External Ca\textsuperscript{2+} Dependency of Synchronous Transmission in *Drosophila synaptotagmin I* Mutants

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**INTRODUCTION**

Synaptotagmin I (Syt I) is considered to be a major Ca\textsuperscript{2+}-sensor for synchronous synaptic transmission (Kidokoro 2003; Koh and Bellen 2003). However, details of its function are yet to be established. The *Drosophila* neuromuscular junction (NMJ) is suitable for studies of Syt I function in vivo because numerous *syt I* mutants are available. Although it is generally agreed that nerve-evoked synaptic transmission is severely impaired in *syt I* mutants (Broadie et al. 1994; DiAntonio and Schwarz 1994; Yoshihara and Littleton 2002), striking differences among reports are impeding our further understanding of Syt I functions.

Three *syt I* mutant alleles are particularly suited for detailed studies (DiAntonio and Schwarz 1994). *syt I\textsuperscript{AD4}* is a null allele. In the homozygous embryos, Broadie et al. (1994) first reported that nerve-evoked synchronous synaptic transmission was severely impaired but not abolished, and the external Ca\textsuperscript{2+} dependency of quantal content in the double logarithmic plot shifted along the Ca\textsuperscript{2+} axis to higher concentrations without changes in the slope (N). This finding seems odd, because, if Syt I were the Ca\textsuperscript{2+} sensor, N, which reflects the number of Ca\textsuperscript{2+} binding sites, should decrease in its absence. However, Yoshihara and Littleton (2002) reported in *syt I\textsuperscript{AD4}* embryos that synchronous synaptic transmission was totally abolished, contradicting the findings in embryos (Broadie et al. 1994; Kidokoro 2003) and in *syt I\textsuperscript{AD3}* third instars (Mackler et al. 2002).

The second *syt I* mutant allele, *syt I\textsuperscript{AD1}*, has a premature stop codon between the two Ca\textsuperscript{2+}-binding domains, resulting in a loss of the second C2B domain (DiAntonio and Schwarz 1994). The first domain, C2A, binds phospholipids Ca\textsuperscript{2+}-dependently, which was thought to be the primary function of Syt I in vesicle fusion (Chapman and Jahn 1994; Davletov and Südhof 1993). However, at *Drosophila* NMJs, Syt IV, a homologue of Syt I, which does not bind phospholipids at C2A (Littleton et al. 1999), substituted for Syt I in synchronous synaptic transmission (Robinson et al. 2002). Thus phospholipid-binding at C2A may not be essential for synaptic transmission. Yoshihara and Littleton (2002) detected synchronous synaptic transmission in *syt I\textsuperscript{AD1}*, but not in *syt I\textsuperscript{AD4}* and implied that C2A is sensing Ca\textsuperscript{2+} for synchronous release, contradicting the finding by Robinson et al. (2002). A major remaining question is which Ca\textsuperscript{2+}-binding domain, C2A or C2B, is sensing Ca\textsuperscript{2+} for nerve-evoked synchronous synaptic transmission.

The third allele, *syt I\textsuperscript{AD3}*, has a mutation from Y(364) to N in C2B (DiAntonio and Schwarz 1994). The equivalent mutants in mouse Syt II, which is almost identical to Syt I, blocked Ca\textsuperscript{2+}-dependent self-oligomerization (Fukuda et al. 2000). Hence this allele provides us an opportunity to reveal the role of Syt I oligomerization in synaptic transmission. In the squid giant-synapse (Fukuda et al. 1995) and in cultured rat neurons (Mochida et al. 1997), inositol high-polyporphosphates, which block Ca\textsuperscript{2+}-dependent self-oligomerization, strongly inhibited synaptic transmission. If Ca\textsuperscript{2+}-dependent Syt I oligomerization was essential for synchronous release, in *syt I\textsuperscript{AD3}* we expect synaptic impairment and smaller N. However, Yoshihara and Littleton (2002) reported an N of 3.5 for both control and *syt I\textsuperscript{AD3}*, suggesting that *syt I\textsuperscript{AD3}* is inhibiting vesicle fusion. These findings led us to conclude that C2B, not C2A, senses Ca\textsuperscript{2+}, and Syt I is a negative regulator of vesicle fusion.

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**METHODS**

**Fly stocks**

Synaptotagmin I (Syt I) mutant alleles used in this study were *syt I\textsuperscript{AD2}* , *syt I\textsuperscript{AD1}*, and *syt I\textsuperscript{AD3}*, which were gifts from Dr. T. L. Schwarz.
Nerve stimulation and calculation of the quantal content

For nerve stimulation, the tip of a microelectrode, which has a resistance of 10–20 MΩ when filled with 4 M K-acetate, was placed in the ventral nerve cord near the exit of segmental nerve, and rectangular pulses of 2-ms duration and about 2-mA were placed in the ventral nerve cord near the exit of segmental nerve, and currents were examined. In those experiments, a single syt I mutants, because muscle contraction was not a serious problem. In those experiments, en bw was used as control.

Preparations and recording conditions

Embryos (19–21 h after fertilization) of mutants and controls were used in this study. Homozygous mutant embryos were selected under a fluorescence stereomicroscope based on a green fluorescence protein marker on the balancer or under a stereomicroscope based on an y marker. Dissecting procedures were the same as described previously (Kidokoro and Nishikawa 1994; Nishikawa and Kidokoro 1995) and carried out in Ca2+-free saline (see Solutions for ionic composition). After treating the dissected preparation with collagenase (1 mg/ml) for 30 s to 1 min, synaptic currents were recorded with patch-clamp techniques in the whole cell configuration from abdominal longitudinal muscle 6 or 12. The membrane potential was usually held at −60 mV, unless stated otherwise. The internal solution contained Cs+ (see Solutions for ionic composition), and the junction potential of electrodes filled with the Cs+ internal solution was −5 mV in normal saline. Thus the true holding potential was −65 mV.

HYPERTONICITY AND Ca2+ IONOPHORE RESPONSES. A hypertonic solution was prepared by adding 420 mM sucrose to the Ca2+-free external solution (see following text for ionic composition). A Ca2+ ionophore, A23187 (10 µM), was dissolved in the HL3 solution (see Solutions for ionic composition) containing 0.5 mM Ca2+, and another Ca2+ ionophore, ionomycin (1 µM), was dissolved in the HL3 medium containing 0.1 mM Ca2+. These solutions were applied to the NMJ by the puff method with a gas pressure of 0.5 kg/cm2 for 11 s. The puff pipette had a tip diameter of 3–5 µm, and the tip was placed within ~20 µm of the junctional area. The quantal synaptic events were counted individually every 0.5 s. The total number of events during each response was counted during a period of 30 s starting at the onset of puff pulse. To apply A23187, the bath solution was the Ca2+-free HL3 solution containing 10 µM A23187, but for the ionomycin experiment, the bath solution (Ca2+-free HL3) did not contain ionomycin.

