DELAYED SYNAPTIC TRANSMISSION IN DROSOPHILA CACOPHONYnull EMBRYOS

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ABSTRACT

Ca$^{2+}$ influx through the *Drosophila* N-type Ca$^{2+}$ channel, encoded by *cacophony* (*cac*), triggers fast synaptic transmission. We now ask whether the *cac* Ca$^{2+}$ channel is the Ca$^{2+}$ channel solely dedicated for fast synaptic transmission. Since 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 upon 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 [Ca$^{2+}$]$_e$ and in majority of cells in 5 mM [Ca$^{2+}$]$_e$. The number of delayed quantal events per stimulus was greater in 5 mM [Ca$^{2+}$]$_e$ than in 1.5 mM. Thus the delayed release is [Ca$^{2+}$]$_e$-dependent. Ten nM PLTXII (a spider toxin analog) depressed the frequency of delayed events, suggesting that voltage-gated Ca$^{2+}$ channels, other than *cac* Ca$^{2+}$ channels, are contributing to them. However, delayed events were not affected by 50 μM La$^{3+}$. The frequency of miniature synaptic currents in *cac* null embryos was ~1/2 of control, while 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* Ca$^{2+}$ channel is indispensable for fast synaptic transmission in normal conditions, and another type of Ca$^{2+}$ channel, non-*cac*, PLTXII-sensitive Ca$^{2+}$ channel, is contributing to delayed release in *cac* null embryos.
INTRODUCTION

Four genes in the Drosophila genome encode voltage-gated Ca\(^{2+}\) channel α1 subunits, which include homologs of mammalian T-type α1 (Dmca1G), L-type α1 (Dmca1D), and N-type α1 (Dmca1A) (Littleton and Ganetzky 2000). These genes are likely to produce multiple types of voltage-gated Ca\(^{2+}\) channels with distinct pharmacological and biophysical properties (Byerly and Leung, 1998; Peng and Wu, 2007). Pharmacologically two types of voltage-gated Ca\(^{2+}\) currents were detected in embryonic Drosophila neurons in culture. A spider toxin, PLTX, blocked both, but another spider toxin, HoTX, suppressed only one of them. The HoTX-sensitive Ca\(^{2+}\) current had a faster inactivation time course. Organic blockers, such as nifedipine and diltiazem, had little effect on these Ca\(^{2+}\) currents (Leung et al, 1989). Voltage-gated Ca\(^{2+}\) 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 Ca\(^{2+}\) channel blocker, and the other to amiloride, a T-type Ca\(^{2+}\) channel blocker (Gielow et al, 1995). Thus pharmacologically at least two types are in neurons and additional two types in muscles.

The Ca\(^{2+}\) channel that is constituted of the α1 subunit encoded by the Dmca1A or cacophony (cac) gene (referred to as the cac Ca\(^{2+}\) channel hereafter) contributes in fast synaptic transmission. In temperature-sensitive cac mutant, cac\(^{TS2}\), flies at 36 °C, the amplitude of nerve-evoked synchronous synaptic currents was depressed to 30% of wild-type (CS) control. Since the amplitude of quantal events, miniature synaptic potentials, was not different in cac\(^{TS2}\) than in wild-type at 36 °C, they concluded that this depression of synaptic transmission was due to a defect in the presynaptic cac Ca\(^{2+}\) channel function at the non-permissive temperature (Kawasaki et al, 2002). This conclusion was supported by Macleod et al (2005) who measured changes in Ca\(^{2+}\) concentrations at the presynaptic bouton in cac\(^{TS2}\) larvae during repetitive nerve stimulation. They found that while at 22 °C the extent of [Ca\(^{2+}\)]\(_i\) elevation induced by 10 Hz stimulation in 0.5 mM [Ca\(^{2+}\)]\(_e\) was not different than 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 [Ca\(^{2+}\)]\(_i\) during 10 Hz stimulation were reduced, but synaptic transmission was not abolished, indicating that either cac Ca\(^{2+}\) channels were not completely blocked at this temperature or that there were other types of Ca\(^{2+}\) channels in the presynaptic terminal supporting remaining fast synaptic transmission. Thus a question remains whether the cac Ca\(^{2+}\) channel is the sole Ca\(^{2+}\) channel for fast synaptic transmission.

