Relative Distribution of Ca\textsuperscript{2+} Channels at the Crayfish Inhibitory Neuromuscular Junction

Tariq N. Allana and Jen-Wei Lin

Department of Biology, Boston University, Boston, Massachusetts 02215

Submitted 22 March 2004; accepted in final form 7 May 2004

Allana, Tariq N. and Jen-Wei Lin. Relative distribution of Ca\textsuperscript{2+} channels at the crayfish inhibitory neuromuscular junction. J Neurophysiol 92: 1491–1500, 2004. First published May 12, 2004; 10.1152/jn.00287.2004. We investigated the Ca\textsuperscript{2+} channel-synaptic vesicle topography at the inhibitory of the crayfish (Procambarus Clarkii) neuromuscular junction (NMJ) by analyzing the effect of different modes of Ca\textsuperscript{2+} channel block on transmitter release. Initial identification of Ca\textsuperscript{2+} channels revealed the presence of two classes, P and non-P-type with P-type channels governing \(~70\%\) of the total Ca\textsuperscript{2+} influx. The remaining Ca\textsuperscript{2+} influx was completely blocked by Cd\textsuperscript{2+} but not by saturating concentrations of \(\omega\)-conotoxins MVIIIC and GVIA, or nifedipine and SNX-482. To examine the relative spatial distribution of Ca\textsuperscript{2+} channels with respect to synaptic vesicles, we compared changes in inhibitory postsynaptic current amplitude and synaptic delay resulting from different spatial profiles of [Ca\textsuperscript{2+}].

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: J.-W. Lin, 5 Cummington St., Boston, MA 02215 (E-mail: jenwelin@bu.edu).
Action potentials (APs) with prolonged duration are ideally suited to investigate the spatial relationship between Ca$^{2+}$ channels and synaptic vesicles for several reasons. First, broad APs generate an optimal condition for resolving synaptic delay because delay varies steeply in the region of low [Ca$^{2+}$]$_i$ (Bollmann et al. 2000; Schneggenburger and Neher 2000). Specifically, the prolonged initial depolarization of broad APs reduces the driving force for Ca$^{2+}$ and results in a small single-channel current and low [Ca$^{2+}$]$_i$ (Taschenberger and von Gersdorff 2000). Varying Ca$^{2+}$ influx when release is probed with broad APs therefore permits detection of changes in synaptic delay resulting from even small variations in local [Ca$^{2+}$]$_i$ (Lin and Faber 2002; Vyshedskiy et al. 2000). Second, by prolonging the duration of Ca$^{2+}$ influx, broad APs enable Ca$^{2+}$ channels located even far from releasable vesicles to activate release. Thus broad APs provide an opportunity to evaluate the distribution of distantly located Ca$^{2+}$ channels, which normally do not participate in release under physiological conditions.

In this report, we find that P- and non-P-type channels contribute unequally the [Ca$^{2+}$] relevant to release. Based on the analysis of amplitude and synaptic delay of release probed with broad and narrow APs, we conclude that although P-type channels are located closer to releasable vesicles than non-P-type channels, these channels overlap partially in their distribution.

METHODS

Preparation and electrophysiology

Crayfish, Procambarus clarkii, were obtained from Carolina Biological (Burlington, NC). Animals were maintained at room temperature, ~22°C, until use. All experiments were performed at room temperature. The typical size of the animals was ~2.5 in, head to tail. The opener muscle of the first walking leg was used for all experiments. A presynaptic electrode penetrated the inhibitory axon (inhibitor) to record APs and pressure inject Ca$^{2+}$-sensitive dye. The AP measuring electrode was 100–300 µm from the terminals on a central muscle at which fluorescence transients were measured. A suction electrode was used to stimulate the inhibitor. Two postsynaptic electrodes, 5–15 MΩ with 1.5–3 M KCl, penetrated a single muscle fiber. A two-electrode voltage-clamp amplifier (GeneClamp 500; Axon Instruments, Foster City, CA) was used to record inhibitory postsynaptic current (IPSC), filtered at 2 kHz.

Physiological saline contained (in mM) 195 NaCl, 5.4 KCl, 13.5 CaCl$_2$, 2.6 MgCl$_2$, and 10 HEPES, titrated to pH 7.4 by NaOH. Solutions with elevated Mg$^{2+}$ concentrations (10, 20, 40 mM) were made by adding MgSO$_4$ and removing the appropriate amount of NaCl to maintain the osmolarity of the solution. When tetraethy lammonium (TEA) chloride was introduced into the physiological saline, an equal amount of NaCl was removed. Saline containing 1 mM 4-aminopyridine (4-AP) and 20 mM TEA was considered the control solution for all toxin-treated experiments unless otherwise stated. Some narrow AP based experiments were carried out in physiological saline containing 0.5 mM 4-AP to enhance neurotransmitter release. Addition of 0.5 mM 4-AP lengthened the duration of the AP by ~100 µs. All chemicals were purchased from Sigma (St. Louis, MO). Toxins ω-Aga IVA, ω-conotoxins MVIIIC and GVIA, and SNX-482 were purchased from either Alamone Labs (Jerusalem, Israel) or Peptides International (Louisville, KY). Stock solutions of all toxins were made in dd H$_2$O, and stored at ~20°C until use.

