

# Calcium Channel Types with Distinct Presynaptic Localization Couple Differentially to Transmitter Release in Single Calyx-Type Synapses

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We studied how  $\text{Ca}^{2+}$  influx through different subtypes of  $\text{Ca}^{2+}$  channels couples to release at a calyx-type terminal in the rat medial nucleus of the trapezoid body by simultaneously measuring the presynaptic  $\text{Ca}^{2+}$  influx evoked by a single action potential and the EPSC. Application of subtype-specific toxins showed that  $\text{Ca}^{2+}$  channels of the P/Q-, N-, and R-type controlled glutamate release at a single terminal. The  $\text{Ca}^{2+}$  influx through the P/Q-type channels triggered release more effectively than  $\text{Ca}^{2+}$  influx through N- or R-type channels. We investigated mechanisms that contributed to these differences in effectiveness. Electrophysiological experiments suggested that individual release sites were controlled by all three subtypes of  $\text{Ca}^{2+}$  channels. Immunocytochemical staining indi-

cated, however, that a substantial fraction of N- and R-type channels was located distant from release sites. Although these distant channels contributed to the  $\text{Ca}^{2+}$  influx into the terminal, they may not contribute to release. Taken together, the results suggest that the  $\text{Ca}^{2+}$  influx into the calyx via N- and R-type channels triggers release less effectively than that via P/Q-type because a substantial fraction of the N- and R-type channels in the calyx is localized distant from release sites.

**Key words:**  $\text{Ca}^{2+}$  channels; presynaptic; calyx of Held; voltage clamp; antibody; immunocytochemistry; P-type  $\text{Ca}^{2+}$  channels; N-type  $\text{Ca}^{2+}$  channels; R-type  $\text{Ca}^{2+}$  channels; synaptotagmin; fura-2; rat

Fast neurotransmitter release depends strongly on the extracellular  $\text{Ca}^{2+}$  concentration (Dodge and Rahamimoff, 1967; Katz, 1969). By changing the  $\text{Ca}^{2+}$  influx through all presynaptic  $\text{Ca}^{2+}$  channels, a quantitative estimate of the relation between intracellular  $\text{Ca}^{2+}$  concentration at the release sites ( $[\text{Ca}_{\text{site}}]$ ) and release may be obtained. This relation is often approximated by:

$$\text{Release} \propto [\text{Ca}_{\text{site}}]^n \quad (1)$$

where  $n$  is between 3 and 4 (for review, see Wu and Saggau, 1997).

$\text{Ca}^{2+}$  channel subtypes have been classified as T-, L-, N-, P-, Q-, and R-type on the basis of their different pharmacological and biophysical properties (Dunlap et al., 1995). Different  $\text{Ca}^{2+}$  channel subtypes have different pore-forming  $\alpha_1$  subunits. The  $\alpha_{1A}$  subunits form P/Q-type,  $\alpha_{1B}$  subunits form N-type, and  $\alpha_{1E}$  subunits form R-type  $\text{Ca}^{2+}$  channels (Dunlap et al., 1995). Most of these  $\text{Ca}^{2+}$  channel subtypes can be blocked by specific toxins. These toxins have a high affinity for  $\text{Ca}^{2+}$  channels and presumably unbind from  $\text{Ca}^{2+}$  channels much slower than inorganic  $\text{Ca}^{2+}$  channel blockers such as  $\text{Cd}^{2+}$  (Yoshikami et al., 1989; Mintz et al., 1995). The toxins can be used to measure the contribution of the different channel subtypes to transmitter release. If both the volume-averaged presynaptic  $\text{Ca}^{2+}$  concentra-

tion ( $[\text{Ca}_{\text{terminal}}]$ ) and release are monitored in experiments in which a  $\text{Ca}^{2+}$  channel subtype is specifically blocked, an estimate for the effectiveness ( $m$ ) of that subtype in controlling transmitter release (Wu and Saggau, 1997) can be obtained from:

$$\text{Release} \propto [\text{Ca}_{\text{terminal}}]^m \quad (2)$$

Relations 1 and 2 have a similar format but different physical meanings. The parameter  $m$  is defined here as a measure of how effectively  $\text{Ca}^{2+}$  influx into a terminal via a particular subtype of  $\text{Ca}^{2+}$  channels triggers release. The parameter  $n$ , most likely, is a measure of the cooperative binding of intracellular  $\text{Ca}^{2+}$  to the  $\text{Ca}^{2+}$  sensor at a release site (Dodge and Rahamimoff, 1967).