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

Solutions

The ionic composition of Ca2+-free saline was (in mM) 140 NaCl, 2 KCl, 6 MgCl2, and 5 HEPES-NaOH (pH 7.1). For nerve stimulation to evoke synaptic currents and for experiments with Ca2+ ionophores, the HL3 solution was used, and the Ca2+ concentration was changed by replacing the same amount of Mg2+. The ionophoric composition of HL3 solution was as follows (in mM): 70 NaCl, 5 KCl, 1.5 CaCl2, 20 MgCl2, 10 NaHCO3, 5 trehalose, 115 sucrose, and 5 HEPES-NaOH (pH, 7.1) (Stewart et al. 1994). The ionophoric composition of high K+ solution was (in mM) 80 NaCl, 62 KCl, 6 MgCl2, and 5 HEPES-NaOH (pH, 7.1). To study the effect of Ca2+, CaCl2 (0.05–0.15 mM) was added by replacing the same amount of MgCl2. The internal solution for the patch pipette had an ionophoric composition of (in mM) 158 CsCl, 5 EGTA, 10 HEPES-NaOH, and 2 ATP (pH 7.1).

Chemicals

TTX and collagenase were purchased from Sigma (St. Louis, MO). A23187 was obtained from Alomone Laboratories (Jerusalem, Israel), and ionomycin was purchased from Calbiochem (La Jolla, CA). A23187 and ionomycin were dissolved in DMSO at 5 mM, and stock solutions were stored at −20°C.

Statistical analyses

For comparison among multiple groups, ANOVA was used with the Scheffé test for linear comparison (Fig. 8, F and G) or the Tukey test. For comparison of two groups, the Student’s t-test was used. In case the parameters were not distributed normally, the nonparametric Kruskal-Wallis test was used together with the Steel-Dwass test.

RESULTS

Nerve-evoked synchronous synaptic transmission in control Mhc1 embryos and its external Ca2+ dependency

In wild-type embryos, prolonged repetitive stimulation of the ventral nerve cord often induced vigorous contraction of body wall muscles and prevented stable synaptic current recording. To avoid muscle contraction, we used a noncontracting myosin mutant, Mhc1 (Mogami et al. 1986), in nerve stimulation experiments. The properties of synaptic transmission at the NMJ in Mhc1 mutant embryos (Fig. 1) are indistinguishable from those in wild-type (Yoshiihara et al. 2000). The dependency of synaptic transmission on the external Ca2+ concentration, [Ca2+]o, was examined in HL3 medium, which has a high Mg2+ concentration (20 mM) (Stewart et al. 1994).
[Ca$^{2+}$]$_o$ was changed by substituting Ca$^{2+}$ for Mg$^{2+}$ in the equivalent amount. With 1.5 mM [Ca$^{2+}$], nerve stimulation at 0.3 Hz evoked robust synaptic currents and the failure rate was close to zero. (Sample current traces are depicted in Fig. 1A2. In this case, no failure was observed during 31 consecutive nerve stimuli. The corresponding frequency histogram is shown in Fig. 1A1.) The initial deflection of synaptic currents occurred between 4 and 12 ms after the onset of stimulus pulse.

The failure rate changed with [Ca$^{2+}$]$_o$. In external solutions with [Ca$^{2+}$]$_o$ between 0.15 and 1 mM, the quantal content was estimated by the failure method, assuming the Poisson statistics (Katz 1969). The quantal content is plotted in the double logarithmic scale against [Ca$^{2+}$]$_o$ in Fig. 1B. The fitted straight line has a slope of 3.01 ± 0.36 (SE; n = 33). All points were used to fit a straight line with the least-square method.

At higher [Ca$^{2+}$]$_o$, the failure rate was small, and it may not be valid to use the failure method to estimate the quantal content assuming the Poisson statistics. Instead, we simply used the mean amplitude of the initial 10 consecutive nerve-evoked synaptic currents and plotted against [Ca$^{2+}$]$_o$. The amplitude of synaptic currents kept increasing at ≤10 mM [Ca$^{2+}$]$_o$ (Fig. 1C). Because the amplitude distribution of minis is skewed in this preparation, the quantal size might not equal the mean amplitude of minis. Thus it may not be appropriate to estimate the quantal content by dividing the mean amplitude of nerve-evoked synaptic currents with the mean amplitude of minis. To circumvent this problem, we normalized the amplitude of nerve-evoked synaptic currents in high concentrations of [Ca$^{2+}$] to that at 1 mM, at which concentration the mean amplitude of nerve-evoked synaptic currents was measured and the quantal content was estimated by the failure method. The composite dose–response curve thus produced is shown in Fig. 1D. At normal 1.5 mM [Ca$^{2+}$], the mean amplitude was 0.95 ± 0.08 (SD) nA (n = 6) at the −35 mV holding potential and the quantal content was estimated to be 4.03 in Fig. 1D. The quantal size is 236 pA (0.95 nA/4.03), which is significantly larger than the mean quantal synaptic event amplitude in the high K$^+$ HL 3 solution (30 or 40 mM K$^+$ and 1.5 mM [Ca$^{2+}$]) in the presence of 3 μM TTX (160.7 ± 17.9 pA, n = 6) at the −35 mV holding potential. A similar discrepancy between the

**FIG. 1.** Nerve-evoked synchronous synaptic transmission at embryonic neuromuscular junctions (NMJs) in Mhc$^1$. A1 and A2: frequency histogram (A1) and sample traces (A2) obtained in a muscle cell of an Mhc$^1$ embryo (control). Number of events/stimulus/bin is plotted against time (time after onset of stimuli pulse). Stimuli were given at an arrow at 0.3 Hz. Each trace was aligned at stimulus, and number of events that fell into each bin was counted, normalized by number of stimuli and plotted on ordinate. Bin width is 8 ms. Asynchronous synaptic currents were rare in control. There were no failures among 31 stimuli at 0.3 Hz, and initial deflection of all evoked events was observed in the time window between 4 and 12 ms after stimulation. Recordings were carried out in HL3 medium containing 1.5 mM Ca$^{2+}$ and 20 mM Mg$^{2+}$. Holding potential was −65 mV. B–D: relationship between quantal content and [Ca$^{2+}$]$_o$ in Mhc$^1$ embryos. B: in Ca$^{2+}$ concentration range between 0.15 and 1 mM, quantal content was estimated by the failure method assuming Poisson statistics. Quantal content, $m = -\ln(n_0/N)$, where $n_0$ is number of failure and N is total number of stimulation. Vertical bars attached to each point are SE. Straight line was fitted to all data points by the least square method. C: in Ca$^{2+}$ concentration range between 1 and 10 mM, amplitude of nerve-evoked synaptic currents recorded at −35 mV is plotted against [Ca$^{2+}$]$_o$. Vertical bars attached to each point are SE. Data points are connected by straight lines. D: composite relation in Ca$^{2+}$ concentration range between 0.15 and 10 mM in the double logarithmic plot. Same data depicted in B and C are replotted. Data in B were converted to quantal content and plotted in the logarithmic scale on the ordinate of D. Diamonds, data in the low Ca$^{2+}$ concentration range shown in B; squares, data in the high Ca$^{2+}$ concentration range shown in C.
quantal size and the mean mini amplitude was observed at NMJs in embryonic Xenopus cultures (Evers et al. 1989). This observation suggests that larger quantal events are preferentially evoked by nerve stimulation.

