Delayed Synaptic Transmission in *Drosophila cacophony* null Embryos

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Ca2+ influx through the *Drosophila* N-type Ca2+ channel, encoded by *cacophony* (ca), triggers fast synaptic transmission. We now ask whether the cac Ca2+ channel is the Ca2+ channel solely dedicated for fast synaptic transmission. Because the *cac* null mutation is lethal, we used cac null embryos to address this question. At the neuromuscular junction in HL3 solution, no fast synchronous synaptic transmission was detected on nerve stimulation. When the wild-type *cac* gene was introduced in the *cac* null background, fast synaptic transmission recovered. However, even in *cac* null embryos, nerve stimulation infrequently induced delayed synaptic events in the minority of cells in 1.5 mM [Ca2+]e and in the majority of cells in 5 mM [Ca2+]e. The number of delayed quantal events per stimulus was greater in 5 mM [Ca2+]e than in 1.5 mM. Thus the delayed release is [Ca2+]e dependent. *Plectreurys* toxin II (PLTXII) (10 nM; a spider toxin analog) depressed the frequency of delayed events, suggesting that voltage-gated Ca2+ channels, other than cac Ca2+ channels, are contributing to them. However, delayed events were not affected by 50 μM La3+. The frequency of miniature synaptic currents in *cac* null embryos was ~1/2 of control, whereas in high K+ solutions, it was ~1/135. The hypertonicity response was ~1/10 of control. These findings indicate that the number of release-ready vesicles is smaller in *cac* null embryos. Taken together, the cac Ca2+ channel is indispensable for fast synaptic transmission in normal conditions, and another type of Ca2+ channel, the non-cac, PLTXII-sensitive Ca2+ channel, is contributing to delayed release in *cac* null embryos.

**INTRODUCTION**

Four genes in the *Drosophila* genome encode voltage-gated Ca2+ channel α1 subunits, which include homologs of mammalian T-type α1 (*Dmca1A*), L-type α1 (*Dmca1D*), and N-type α1 (*Dmca1A*) (Littleton and Ganetzky 2000). These genes are likely to produce multiple types of voltage-gated Ca2+ channels with distinct pharmacological and biophysical properties (Byerly and Leung 1988; Peng and Wu 2007). Pharmacologically, two types of voltage-gated Ca2+ currents were detected in embryonic *Drosophila* neurons in culture. A spider toxin, *Plectreurys* toxin (PLTX), blocked both, but another spider toxin, *Hololena* toxin (HoTX), suppressed only one of them. The HoTX-sensitive Ca2+ current had a faster inactivation time course. Organic blockers, such as nifedipine and diltiazem, had little effect on these Ca2+ currents (Leung et al. 1989). Voltage-gated Ca2+ currents in *Drosophila* muscles were insensitive to PLTX (Leung et al. 1989) and had two components, one of which was sensitive to nifedipine, an L-type Ca2+ channel blocker, and the other to amiloride, a T-type Ca2+ channel blocker (Gielow et al. 1995). Thus pharmacologically, at least two types are in neurons and there are an additional two types in muscles.

The Ca2+ channel that is made up of the α1 subunit encoded by the *Dmca1A* or *cacophony* (cac) gene (referred to as the cac Ca2+ channel hereafter) contributes to fast synaptic transmission. In temperature-sensitive, cac mutant, cacTS2 flies at 36°C, the amplitude of nerve-evoked synchronous synaptic currents was depressed to 30% of wild-type (CS) control. Because the amplitude of quantal events, miniature synaptic potentials, was not different in cacTS2 than in wild types at 36°C, the authors concluded that this depression of synaptic transmission was caused by a defect in the presynaptic cac Ca2+ channel function at the nonpermissive temperature (Kawasaki et al. 2000). This conclusion was supported by Macleod et al. (2006), who measured changes in Ca2+ concentrations at the presynaptic bouton in *cac*TS2 larvae during repetitive nerve stimulation. They found that, while at 22°C, the extent of [Ca2+]e elevation induced by 10-Hz stimulation in 0.5 mM [Ca2+]e was not different from in a control (CS), at 36°C, it was reduced to 20% of CS. Thus in *cac*TS2 at 36°C, the amplitude of nerve-evoked synaptic currents and the elevation of [Ca2+]e during 10-Hz stimulation were reduced, but synaptic transmission was not abolished, indicating that either cac Ca2+ channels were not completely blocked at this temperature or that there were other types of Ca2+ channel in the presynaptic terminal supporting remaining fast synaptic transmission. Thus a question remains of whether the cac Ca2+ channel is the sole Ca2+ channel for fast synaptic transmission.