The values of  $m$  for different subtypes of  $\text{Ca}^{2+}$  channels may differ in single terminals and can be different from  $n$  (Mintz et al., 1995; Wu et al., 1998). These differences may provide information about the spatial organization of release sites and  $\text{Ca}^{2+}$  channels. For example, if each release site is controlled by the  $\text{Ca}^{2+}$  domain near a single open  $\text{Ca}^{2+}$  channel, reducing the number of active  $\text{Ca}^{2+}$  channels by applying a channel subtype-specific toxin will cause a linear reduction of release, resulting in  $m = 1$  (Yoshikami et al., 1989; Augustine et al., 1991). However, evidence suggests that multiple  $\text{Ca}^{2+}$  channels contribute to release in a single release site in many central synapses (Mintz et al., 1995; Borst and Sakmann, 1996, 1999; Wu and Saggau, 1997). When a release site is controlled by multiple  $\text{Ca}^{2+}$  channels, several mechanisms may be responsible for different values of  $m$ . As an example, assume that two subtypes of  $\text{Ca}^{2+}$  channels (types 1 and 2), with each contributing one half of the total  $\text{Ca}^{2+}$  influx, are present in a terminal with two release sites. If the channel subtypes are completely segregated, with one subtype being present exclusively on the first release site and the other one only on the

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second, blocking one subtype of Ca<sup>2+</sup> channels will reduce both Ca<sup>2+</sup> influx and release by half, meaning that  $m$  for both subtypes would be only 1. The value  $m$  thus depends on the distribution of a subtype of Ca<sup>2+</sup> channels at different release sites (Mintz et al., 1995; Reid et al., 1998; see Appendix). An alternative mechanism applies when type 1 Ca<sup>2+</sup> channels are located so far from the release sites that they do not contribute to release. In that case,  $m$  is zero for type 1 and infinity for type 2 channels when these channel types are blocked. If type 1 Ca<sup>2+</sup> channels are, on average, more distantly located from the release sites than type 2 channels,  $m$  for type 1 will be lower than for type 2. Thus,  $m$  also depends on the distance between the channel and the release sites (Artalejo et al., 1994).

Here, we investigated mechanisms underlying differences in  $m$  of Ca<sup>2+</sup> channel subtypes at the medial nucleus of the trapezoid body (MNTB) (Wu et al., 1998). In contrast to other studies that determined the effectiveness of a Ca<sup>2+</sup> channel subtype from a population of synapses (Wu and Saggau, 1994; Mintz et al., 1995), we measured  $m$  by simultaneously recording the presynaptic Ca<sup>2+</sup> influx and release at single synapses (Wu et al., 1998). Because of the large size of the presynaptic calyx, the subcellular distribution of different subtypes of Ca<sup>2+</sup> channels can be studied with immunocytochemical techniques (Westenbroek et al., 1992, 1995; Yokoyama et al., 1995). We found that transmitter release from the calyx was controlled by P/Q-, N-, and R-type channels. However, the effectiveness of P/Q-type Ca<sup>2+</sup> channels was higher than of N- and R-type channels. This difference was most likely caused by a larger fraction of N- and R-type than P/Q-type channels being located distant from release sites.