Application of toxins

Solutions containing ω-Aga IVA at 1, 5, and 10 nM, as well as Mg$^{2+}$ and Cd$^{2+}$, were circulated through a 5-ml chamber four to five times to ensure equilibration and complete washout of previous solutions. Experiments using saturating concentrations of toxins (ω-Aga IVA, 100–200 nM; ω-conotoxins MVIIIC, 1.5 µM; ω-conotoxin GVIA, 2 µM; Nifedipine, 5 µM and SNX-482, 100 nM) were carried out in custom-made chambers with a volume of ~1 ml. Toxins were applied directly above the preparation. The solution in the chamber was then mixed gently using a micropipette. It is likely that the immediate concentration of toxin the preparation was exposed to was greater than the final concentration, which was calculated based on the volume of the chamber. In all cases, the effect of the drugs remained constant for up to 1 h after application.

Photometric measurement of calcium transients

The inhibitory axon was penetrated with an electrode containing 2.5–5 mM Magnesium Orange K$^+$ salt (MgOrg, K$_0$ = 12 µM for Ca$^{2+}$) and 400 mM K$^+$ Methansulphonate, with a final resistance of 15–30 MΩ. The dye was pressure injected until varicosities close to the injection site were clearly visible. Experiments commenced 15–20 min after dye injection was completed, i.e., after the fluorescence level had stabilized. We visually compared and photographed the fluorescence intensity of the injected axon with a micropipette of similar diameter filled with dye of known concentration. The calibration solution was prepared by dissolving the dye in a standard buffer with a final [Ca$^{2+}$] of 100 nM (Ca$^{2+}$ Calibration Buffer Kit with 1 mM Mg$^{2+}$, Molecular Probes, C3721). With this criterion, a typical injection resulted in an intra-axonal concentration diluted by a factor of ~10 compared with the solution in the injection electrode, i.e., a final concentration of 200–400 µM.

When the effect of EGTA was investigated, 20 mM EGTA was included in the axon electrode, along with 2.5 mM MgOrg and 400 mM K$^+$ methansulphonate. This solution was pressure injected into the inhibitory axon. We assumed that EGTA was diluted to a similar extent as MgOrg (~10 times), i.e., to a final EGTA concentration of ~2–4 mM.

Photometric measurement of Ca$^{2+}$ transients in this preparation has been described previously (Vyshedskiy and Lin 2000). Briefly, a photodiode (S5973-01; Hamamatsu, Bridgewater, NJ) was used to record fluorescence transients on an upright microscope (Axioskop; Zeiss, Oberkochen, Germany) with ×40 or ×60 water-immersion lens. The photocurrent was measured using a single-channel head stage, coupled to Geneclamp 500. A 150-W Xenon lamp was powered by an Optiquip 1600 power supply with 770 Lamphouse. Illumination was gated by a shutter (Uniblitz; Vincent Associates) with a typical duration of ~500 ms and repeated at 0.3 Hz. The specifications of the filter set were as follows: excitation, D535/45; dichroic, 570DRLP; emission, OG590 (Omega Optical, Brattleboro, VT). The area of illumination was restricted by an iris diaphragm customized to allow an illumination diameter of ~50 µm with a ×40 objective, which typically encompassed one to five varicosities on the upper surface of a central muscle fiber. Fluorescence transients are presented as $\Delta F/F_0 = (F(t) - F_{rest})/F_{rest}$, where $F_{rest}$ represents the fluorescence intensity of stained varicosities in the absence of activity. Typically, corrections were not made for background fluorescence levels in unstained region.

Data analysis

All traces used are the average of 80–120 traces. Averaged control IPSC amplitude varied considerably [43.7 ± 24.9 (SD) nA, n = 53] from preparation to preparation. Standard deviations of 100 ms (2,000 points) of IPSC baseline were used to estimate background noise. In randomly chosen experiments, the SD was very low (0.13 ± 0.01 nA,
**RESULTS**

**ω-Aga IVA eliminates release but not Ca<sup>2+</sup> transients**

Because previous voltage-clamp studies have suggested that only P-type channels are present at the inhibitory NMJ (Wright et al. 1996), we used the photometric method to examine the effect of ω-Aga IVA on the presynaptic Ca<sup>2+</sup> influx. Saturating concentration of ω-Aga IVA (100 nM) completely eliminated release (2.9 ± 1.4% of control, n = 6, Fig. 1A1), but presynaptic Ca<sup>2+</sup> influx persisted at ~40% (Fig. 1A2). The toxin also modified the shape of the AP by slightly enhancing the afterdepolarization (Fig. 1B3, ↓), presumably by reducing Ca<sup>2+</sup>-activated outward currents. The mechanism underlying the difference in shapes of the APs was not pursued. Similar results were observed in four preparations. Recordings in Fig. 1B show synaptic transmission and Ca<sup>2+</sup> influx in response to the first AP on an expanded time scale.