Multiple types of Ca2+ channel contribute to fast synaptic transmission at the rat Calyx of Held synapse. Although the P/Q type is predominant, both N- and R-types also support fast synaptic transmission (Wu et al. 1998, 1999). Similarly, at *Xenopus* neuromuscular junctions formed in culture, in addition to N-type Ca2+ channels, L-type Ca2+ channels efficiently contribute to fast synaptic transmission (Sand et al. 2001). The obvious questions arise. Why are there multiple types of Ca2+ channel in the presynaptic terminal? Does each of them have a specific function for synaptic transmission? On the other hand, dihydropyridine-sensitive L-type Ca2+ channels are predominant and support synaptic transmission in hair cells of the chicken cochlea (Kollmar et al. 1997; Roberts et al. 1990) and in bipolar cells in the goldfish retina (Tachibana et al. 1993). Apparently one type of Ca2+ channel is sufficient to support synaptic transmission in these synapses. Ca2+ influx in the presynaptic terminal is not only necessary for exocytosis but also required for endocytosis (Ceccarelli and Hurlbut 1980; Neale et al. 1999). At the *Drosophila* neuromuscular junction (NMJ), some blockers of voltage-gated Ca2+ channels inhibit endocytosis at concentrations that hardly affect exocytosis. A Ca2+ channel blocker, flunarizine, at 50 μM inhibits the rate of endocytosis by ~90% in the absence of synaptic stimulation (Strom et al. 2000).
µM blocked endocytosis, whereas exocytosis was only slightly depressed. Similarly, a trivalent cation, La$^{3+}$, at 50 µM reduced endocytosis to 20% of control, whereas exocytosis was not affected. On the other hand, Plectreursy toxin II (PLTXII), a synthetic analog of PLTX (Bodi et al. 1995), strongly depressed both endocytosis and exocytosis at 5 nM. These findings suggest that there are at least two types of Ca$^{2+}$ channel, namely flunaridine/La$^{3+}$-sensitive and PLTXII-sensitive types of Ca$^{2+}$ channel, in the presynaptic terminal (Kuroni et al. 2004). PLTX blocks synaptic transmission at the Drosophila NMJ at nanomolar concentrations (Branton et al. 1987), and cac mutations depress synaptic transmission (Kawasaki et al. 2000). Therefore it is most likely that cac Ca$^{2+}$ channels are sensitive to this blocker. In addition, PLTXII may block another type of Ca$^{2+}$ channel as suggested by work in cultured Drosophila neurons (Leung et al. 1989).

We wished to find whether the cac Ca$^{2+}$ channel is the Ca$^{2+}$ channel solely designated for fast synaptic transmission at the Drosophila NMJ. Because the cac$^{null}$ mutation is lethal, we examined synaptic transmission in embryos and found that the cac Ca$^{2+}$ channel is necessary and sufficient for fast synaptic transmission. However, in the absence of cac Ca$^{2+}$ channels, another type of Ca$^{2+}$ channel supports delayed release of transmitter with a unique slow time course. PLTXII (5 nM) blocked the delayed release, but 50 µM La$^{3+}$ did not. Hence this type of Ca$^{2+}$ channel is called a non-cac, PLTXII-sensitive Ca$^{2+}$ channel. We found an indication that this type of Ca$^{2+}$ channel is localized close to the transmitter release site.

**METHODS**

**Fly strains**

 cac$^{null}$ embryos were selected from those produced by the strains described in Kawasaki et al. (2004) [w elav Gal4 l(1) L1$^{3hc129}$ sd f/FM7i-GFP; a gift from Dr. R. Ordway]. They did not have a green fluorescent protein (GFP) marker [w elav Gal4 l(1) L1$^{3hc129}$ sd f/F]; and are referred to as cac$^{null}$ in the text. Rescued strains were either w elav Gal4 l(1) L1$^{3hc129}$ sd f/+; UAS-cac1-EGFP786C or elav Gal4 l(1) L1$^{3hc129}$ sd f+; UAS-cac1-EGFP786C. These strains have the cac gene tagged with enhanced green fluorescence protein (EGFP). Synaptic transmission in the former construct was examined and found to be indistinguishable from the control (Kawasaki et al. 2004). The former construct has one copy of cac1-EGFP, whereas the latter has two. However, we did not find any difference in synaptic transmission nor in imaging of cac Ca$^{2+}$ channel clusters (data not shown). Thus we used them interchangeably, but the majority of experiments were done with the former construct and referred to as cac$^{null}$-EGFP. As controls, we used heterozygote embryos that had a balancer with a GFP marker (cac$^{null}$-GFP) or wild-type (CS) embryos.

**Electrophysiology**

Whole cell patch-clamp recordings from abdominal muscle 6 in embryos (19–21 h after fertilization) of mutants and controls were carried out as described elsewhere (Tamura et al. 2007). Dissecting procedures were the same as described previously (Kidokoro and Nishikawa 1994; Nishikawa and Kidokoro 1995) and carried out in Ca$^{2+}$-free saline. After treating the dissected preparation with collagenase (1 mg/ml) for 30 s to 2 min, synaptic currents were recorded with patch-clamp techniques in the whole cell configuration from abdominal longitudinal muscle 6. The series resistance of the recording electrode, which varied between 5 and 30 MΩ, was compensated at an 80% level. The membrane potential was held at −60 mV. The internal solution contained Cs$^+$, and the junction potential of electrodes filled with the Cs$^+$ internal solution was −5 mV in HL3 solution. Thus the true holding potential was −65 mV.