## MATERIALS AND METHODS

Slices were cut from the brainstems of 8- to 10-d-old Wistar rats, transferred to a recording chamber and perfused at room temperature (23–24°C) with a solution containing (in mM): 125 NaCl, 2.5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 25 dextrose, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 0.4 ascorbic acid, 3 *myo*-inositol, 2 sodium pyruvate, and 25 NaHCO<sub>3</sub>, pH 7.4, when bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (Borst et al., 1995). Whole-cell current-clamp recordings from terminals were made with an AxoClamp-2B (Axon Instruments, Foster City, CA) amplifier and glass pipettes (8–12 MΩ) containing (in mM): 115 potassium gluconate, 20 KCl, 4 MgATP, 10 Na<sub>2</sub>-phosphocreatine, 0.3 GTP, 10 HEPES, and 0.05 fura-2 (Molecular Probes, Eugene, OR), pH 7.2, adjusted with KOH. Apart from serving as a Ca<sup>2+</sup> indicator, the fura-2 fluorescence was also used at the end of the experiment to confirm the presynaptic origin of the recording. Whole-cell voltage-clamp recordings from postsynaptic cells were made with an Axopatch-200A amplifier. Pipettes had a resistance of 1.5–2 MΩ and were filled with the same solution as the pipettes used for presynaptic recordings, except they contained 0.5 mM EGTA instead of 50 μM fura-2. Series resistance in postsynaptic recordings (<15 MΩ) was always compensated to 98% (lag, 10 μsec).

Whole-cell Ca<sup>2+</sup> current recordings from terminals were made with an Axopatch-200A amplifier (Axon Instruments) and pipettes with a resistance of 4–6 MΩ that contained the same intracellular solution as used for current-clamp recordings except that potassium was replaced by cesium. In addition, 1 μM tetrodotoxin and 0.1 mM 3,4-diaminopyridine (Sigma, St. Louis, MO) were added to the extracellular solution, and 20 mM NaCl was replaced with 20 mM tetraethylammonium chloride (Sigma) to block sodium and potassium channels (Borst et al., 1995). Series resistance (<35 MΩ) compensation was set at 90% with a lag of 10 μsec, and prediction was set at 60%. Subtraction of the passive response was by the P/5 method. Terminals with a capacitance of <35 pF were selected to avoid long axons (Borst and Sakmann, 1998). For the waveform command, we used the presynaptic action potential displayed in Figure 2A of Borst et al., 1995, interpolated to 20 μsec per point with a cubic spline. Potentials were corrected for a liquid junction potential of –11 mV between the extracellular and the pipette solution. Holding potential in voltage-clamp experiments was –80 mV. Potentials or currents were

low-pass filtered at 2–5 kHz and digitized at 20–50 kHz with a 16-bit analog-to-digital converter (Instrutech, Great Neck, NY).