Next, release and Ca<sup>2+</sup> influx were examined in saline containing the K<sup>+</sup> channel blockers TEA (20 mM) and 4-AP (1 mM). ω-Aga IVA (100 nM) failed to completely block release (55.0 ± 5.5% of control, n = 4) although synaptic delay was significantly increased (2.3 ± 0.3 ms, n = 4, Figs. 2A1 and 6B). Ca<sup>2+</sup> transients in control and toxin conditions had a similar onset (Fig. 2A2, ↓), but strikingly different rising phases. The slower rising phase of the Ca<sup>2+</sup> transient in the presence of ω-Aga IVA suggests a smaller Ca<sup>2+</sup> influx, confirming the block of Ca<sup>2+</sup> channels by the toxin (Fig. 2, A2). The Ca<sup>2+</sup> transient peak amplitude decreased slightly (84.7 ± 6.0% of control, n = 6), presumably due to the substantial increase in AP duration (Fig. 2A3). Bath application of 1 mM Cd<sup>2+</sup> led to complete suppression of release and Ca<sup>2+</sup> transients in four preparations. These results are contrary to (Wright et al. 1996) in which 100 nM ω-Aga IVA completely blocked I<sub>Ca</sub>, and release activated by a 6-ms voltage step.

The prolongation of AP duration probably reflects a Ca<sup>2+</sup>-activated Cl<sup>−</sup> current, rather than I<sub>K(Ca)</sub>, because the latter would be blocked in the presence of 20 mM TEA. Cd<sup>2+</sup> applied in the absence of ω-Aga IVA also increased the duration of the AP (n = 4), suggesting that the increased AP duration accompanying ω-Aga IVA results because of the decrease in a Ca<sup>2+</sup>-activated current rather than from an unknown effect of the toxin.

To account for the difference in AP durations between control and ω-Aga IVA conditions (Fig. 2A3), we compared release and Ca<sup>2+</sup> influx while the presynaptic waveforms were similar. Specifically, we measured Ca<sup>2+</sup> transient amplitude 2.5 ms after the steepest point on the rising phase of the AP, a time point before APs diverged significantly (<5 nV; Fig. 2A, - - -) (Vyshedskiy et al. 2000). IPSP amplitude was measured 0.5 ms after the Ca<sup>2+</sup> transient amplitude measurement to allow for synaptic delay (Vyshedskiy et al. 2000). Using these criteria, changes in Ca<sup>2+</sup> influx and release can be attributed solely to the mode or extent of the Ca<sup>2+</sup> channel block. We found that addition of 100 nM ω-Aga IVA blocked Ca<sup>2+</sup> transients to 30.2 ± 3.3% (n = 6) of control values, a level similar to that observed with narrow APs (37.5 ± 3.6% of control, n = 4, 0.1<P<0.2). IPSP amplitude at 3 ms was also completely suppressed (1.0 ± 0.6% of control, n = 4) with broad APs, similar to the results when release was probed with narrow APs. The same criteria mentioned in the preceding text were used for all recordings obtained with broad APs.

The incomplete block of IPSPs and Ca<sup>2+</sup> transient is unlikely to be due to poor access of toxin to Ca<sup>2+</sup> channels. Figure 2B illustrates the time course of the toxin’s effect in a different preparation from that shown in Fig. 2A. Release and

![FIG. 1. ω-Aga IVA (100 nM) completely eliminates release but not Ca<sup>2+</sup> influx in physiological saline. A. 1–3: inhibitory postsynaptic potentials (IPSPs), Ca<sup>2+</sup> transients, and presynaptic action potentials (APs), respectively, in the absence (−−−) or presence of 100 nM ω-Aga IVA (· · ·). B: release and Ca<sup>2+</sup> transients evoked by the 1st AP displayed at higher time resolution. Although release probed by narrow APs is completely eliminated (B1), a significant fraction of the Ca<sup>2+</sup> transient still remains (B2), suggesting the presence of ω-Aga IVA-resistant channels. The arrow (↓) indicates that the toxin slightly increased the afterdepolarization (B3). All recordings are an average of 100 trials.](http://jn.physiology.org/lookup/doi/10.1152/jn.01330.2000)
Ca\(^{2+}\) influx were blocked to their final levels instantaneously and remained at these values for up to 60 min (Fig. 2B, top). \(\omega\)-Aga IVA could not be washed out. The fast onset and stability of block, as well as the immediate increase in AP duration (Fig. 2B, bottom), suggest that the toxin had good access to the channels. Increasing toxin concentration to 200 nM did not further increase the Ca\(^{2+}\) transient block (23.0 ± 5.2% of control, \(n = 3\)) already achieved by 100 nM \(\omega\)-Aga IVA (0.2 < \(P < 0.4\)). Finally, \(\omega\)-Aga IVA purchased from two different vendors, Alamone Labs (\(n = 6\)) and Peptides International (\(n = 5\)), provided the same results. The persistence of Ca\(^{2+}\) influx even after saturating concentrations of toxin were applied argues for the presence of more than one type of pharmacologically defined Ca\(^{2+}\) channel (see METHODS for details on toxin application).