Nerve-evoked synchronous synaptic transmission in syt I mutant embryos and its external Ca\(^{2+}\) dependency

In a double mutant, Mhc\(^{1}\) syt I\(^{AD4}\), stimulation of the ventral nerve cord rarely evoked synaptic currents in HL3 medium containing 2 mM Ca\(^{2+}\), whereas asynchronous synaptic events were readily observed between stimuli (Fig. 2A1, frequency histogram; Fig. 2A2, sample traces). However, even in Mhc\(^{1}\) syt I\(^{AD4}\), occasionally the initial deflection of synaptic currents was observed at the right timing between 4 and 12 ms after stimulation (Fig. 2A2, a synaptic current in the top trace), although the intervals between the stimulus and onset of synaptic currents were more variable in Mhc\(^{1}\) syt I\(^{AD4}\) than those in the control. It is possible that asynchronous quantal release or spontaneous events occurred during this time window. To test this possibility, we constructed an event frequency histogram as shown in Fig. 2A1. The number of events in the bin between 4 and 12 ms after stimulation was significantly larger than the entry of other bins in HL3 medium containing 2 mM [Ca\(^{2+}\)] (the number of entry in this bin deviated more than 3 times the SD from the mean number of entry in other bins). Therefore we concluded that some of these synaptic events within the window were synchronized to nerve stimulation. The average number of events in the window between 4 and 12 ms was 0.078 ± 0.030 events/stimulus, and the average of following bins was 0.017 ± 0.008 events/stimulus in five cells. Thus nerve-evoked synchronous synaptic transmission did occur in syt I–null mutant embryos. To estimate the number of events evoked by nerve stimulation in the case shown in Fig. 2A1, we subtracted the mean number of events in the following 11 bins (3.1) from the number of entry in this bin (13) during 193 nerve stimulations. Thus we calculated the failure rate to be 0.95 (average in 5 cells, 0.95 ± 0.04). The same procedure was used to assess the failure rate in the following experiments to construct the curves shown in Fig. 4.

Contrary to our finding described above, Yoshihara and Littleton (2002) detected no nerve-evoked synchronous release in Mhc\(^{1}\) syt I\(^{AD4}\) embryos. Because this is a crucial point to determine which domain of the Syt I molecule senses Ca\(^{2+}\), we further examined the cause of this discrepancy. Although the experimental arrangements were similar, their binning procedure in constructing event frequency histograms was different from ours. (Their first bin was between 0 and 10 ms, in which synchronous events were expected to enter, and the bin width thereafter was 10 ms.) To examine more closely the timing of quantal events after nerve stimulation, we constructed an event frequency histogram with a bin width of 1 ms as shown in Fig. 2B1. In this experiment, we stimulated the nerve at 1 Hz in 5 mM [Ca\(^{2+}\)]\(_{\text{e}}\) to collect enough events for analysis (this experimental condition was different from those used to construct the quantal content vs. [Ca\(^{2+}\)]\(_{\text{e}}\) curves shown in Fig. 4.). Entry of quantal events increased at the fifth bin and peaked at the sixth bin after stimulation. The event frequency declined thereafter to a quasi-steady level (3.6 events/bin), which is higher than that before stimulation (0.9 events/bin), with a time constant of about 2 ms. For comparison, in Fig. 2C, we plotted an event frequency histogram for Mhc\(^{1}\) at 0.7 mM Ca\(^{2+}\). (To obtain enough events, data from 5 cells were pooled.) The time-course of synchronized events between 4 and 12 ms was indistinguishable from that for Mhc\(^{1}\) syt I\(^{AD4}\), but the following asynchronous events were much less.

We next analyzed the same set of data for Mhc\(^{1}\) syt I\(^{AD4}\) with a bin width of 8 ms as in our routine procedure. (The first bin after stimulation is set between 4 and 12 ms.) The entry in the first bin was 55 and the mean number of entry in other bins between 12 and 100 ms was 18.2 ± 2.1 events/bin during 238 stimuli (Fig. 2B2). The failure rate is 0.845 and the quantal content is 0.17. Similar numbers for quantal content, 0.17 and 0.14, were obtained in two other cells under the same condition. These numbers are in accordance with those shown in Fig. 4 for Mhc\(^{1}\) syt I\(^{AD4}\) in 5 mM [Ca\(^{2+}\)]\(_{\text{e}}\) at 0.3 Hz stimulation (0.16 ± 0.08, n = 5).

When the same set of data were processed with the binning method adopted by Yoshihara and Littleton (2002), the first bin (0-10 ms) contained 50 events, and the mean number of entry in the following bins was 23.4 ± 5.0 events/bin (Fig. 2B3). Thus the first bin still contains significantly larger entry than others, but synchronous release is less prominent compared with Fig. 2B2. The failure rate is 0.89, and the quantal content is 0.11, which is smaller than our estimate. Although significant synchronous release is detected even with their binning procedure with this set of data obtained at 5 mM [Ca\(^{2+}\)]\(_{\text{e}}\), they probably missed nerve-evoked synchronous events at lower [Ca\(^{2+}\)]\(_{\text{e}}\) (<4 mM), which they used.

We next examined the Ca\(^{2+}\) dependency of the failure rate in Mhc\(^{1}\) syt I\(^{AD4}\) with the same experimental condition as Fig. 2A. The failure rate was measured as described above at each [Ca\(^{2+}\)]\(_{\text{e}}\) and the quantal content was calculated. The mean failure rate changed with [Ca\(^{2+}\)]\(_{\text{e}}\) between 0.3 and 10 mM Ca\(^{2+}\). When the quantal content was plotted against [Ca\(^{2+}\)]\(_{\text{e}}\) in the double logarithmic scale, a straight line fitted reasonably well between 0.3 and 5 mM (Fig. 4, yellow triangles). The slope, N, is 0.95 ± 0.36 (n = 21), which is smaller than 3.01 ± 0.36 (n = 33) in Mhc\(^{1}\) (Fig. 4, blue diamonds; P < 0.01). Thus we conclude that the quantal content of nerve-evoked synchronous synaptic currents in Mhc\(^{1}\) syt I\(^{AD4}\) is external Ca\(^{2+}\)-dependent but the apparent cooperativity, N, is less than in the control. At 1 mM [Ca\(^{2+}\)]\(_{\text{e}}\), the quantal content in Mhc\(^{1}\) syt I\(^{AD4}\) is ~1/60 of that in Mhc\(^{1}\). At least a part of this small quantal content in the mutant is due to the smaller number of vesicles that locate immediately adjacent to the presynaptic membrane (Reist et al. 1998), but the smaller N cannot be explained by this defect. Rather, it suggests that, in the absence of Syt I, the mutant lacks a major Ca\(^{2+}\) sensor, and the remaining Ca\(^{2+}\) dependency derives from another Ca\(^{2+}\) sensor that has probably only one Ca\(^{2+}\) binding site.