Multiple types of Ca\(^{2+}\) channels contributing to fast synaptic transmission at the rat Calyx of Held synapses. 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 Ca\(^{2+}\) channels, L-type Ca\(^{2+}\) channels efficiently contribute to fast synaptic transmission (Sand et al, 2001). Then obvious questions arise; Why are there multiple types of Ca\(^{2+}\) channels in the presynaptic terminal? And does each of them have a specific function for synaptic transmission? On the other hand, dihydropyridine-sensitive L-type Ca\(^{2+}\) channels are predominant and support synaptic transmission in hair cells of the chicken cochlea (Roberts et al, 1990; Kollmar et al, 1997) 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 in these synapses.

Ca\(^{2+}\) 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 Ca\(^{2+}\) channels inhibit endocytosis at concentrations that hardly affects exocytosis. A Ca\(^{2+}\) channel blocker, flunarizine at 50 \(\mu\)M, blocked endocytosis while exocytosis was only slightly depressed. Similarly, a trivalent cation, La\(^{3+}\), at 50 \(\mu\)M, reduced endocytosis to 20% of control while exocytosis was not affected. On the other hand, 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\(^{2+}\) channels, namely flunaridine/La\(^{3+}\)-sensitive and PLTXII-sensitive types of Ca\(^{2+}\) channels, in the presynaptic terminal (Kuromi et al., 2004). PLTX blocks synaptic transmission at the *Drosophila* NMJ at nM concentrations (Branton et al., 1987) and *cac* mutations depress synaptic transmission (Kawasaki et al., 2002). 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 the 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. Since 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. Five nM PLTXII blocked the delayed release but 50 \(\mu\)M La\(^{3+}\) did not. Hence this type of Ca\(^{2+}\) channel is called 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.


**MATERIALS AND 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) L13^{HCl29} sd f/FM7i-GFP;; a gift from Dr. R. Ordway). They did not have a GFP marker (w elav Gal4 l(1) L13^{HCl29} sd f/Y;; and are referred to as *cac null* in the text. Rescued strains were either w elav Gal4 l(1) L13^{HCl29} sd f/+;;UAS-cac1-EGFP786C/+ or elav Gal4 l(1) L13^{HCl29} sd f;;UAS-cac1-EGFP786C. These strains have the *cac* gene tagged with EGFP. Synaptic transmission in the former construct was examined and found indistinguishable from the control (Kawasaki et al, 2004). The former construct has one copy of *cac1-EGFP* while the latter has two. But, 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 WT-EGFP*. As controls we used heterozygote embryos that had a balancer with a GFP marker (*cac null/+*) or wild-type, CS, embryos.

*Electrophysiology;* Whole-cell patch-clamp recordings from abdominal muscles 6 or 7 in embryos (19-21 hr 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 (for ionic composition, see below) 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 has a resistance of 10 to 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 about 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. Then 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 ms 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 DNJM by the puff method with a gas pressure of 0.5 Kg/cm\(^2\) for 11s. 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.

Solutions: The ionic composition of Ca\(^{2+}\)-free saline used for dissection of embryos was (in mM): NaCl, 140; KCl, 2; MgCl\(_2\), 6; HEPES-NaOH, 5 (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): NaCl, 70; KCl, 5; CaCl\(_2\), 1.5; MgCl\(_2\), 20; NaHCO\(_3\), 10; Trehalose, 5; sucrose, 115; HEPES-NaOH, 5 (pH, 7.1) (Stewart et al, 1994). Ca\(^{2+}\)-free HL3 solution used to record the hypertonicity response had following ionic composition; NaCl, 70; KCl, 5; MgCl\(_2\), 21.5; NaHCO\(_3\), 10; Trehalose, 5; sucrose, 115; HEPES-NaOH, 5. The ionic composition of high K\(^+\) solution was (in mM): NaCl, 80; KCl, 62; CaCl\(_2\), 0.15; MgCl\(_2\), 6; HEPES-NaOH, 5 (pH, 7.1). The internal solution for the patch pipette had the ionic composition (in mM); CsCl, 158; EGTA, 5; HEPES-NaOH, 10; ATP, 2 (pH 7.1).