To characterize the \(\omega\)-Aga IVA resistant channels, we tested for the presence of N-, L-, and R-type (only Cav2.3) channels. N- and L-type channels have been found in Aplysia (Edmonds et al. 1990) and in the crayfish swimmeret motorneuron (Chrachri 1995). We used the N-type channel blocker GVIA (2 \(\mu\)M), P/Q-type blocker MVIIIC (1.5 \(\mu\)M), L-type blocker nifedipine (5 \(\mu\)M), and R-type channel blocker SNX-482 (100 nM). All toxins were tested with broad APs. \(\omega\)-conotoxins GVIA and MVIIIC had no effect on release or Ca\(^{2+}\) influx, and remained at these values for up to 60 min (Fig. 2.). Although the onset of the Ca\(^{2+}\) transients are the same (\(\downarrow\), A2), the slope of the rising phase of the Ca\(^{2+}\) transient is shallower than control, suggesting a smaller Ca\(^{2+}\) influx. More, 2.5 ms after the steepest point on the rising phase of the AP, the time point at which Ca\(^{2+}\) transient amplitude was measured. B: \(\omega\)-Aga IVA reaches synapses instantaneously. Top: time course of 100 nM \(\omega\)-Aga IVA-mediated block of Ca\(^{2+}\) transient (2.5 ms) and release (3 ms). \(\downarrow\), toxin application. Bottom: time course of increase in AP duration measured at −20 mV. Recordings remained stable for up to 60 min. All recordings were made in the presence of 1 mM 4-aminopyridine (4-AP), 20 mM TEA and 100 nM \(\omega\)-Aga IVA. Traces in A are an average of 100 trials.

**FIG. 2.** \(\omega\)-Aga IVA (100 nM) does not completely eliminate release or Ca\(^{2+}\) transients probed by broad APs. A: I–3, IPSPs, Ca\(^{2+}\) transients, and presynaptic APs, respectively, in the absence (−) and presence of 100 nM \(\omega\)-Aga IVA (⋯⋯). Release in the presence of the toxin is significantly delayed and reduced (A1). Ca\(^{2+}\) transient peak amplitude remains unaffected after toxin application (A2), presumably due to the increased AP duration (A3).

**Mg\(^{2+}\) and \(\omega\)-Aga IVA reduce release, Ca\(^{2+}\) influx, and increase synaptic delay**

Mg\(^{2+}\) and \(\omega\)-Aga IVA allow us to alter the local [Ca\(^{2+}\)]\(_{i}\) profiles in two distinct ways. Specifically, addition of Mg\(^{2+}\) effectively reduces the average single-channel current by a flickering block mechanism (Lansman et al. 1986), while \(\omega\)-Aga IVA stabilizes channels in the closed state (McDonough et al. 1997) and therefore completely eliminates single-channel current. (Reducing [Ca\(^{2+}\)]\(_{i}\) by Mg\(^{2+}\) substitution was not used because this procedure introduced uncontrolled spontaneous firing of the inhibitor and muscle contraction in some preparations.) We found that increasing [Mg\(^{2+}\)]\(_{i}\) (10, 20, and 40 mM) led to a progressive shallowing of the slope of the rising phase of the Ca\(^{2+}\) transient. Peak Ca\(^{2+}\) transient amplitude was also reduced at all [Mg\(^{2+}\)]\(_{i}\). In addition, a decrease in peak IPSC amplitude was observed. A detectable increase in synaptic delay also occurred in all Mg\(^{2+}\)-treated preparations (Fig. 4, insets). This increase in synaptic delay did not appear to be due to the slight increase in AP duration because the changes in delay occurred well before the AP diverged significantly.