The apparent cooperativity of 0.95 that we obtained for Mhc\(^{1}\) syt I\(^{AD4}\) is smaller than that reported by Broadie et al. (1994) (1.7) in svt I\(^{AD4}\) embryos. On the other hand, in control embryos, they reported 1.8 for N, which is much smaller than ours (3.01) and those reported by others in Drosophila NMJs (Littleton et al. 1994; Stewart et al. 2000; Yoshihara and Littleton 2002; Yoshihara et al. 2000). The finding by Broadie et al. (1994), that N is the same between wild-type and svt I\(^{AD4}\), is inconsistent with the generally accepted notion that Syt I is a major Ca\(^{2+}\) sensor.
FIG. 2. Synchronous and asynchronous nerve-evoked events in an Mhc<sup>1</sup> syt<sup>AD4</sup> embryo. A1 and A2: frequency histogram (A1) and sample traces (A2) of synaptic events evoked by nerve stimulation obtained in a muscle cell of an Mhc<sup>1</sup> syt<sup>AD4</sup> embryo. Asynchronous synaptic events were frequently observed that are depicted in bins 12 ms or later after stimulation. There were also spontaneous events that are represented in bins preceding stimulation. Total number of stimuli was 193. Sample current traces are shown in A2. Recordings were carried out in HL3 medium containing 2 mM Ca<sup>2+</sup> and 19.5 mM Mg<sup>2+</sup>. Holding potential was –65 mV and stimulus frequency was 0.3 Hz. Data obtained in this experimental condition in various [Ca<sup>2+</sup>]e were plotted in Fig. 4. B1: timing of quantal events, measured at the initial deflection, after nerve stimulation in an Mhc<sup>1</sup> syt<sup>AD4</sup> embryo. Event frequency histogram was constructed from a different set of data with a bin width of 1 ms. A total of 238 stimuli was delivered at 1 Hz in 5 mM [Ca<sup>2+</sup>]e. There was a delay of 4 ms after nerve stimulation before synchronized events occurred, which is likely to be due to a sum of time for generation of an action potential, its conduction time along the motor nerve, and synaptic delay. Events before stimulation were spontaneous events. Asynchronous delayed events after stimulation were also observed. B2: event frequency histogram was constructed using the same set of data as B1 with a bin width of 8 ms, and the 1st bin was set between 4 and 12 ms as it was routinely done with the data depicted in A. Number of entry in the 1st bin after stimulation (55 events) was significantly larger than those in the following 11 bins (18.2 ± 2.1 events/bin). Synchronous events occurred 36.8 (55–18.2) times during 238 stimuli, and failure rate was 0.845. Quantal content was 0.17, which fits well with the value depicted in Fig. 4 at 5 mM [Ca<sup>2+</sup>]e for Mhc<sup>1</sup> syt<sup>AD4</sup> stimulated at 0.3 Hz. B3: event frequency histogram with a bin width of 10 ms. First bin was set between 0 and 10 ms according to the procedure reported by Yoshihara and Littleton (2002). The same set of data as B1 was used. Number of entry in the 1st bin was 50, which was also significantly larger than those in the following 9 bins (23.4 ± 5.0 events/bin). However, synchronous events were less conspicuous in this plot compared with those in B2, and calculated quantal content was smaller (0.11). C: timing of quantal events after nerve stimulation in Mhc<sup>1</sup> embryos at 0.7 mM Ca<sup>2+</sup>. Bin width is 1 ms. Quantal events in 5 cells were pooled to construct histogram.
We next examined other syt I alleles with the same method as used for Mhc syt I<sup>AD4</sup>. In Mhc syt I<sup>AD1</sup>, in which the C2B domain is completely missing due to insertion of a premature stop cordon while the C2A domain remains, nerve-evoked synchronous synaptic transmission rarely occurred as in Mhc syt I<sup>AD4</sup>, but asynchronous release was less frequent compared with that observed in Mhc syt I<sup>AD4</sup> (Fig. 3A; event frequency histogram in A1 and sample traces in A2). The double logarithmic plot in Fig. 4 (green squares) yielded a slope of 1.06 ± 0.42 (n = 23; a straight line was fitted between 0.5 and 5 mM), which is not different from in Mhc syt I<sup>AD4</sup> (P > 0.05), suggesting that the remaining C2A domain does not contribute to Ca<sup>2+</sup>-sensing for synchronous synaptic transmission.

In Mhc syt I<sup>AD3</sup> embryos, in which one amino acid is substituted in the C2B domain, nerve-evoked synchronous synaptic transmission was also reduced, but in a solution containing 2 mM [Ca<sup>2+</sup>]e, synaptic transmission often occurred (Fig. 3B1, frequency histogram; Fig. 3B2, sample traces). Forty-one events occurred in the window between 4 and 12 ms during 100 stimuli while the average entry in other bins was 0.36. Thus the failure rate was 0.59. The average of six cells was 0.68 ± 0.15. In the double logarithmic plot of the quantal content versus [Ca<sup>2+</sup>]e between 0.3 and 5 mM, the slope was 1.54 ± 0.34 (n = 31), and the line (Fig. 4, red circles) was shifted to the right and/or downward relative to that of the control (blue diamonds). This value of N was significantly

**FIG. 3.** Nerve-evoked synchronous events in an Mhc<sup>1</sup> syt I<sup>AD1</sup> embryo (A) and in an Mhc<sup>1</sup> syt I<sup>AD3</sup> embryo (B). A: event frequency histogram for Mhc<sup>1</sup> syt I<sup>AD1</sup> (A1) and sample traces (A2). Histogram was constructed as described for Mhc<sup>1</sup> syt I<sup>AD4</sup> in Fig. 2A. Stimulus frequency was 0.3 Hz. Asynchronous events were less frequent than in Mhc<sup>1</sup> syt I<sup>AD4</sup>. Total number of stimuli was 46. Entry in the 1st bin (between 4 and 12 ms) was 11. Recordings were carried out in HL3 medium containing 5 mM Ca<sup>2+</sup> and 16.5 mM Mg<sup>2+</sup>. B: event frequency histogram for Mhc<sup>1</sup> syt I<sup>AD3</sup> (B1) and sample traces (B2). Stimulus frequency was 0.3 Hz. Total number of stimuli was 100. Recordings were carried out in HL3 medium containing 2 mM Ca<sup>2+</sup> and 19.5 mM Mg<sup>2+</sup>.

