Kashani *et al.* 2001), while larger changes in osmolarity decreased evoked release (Thesleff, 1959; Hubbard *et al.* 1968; Kita & Van der Kloot, 1977). These results are in accord with our findings in the embryonic *Drosophila* neuromuscular junction. Although the predominant effect of hypertonicity on nerve-evoked release at $\geqslant$ 200 mM sucrose was depression, we did observe the enhancement of spontaneous release at a low sucrose concentration (50 mM sucrose; Fig. 3*B*).

In cultured hippocampal synapses, the amplitude of nerve-evoked synaptic currents was reduced shortly after puff application of hypertonic solutions, and recovered as the interval between the puff pulse and nerve stimulation increased. The evoked current amplitude recovered with a time constant of about 8 s. Based upon the assumption that the application of hypertonicity depleted vesicles from the readily releasable pool, this time constant was interpreted as the rate of refilling of the pool (Rosenmund & Stevens, 1996). However, this interpretation does not seem to be valid, at least in our preparation, for the following reasons. First, the maximum hypertonicity response does not deplete the readily releasable pool (Fig. 4*B* and see Discussion in Suzuki *et al.* 2002). Second, the depressing effect of hypertonicity on voltage-gated Ca^{2+} channels (Figs 5 and 6) should reduce the amplitude of nerve-evoked synaptic currents upon application of a hypertonic solution. Third, the depression of quantal release can occur without enhancement of quantal release (Fig. 4*A*).

We suggest that the depressing effect of hypertonicity on vesicle fusion results from the inhibitory effect of hypertonicity on voltage-gated Ca^{2+} channels. Depression of synaptic transmission was observed only when voltage-gated Ca^{2+} channels were activated either with electrical nerve stimulation or with high-K^+-induced depolarization, and was not detected when the mSC frequency was elevated with forskolin in Ca^{2+}-free saline. Moreover, the mSC frequency was not depressed in normal saline (Fig. 1*D*), in which voltage-gated Ca^{2+} channels were presumably not activated. Furthermore, in cultured embryonic *Drosophila* neurons, the Ca^{2+} signals induced by a high K^+ solution were inhibited by hypertonicity (Fig. 6*E*). Our interpretation is in accord with the reports on pituitary cells (Matzner *et al.* 1996) and in cultured hippocampal neurons (Rosenmund & Stevens, 1996), in which hypertonicity depresses voltage-gated Ca^{2+} currents. However, in the latter case the inhibition was observed only during application of a hypertonic solution (300 mM sucrose), and the current recovered immediately after the puff (Fig. 5*D* in Rosenmund & Stevens, 1996). Thus, it is not clear whether the prolonged inhibition of synaptic currents after application of hypertonicity, which Rosenmund & Stevens (1996) observed and interpreted as the refilling time course of the readily releasable pool, can be explained by the mechanism we postulated.

In the study presented here, we found that 50 mM sucrose produced a clear enhancement of mSC frequency only in the presence of external Ca^{2+}, and that the response was greater in cells in which the background mSC frequency was higher, and presumably $[Ca^{2+}]_i$ was higher (Fig. 3*D*). These findings suggest that the hypertonicity-induced increase in mSC frequency is augmented by Ca^{2+}. On the other hand, the enhancement induced by high concentrations of sucrose remained even when the presynaptic neuron was injected with high concentrations of BAPTA, which blocked nerve-induced synaptic currents (Rosenmund & Stevens, 1996; Mochida *et al.* 1998). Thus, at least a part of the enhancing hypertonicity response is independent of Ca^{2+}.

In all those studies in which BAPTA was used to reduce $[Ca^{2+}]_i$, the hypertonicity response was evoked by use of solutions of very high tonicity (500 mM sucrose was added to normal saline). On the other hand, at rat neuromuscular junctions, Losavio & Muchnik (1997) compared the hypertonicity response induced by a solution with

relatively low hypertonicity (200 mosmol above normal saline) before and after application of EGTA-AM or BAPTA-AM, and found that although the hypertonicity response remained, the amplitude of the response was reduced. Similar results were obtained at the frog neuromuscular junction with solutions of 10–20 mosmol above normal saline (Kashani *et al.* 2001). These results are in accord with our finding that Ca^{2+} enhances the hypertonicity response induced by a solution with relatively low hypertonicity (Fig. 3).

The hypertonicity-induced enhancement of spontaneous release and the enhancement of nerve-evoked synaptic transmission by mechanical stretch have many properties in common. Both responses remain in the absence of external Ca^{2+} and even after the internal Ca^{2+} is strongly buffered to a low level (Chen & Grinnell, 1997; Rosenmund & Stevens, 1996; Mochida *et al.* 1998), and both are reduced by treatment with RGD peptides (Chen & Grinnell, 1995, 1997; Kashani *et al.* 2001; Suzuki *et al.* 2002). Responses to hypertonicity and stretch co-varied in magnitude among different muscle fibres in the frog (Kashani *et al.* 2001). Thus it seems likely that these responses, at least to some degree, share a common underlying mechanism. Because of the close temporal relationship between stretch and the enhancement of synaptic transmission, the stretch effect is considered to be purely mechanical (Chen & Grinnell, 1997). On the other hand, the elimination of external Ca^{2+} reduced by half the stretch effect on synaptic transmission, and buffering external Ca^{2+} with BAPTA further reduced it to one-fifth of that recorded in normal saline (Chen & Grinnell, 1997). These results are in accord with our finding that the enhancing hypertonicity response is augmented by Ca^{2+}.

We have proposed the following hypothesis for the enhancing effect of hypertonicity on release: independent of Ca^{2+}, hypertonicity facilitates vesicle fusion as well as the recruitment of vesicles for release. The magnitude of the hypertonicity response may be determined mainly by factors such as the intensity and time course of the enhanced rate of vesicle fusion induced by hypertonicity and the recruitment rate of vesicles for release (Suzuki *et al.* 2002). Our observation that external Ca^{2+} augments the enhancing hypertonicity response (Fig. 3) is in accord with the idea that the recruitment of vesicles to the release sites is facilitated by Ca^{2+} (Von Ruden & Neher, 1993; Von Gersdorff & Matthews, 1997; Wang & Kaczmarek, 1998).

The hypertonicity response appears to be complex. The enhancing effect of release has at least two components, integrin-mediated and integrin-independent. The integrin-mediated response is coupled to the cAMP/PKA cascade (Suzuki *et al.* 2002). The depressing hypertonicity response is independent of the enhancing effect and is probably caused by the inhibitory effect of hypertonicity on presynaptic voltage-gated Ca^{2+} channels. Knowing these properties of the hypertonicity response, we can now use it as a tool to dissect presynaptic events in mutants.

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