Increasing concentrations of \(\omega\)-Aga IVA (1, 5, and 10 nM) also led to a significant decrease in the slope of the rising phase of the Ca\(^{2+}\) transient (Fig. 5, middle). Because the onset of the Ca\(^{2+}\) transient was unaffected by the addition of \(\omega\)-Aga IVA, the rate of non-P-type channel opening should be comparable to that of P-type channels. Ca\(^{2+}\) transients measured at 2.5 ms were substantially reduced compared with control conditions, even though the peak Ca\(^{2+}\) transient remained unchanged. The lack of any effect on the peak Ca\(^{2+}\) transient in the presence of \(\omega\)-Aga IVA can be explained by the toxin’s ability to increase the duration of the AP (Fig. 5, bottom). Peak IPSC amplitude was reduced, despite the fact that the peak Ca\(^{2+}\) transient peaks remained unaffected by the addition of \(\omega\)-Aga IVA.
Addition of \(\omega\)-Aga IVA at all tested concentrations increased synaptic delay significantly (Fig. 5, insets). As in the case of \(\text{Mg}^{2+}\) block, this increase in delay could not be attributed to changes in AP duration because delay occurred either before or right around when the AP diverged significantly from control (Fig. 5C, dashed vertical line). The increase in delay was similar for 10 and 100 nM \(\omega\)-Aga IVA (0.1 < \(P < 0.2\), Fig. 6B), suggesting that at 10 nM the toxin reached near saturating levels. The effectiveness of \(\omega\)-Aga IVA block of \(\text{Ca}^{2+}\) influx at concentrations as low as 1 nM suggests that the toxin targets P- rather than Q-type channels (see Discussion).

**Comparison of \(\text{Mg}^{2+}\) and \(\omega\)-Aga IVA block of IPSC and synaptic delay**

We first evaluated the relationship between \(\text{Ca}^{2+}\) influx and IPSC amplitude (Mintz et al. 1995; Wu et al. 1999). A supra-linear relationship emerged when \(\text{Ca}^{2+}\) influx was blocked by either \(\omega\)-Aga IVA or \(\text{Mg}^{2+}\) (Fig. 6A). The \(\text{Mg}^{2+}\)
data were well fit by the Hill equation, yielding a Hill coefficient of 3.87. However, despite its supralinearity, we were unable to fit the ω-Aga IVA data set with the Hill equation.

Compared with the changes in IPSC amplitude, Δdelay appeared to be more sensitive to changes in Ca^{2+} influx. [ΔDelay is the difference in synaptic delay, measured 5 SD below baseline (see METHODS), before and after addition of the Ca^{2+} channel blockers.] This was most clearly evident for 5 and 10 nM ω-Aga IVA conditions. Although IPSC amplitude [3.8 ± 0.5% of control, n = 5 for 5 nM (Fig. 6A, Y), and 3.0 ± 1.3% of control, n = 9 for 10 nM (Fig. 6A, †)] was not significantly different at these concentrations (0.5 < P < 0.9, Fig. 6A), Δdelay increased from 1.3 ± 0.03 ms (n = 5) to 1.9 ± 0.09 ms (n = 9) in 10 nM ω-Aga IVA (P < 0.001, Fig. 6B). We therefore also evaluated Δdelay in the presence of Mg^{2+} and ω-Aga IVA.

For small changes in Ca^{2+} influx (10 mM, 20 mM Mg^{2+} or 1 nM ω-Aga IVA), Mg^{2+} and ω-Aga IVA increased Δdelay by comparable amounts, suggesting that selectively blocking P-type channels did not result in a reduction in local [Ca^{2+}], any more than decreasing the single-channel current with Mg^{2+}. Therefore non-P-type channels could “compensate” for the loss of P-type channels at these toxin concentrations, suggesting that these channels overlap. However, Δdelay differed significantly (P < 0.001) between 5 nM ω-Aga IVA and 40 mM Mg^{2+}, even though Ca^{2+} influx reduction in these conditions was similar (0.5 < P < 0.9, data points in --- box). This observation is consistent with the significantly lower IPSC amplitude in 5 nM ω-Aga IVA [3.8 ± 0.5% of control (n = 5), †] compared with 40 mM Mg^{2+} [11.5 ± 1.3% of control (n = 17), P < 0.01, Fig. 6A, *]. Thus because the onset of channel opening appear to be similar for both types of channels (Figs. 2 and 5), the difference in Δdelay is best attributed to difference in local [Ca^{2+}], and the separation between Ca^{2+} source and releasable vesicles. We conclude that though P-type channels are probably located closer than non-P-type to synaptic vesicles, these channels overlap in their distributions.

**EGTA reduces IPSC amplitude but does not increase synaptic delay**

To further test the hypothesis that P-type channels are closer than non-P-type channels to releasable vesicles, we examined the effect of the slow Ca^{2+} buffer EGTA on transmitter release using narrow APs. Because Ca^{2+} influx during narrow APs is short lived, we reasoned that Ca^{2+} influx through only those channels closest to releasable vesicles would be able to trigger release. Therefore because of its slow kinetics, EGTA would be unable to affect release. Fig. 7A, inset, illustrates that EGTA could not inhibit the IPSC. The presence of EGTA was confirmed by reduction in facilitation, as well as a slight increase in the afterdepolarization of the AP (data not shown). The same observation was made in three preparations.