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

Chemicals: Tetrodotoxin (TTX) and collagenase were purchased from Sigma (St. Louis, MO, USA). 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 non-parametric test (Steel-Dwass test).
RESULTS

In temperature-sensitive cacophony (cac) mutant (cacTS2) flies nerve-evoked synaptic currents were strongly depressed at non-permissive temperatures (30% of control at 36 °C). Thus it was concluded that the cac gene encodes the primary Ca\(^{2+}\) channel supporting fast synaptic transmission (Kawasaki et al, 2002). However, this result does not tell us whether the cac Ca\(^{2+}\) channel is the sole Ca\(^{2+}\) channel that supports fast synaptic transmission because remaining synaptic transmission at non-permissive temperatures could be due to a remaining cac Ca\(^{2+}\) channel function at 36 °C or due to a contribution of other types of Ca\(^{2+}\) channels. To address this question we need to examine synaptic transmission in cacnull animals. The cacophonynull mutation is embryonic lethal (Smith et al, 1996). Morphologically, however, the neuromuscular junction (NMJ) forms normally up to late embryonic stages (Rieckhof et al, 2003). Thus, we examined synaptic transmission at the NMJ in late-stage cacnull embryos.

In cacnull embryos nerve-evoked fast synaptic transmission is absent in HL solution

In HL3 solution (1.5 mM [Ca\(^{2+}\)]e) no synaptic events were evoked between 2 and 8 ms after nerve stimulation in cacnull embryos (Fig. 1A). This result was confirmed in 8 cells (at least 100 stimuli at 0.3 or 10 Hz were delivered in each cell). We believe that synapses were formed even in these embryos since 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 non-synchronous synaptic events. The amplitudes of these quantal events (183±76 pA, mean±SD, in 6 cells; since 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 to be due to release of glutamate from individual synaptic vesicles. Thus we conclude that the NMJ is formed in cacnull embryos and the absence of fast synaptic transmission in cacnull embryos is due to a lack of Ca\(^{2+}\) influx through cac Ca\(^{2+}\) channels responding to nerve action potentials and not due to other developmental defects, such as absence of postsynaptic glutamate receptors or a lack of synaptic vesicles.

Under the same experimental condition, fast robust synaptic currents were always evoked in heterozygous embryos (cacnull/+), 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\(^{2+}\)]e. The mean amplitude of initial five synaptic currents during continuous stimulation at 0.3 Hz was 1164±1265 pA (n=15), which is smaller than that in a control strain with non-contracting muscles under the same experimental condition (Mhc, ~ 2 nA, Okamoto et al, 2006).

Fast synchronous synaptic transmission was rescued in a transformant in which the wild-type cac gene (tagged with EGFP) was expressed in the cacnull background (cacWT-EGFP, Fig. 1B). The failure rate in the solution containing 1.0 mM [Ca\(^{2+}\)]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). 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 \textit{cac} gene is sufficient to restore fast synaptic transmission.

\textbf{Delayed synaptic events after nerve stimulation is [Ca$^{2+}$]$_{e}$-dependent}

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

\textbf{The time course of delayed release}

To closely examine the time course of delayed release we combined data obtained in 9 cells in 5 mM [Ca$^{2+}$]$_{e}$. The nerve was stimulated at 0.3 Hz. As shown in Fig. 2A2, the delayed quantal events appeared around 8 ms after nerve stimulation, peaked at around 13 ms and slowly decayed during the period of 100 ms.

In Fig. 2B and C, composite event frequency histograms for the rescued construct (\textit{cac}\textsuperscript{WT-EGFP}) and the heterozygous control (\textit{cac}\textsuperscript{null/+}) in HL3 solution with 1 mM [Ca$^{2+}$]$_{e}$ 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 \textit{cac}\textsuperscript{null} embryos with that in rescued constructs or heterozygotes, because the experimental condition was different ( [Ca$^{2+}$]$_{e}$ was 5 mM in the experiment with \textit{cac}\textsuperscript{null} embryos while 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 where 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 the rising phase of about 5 ms after the delay of 8 ms, which was not observed in hippocampal neurons. Thus it is not clear at this moment whether the delayed release in hippocampal neurons is the same in the underlying mechanism as the one that we observed in our experiments.

\textbf{Delayed release was depressed by 10 nM PLTXII, but not by 50 μM La$^{3+}$}

Even in 5 mM [Ca$^{2+}$]$_{e}$ only one out of 7 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 [Ca$^{2+}$]$_{e}$(1.65±2.82, n=15) at p=0.05 (non-parametric test). Thus we conclude that PLTXII depressed delayed release.