**Nerve stimulation**

For nerve stimulation, the tip of a microelectrode, which had a resistance of 10–20 MΩ after being filled with 4 M K-acetate, was placed in the ventral nerve cord near the exit of the segmental nerve, and rectangular pulses of 1 ms in duration and ~2 µA in intensity were delivered at 0.3 Hz. In the case of cac$^{null}$ embryos, each stimulus did not produce a synaptic current, which made it difficult to judge whether the stimulation was effective. However, even in those cases, tetanic stimulation (10 Hz for 2 s) invariably increased asynchronous release, indicating the effectiveness of stimulation. The stimulus frequency was switched to 0.3 Hz to collect data.

**Quantification of delayed release**

Delayed release started to appear at 8 ms after stimulation and lasted tens of milliseconds in cac$^{null}$ embryos. To quantify delayed release, we counted the number of events between 8 and 50 ms and normalized with the number of stimuli. With 1.5 mM [Ca$^{2+}$]$_e$, spontaneous events were rare, but with 5 mM in some cells, asynchronous spontaneous events were more frequent and expected to occur during the period between 8 and 50 ms. To correct the contamination of spontaneous events, the frequency of synaptic events preceding stimulation pulses were counted during the 42-ms period and subtracted from the number of events during the period of 8 and 50 ms.

**Hypertonic response**

A hypertonic solution was prepared by adding 420 mM sucrose to the Ca$^{2+}$-free external solution. The solution was applied to the Drosophila NMJ by the puff method with a gas pressure of 0.5 kg/cm$^2$ 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 quanta of 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.

**Solutions**

The ionic composition of Ca$^{2+}$-free saline used for dissection of embryos was (in mM) 140 NaCl, 2 KCl, 6 MgCl$_2$, and 5 HEPES-NaOH (pH 7.1). For nerve stimulation to evoke synaptic currents, HL3 solution was used, and [Ca$^{2+}$]$_e$ was changed by substituting the equivalent amount of Mg$^{2+}$. The ionic composition of HL3 solution was as follows (in mM): 70 NaCl, 5 KCl, 1.5 CaCl$_2$, 20 MgCl$_2$, 10 NaHCO$_3$, 5 Trehalose, 115 sucrose, and 5 HEPES-NaOH (pH 7.1) (Stewart et al. 1994). Ca$^{2+}$-free HL3 solution used to record the hypertonicity response had following ionic composition (in mM): 70 NaCl, 5 KCl, 21.5 MgCl$_2$, 10 NaHCO$_3$, 5 Trehalose, 115 sucrose, and 5 HEPES-NaOH. The ionic composition of high K$^+$ solution was (in mM) 80 NaCl, 62 KCl, 0.15 CaCl$_2$, 6 MgCl$_2$, and 5 HEPES-NaOH (pH 7.1). The internal solution for the patch pipette had the following ionic composition (in mM): 158 CsCl, 5 EGTA, 10 HEPES-NaOH, and 2 ATP (pH 7.1).

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

**Chemicals**

TTX and collagenase were purchased from Sigma (St. Louis, MO). PLTXII was obtained from Peptide Research Institute (Osaka, Japan).
Statistical analyses

For comparison among multiple groups, ANOVA was used with the Tukey test. For comparison of two groups, Student’s t-test was used. The frequency of minis and the number of events during delayed release varied in a wide range among cells examined and did not distribute normally. The comparison of this parameter in various preparations was done with a nonparametric test (Steel-Dwass test).

RESULTS

In temperature-sensitive cacophony (cac) mutant (cac\textsuperscript{TS2}) flies, nerve-evoked synaptic currents were strongly depressed at nonpermissive temperatures (30% of control at 36°C). Thus it was concluded that the cac gene encodes the primary Ca\textsuperscript{2+} channel supporting fast synaptic transmission (Kawasaki et al. 2000). However, this result does not tell us whether the cac Ca\textsuperscript{2+} channel is the sole Ca\textsuperscript{2+} channel that supports fast synaptic transmission, because remaining synaptic transmission at nonpermissive temperatures could be caused by remaining cac Ca\textsuperscript{2+} channel function at 36°C or because of a contribution of other types of Ca\textsuperscript{2+} channel. To address this question, we need to examine synaptic transmission in cac\textsuperscript{null} animals. The cacophony\textsuperscript{null} mutation is embryonic lethal (Smith et al. 1996). Morphologically, however, the NMJ forms normally up to late embryonic stages (Rieckhof et al. 2003). Thus we examined synaptic transmission at the NMJ in late-stage cac\textsuperscript{null} embryos.