This distribution of P- and non-P-type channels was further tested by evaluating the effect of EGTA on release activated by broad APs. Specifically, we reasoned that synaptic delay would not be affected in the presence of EGTA when release was dominated by P-type channels. Similar to the situation with narrow APs, the proximity of P-type channels to releasable vesicles, as well as EGTA’s slow on-rate, would prevent effective buffering of Ca^{2+} ions by EGTA before the onset of release. Figure 7B shows that in fact synaptic delay remained unchanged before (1.7 ± 0.1 ms, n = 4) and after EGTA injection (1.8 ± 0.1 ms, n = 4, 0.5 < P < 0.9).

The proposed distribution of Ca^{2+} channels also predicts that in the presence of EGTA synaptic delay should be impacted more severely when non-P-type channels predominate as the source of Ca^{2+} influx. In this situation, EGTA will effectively buffer Ca^{2+} influx given that non-P-type channels are located further from releasable vesicles. We applied 5 nM ω-Aga IVA (block of ~75% of P-type channels) and measured...
the changes in delay (Δdelay) in the presence and absence of EGTA. ω-Aga IVA (5 nM) alone yielded a Δdelay of 1.3 ± 0.03 ms (n = 5). In separate preparations, when the inhibitor was applied with EGTA, 5 nM ω-Aga IVA mediated Δdelay was 2.1 ± 0.3 ms (n = 6; Fig. 7C). This difference in Δdelay before and after EGTA was significant (P < 0.05). Therefore EGTA’s selective lengthening of synaptic delay dominated by non-P-type channels is consistent with our hypothesis.

**DISCUSSION**

Evaluating the effect of Ca²⁺ channel blockers on release is a widely used method to determine the distribution of Ca²⁺ channels and releasable vesicles when multiple Ca²⁺ channels contribute to synaptic transmission (Dietrich et al. 2003; Mintz et al. 1995; Wu et al. 1999). Specifically, the effect of Ca²⁺ channel blockers on synaptic transmission is determined when blockers are applied either together or in isolation. When the sum of the percentage block of synaptic transmission resulting from application of blockers individually is >100%, it is taken to suggest that multiple classes of Ca²⁺ channels govern release at individual release sites. The success of this approach depends critically on the availability of selective blockers for all types of Ca²⁺ channels involved in release. In this report, we uncover the presence of two classes of Ca²⁺ channels, P and non-P type, at the inhibitory crayfish NMJ. Because non-P-type channels are insensitive to any of the known Ca²⁺ channel blockers, we are unable to use the above-stated pharmacological approach. Instead, using broad APs, we demonstrate that the Ca²⁺ channel-synaptic vesicle topography can be inferred by analyzing changes in synaptic delay (Δdelay) resulting from the nonspecific (Mg²⁺) or selective (ω-Aga IVA) block of Ca²⁺ channels.

Application of Mg²⁺ and ω-Aga IVA resulted in similar increases in Δdelay when total Ca²⁺ influx was blocked <40%. This suggests that P- and non-P-type channels overlap considerably in their distribution. However, because Δdelay was considerably longer in the presence of ω-Aga IVA than Mg²⁺ when total Ca²⁺ influx block was ~50%, we propose that synaptic vesicles are located closer to P- than non-P-type channels. Further support for this conclusion comes from the findings that I) although non-P-type channels contribute ~30% Ca²⁺ influx, they do not trigger release in physiological conditions and 2) EGTA can prolong synaptic delay mediated by non-P-type but not P-type channels when release is activated by broad APs.

**Use of fluorescence transients as an indicator of Ca²⁺ influx**

Fluorescence transients have been used as reliable indicators of Ca²⁺ influx at many mammalian synapses (Atluri and Regehr 1996; Wu et al. 1999). For these transients to accurately represent Ca²⁺ influx, errors arising from dye saturation must be considered. We examined this issue for the time point at which Ca²⁺ transient measurements were made, i.e., 2.5 ms after the steepest point on the rising phase of the AP. We believe our measurement is reliable for several empirical reasons. First, given that a single narrow AP raises intracellular-free Ca²⁺ by tens of nanomolar (unpublished data; Tank et al. 1995) at the crayfish NMJ, it is unlikely that Ca²⁺ influx would saturate MgOrg (Kₐ = 12 μM for Ca²⁺). Because 100 nM ω-Aga IVA blocks Ca²⁺ transients activated by narrow and broad APs (at 2.5 ms) by a similar amount (~30%), we conclude that potential errors due to MgOrg saturation at 2.5 ms are unlikely. Second, we found that the percent reduction of Ca²⁺ transient measured at 2.5 ms was linearly related to the percent reduction of Ca²⁺ influx measurement 50 ms after a burst of 10 narrow APs, under the condition of increasing [Mg²⁺]o. The latter time point represents a condition in which not only is the [Ca²⁺]o, unlikely to saturate MgOrg, but Ca²⁺, MgOrg, and endogenous buffers are in spatial and chemical equilibrium. The linearity of the relationship therefore also suggests that Ca²⁺ transients measured at 2.5 ms are a reasonable indicator of the fractional change in Ca²⁺ influx.