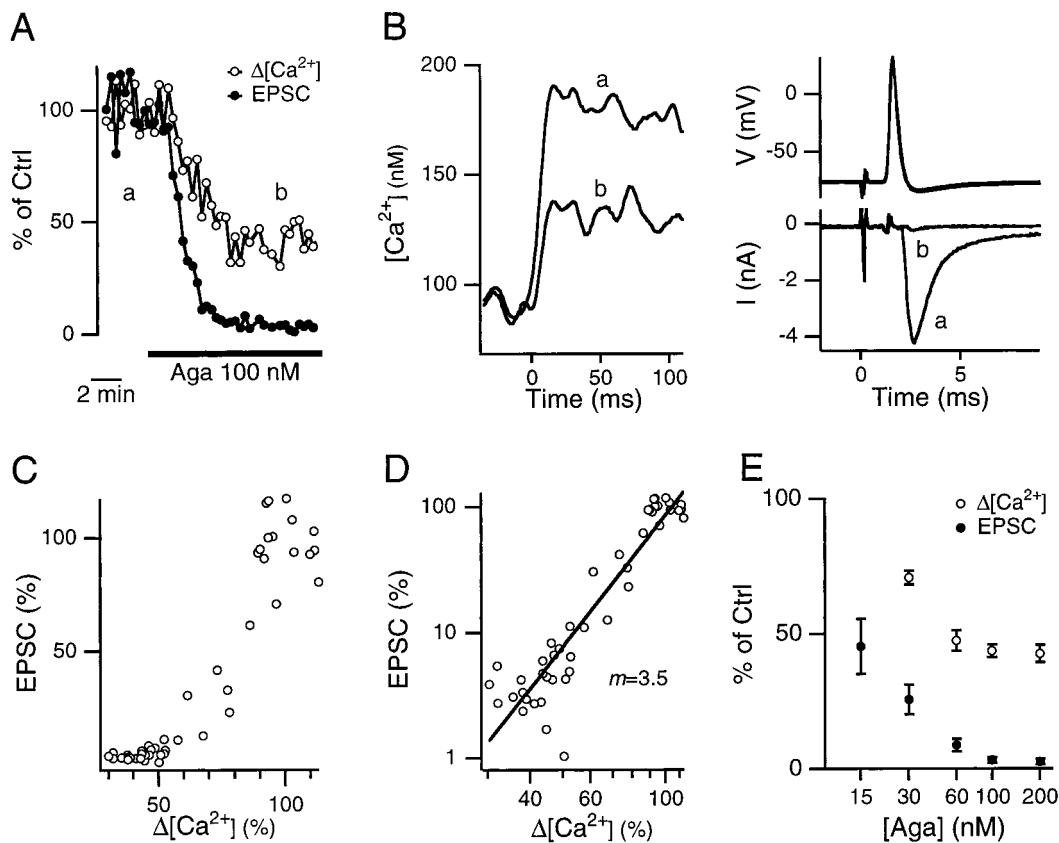
The optical recording system comprised an upright epifluorescence microscope (Axioskop; Achroplan 40×, NA 0.75, Zeiss), equipped with a polychromatic illumination system (TILL Photonics, Munich, Germany), a dichroic mirror (400 nm), a long-pass (415 nm) emission filter, and two photodiodes at the image plane for signal and background subtraction, respectively. Excitation light was coupled to the microscope via a light guide. Fura-2 measurements of Ca<sup>2+</sup> concentration were made by forming ratios between two continuous recordings (100–500 msec) of fluorescence (after background subtraction) at two excitation wavelengths (357 and 380 nm) with an interval of ~10 msec (Grynkiewicz et al., 1985). Calibration parameters were obtained from *in vivo* calibrations as described in Helmchen et al. (1997). Fluorescence signals recorded by the photodiode (Hamamatsu) were filtered at 30 Hz (8-pole Bessel filter). Since the Ca<sup>2+</sup> transient evoked by an action potential decays with a time constant >400 msec in the presence of 50 μM fura-2 (Helmchen et al., 1997), filtering this transient at 30 Hz did not affect the measurement of its amplitude. Fura-2 was far from saturation during single action potentials, because (1) the peak Ca<sup>2+</sup> concentrations evoked by single action potentials were usually less than the K<sub>d</sub> (273 nM) of fura-2 measured *in vivo* (Helmchen et al., 1997), and (2) paired-pulse action potential stimulation (interval: 50 msec) caused approximately the same Ca<sup>2+</sup> influx for each action potential (data not shown). The large Ca<sup>2+</sup> influx during a presynaptic action potential (Borst and Sakmann, 1996) and the favorable surface-to-volume ratio of the terminal permitted us to resolve Ca<sup>2+</sup> influx during single action potentials, and to measure the relation between release and presynaptic Ca<sup>2+</sup> influx at the level of a single synapse.

For simultaneous presynaptic and postsynaptic recordings, only synapses in which the postsynaptic cells discharged an action potential in response to afferent stimulation were selected for recording (Borst et al., 1995). Single afferent stimuli were applied via a bipolar electrode (3–30 V, 100 μsec) placed at the midline of the trapezoid body. Stimulation interval was 20–30 sec, which is sufficiently long to avoid synaptic depression (Von Gersdorff et al., 1997).

The degree of reduction in the presynaptic Ca<sup>2+</sup> influx and release during application of a toxin was evaluated after its effect reached equilibrium. Data were expressed as mean ± SEM. ω-Agatoxin-IVA (Aga) was a gift from Dr. N. A. Saccomano, Pfizer, Groton, CT; ω-conotoxin-GVIA (Ctx) was purchased from Bachem (Bubendorf, Germany) and Research Biochemicals (Natick, MA); and ω-conotoxin-MV1C was from Research Biochemicals. The toxins were applied in the presence of 0.1 mg/ml cytochrome C to block nonspecific binding sites. Cytochrome C did not significantly affect the EPSC ( $n = 16$ ;  $p > 0.5$ ), or Δ[Ca<sup>2+</sup>] ( $n = 3$ ;  $p > 0.5$ ; paired  $t$  test).