In cac\textsuperscript{null} embryos, nerve-evoked fast synaptic transmission is absent in HL3 solution

In HL3 solution (1.5 mM [Ca\textsuperscript{2+}]\textsubscript{e}), no synaptic events were evoked between 2 and 8 ms after nerve stimulation in cac\textsuperscript{null} embryos (Fig. 1A). This result was confirmed in eight cells (≥100 stimuli at 0.3 or 10 Hz were delivered in each cell). We believe that synapses were formed even in these embryos because quantal synaptic events were observed, although infrequently, in all cells examined. The effectiveness of stimulation was ascertained in each cell by stimulation at 10 Hz for 2 s, which increased the frequency of nonsynchronous synaptic events. The amplitudes of these quantal events [183 ± 76 (SD) pA in 6 cells; because the number of events are small, those evoked at 0.3 and 10 Hz are pooled] were similar to those of miniature synaptic currents (minis) recorded in the presence of TTX as described below. These events are most likely caused by release of glutamate from individual synaptic vesicles. Thus we conclude that the NMJ is formed in cac\textsuperscript{null} embryos and the absence of fast synaptic transmission in cac\textsuperscript{null} embryos is caused by a lack of Ca\textsuperscript{2+} influx through cac Ca\textsuperscript{2+} channels responding to nerve action potentials and not from other developmental defects, such as the absence of postsynaptic glutamate receptors or a lack of synaptic vesicles.

Under the same experimental conditions, fast robust synaptic currents were always evoked in heterozygous embryos (cac\textsuperscript{null+/+}; Fig. 1C). The time between the onset of stimulus current pulse and the onset of synaptic current was 5.3 ± 1.5 ms (n = 15; range, 3.9–8.1 ms), and the failure rate was 0.06 ± 0.09 (n = 14) in HL3 with 1.0 mM [Ca\textsuperscript{2+}]\textsubscript{e}. The mean amplitude of the initial five synaptic currents during continuous stimulation at 0.3 Hz was 1,164 ± 1,265 pA (n = 15), which is smaller than that in a control strain with noncontracting muscles under the same experimental condition (Mhc, ~2 nA; Okamoto et al. 2005).

Fast synchronous synaptic transmission was rescued in a transformant in which the wild-type cac gene (tagged with EGFP) was expressed in the cac\textsuperscript{null} background (cac\textsuperscript{WT-EGFP}; Fig. 1B). The failure rate in the solution containing 1.0 mM [Ca\textsuperscript{2+}]\textsubscript{e} was 0.13 ± 0.13 (n = 9). The mean amplitude was 519 ± 339 pA (n = 11). The synaptic delay was 6.3 ± 1.6 ms (n = 8; range, 3.8–9.2 ms). These three parameters varied in a large range and were not statistically different from corresponding values in heterozygotes. Thus we conclude that expression of the cac gene is sufficient to restore fast synaptic transmission.

Delayed synaptic events after nerve stimulation is [Ca\textsuperscript{2+}]\textsubscript{e} dependent

Delayed synaptic quantal events in cac\textsuperscript{null} embryos were observed in one half of cases in HL3 solution containing 1.5 mM [Ca\textsuperscript{2+}]\textsubscript{e} (3 of 6 cells tested; 100 stimuli were given at 0.3 Hz in each cell; Fig. 2A). However, in the presence of 5 mM [Ca\textsuperscript{2+}]\textsubscript{e}, delayed events after nerve stimulation were detected more frequently (in 14 of 15 cells examined; Figs. 1A2 and 2A2). The average number of events/stimulus within the window between 8 and 50 ms was varied in a wide range of 0.01 ± 0.01/stimulus (n = 6 cells; range, 0–0.03; Fig. 2A1) in 1.5 mM [Ca\textsuperscript{2+}]\textsubscript{e} and 1.65 ± 2.82/stimulus in 5 mM [Ca\textsuperscript{2+}]\textsubscript{e} (n = 15; range, 0–5.93; Fig. 2A2). These two numbers are statistically different at P = 0.05 (nonparametric test). Thus we conclude that the occurrence of delayed quantal events is [Ca\textsuperscript{2+}]\textsubscript{e} dependent.

Time course of delayed release

To closely examine the time course of delayed release, we combined data obtained in nine cells in 5 mM [Ca\textsuperscript{2+}]\textsubscript{e}. The nerve was stimulated at 0.3 Hz. As shown in Fig. 2A2, the delayed quantal events appeared ~8 ms after nerve stimulation, peaked at ~13 ms, and slowly decayed during the period of 100 ms.

In Fig. 2, B and C, composite event frequency histograms for the rescued construct (cac\textsuperscript{WT-EGFP}) and the heterozygous control (cac\textsuperscript{null/+}) in HL3 solution with 1 mM [Ca\textsuperscript{2+}]\textsubscript{e} are shown. After synchronized release between 2 and 8 ms, delayed release was evident.

It is difficult to quantitatively compare the time course of delayed release in cac\textsuperscript{null} embryos with that in rescued constructs or heterozygotes, because the experimental condition was different ([Ca\textsuperscript{2+}]\textsubscript{e} was 5 mM in the experiment with cac\textsuperscript{null} embryos, whereas it was 1 mM in rescued constructs and heterozygotes), and synchronized release occurred in the latter but not in the former.