On the other hand, 50 μM La$^{3+}$ 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 [Ca$^{2+}$]$_{e}$ stimulated at 10 Hz, which is not statistically different than in the control (0.07±0.08/stimulus n=9, range 0–0.25 in 1.5 mM [Ca$^{2+}$]$_{e}$ stimulated at 10 Hz). Thus we conclude that 50 μM La$^{3+}$
does not inhibit delayed release. La$^{3+}$ at 50 μM is known to block endocytosis at the *Drosophila* NMJ by blocking one type of voltage-gated Ca$^{2+}$ channel in the presence of 1.5 mM [Ca$^{2+}$]$_e$ (Kuromi et al, 2004).

**Bursting of quantal events after nerve stimulation in the presence of 5 mM [Ca$^{2+}$]$_e$**

In the minority of cells nerve stimulation induced bursting of quantal events as shown in Fig. 3A (4 cells out of 15 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 while 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).

The number of events in one burst also varied considerably, but some of them had more than 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 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 cases out 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 (Fig. 1B, C and Fig. 2B, 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 5 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 approximately 10 in the non-contracting myosin mutant under the same experimental condition (*Mhc*, Okamoto et al, 2006). Thus fast synaptic transmission in cac$^{null}$ embryos was extremely weak and occurred in 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 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<sup>null</sup> embryos in HL3 solution. Next we examined how the lack of cac Ca<sup>2+</sup> channels affects spontaneous synaptic vesicle release. In HL3 solution containing 1.5 mM [Ca<sup>2+</sup>]<sub>e</sub> and 3 µM TTX miniature synaptic currents (minis) occurred infrequently in cac<sup>null</sup> embryos. The mean frequency in cac<sup>null</sup> embryos was 0.8±0.5/min (n=10), while that in the control was 1.8±1.5/min (n=13, cac<sup>WT-EGFP</sup>) (Fig. 5A). These values are significantly different at p=0.05 (non-parametric test). This finding suggests either that a lack of cac Ca<sup>2+</sup> channels depresses the mini frequency or that the population of vesicles that are ready to be released spontaneously is smaller in cac<sup>null</sup> embryos.

Mini amplitudes were variable and the amplitude histogram was skewed toward larger amplitudes in cac<sup>null</sup> embryos similar to that observed in control embryos. These properties are similar to those observed in wild-type embryos reported previously (Kidokoro and Nishikawa 1995; Zhang et al, 1999; Hou et al, 2003).

The mean amplitude in cac<sup>null</sup> embryos was 108±41 pA (n=10, the number of preparations), whereas that in the control was 113±44 pA (n=13, cac<sup>WT-EGFP</sup>). 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 cac<sup>null</sup> embryos.

**The frequency of quantal synaptic events in high K<sup>+</sup> solution is greatly depressed**

In high K<sup>+</sup> solutions, the presynaptic terminal membrane is continuously depolarized and voltage-gated Ca<sup>2+</sup> channels open asynchronously unlike synchronized opening induced by nerve stimulation. We next examined the contribution of cac Ca<sup>2+</sup> channels in the elevation of Ca<sup>2+</sup> concentration in the vicinity of release site in high K<sup>+</sup> solutions. The frequency of quantal synaptic events increased significantly in high K<sup>+</sup> HL3 solution (62 mM K<sup>+</sup>) even in cac<sup>null</sup> embryos (11±9/min, n=4) compared with the mini frequency in HL3 solution (0.8±0.5/min). But it was still significantly lower than that in the control (CS), 1481±1451 /min (n=4) (Fig. 5B).

Thus it appears that Ca<sup>2+</sup> influx through cac Ca<sup>2+</sup> channels strongly contributes in an increase of quantal synaptic event frequency in high K<sup>+</sup> solutions in control embryos while that through non-cac Ca<sup>2+</sup> channels is minor.

**The hypertonicity response is ~1/10 of control**

The lower frequency of minis and quantal events in high K<sup>+</sup> solution in cac<sup>null</sup> embryos could be due to 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). Since Ca<sup>2+</sup> is not required for this response (Rosenmund and Stevens 1996; Mochida et al, 1998), the population of release-ready vesicles can be assessed regardless of Ca<sup>2+</sup> 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<sup>2+</sup>-free HL3 in the control (cac<sup>WT-EGFP</sup>) (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 (Suzuki et al, 2002a,b; Okamoto et al, 2005). On the other hand, the
hypertonicity response in \textit{cac}^{\text{null}} \textit{embryos} was approximately 1/10 of the control (total number of events, 26\(\pm\)22, n=9; peak frequency, 12\(\pm\)9/s, n=15).