For the immunocytochemical studies, 10-d-old Wistar rats were anesthetized with Nembutal and then intracardially perfused with 4% paraformaldehyde in PB (0.1 M sodium phosphate, pH 7.4) containing 0.34% L-lysine and 0.05% sodium *m*-periodate (McLean and Nakane, 1974). The brains were removed immediately from the cranium, post-fixed for 2 hr, and then cryoprotected by sinking in 10 (w/v) and 30% (w/v) sucrose in PB at 4°C for 72 hr. Coronal sections (40 μm) were cut on a sliding microtome and processed for immunocytochemistry using the avidin-biotin complex method.

Free-floating sections were processed for immunocytochemistry by rinsing the tissue in 0.1 M Tris buffer (TB) for 15 min, 0.1 M Tris-buffered saline (TBS) for 15 min, blocking in 2% avidin in TBS for 30 min, rinsing in TBS for 30 min, blocking in 2% biotin in TBS for 30 min, and rinsing in TBS for 30 min. The tissue was then incubated in affinity-purified anti-peptide antibody anti-CNA5 (against α<sub>1A</sub> subunit, diluted 1:15), anti-CNB2 (against α<sub>1B</sub> subunit, diluted 1:15), or anti-CNE2 (against α<sub>1E</sub> subunit, diluted 1:15) simultaneously with anti-synaptotagmin (diluted 1:200) for 1 hr at room temperature followed by 36 hr at 4°C. All antibodies were diluted in 0.1 M TBS containing 0.075% Triton X-100 and 1% normal goat serum. The sections were rinsed for 1 hr in TBS and then incubated in a solution containing biotinylated goat anti-rabbit IgG (for anti-CNA5, anti-CNB2, or anti-CNE2 antibodies) diluted 1:300 and anti-mouse IgG Texas Red (for anti-synaptotagmin antibody) diluted 1:200 for 1 hr at 37°C. Tissue sections were then rinsed in TBS for 1 hr and incubated in avidin D-fluorescein (diluted 1:300) and anti-mouse IgG Texas Red (diluted 1:200) for 1 hr at 37°C, rinsed in TBS for 10 min, rinsed in TB for 20 min, mounted onto gelatin-coated slides, and then coverslipped using Vectashield.



**Figure 1.** Effect of Aga on presynaptic  $\Delta[\text{Ca}^{2+}]$  and EPSC. *A*, Aga (100 nM) reduced concurrently both the  $\Delta[\text{Ca}^{2+}]$  and the EPSC evoked by single presynaptic action potentials. The values of  $\Delta[\text{Ca}^{2+}]$  and of the EPSCs are given relative to their respective average values before application of the toxin. *B*, Sample traces of the presynaptic  $\text{Ca}^{2+}$  influx (*left*), the presynaptic action potential (*top right*), and the EPSC (*bottom right*) before (*a*) and after (*b*, as indicated in *A*) Aga application. The stimulus was given at time 0. Each trace was taken from a single sweep (same for all the following figures). *C*, *D*, EPSCs plotted against the  $\Delta[\text{Ca}^{2+}]$  from panel *A* on linear (*C*) and double logarithmic (*D*) scales. The slope of the linear regression line in *D* was 3.5. *E*, Summary of the dose-dependent effect of Aga on the  $\Delta[\text{Ca}^{2+}]$  and the EPSC (each value was obtained from three to nine cells).

The immunofluorescent staining was viewed using a Bio-Rad (Hercules, CA) MRC 600 microscope located in the imaging facility of the W. M. Keck Center for Research in Neural Signaling at the University of Washington. The thickness of optical sections was typically 1  $\mu\text{m}$ . Tissue sections double-labeled for N-type and P/Q-type  $\text{Ca}^{2+}$  channels were treated the same as above but were incubated in anti-CNB2 (made in goat; diluted 1:15) and anti-CNA5 (made in rabbit; diluted 1:15), followed by incubation in biotinylated anti-goat IgG (diluted 1:300) and anti-rabbit IgG labeled with Texas Red (diluted 1:200). Sections were then incubated in avidin D-fluorescein (diluted 1:300) and anti-rabbit IgG labeled with Texas Red (diluted 1:200) for 1 hr at 37°C. To determine the level of nonspecific staining, some of the control sections were incubated without one or both of the primary antibodies, or replaced by normal rabbit serum. All staining reported here was abolished in the absence of primary antibody. The generation, purification, and characterization of anti-CNA5, anti-CNB2, and anti-CNE2 have been reported previously (Westenbroek et al., 1992; Yokoyama et al., 1995; Sakurai et al., 1996). The anti-synaptotagmin antibody was a generous gift of Dr. Masami Takahashi, Mitsubishi-Kasei Life Sciences Institute, Toyko, Japan (Yoshida et al., 1992).