In cultured hippocampal neurons, the event frequency histograms were fit with two exponentials, and the longer component was called the delayed release and had a time constant of ~100 ms (Goda and Stevens 1994). The time course of delayed event frequency shown in Fig. 2A2 had a rising phase of ~5 ms after the delay of 8 ms, which was not observed in hippocampal neurons. Thus it is not clear whether the delayed release in hippocampal neurons is the same in the underlying mechanism as the one that we observed in our experiments.
Delayed release was depressed by 10 nM PLTXII but not by 50 μM La³⁺.

Even in 5 mM [Ca²⁺]ₑ, only one of seven cells examined showed delayed release in the presence of 10 nM PLTXII. The average number of events was 0.04 ± 0.12/stimulus (n = 7; range, 0–0.31). This value is statistically smaller than that for the control in 5 mM [Ca²⁺]ₑ (1.65 ± 2.82; n = 15) at P = 0.05 (nonparametric test). Thus we conclude that PLTXII depressed delayed release.

On the other hand, 50 μM La³⁺ did not affect the delayed release. The number of delayed events was 0.57 ± 0.57/stimulus (n = 4; range, 0–1.3) in 1.5 mM [Ca²⁺]ₑ stimulated at 10 Hz, which is not statistically different from in the control (0.07 ± 0.08/stimulus; n = 9; range, 0–0.25 in 1.5 mM [Ca²⁺]ₑ stimulated at 10 Hz). Thus we conclude that 50 μM La³⁺ does not inhibit delayed release. La³⁺ at 50 μM is known to block endocytosis at the Drosophila NMJ by blocking one type of voltage-gated Ca²⁺ channel in the presence of 1.5 mM [Ca²⁺]ₑ (Kuromi et al. 2004).

**Bursting of quantal events after nerve stimulation in the presence of 5 mM [Ca²⁺]ₑ**

In the minority of cells, nerve stimulation induced bursting of quantal events as shown in Fig. 3A (4 of 15 cells...
examined). Here we defined a burst as a sequence of quantal events, the number of which exceeded 10 per stimulus during the period between 8 and 50 ms after nerve stimulation. Even in the same cell, some stimuli evoked bursting of events, whereas the majority of stimuli induced sporadic delayed quantal release as depicted in Fig. 1A2. (The percentage of stimuli that evoked a burst in 4 cells was 36 ± 35%). The delay of the first event in the burst varied considerably as shown in Fig. 3B but is in the same range as solitary delayed events (Fig. 3C).

FIG. 2. Composite event frequency histograms of nerve-evoked events in cacnull and controls. Composite event frequency histograms from multiple cells were constructed for each genotype of embryos by adding and normalizing the histograms obtained in multiple cells. The number of events per stimulus per cell is plotted in the ordinate against time after nerve stimulation. A1: composite event frequency histogram constructed from the data obtained in 6 cells in cacnull embryos in the presence of 1.5 mM [Ca^{2+}]_e. A2: composite event frequency histogram constructed from the data obtained in 9 cells in cacnull embryos in the presence of 5 mM [Ca^{2+}]_e. B: composite event frequency histogram constructed from the data obtained in 16 cells from cacWT-EGFP embryos in the presence of 1 mM [Ca^{2+}]_e. C: composite event frequency histogram constructed from the data obtained in 10 cells from heterozygotic embryos, cacnull/+, in the presence of 1 mM [Ca^{2+}]_e.
The number of events in one burst also varied considerably, but some of them had 40 events, which is greater than the maximal total number of events induced by hypertonic solution described below. This finding indicates that, in this preparation, the maximal hypertonicity response does not represent the size of the readily releasable pool as defined by Rosenmund and Stevens (1996) in cultured mammalian neurons.

**Rare fast synchronous synaptic transmission in 5 mM [Ca\(^{2+}\)]\(_e\)**

In 5 of 15 cells examined in cac\(^{null}\) embryos, some quantal events occurred between 2 and 8 ms after stimulation in 5 mM [Ca\(^{2+}\)]\(_e\), as shown in Fig. 4A, when the nerve was stimulated at 0.3 Hz. This is the period in which fast synaptic events occur in control embryos under this experimental condition (Figs. 1, B and C, and 2, B and C). The frequency of fast events in this cell (0.08/stimulus during the period of 6 ms) was significantly more frequent than that expected from accidental occurrence of spontaneous events (0.021 ± 0.015/stimulus). After subtracting the background frequency during this window, the quantal content in this case was calculated to be 0.06. In five cases, the mean quantal content was 0.08 ± 0.04.

The quantal content in control embryos in 5 mM [Ca\(^{2+}\)]\(_e\) cannot be measured by the failure method because the failure rate is extremely low. Previously, we estimated it from the mean amplitude to be ~10 in the noncontracting myosin mutant under the same experimental condition (Mhc; Okamoto et al. 2005). Thus fast synaptic transmission in cac\(^{null}\) embryos was extremely weak and occurred in the minority of cells.