The small number of synaptic vesicles in the vicinity of release site in \textit{cac}^{\text{null}} \textit{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\(^{2+}\)]\(_{e}\) (Fig 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 \(\omega\)-agatoxin IVA, inhibited fast synaptic transmission to 3% while Ca\(^{2+}\) influx in the whole terminal was depressed only to 44% of the control, while an N-type Ca\(^{2+}\) channel blocker, 1 \(\mu\)M \(\omega\)-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\(^{2+}\) channels, 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 (Roberts et al, 1990; Kollmar et al, 1997) 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 neuromuscular junction. Then the contribution of multiple Ca\(^{2+}\) channels for fast synaptic transmission is not the arrangement of necessity. Other types of Ca\(^{2+}\) channels 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\(^{2+}\)]\(_{e}\) and more frequently in 5 mM [Ca\(^{2+}\)]\(_{e}\). The delay was around 8 ms after nerve stimulation, the frequency peaked at around 15 ms and events continued for tens of ms (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\(^{2+}\)]\(_{e}\)-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\(^{2+}\) channels). 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 ms (DiGregorio and Vergara, 1997). Since 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\(^{2+}\)]\(_{e}\) after single action potentials, which is expected to decline monotonously. A similar argument also eliminates the possibility that the slow time course is due to diffusion of Ca\(^{2+}\) from distant Ca\(^{2+}\) channels from the release site.
However, since the delayed release is loosely synchronized to nerve stimulation and depends on \([\text{Ca}^{2+}]_{\text{e}}\), it is possible that \(\text{Ca}^{2+}\) influx during single action potentials through \(\text{Ca}^{2+}\) channels is triggering \(\text{Ca}^{2+}\) release from internal \(\text{Ca}^{2+}\) stores and \(\text{Ca}^{2+}\) released from the store induces delayed vesicle fusion.

There are numerous observations that support the idea that \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release at the presynaptic terminal modulates synaptic transmission (reviewed by Collin et al., 2005). There seem to be multiple mechanisms for \(\text{Ca}^{2+}\) release from internal stores. But at the frog neuromuscular junction there is a good indication that \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release from ryanodine-sensitive internal \(\text{Ca}^{2+}\) 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 (Fig. 2 and Fig. 3). Taken together, we suggest that in \text{cacnull}\) embryos activation of non-\text{cac}, PLTXII-sensitive \(\text{Ca}^{2+}\) channels triggers \(\text{Ca}^{2+}\) release from internal \(\text{Ca}^{2+}\) stores and supports delayed quantal release.

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

**Fast synaptic transmission in \text{cacnull} embryos**

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

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

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

**Localization of \(\text{Ca}^{2+}\) 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, while 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 EM studies were successful to demonstrate the precise location of cac Ca\(^{2+}\) channels within the active zone. Non cac, PLTXII-sensitive Ca\(^{2+}\) channel that supports delayed quantal release in cac\(^{null}\) embryos may locate close to the release site as our observation of fast quantal release in minority of cells in cac\(^{null}\) embryos indicates (Fig. 4). Further remaining questions are; 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 DNMJ will continue to be one of favorable preparations to answer these questions.
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GRANTS

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**FIGURE LEGENDS**

**Figure 1.** Nerve-evoked synaptic currents in cacnull and controls

A1. Event frequency histogram after nerve stimulation in a cacnull embryo in HL3 solution with 1.5 mM [Ca²⁺]ₐ. Stimulus was given at 0 indicated on the abscissa and time after or before stimulus is indicated in ms. On the ordinate the number of events in 1 ms bin is shown after normalization for the number of stimuli. Sample traces are shown in the right panel. Only one event occurred in the top trace with a delay of approximately 25 ms.

A2. Event frequency histogram in a cacnull embryo in HL3 solution with 5 mM [Ca²⁺]ₐ. Delayed events starting around 10 ms after stimulation were evident. Sample traces are shown in the right panel.

B. Event frequency histogram in a control, cacWT-EGFP, embryo in HL3 with 1 mM [Ca²⁺]ₐ. Synchronous events starting at 5 ms after stimulation are shown. Sample traces are shown in the right panel.