**P- and non-P-type channels govern release the crayfish NMJ**

In the presence of up to 200 nM ω-Aga IVA, Ca²⁺ influx at the inhibitory terminals was reduced to ~30% of control...
values, confirming that the majority of channels at this synapse are P type. This result contradicts previous work at this synapse that showed that 100 nM ω-Aga IVA completely eliminated \(I_{\text{Ca}}\) (Wright et al. 1996). These conflicting results could be due to the different methods used to detect \(\text{Ca}^{2+}\) influx (fluorescence imaging vs. presynaptic voltage clamp) and/or the presence of \(\text{Ca}^{2+}\)-activated \(\text{Ca}^{2+}\) release. However, the latter possibility is unlikely because depletion of intracellular stores of \(\text{Ca}^{2+}\) with thapsigargin (2 μM) did not significantly change the amplitude or time course of the fluorescence transient (unpublished data; Tang and Zucker 1997), indicating that the \(\text{Ca}^{2+}\) transients recorded here result solely from \(\text{Ca}^{2+}\) influx through voltage-gated channels.

Our conclusion that ω-Aga IVA-sensitive channels are P rather than Q type is based on a report (Randall and Tsien 1995) that showed that ω-Aga IVA selectively blocks only P-type channels at low nanomolar concentrations. At the crayfish NMJ 1 nM ω-Aga IVA blocks ~37% of total \(\text{Ca}^{2+}\) influx, which corresponds to ~50% block of ω-Aga IVA-sensitive channels. This estimated \(K_p\) of ω-Aga IVA is identical to that found in rat cerebellar Purkinje cell (Mintz et al. 1992).

To identify the ω-Aga IVA-resistant \(\text{Ca}^{2+}\) channels, we applied ω-conotoxins GVIA and MVIIC, SNX-482, and nifedipine. None of these toxins had any effect on AP shape, peak \(\text{Ca}^{2+}\) transient, or the time course or amplitude of IPSP (Fig. 3). These data suggest that the non-P-type channels are not N-, R-, or L-type \(\text{Ca}^{2+}\) channels. The ineffectiveness of ω-conotoxin GVIA and ω-conotoxin MVIIC could be due to the high-divalent cation concentration (~15 mM) of the crayfish control solution (Albillos et al. 1996; Hernandez-Guijo et al. 1998; Liang and Elmslie 2002). To address this issue, we incubated the preparation in saturating concentrations of ω-conotoxins GVIA and MVIIC in \(\text{Ca}^{2+}\)- and Mg\(^{2+}\)-free saline for 5–20 min and then tested for the action of the toxin on returning to control saline. During the incubation period, the divalent cation free saline depolarized the presynaptic axon by >10 mV and caused spontaneous muscle contraction, thereby preventing accurate interpretation of the results.

**\(\text{Ca}^{2+}\) channels—synaptic vesicle organization at the crayfish inhibitor**

To determine the \(\text{Ca}^{2+}\) channel–synaptic vesicle topography, we first evaluated the relationship between IPSC amplitude and \(\text{Ca}^{2+}\) influx (Fig. 6A). IPSC amplitude varied nonlinearly as a function of \(\text{Ca}^{2+}\) influx, regardless of whether \(\text{Ca}^{2+}\) influx was blocked by reducing single-channel current or by decreasing the number of channels. This finding shows that
selective block of P-type channels, like Mg$^{2+}$, reduces $[\text{Ca}^{2+}]$, at the release site gradually, thus supporting a topography in which multiple Ca$^{2+}$ channels trigger vesicle fusion. Additional evidence for this topography came from the relationship between $\Delta$delay and Ca$^{2+}$ influx. Specifically, $\Delta$delay in 10 mM, 20 mM Mg$^{2+}$ and 1 nM α-Aga IVA were similar for comparable changes in total Ca$^{2+}$ influx (Fig. 6B). The best interpretation of this observation is that the Ca$^{2+}$ sensors on the vesicles responds to a summed Ca$^{2+}$ signal from both P- and non-P-type channels.

The significant difference in $\Delta$delay in the presence of 40 mM Mg$^{2+}$ and 5 nM α-Aga IVA (Fig. 6B), - - -), despite the comparable changes in Ca$^{2+}$ influx, suggest that the two classes of Ca$^{2+}$ channels regulating release do not contribute equally to rapid synaptic transmission. Specifically, the inability of non-P-type channels to sustain release when ~75% of P-type channels are blocked (5 mM α-Aga IVA), places non-P-type channels further away than P-type channels from the synaptic vesicle. This distribution of P- and non-P-type channels is consistent with additional findings that 100 nM α-Aga IVA completely eliminated release in physiological saline (Fig. 1), EGTA did not affect fast synaptic transmission when narrow APs were used [Fig. 7A; EGTA was also unable to suppress release mediated by the first AP of a train at the crayfish excitatory NMJ (Hochner et al. 1991)], and EGTA increased $\Delta$delay only when a majority of P-type channels were blocked (Fig. 7C). In conclusion, our results suggest the presence of at least two pharmacologically distinct Ca$^{2+}$ channel populations at the crayfish inhibitory NMJ: P- and non-P-type channels. P-type channels appear to be located closer than non-P-type channels to synaptic vesicles, and dominate synaptic transmission under physiological conditions.