When the stimulus frequency was increased to 10 Hz, synchronized events occurred more frequently in this cell, as shown in Fig. 4B. The quantal content in this case was 0.52, which is still very small compared with the control as mentioned above. This was the only case in which facilitation of synchronous fast release with 10-Hz stimulation was observed (15 cells examined).

Although fast synchronous release was rare in cac\(^{null}\) embryos, the observation of these events indicates that non-cac Ca\(^{2+}\) channels responsible for delayed release are located close to the release site, and Ca\(^{2+}\) influx through those channels could evoke fast synchronous synaptic currents when a favorable condition, such as 5 mM [Ca\(^{2+}\)]\(_e\) and 10-Hz stimulation, is provided.

**Miniature synaptic currents**

As described above, after nerve stimulation, no fast synaptic currents were evoked, but delayed release remained in cac\(^{null}\) embryos in HL3 solution. Next, we examined how the lack of cac Ca\(^{2+}\) channels affects spontaneous synaptic vesicle release. In HL3 solution containing 1.5 mM [Ca\(^{2+}\)]\(_e\) and 3 μM TTX, miniature synaptic currents (minis) occurred infrequently in cac\(^{null}\) embryos. The mean frequency in cac\(^{null}\) embryos was 0.8 ± 0.5/min (n = 10), whereas in the control was 1.8 ± 1.5/min (n = 13, cac\(^{WT-EGFP}\); Fig. 5A). These values are significantly different at P = 0.05 (nonparametric test). This finding suggests either that a lack of cac Ca\(^{2+}\) channels depresses the mini frequency or that the population of vesicles that are ready to be released spontaneously is smaller in cac\(^{null}\) embryos.

Mini amplitudes were variable, and the amplitude histogram was skewed toward larger amplitudes in cac\(^{null}\) embryos similar to that observed in control embryos. These properties are similar to those observed in wild-type embryos reported pre-
viously (Hou et al. 2003; Kidokoro and Nishikawa 1994; Zhang et al. 1999).

The mean amplitude in $cacnull$ embryos was $108 \pm 41$ pA ($n = 10$, the number of preparations), whereas that in the control was $113 \pm 44$ pA ($n = 13$, $cacWT$-EGFP). These two values are not significantly different ($P > 0.05$). Thus as suggested above, the properties of postsynaptic glutamate receptor channels and their density in the postsynaptic membrane are not likely to be altered in $cacnull$ embryos.

Frequency of quantal synaptic events in high K$^+$ solution is greatly depressed

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. We next examined the contribution of $cac$ Ca$^{2+}$ channels in the elevation of Ca$^{2+}$ concentration in the vicinity of release site in high K$^+$ solutions.

The frequency of quantal synaptic events increased significantly in high K$^+$ HL3 solution (62 mM K$^+$) even in $cacnull$ embryos (11 ± 9/min, $n = 4$) compared with the mini frequency in HL3 solution (0.8 ± 0.5/min). However, it was still significantly lower than that in the control (CS; 1,481 ± 1,451/min; $n = 4$; Fig. 5B).

Thus it seems that Ca$^{2+}$ influx through $cac$ Ca$^{2+}$ channels strongly contributes in an increase of quantal synaptic event frequency in high K$^+$ solutions in control embryos, whereas that through non-$cac$ Ca$^{2+}$ channels is minor.

Hypertonicity response is $\sim 1/10$ of control

The lower frequency of minis and quantal events in high K$^+$ solution in $cacnull$ embryos could be caused by fewer release-ready synaptic vesicles at the NMJ. To assess the population of release-ready vesicles, we next examined the hypertonicity response. The quantal event frequency increases with puff application of hypertonic solutions at embryonic NMJs (Suzuki et al. 2002a,b). Because Ca$^{2+}$ is not required for this response (Mochida et al. 1998; Rosenmund and Stevens 1996), the population of release-ready vesicles can be assessed regardless of Ca$^{2+}$ influx at the release site. We used a hypertonic...
solution that contains 420 mM sucrose, which induces the maximal response in Drosophila embryos (Suzuki et al. 2002a).

The hypertonicity response evoked with 420 mM sucrose added to Ca\(^{2+}\)-free HL3 in the control (cac\(^{WT-EGFP}\); Fig. 6; total number of events, 265 ± 120; n = 5; peak frequency, 68 ± 14/s; n = 8) was not different from the previously reported maximal values for wild-type embryos (Okamoto et al. 2005; Suzuki et al. 2002a,b). On the other hand, the hypertonicity response in cac\(^{null}\) embryos was ~1/10 of the control (total number of events, 26 ± 22; n = 9; peak frequency, 12 ± 9/s; n = 15).

The small number of synaptic vesicles in the vicinity of release site in cac\(^{null}\) embryos, which is suggested by the smaller hypertonicity response, could account for the lower mini frequency described above, but the total lack of fast synaptic transmission in HL3 solution cannot be explained.