C. Event frequency histogram in another control, cacnull/+, embryo in HL3 with 1 mM [Ca²⁺]ₐ. Synchronous events starting at 3 ms after stimulation are shown. Sample traces are shown in the right panel.

**Figure 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 [Ca²⁺]ₐ.

A2. Composite event frequency histogram constructed from the data obtained in 9 cells in cacnull embryos in the presence of 5 mM [Ca²⁺]ₐ.

B. Composite event frequency histogram constructed from the data obtained in 16 cells from cacWT-EGFP embryos in the presence of 1 mM [Ca²⁺]ₐ.

C. Composite event frequency histogram constructed from the data obtained in 10 cells from heterozygotic embryos, cacnull/+, in the presence of 1 mM [Ca²⁺]ₐ.

**Figure 3.** Bursting of quantal events during 10 Hz stimulation in 5 mM [Ca²⁺]ₐ in cacnull embryos

A. Traces showing bursting of quantal events. This bursting behavior was observed in a cell stimulated at 0.3 Hz in 5 mM [Ca²⁺]ₐ.

B. The latency histogram of the first event in a burst after stimulation in the same cell shown in A.

C. The latency histogram of delayed quantal events in the same cell. Some stimuli evoked bursts while others induced isolated quantal events as seen in majority of cells.

**Figure 4.** Rare, fast synchronized events in a cacnull embryo.
A. a cell in cacnull embryos at 0.3 Hz in 5 mM [Ca\(^{2+}\)]e had relatively frequent spontaneous as well as delayed release. Sample current traces are shown on the right. In this cell there were 9 quantal events during a 6-ms interval between 4 and 10 ms after the onset of stimulus pulse, which is significantly larger than 2.1±1.5 events/6 ms in the periods preceding stimulation. Stimuli were given 100 times at 0.3 Hz.

B. when stimulated at 10 Hz the same cell in A produced more frequent fast quantal events. These events are loosely synchronized to stimuli in most of cases with longer delays compared with those in control embryos. But some quantal events occurred during the period between 4 and 8 ms, which is similar to fast synchronized events seen in control embryos (Fig. 2B and C).

**Figure 5.** Mean frequencies of minis and quantal events in normal and high K\(^+\) saline in cacnull and control embryos.
A. Mean frequencies of minis in cacnull and control, cacWT-EGFP, embryos in HL3 with 1.5 mM [Ca\(^{2+}\)]e and 3µM TTX. An asterisk indicates a significant difference at 0.05.

B. Mean frequencies of quantal events in high K\(^+\) saline (HL3 with 62 mM K\(^+\)) in cacnull and control, cacWT-EGFP, embryos. An asterisk indicates a significant difference at 0.05.

**Figure 6.** Hypertonicity responses in cacnull and control, cacWT-EGFP, embryos.
A. Hypertonic solution (450 mM sucrose was added to Ca\(^{2+}\)-free HL3 solution) was puff-applied for 11 s in 15 cacnull embryos in Ca\(^{2+}\)-free HL3 and all data were combined. The induced events were individually counted.

B. The hypertonicity response in control, cacWT-EGFP, embryos. In 8 cells the response was examined and all data were combined.

C. The peak frequency of events recorded in cacnull (n=15) and control, cacWT-EGFP, (n=8) embryos. These values are statistically different at p=0.05.

D. The total number of events recorded in cacnull (n=9) and control, cacWT-EGFP, (n=5) embryos. These values are statistically different at p=0.05.
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A1

$cac^{null}$

1.5mM Ca$^{2+}$

A2

$cac^{null}$

5mM Ca$^{2+}$

B

Control, $cac^{WT-EGFP}$

1mM Ca$^{2+}$

C

Control, $cac^{null}/+$

1mM Ca$^{2+}$
A

$\text{cac}^{\text{null}}$

$0.3\text{Hz, 5mM Ca}^{2+}$

B

$\text{cac}^{\text{null}}$

$10\text{Hz, 5mM Ca}^{2+}$
A

Frequency (events/sec)

$\text{cac}^{\text{null}}$

$\text{cac}^{\text{WT-EGFP}}$

13

B

Frequency (events/sec)

$\text{cac}^{\text{null}}$

$\text{control, Canton-S}$

4