**FIG. 4.** Relationship between quantal content and external Ca<sup>2+</sup> concentration in the double logarithmic plot in Mhc<sup>1</sup> and Mhc<sup>1</sup> syt I<sup>AD</sup> mutant embryos. All data depicted in this figure were obtained in the experimental condition described in Figs. 1B, 2A, and 3, and data described in Fig. 2B and C were not included. Quantal content was estimated by the failure method assuming the Poisson distribution of evoked events except for those for high external Ca<sup>2+</sup> concentrations (between 1.5 and 10 mM) for Mhc<sup>1</sup>. (In this range, quantal content was calculated as described in the text.) Quantal content, \( m = -\ln(n_0/N) \), where \( n_0 \) is number of failure and \( N \) is total number of stimulation. Vertical bars attached to each point are SE. Straight line was fitted to all data points (not means) by the least square method between 0.15 and 1 mM for Mhc<sup>1</sup> and between 0.5 and 5 mM for Mhc<sup>1</sup> syt I<sup>AD3</sup>, Mhc<sup>1</sup> syt I<sup>AD4</sup>, and Mhc<sup>1</sup> syt I<sup>AD7</sup>. Blue diamonds, Mhc<sup>1</sup> replotted from Fig. 1D; green squares, Mhc<sup>1</sup> syt P<sup>AD1</sup>, red circles, Mhc<sup>1</sup> syt P<sup>AD2</sup>, yellow triangles, Mhc<sup>1</sup> syt P<sup>AD4</sup>. Number of cells examined was between 4 and 6 for each Ca<sup>2+</sup> concentration.
smaller than that in the control and larger than that in Mhc^1 syt^IAD4 (P < 0.05).

In all, nerve-evoked synaptic transmission was severely impaired in syt I mutants, but even in syt I-null mutant embryos, it was not abolished. The Ca^{2+} dependency of quantal content, N, was distinctly smaller in Mhc^1 syt^IAD4 and in Mhc^1 syt^IAD1, whereas in Mhc^1 syt^IAD3, N was also statistically smaller than that in the control, but larger than that in Mhc^1 syt^IAD4. These findings suggest that C2B, not C2A, is sensing Ca^{2+} for nerve-evoked synchronous release.

Minis in normal saline and quantal synaptic events in high K^{+} solutions in syt I mutant embryos

Although nerve-evoked synchronous synaptic transmission was rarely observed in HL3 medium in Mhc^1 syt^IAD4 embryos, minis were readily observed. The mini frequency was counted during a period of 10 min in Mhc^1 syt^IAD4 in HL3 medium containing 3 μM TTX. The frequency was low and ranged between 0.3 and 1.4/min (0.8 ± 0.4/min, n = 11). In the control embryos (cn bw; parental line), the frequency was 2.1 ± 1.6/min (n = 7). These values are not statistically different (P > 0.05). This result is in contrast to the report by Broadie et al. (1994), who reported more than fourfold higher frequencies of minis in syt I AD4 embryos than in the control, but in accordance with the report by Yoshihara and Littleton (2002). This discrepancy might have come from an inadvertent bias in sampling because the mini frequency varies in a wide range among cells.

In high K^{+} solutions, the presynaptic terminal membrane is continuously depolarized and voltage-gated Ca^{2+} channels open asynchronously, unlike synchronized opening induced by nerve stimulation. Nevertheless, an elevation of the cytosolic Ca^{2+} concentration is most likely to be detected by Syt I. Thus the study of high K^{+}-induced synaptic events may reveal the Syt I function at the steady state, namely, its negative regulatory role.

We measured the quantal synaptic event frequency in high K^{+} medium (including 62 mM K^{+}, 3 μM TTX, and 0 – 0.15 mM [Ca^{2+}]_e) to assess its external [Ca^{2+}] dependency. Because the muscle contraction was not a serious problem in this recording condition, we simply used single mutants without an additional mutation in Mhc in this series of experiments. The amplitude of quantal synaptic events varied in a wide range in all syt I mutants as in controls (Fig. 5A, sample traces). The amplitude histograms are all skewed toward large amplitudes (Fig. 5B). The mean amplitude in syt I mutant embryos was not different from in cn bw (Fig. 5C), suggesting no detectable postsynaptic alteration in syt I mutant embryos.

We next examined the dependency of quantal synaptic event frequency on [Ca^{2+}]_e in syt I mutant embryos in 62 mM K^{+} solution and quantal synaptic currents in syt I mutants and a control. Quantal synaptic events were recorded in high K^{+} (62 mM) saline with 0.1 mM Ca^{2+} and 3 μM TTX at the holding potential of –65 mV. A: sample current traces from syt I mutant embryos of syt I^AD4, syt I^AD1, syt I^AD3 and cn bw as indicated. B: amplitude histograms from embryos of syt I^AD4, syt I^AD1, syt I^AD3, and cn bw. C: mean amplitudes of quantal synaptic events (pA) in syt I mutant embryos and controls. Vertical bars attached to each column are SE, and numbers are numbers of cells examined.
saline containing 3 μM TTX (high K⁺-induced quantal synaptic events; Fig. 6). In the absence of external Ca²⁺, the event frequency was not different among syt I mutants and a control (Fig. 6A). The event frequency increased with [Ca²⁺]o in all syt I mutants as in the control, but the Ca²⁺ dependency of increase was different (Fig. 6A). Even in syt IAD4 (Fig. 6A and B, yellow triangles), the event frequency increased with [Ca²⁺]o, indicating the existence of a Ca²⁺ sensor other than Syt I for vesicle release.

Interestingly, the Ca²⁺ dependency was least in syt IAD3 (Fig. 6A and B, red circles). The high K⁺-induced quantal synaptic event frequency in syt IAD3 was significantly lower than that in syt IAD4 (Fig. 6B, yellow triangles), suggesting that the mutant Syt I(AD3) is inhibiting fusion of vesicles.

### Ca²⁺ ionophore response in Mhc1 syt IAD4

Thus far we found that the responsiveness of quantal synaptic event frequency in syt IAD4 to high K⁺ stimulation and that in Mhc1 syt IAD4 to nerve stimulation were markedly reduced compared with those in the control and interpreted the finding as a result of absence of a major Ca²⁺ sensor. However, because it is known that Syt I interacts with presynaptic Ca²⁺ channels through the SNARE complex (Catterall 1999), it is possible that the observed defect in synaptic transmission is due to smaller amount of Ca²⁺ influx during depolarization in syt IAD4. To test this possibility, we bypassed voltage-gated Ca²⁺ channels with Ca²⁺ ionophores and directly measured the relative Ca²⁺ sensitivity of the vesicle fusion machinery in syt IAD4 embryos. As shown in Fig. 7, puff application of A23187 with 0.5 mM [Ca²⁺] produced extensive release of vesicles in Mhc1 (Fig. 7A). In contrast, the same procedure in Mhc1 syt IAD4 evoked a small response (Fig. 7A). The combined data obtained in five cells in Mhc1 and in seven cells in Mhc1 syt IAD4 are depicted in Fig. 7D and C, respectively. The total number of events in Mhc1 syt IAD4 was 1/7 of that in Mhc1. A similar result was obtained with another ionophore, ionomycin (Fig. 7, E and F). The total number of events was 1/11 in Mhc1 syt IAD4 of that in Mhc1. Thus the responsiveness to elevated [Ca²⁺]o is markedly reduced in syt IAD4, suggesting that the malfunction of voltage-gated Ca²⁺ channels is not the major cause for reduced synaptic responses in syt IAD4 mutants. However, the smaller response to Ca²⁺ ionophores in syt IAD4 is, at least partly, due to a smaller number of synaptic vesicles in the vicinity of presynaptic release sites (Reist et al. 1998).