**DISCUSSION**

**cac Ca\(^{2+}\) channels are necessary and sufficient for fast synaptic transmission**

In cac\(^{null}\) embryos, no nerve-evoked fast synaptic transmission was observed in HL3 solution with 1.5 mM [Ca\(^{2+}\)]\(_{c}\) (Figs. 1A1 and 2A1). Thus we conclude that cac-Ca\(^{2+}\) channels are necessary for fast synaptic transmission. When the wild-type cac gene, tagged with EGFP, was expressed in the cac\(^{null}\) background, clusters of EGFP were observed at the active zones, and synaptic transmission was restored (Kawasaki et al. 2004). In the embryos of this transformant, we found that fast synaptic transmission was recovered to 44% of the control (heterozygotes, cac\(^{null/+}\)). Thus we conclude that the presence of cac-Ca\(^{2+}\) channels is sufficient for restoration of fast synaptic transmission.

Multiple types of voltage-gated Ca\(^{2+}\) channels are contributing to fast synaptic transmission in other systems. For example, in the Calyx of Held synapse, a specific blocker for the P/Q type Ca\(^{2+}\) channel, 100 nM ω-agatoxin IVA, inhibited fast synaptic transmission to 3%, whereas Ca\(^{2+}\) influx in the whole terminal was depressed only to 44% of the control, and an N-type Ca\(^{2+}\) channel blocker, 1 μM ω-conotoxin-GVIA, also reduced synaptic currents to 64% and Ca\(^{2+}\) influx to 73% (Wu et al. 1999). In addition to P/Q and N types of Ca\(^{2+}\) channel, R type Ca\(^{2+}\) channels also contribute to fast synaptic transmission in the Calyx of Held synapse (Wu et al. 1998). At Xenopus neuromuscular synapses formed in culture, L-type Ca\(^{2+}\) channels together with N-type are contributing in fast synaptic transmission (Sand et al. 2001).

On the other hand, L-type Ca\(^{2+}\) channels are the sole Ca\(^{2+}\) channel that supports synaptic transmission in hair cells of the chicken cochlea (Kollmar et al. 1997; Roberts et al. 1990) and in bipolar cells in the goldfish retina (Tachibana et al. 1993). Apparently one type of Ca\(^{2+}\) channel is sufficient to support synaptic transmission at these synapses as well as at the Drosophila embryonic NMJ. The contribution of multiple Ca\(^{2+}\) channels for fast synaptic transmission is not the arrangement of necessity. Other types of Ca\(^{2+}\) channel that have different roles might be distributed close to the transmitter release site by chance.

**Delayed synaptic events after nerve stimulation in cac\(^{null}\) embryos**

In cac\(^{null}\) embryos, no fast synaptic transmission was observed in HL3 solution, but delayed quantal events were detected occasionally in HL3 with 1.5 mM [Ca\(^{2+}\)]\(_{c}\) and more frequently in 5 mM [Ca\(^{2+}\)]\(_{c}\). The delay was around 8 ms after nerve stimulation, the frequency peaked at ~15 ms, and events continued for tens of milliseconds (Fig. 2A2). The quantal delayed events were blocked by PLTXII, an analog of spider toxin PLTX that has been shown to block voltage-gated Ca\(^{2+}\) channels in cultured Drosophila neurons (Leung et al. 1989), and their occurrence was [Ca\(^{2+}\)]\(_{c}\) dependent. Thus the delayed release is most likely to be supported by another type of voltage-gated Ca\(^{2+}\) channel (non-cac, PLTXII sensitive Ca\(^{2+}\) channel). This slow time course of vesicle release cannot be explained by fast Ca\(^{2+}\) influx during single action potentials, which is expected to subside within a few milliseconds (Di-

![FIG. 6. Hypertonicity responses in cac\(^{null}\) and control, cac\(^{WT-EGFP}\), embryos. A: hypertonic response (450 mM sucrose was added to Ca\(^{2+}\)-free HL3 solution) was puff-applied for 11 s in 15 cac\(^{null}\) embryos in Ca\(^{2+}\)-free HL3 and all data were combined. The induced events were individually counted. B: the hypertonicity response in control, cac\(^{WT-EGFP}\), embryos. In 8 cells, the response was examined, and all data were combined. C: the peak frequency of events recorded in cac\(^{null}\) (n = 15) and control, cac\(^{WT-EGFP}\) (n = 8), embryos. These values are statistically different at P = 0.05. D: the total number of events recorded in cac\(^{null}\) (n = 5) and control, cac\(^{WT-EGFP}\) (n = 9) embryos. These values are statistically different at P = 0.05.](http://jn.physiology.org/)

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Gregorio and Vergara 1997). Because the delayed event frequency histogram has a rising phase between 8 and 15 ms (Fig. 2A2), it is difficult to explain their occurrence with residual [Ca\(^{2+}\)]\(_i\) after single action potentials, which is expected to decline monotonously. A similar argument also eliminates the possibility that the slow time course is caused by diffusion of Ca\(^{2+}\) from distant Ca\(^{2+}\) channels from the release site. However, because the delayed release is loosely synchronized to nerve stimulation and depends on [Ca\(^{2+}\)]\(_i\), it is possible that Ca\(^{2+}\) influx during single action potentials through Ca\(^{2+}\) channels is triggering Ca\(^{2+}\) release from internal Ca\(^{2+}\) stores, and Ca\(^{2+}\) released from the store induces delayed vesicle fusion.