Implications of multiple domains for the affinity of the Ca$^{2+}$ sensor

The effect of EGTA on release, as well as the differing release probabilities at the crayfish NMJ and calyx of Held, helps constrain the nature of the Ca$^{2+}$ sensor at the crayfish NMJ. Specifically, whereas 2–4 mM EGTA does not affect release at the crayfish NMJ, EGTA at concentrations even as low as 1 mM can reduce release at the calyx to ~60% of control values (Borst and Sakmann 1996). This result argues that Ca$^{2+}$ channels couple more closely to releasable vesicles at the crayfish NMJ than at the calyx. However, the release probability at the crayfish NMJ is known to be lower than at the calyx because the former is a strongly facilitating synapse whereas the latter exhibits pronounced depression (Atwood and Wojtowicz 1986; Borst et al. 1995). Taken together, lower release probability and closer coupling of Ca$^{2+}$ channels with synaptic vesicles at the crayfish NMJ suggests that the affinity of the Ca$^{2+}$ sensor at the this synapse is probably lower than that of the calyx of Held (~10 μM, Bollmann et al. 2000; ~25 μM, Schneegansburger and Neher 2000).

Multiple classes of Ca$^{2+}$ channels contribute to release

Multiple classes of Ca$^{2+}$ channels are found in many preparations, such as the rat cerebellum (Mintz et al. 1995; Reggehr and Mintz 1994), calyx of Held (Wu et al. 1999), mouse hippocampus (Qian and Noebels 2001), mossy fiber terminals (Castillo et al. 1994; Honda et al. 2000), crayfish excitator (Troncone et al. 2003), and the crab slow and fast closer excitator muscles (Rathmayer et al. 2002). Similar to the results presented here, P-type channels found in these preparations control a majority of release (>80%), despite the fact that non-P-type channels contribute a sizable fraction of Ca$^{2+}$ influx (50–70%). The effectiveness of P-type channels in triggering release is attributed to their proximity to synaptic vesicles. Such placement of P-type channels correlates well with mounting evidence that these channels contain a “synaptic protein interaction” or synprint site (Mochida et al. 2003; Rettig et al. 1996). A similar interaction might exist at the inhibitory crayfish neuromuscular junction between P-type channels and synaptic vesicles, although additional experiments are required to validate this possibility.

The functional significance of non-P-type channels to synaptic vesicles in our preparation is intriguing. Given the proximity and the significant fraction (~30%) of non-P-type channels, it is reasonable to assume that they are involved in processes that require high [Ca$^{2+}$], around the active zone. The range of possibilities includes, but are not limited to, being involved in supplying a “facilitation sensor” (Matveev et al. 2002; Tang et al. 2000) or local buffer (Felmy et al. 2003) with adequate Ca$^{2+}$ to initiate the facilitation process. Alternatively, non-P-type channels might be required for regulation of Ca$^{2+}$, for long-term synaptic strength (Breustedt et al. 2003; Dietrich et al. 2003), such as augmentation and post-tetanic potentiation, both of which are present at the crayfish NMJ. It is also possible that Ca$^{2+}$ entering through these channels might be important for the regulation of endocytosis during periods of sustained activity (Beutner et al. 2001). Last, modulation of non-P-type channels, either by affecting their numbers or their coupling with synaptic vesicles, might also serve as a mechanism for finer control of transmitter release (Zamponi and Snutch 1998).

Acknowledgments

We thank I. Mintz and S. McDonough for insightful suggestions and advice. We also thank J. Kim for helping with data entry and A. Hooper for helping proofread this manuscript.

Grants

This work was supported by National Institutes of Health Grant 31707 to J.-W. Lin.

References


Albillos A, Garcia AG, Olivera B, and Gandia L. Re-evaluation of the P/Q Ca$^{2+}$ channel component of Ba$^{2+}$ currents in bovine chromaffin cells superfused with solutions containing low and high Ba$^{2+}$ concentrations. Pfluegers 432: 1030–1038, 1996.

Athluri PP and Regehr WG. Determinants of the time course of facilitation at the granule cell to Purkinje cell synapse. J Neurosci 16: 5661–5671, 1996.


CA$^{2+}$ CHANNEL DISTRIBUTION AT CRAYFISH NMJ

J Neurophysiol • VOL. 92 • SEPTEMBER 2004 • www.jn.org