### Hypertonicity response in syt I mutant embryos

It is possible that lower quantal contents and lower high K⁺-induced quantal synaptic event frequencies in syt I mutants are due to a smaller number of release-ready vesicles. To assess the population of release-ready vesicles, we next examined Ca²⁺-independent vesicle fusion induced by hypertonicity. The quantal event frequency increases with puff application of hypertonic solutions in Ca²⁺-free saline (Suzuki et al. 2002a). Ca²⁺ is not required for this response because it persisted even after BAPTA was introduced in the presynaptic terminal (Mochida et al. 1996; Rosenmund and Stevens 1996). Although the number of quantal events during a hypertonicity response was defined to be the size of readily releasable pool in cultured hippocampal neurons (Rosenmund and Stevens 1996), it does not represent a defined pool of vesicles at the NMJ of Drosophila embryos because, even after maximal hypertonicity response the frequency of spontaneous quantal events did not change. On the other hand, the hypertonicity response was enhanced with procedures that increase the size of the releasable pool (Suzuki et al. 2002a,b). Therefore the hypertonicity response is a useful tool to assess the relative population of release-ready vesicles.

In synapses formed between cultured mouse neurons completely lacking Syt I (Geppert et al. 1994) and in autaptic synapses prepared from newborn syt I knock-in mice with one amino acid substitution in the C2A domain (R233Q) (Fernández-Chacón et al. 2001), the hypertonicity response remained unaffected, although nerve-evoked synchronous synaptic transmission was severely impaired.

Puff application of 420 mM sucrose (420 mM sucrose was added to Ca²⁺-free saline) induced robust responses in control embryos of Mhc1 (Fig. 8A) and cn bw (Fig. 8B), which are similar to those in wild-type (Suzuki et al. 2002a). In contrast, only rudimental responses were observed in Mhc1 syt IAD4 embryos (Fig. 8C). The total number of events and the peak frequency during the hypertonicity response in population of cells examined are shown in Fig. 8F and G, respectively. Both parameters are significantly smaller than those in Mhc1 or cn

![Fig. 6](http://jn.physiology.org/)

**Fig. 6.** Relationship between frequency of quantal synaptic events and external Ca²⁺ concentration in high K⁺ saline. Quantal synaptic events were recorded in external solution containing 62 mM K⁺, 3 μM TTX, and various Ca²⁺ concentrations in embryos of syt IAD4, syt IAD1, syt IAD3, and cn bw. Events were counted during 10 min of recording in majority of cells, but when quantal synaptic event frequency was high, recording time was shortened to 3 min. A: mean quantal synaptic event frequency was plotted against external Ca²⁺ concentration. b: data for syt IAD4 and syt IAD3 in A are replotted to show difference between these strains of syt I mutant. Regression lines were drawn for syt IAD4 (yellow broken line) and for syt IAD3 (red dotted line) with the least square method. These two regression lines are statistically different (P < 0.05).
bw. The total number of events in Mhc\textsuperscript{1} syt \textsuperscript{AD4} is \(< 1/10\) of controls (Fig. 8F). These results are qualitatively similar to those reported by Yoshihara and Littleton (2002). In syt \textsuperscript{AD4} first instars, the number of vesicles immediately adjacent to the presynaptic membrane, including morphologically docked vesicles, is reduced to \(< 1/24\) of wild-type (Reist et al. 1998), which is most likely to reflect on the reduced hypertonicity response observed here.

Similarly, the hypertonicity response in Mhc\textsuperscript{1} syt \textsuperscript{AD1} was smaller (Fig. 8, D, F, and G) compared with controls. However, it was slightly but significantly larger than that in Mhc\textsuperscript{1} syt \textsuperscript{AD4} (Fig. 8, F and G, +), suggesting that the remaining C2A domain in syt \textsuperscript{AD1} is contributing in recruitment of vesicles to release sites.

In contrast, the hypertonicity response in Mhc\textsuperscript{1} syt \textsuperscript{AD3} was not different from in controls (Fig. 8, E–G). The distribution of synaptic vesicles in heterozygotic first instars of syt \textsuperscript{AD3}/syt \textsuperscript{AD4} was examined in electron microscopy (Reist et al. 1998). The number of morphologically docked vesicles in this mutant is less than one-half of wild-type, but vesicles in the vicinity of presynaptic membrane are about 70% of wild-type. Because in this heterozygotes, syt \textsuperscript{AD4} is probably contributing minor number of vesicles (Reist et al. 1998), syt \textsuperscript{AD3} must be providing vesicles at the level similar to wild-type syt I. If that is the case, we expect a wild-type level of synaptic vesicles in the vicinity of presynaptic membrane in syt \textsuperscript{AD3} homozygotes. Thus our findings with the hypertonicity response in Mhc\textsuperscript{1} syt I embryos fit reasonably well with the morphological observation.

**DISCUSSION**

In this study, we re-examined synaptic transmission at embryonic NMJs of Drosophila syt I mutants in various external Ca\textsuperscript{2+} concentrations. Our findings are as follows. 1) In a null allele, Mhc\textsuperscript{1} syt \textsuperscript{AD4}, nerve-evoked synchronous synaptic transmission was severely reduced but not abolished. Apparent Ca\textsuperscript{2+} cooperativity, \(N\), was 0.95 and significantly smaller than in control (3.01). Synchronous synaptic events were observed at right timing after nerve stimulation. 2) In Mhc\textsuperscript{1} syt \textsuperscript{AD1}, in which only the C2A domain remains, synaptic transmission was similar to that in Mhc\textsuperscript{1} syt \textsuperscript{AD4}. \(N\) was 1.06. Thus the
remaining C2A does not contribute to synchronous release. 3) In Mhc1 syt PAD3, in which one amino acid is substituted in the C2B domain and Ca2+-dependent self-oligomerization might be impaired, synchronous release was impaired, and N was 1.54 and significantly smaller than in the control but larger than that in Mhc1 syt PAD4 or in Mhc1 syt PAD1. 4) Quantal events in high K+ solutions in syt PAD3 were less frequent than in syt PAB4, suggesting that Syt I(AD3) is inhibiting vesicle fusion.

With these findings, we reached substantially different conclusions from previous reports.

**External Ca2+ dependency of nerve-evoked synchronous synaptic transmission in control Mhc1 embryos**

In wild-type Drosophila embryos, Brodie et al. (1994) reported a small value (1.8) of the apparent Ca2+ cooperativity...
for synchronous synaptic transmission and attributed it to the immaturity of embryonic synapse. We re-examined nerve-evoked synchronous synaptic transmission in a noncontracting mutant, Mhc\(^1\) (Mogami et al. 1986), in a wide range of external Ca\(^{2+}\) (0.15–10 mM). Between 0.15 and 1 mM Ca\(^{2+}\), the relation between the quantal content and external Ca\(^{2+}\) concentration was linear in the double logarithmic plot with a slope of 3.01, which is considerably larger than the value reported by Broadie et al. (1994), but similar to those reported by others in embryonic Drosophila NMJs (~4, Yoshihara et al. 2000; 3.5, Yoshihara and Littleton 2002) and in third-instar Drosophila NMJs (3.5, Littleton et al. 1994; 3.5, Stewart et al. 2000), indicating that embryonic NMJs have similar N values as mature synapses, and at least four Ca\(^{2+}\) binding sites are involved in synchronous nerve-evoked synaptic transmission in Drosophila NMJs.