There are numerous observations that support the idea that Ca\(^{2+}\)-induced Ca\(^{2+}\) release at the presynaptic terminal modulates synaptic transmission (reviewed by Collin et al. 2005). There seem to be multiple mechanisms for Ca\(^{2+}\) release from internal stores. However, at the frog NMJ, there is a good indication that Ca\(^{2+}\)-induced Ca\(^{2+}\) release from ryanodine-sensitive internal Ca\(^{2+}\) stores occurs close to the transmitter release site (Narita et al. 2000). A similar mechanism might be responsible for the delayed release and the bursting behavior of quantal release observed in this study (Figs. 2 and 3). Taken together, we suggest that, in cacnull embryos, activation of non-cac, PLTXII-sensitive Ca\(^{2+}\) channels triggers Ca\(^{2+}\) release from internal Ca\(^{2+}\) stores and supports delayed quantal release.

In some cells, we observed bursting of quantal events in the presence of 5 mM [Ca\(^{2+}\)]\(_i\) (Fig. 3). These bursts occurred with similar latencies after stimulation as delayed release. These bursting events might also be explained by release of Ca\(^{2+}\) from internal stores. After priming, Ca\(^{2+}\)-induced Ca\(^{2+}\) release from ryanodine-sensitive internal Ca\(^{2+}\) stores may occur regeneratively and produce a burst of quantal events (Narita et al. 2000).

**Fast synaptic transmission in cacnull embryos**

In rare cases, we observed fast synaptic quantal release in cacnull embryos (Fig. 4). The latency of fast events in these cells was not different from in control embryos (2–8 ms). Although these fast events occurred rarely in HL3 with 1.5 mM [Ca\(^{2+}\)]\(_i\), they were observed more frequently with 5 mM [Ca\(^{2+}\)]\(_i\). This finding suggests that the non-cac, PLTXII-sensitive Ca\(^{2+}\) channel is closely located to the transmitter release site and directly, or indirectly through the Ca\(^{2+}\)-induced release mechanism (Narita et al. 2000), evokes fast release.

**Multiple types of Ca\(^{2+}\) channel in the presynaptic terminal at the Drosophila NMJ**

Although the cac Ca\(^{2+}\) channels are the sole voltage-gated Ca\(^{2+}\) channel participating in fast synaptic transmission at the Drosophila NMJ, lines of evidence indicate multiple types of Ca\(^{2+}\) channel in the presynaptic terminal. Pharmacologically, another type of Ca\(^{2+}\) channel has been identified at the presynaptic nerve terminal, which is sensitive to La\(^{3+}\) or flunarizine and participates in endocytosis (Kuromi et al. 2004). Although cac Ca\(^{2+}\) channels are sensitive to PLTXII because we did not observe fast synaptic transmission in control embryos (cac WT-EGFP) in the presence of 10 nM PLTXII (unpublished observations), we also found that 10 nM PLTXII blocks delayed release in cacnull embryos. This finding indicates that yet another type of PLTXII-sensitive Ca\(^{2+}\) channel resides in the presynaptic terminal. Thus there are at least three types of Ca\(^{2+}\) channel in the presynaptic terminal: cac Ca\(^{2+}\) channel, non-cac, PLTXII-sensitive Ca\(^{2+}\) channel, and La\(^{3+}\)-sensitive Ca\(^{2+}\) channel.

**Localization of Ca\(^{2+}\) channels in the presynaptic terminal**

In the Calyx of Held synapse, the major contributor, P/Q type, to fast synaptic transmission is localized at the release site, whereas others are diffusely distributed in the presynaptic terminal (Wu et al. 1999). Thus the location of channels is crucial to effectively participate in fast synaptic transmission. cac Ca\(^{2+}\) channels tagged with EGFP were imaged and found to be localized in the active zone, which is defined as the presynaptic area facing the postsynaptic density (Kawasaki et al. 2004; Kittel et al. 2006). Unfortunately, to date, no electron microscope EM studies have successfully shown the precise location of cac Ca\(^{2+}\) channels within the active zone. A non-cac, PLTXII-sensitive Ca\(^{2+}\) channel that supports delayed quantal release in cacnull embryos may locate close to the release site, as our observation of fast quantal release in the minority of cells in cacnull embryos indicates (Fig. 4). Further remaining questions are as follows. Where do La\(^{3+}\)-sensitive Ca\(^{2+}\) channels, which are contributing to endocytosis (Kuromi et al. 2004), locate? Do non-cac, PLTXII-sensitive Ca\(^{2+}\) channels have a specific function for synaptic transmission in wild-type animals in normal conditions? The Drosophila NMJ will continue to be one of the favorable preparations to answer these questions.

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