The amplitude of synaptic current gradually increased within the range between 1.5 and 10 mM (Figs. 1 and 4). At high Ca\(^{2+}\) concentrations, the Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels is expected to saturate (Hagiwara and Takahashi 1967). In fact, in all syt I mutants, the quantal content at 10 mM deviated from the linearity in the double logarithmic plot (Fig. 4), suggesting that Ca\(^{2+}\) influx is reaching saturation at this concentration. This prevented us from using higher Ca\(^{2+}\) concentrations for assessing a saturation of Ca\(^{2+}\) binding to Syt I.

**External Ca\(^{2+}\) dependency of synchronous synaptic transmission in Mhc\(^1\) syt P\(_{AD4}\) and Mhc\(^1\) syt P\(_{AD1}\) embryos**

In Mhc\(^1\) syt P\(_{AD4}\) (syt P\(_{AD1}\)) embryos, synchronous synaptic events were rare but occasionally observed at the right time window (between 4 and 12 ms) after stimulation (Fig. 2). The quantal content increased with external [Ca\(^{2+}\)] with a slope of 0.95 in the double logarithmic plot (Fig. 4). In contrast, Yoshihara and Littleton (2002) did not detect nerve-evoked synchronous synaptic transmission in Mhc\(^1\) syt P\(_{AD4}\). As described in RESULTS (Fig. 2), it is likely that they missed synchronous synaptic events in the binning procedure to construct the event frequency histogram. In agreement with our result, nerve-evoked synchronous events were detected in Drosophila embryos (Broadie et al. 1994) and in third-instar larvae in the absence of syt I (Mackler et al. 2002). Also, in neuronal cell cultures from syt I knockout mice, Geppert et al. (1994) observed synchronous quantal events. Thus we conclude that Syt I is not the sole Ca\(^{2+}\) sensor for nerve-evoked synchronous release.

The findings in Drosophila and mice that quantal events do occur synchronized to nerve stimulation in the right window in the absence of Syt I indicate that voltage-gated Ca\(^{2+}\) channels open in proper timing in the mutant. However, we cannot exclude the possibility that the amount of Ca\(^{2+}\) influx is less due to absence of interaction between Syt I and SNARE proteins and/or Ca\(^{2+}\) channels (Catterall 1999). In addition, we showed using Ca\(^{2+}\) ionophores that the response in Mhc\(^1\) syt P\(_{AD4}\) embryos is ~1/10 of the control (Fig. 7), and in first instars of syt P\(_{AD4}\), the number of vesicles adjacent to the presynaptic membrane including morphologically docked vesicles is ~24% of controls (Reist et al. 1998). These factors are likely to be contributing to small quantal contents in Mhc\(^1\) syt P\(_{AD4}\). On the other hand, the smaller N in the mutant cannot be explained with this factor, suggesting that the defect in Mhc\(^1\) syt P\(_{AD4}\) in synaptic transmission is at least partly due to impairment in Ca\(^{2+}\) sensing. Therefore we conclude that Syt I is a major Ca\(^{2+}\) sensor, but in its absence, another Ca\(^{2+}\) sensor supports residual synchronous synaptic transmission.

We expected the apparent Ca\(^{2+}\) cooperativity, \(N\), to decrease in syt P\(_{AD1}\) mutants if Syt I were a major Ca\(^{2+}\) sensor for synchronous release and indeed found in Mhc\(^1\) syt P\(_{AD4}\) that it was 0.95 in contrast to 3.01 in the control. Our result is in accordance with that reported by Littleton et al. (1994), i.e., 1.5 or 1.6 for syt I hypomorphic third-instar larvae and 3.5 for wild-type. In contrast, Broadie et al. (1994) reported that the quantal content versus [Ca\(^{2+}\)]\(_c\) curve in syt P\(_{AD4}\) embryos shifted to higher Ca\(^{2+}\) concentrations without changing the slope. This finding indicates that in the absence of Syt I the affinity of Ca\(^{2+}\) binding sites greatly reduced without a decrease in the number of Ca\(^{2+}\) binding sites. Their finding is hard to reconcile with the generally accepted idea that Syt I is a major Ca\(^{2+}\) sensor for synchronous transmitter release.

In Mhc\(^1\) syt P\(_{AD4}\) embryos, the nerve-evoked synchronous release was also severely impaired but not abolished. The value of \(N\) was 1.06, which is similar to 0.77 reported by Yoshihara and Littleton (2002) and not different from in Mhc\(^1\) syt P\(_{AD4}\), suggesting that the remaining C2A domain is not contributing to Ca\(^{2+}\) sensing for synchronous release. This conclusion contradicts the report by Yoshihara and Littleton (2002), who did not detect synchronous release in Mhc\(^1\) syt P\(_{AD4}\) and attributed synchronous release found in Mhc\(^1\) syt P\(_{AD1}\) to the remaining C2A. Because we unambiguously showed synchronous release in Mhc\(^1\) syt P\(_{AD4}\) in this study, their argument is no longer valid. In wild-type Syt I, the C2A domain may be contributing to synchronous synaptic transmission together with C2B (Fernández-Chacón et al. 2001), but without the latter, it may no longer function as a part of Ca\(^{2+}\) sensor.

**Nerve-evoked synchronous synaptic transmission in syt P\(_{AD3}\) mutant embryos**

A Y312N mutation in mouse Syt II, equivalent to the Drosophila AD3 mutation, prevents Ca\(^{2+}\)-dependent self-oligomerization (Fukuda et al. 2000), and Syt II is highly homologous to Syt I (Fernandez et al. 2001; Sugita et al. 2002). It is plausible that the ~20-fold reduction of synaptic transmission in Mhc\(^1\) syt P\(_{AD3}\) embryos (at 1 mM [Ca\(^{2+}\)]\(_c\)) and a decrease in the apparent Ca\(^{2+}\) cooperativity (Fig. 4) reflects the absence of oligomerization of Syt I. The Ca\(^{2+}\)-dependent oligomerization is conserved in all members of Syt family and considered to facilitate vesicle fusion. It is mediated by a polylsine motif in the C2B domain. A mutation of these two lysine residues to alanines prevents Ca\(^{2+}\)-dependent oligomerization of Syt I (K326, 327A mutation in the rat sequence; Chapman et al. 1998). Inhibition of Syt oligomerization by various reagents, such as antibodies against the C2B sequence or inositol polyphosphates, reduces transmitter release in squid giant synapses (Lilins et al. 1994) and in cultured rat sympathetic neurons (Mochida et al. 1996). The relation between catecholamine release and Ca\(^{2+}\) concentration was examined in permeabilized bovine adrenal chromaffin cells after treatment with IP6, which prevents Ca\(^{2+}\)-dependent oligomerization of Syt. This treatment inhibited catecholamine secretion to ~1/5 at 5 \(\mu\)M [Ca\(^{2+}\)], and the relation between secretion and [Ca\(^{2+}\)]
became less steep compared with control (Ohara-Imaizumi et al. 1997). Thus Ca\(^{2+}\)-dependent Syt oligomerization might be an essential step for vesicle fusion, and the synaptic phenotypes in Mhc\(^{-}\) syt I\(^{AD3}\) embryos that we found in this study may reflect its absence in this mutant.

However, Mackler and Reist (2001) found a mild inhibition of nerve-evoked synaptic potentials (~60% of the control at 1.5 mM [Ca\(^{2+}\)]\(_i\)) in a Drosophila transformant in which three lysine residues in the polylysine motif were changed to glutamines. This mutation in mouse Syt II has been shown to block Ca\(^{2+}\)-dependent oligomerization in vitro (Fukuda et al. 2000). Therefore the strong inhibition of synaptic transmission in Mhc\(^{-}\) syt I\(^{AD3}\) embryos (~5% of the control at 1 mM [Ca\(^{2+}\)]\(_i\)) could be due to an additional effect of this mutation on synchronous synaptic transmission, such as on Syt I interaction with phospholipids, Syx, or Ca\(^{2+}\) channels. Alternatively, the mild inhibition in the mutant of lysine residues found in third-instar larvae (Mackler and Reist 2001) is the result of developmental compensation for weak synaptic transmission.

Ca\(^{2+}\)-dependent oligomerization of Syt I came into question when Ubach et al. (2001) found that bacterial contaminants tightly bound to the C2B domain were responsible for this activity, and highly purified C2A-C2B fragments did not oligomerize in a Ca\(^{2+}\)-dependent manner. However, a subsequent study showed that, in the presence of phospholipids, purified C2A-C2B fragments do oligomerize Ca\(^{2+}\)-dependently (Wu et al. 2003). Thus in the natural environment, it is plausible that Ca\(^{2+}\)-dependent oligomerization of Syt I occurs preceding vesicle fusion.

**Frequencies of minis in syt I mutants**

The role of Syt I as a negative regulator of vesicle fusion has been postulated when higher mini frequencies were found in third-instar syt I mutants (DiAntonio and Schwarz 1994; Littleton et al. 1994). However, the mini frequency did not change when Syt I was acutely inactivated by UV irradiation in a Drosophila transformant (Marek and Davis 2002), and higher mini frequencies in normal saline were not found in syt I-null embryos (Yoshihara and Littleton 2002). These findings led Yoshihara and Littleton (2002) to suggest that the higher mini frequencies observed in third instars are the result of developmental compensation. However, the mini frequency is a function of the release probability of release-ready vesicles and their number. Because, in first instars of syt I\(^{AD4}\), the number of vesicles adjacent to the presynaptic membrane is markedly reduced (Reist et al. 1998), and in Mhc\(^{-}\) syt I\(^{AD4}\) embryos, the hypertonicity response was ~1/10 of the controls (Fig. 8), the unchanged mini frequency found in syt I\(^{AD4}\) in normal saline indicates that the release probability of release-ready vesicles is higher in this mutant compared with controls, suggesting that Syt I is negatively controlling the mini frequency.

Furthermore, in high K\(^{+}\) saline, the quantal synaptic event frequency in syt I\(^{AD3}\) was significantly lower than that in syt I\(^{AD4}\) (Fig. 6, A and B), which suggests strongly that the mutant Syt I (AD3) protein is inhibiting vesicle fusion. In this case, the number of vesicles in a close proximity of release sites is larger in syt I\(^{AD3}\) than in syt I\(^{AD4}\), indicating that the release probability of release-ready vesicles in syt I\(^{AD3}\) is even lower than it appears in the quantal synaptic event frequency assay. This is not due to less elevation of [Ca\(^{2+}\)]\(_i\) in high K\(^{+}\) solutions in syt I\(^{AD3}\) because significantly lower frequencies of quantal synaptic events in Mhc\(^{-}\) syt I\(^{AD3}\) than in Mhc\(^{-}\) syt I\(^{AD4}\) were reported when [Ca\(^{2+}\)]\(_i\) was elevated using a Ca\(^{2+}\) ionophore (Yoshihara and Littleton 2002). Therefore we conclude that Syt I(AD3) is preventing high K\(^{+}\)-induced vesicle fusion.

**A role of Syt I in synaptic vesicle recruitment**

Even after maximal hypertonicity responses, spontaneous synaptic events continue uninterrupted. Therefore the hypertonicity response does not deplete the readily releasable pool (Suzuki et al. 2002a,b). Thus the hypertonicity response cannot be interpreted in this preparation in the same way as in cultured hippocampal neurons (Rosenmund and Stevens 1996; Stevens and Tsujimoto 1995). Because the number of quanta released during the maximal hypertonicity response in wild-type embryos (240 quanta; Suzuki et al. 2002a) is far larger than the number of release sites (30; Schuster et al. 1996), and each release site has less than one morphologically docked vesicle (Reist et al. 1998), it is likely that the hypertonicity response reflects not only the number of docked vesicles but also those that are readily recruitable for release. On the other hand, the hypertonicity response was enhanced with procedures that increase the size of releasable pool (Suzuki et al. 2002a). It is reasonable to assume that the hypertonicity response reflects a population of vesicles in the vicinity of release sites.

In synapses formed in culture between neurons obtained from homozygous syt I-knockout mice, the hypertonicity response was indistinguishable from that in wild-type (Geppert et al. 1994). In contrast, we found that the hypertonicity responses in Mhc\(^{-}\) syt I\(^{AD4}\) and Mhc\(^{-}\) syt I\(^{AD3}\) were dramatically reduced compared with the controls, whereas that in Mhc\(^{-}\) syt I\(^{AD2}\) was unchanged (Fig. 8). Syt I binds to AP-2 with a high affinity and is involved in endocytosis, and the binding site of Syt I for AP-2 is in the C2B domain (Zhang et al. 1994). Syt I(AD1) does not have the C2B domain, and the hypertonicity response in this mutant was rudimental, although slightly larger than that in syt I\(^{AD4}\). In contrast, Syt I(AD3) has the C2B domain with one amino acid substitution, and the hypertonicity response was not different from in controls. Biochemically Syt I(AD1) does not bind to AP-2, whereas Syt I(AD3) does, and in electron microscopy, synaptic vesicles in syt I\(^{AD4}\) are depleted in the distaminergic synapses between photoreceptors and lamina neurons after prolonged constant light stimulation, whereas in syt I\(^{AD3}\), they are not depleted (Littleton et al. 2001). Thus the hypertonicity response reflects the population of releasable vesicles and small hypertonicity responses in Mhc\(^{-}\) syt I\(^{AD4}\) and Mhc\(^{-}\) syt I\(^{AD3}\) suggest that, without C2B, vesicle recruitment is impaired.

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