## RESULTS

### P/Q-type channels control release effectively

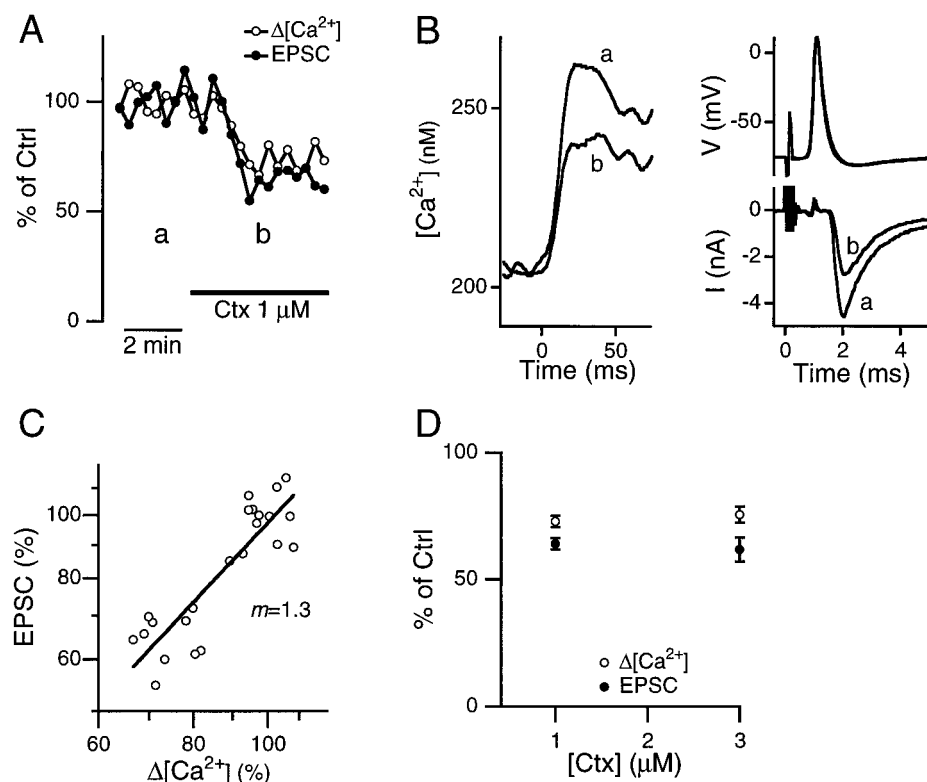
The presynaptic  $\text{Ca}^{2+}$  influx and the corresponding EPSCs evoked by single action potentials were simultaneously recorded at single calyx-type synapses. Application of the P/Q-type  $\text{Ca}^{2+}$  channel blocker  $\omega$ -agatoxin-IVA (Aga, 15–200 nM) reduced both the amplitude of the presynaptic  $\text{Ca}^{2+}$  influx ( $\Delta[\text{Ca}^{2+}]$ ) and the

EPSC without significantly affecting the presynaptic resting membrane potential, the shape of the action potential, or the resting intracellular  $\text{Ca}^{2+}$  concentration (Fig. 1*A, B*). This effect could not be reversed after >30 min of wash out (data not shown). The relation between the EPSC and the  $\Delta[\text{Ca}^{2+}]$  was nonlinear (Fig. 1*C*) and could be fit with relation (2), where  $m = 3.7 \pm 0.2$  ( $n = 5$  pairs of presynaptic and postsynaptic recordings, Fig. 1*D*). The effect of Aga was dose-dependent. It reached a plateau at 100 nM (Fig. 1*E*), at which  $\Delta[\text{Ca}^{2+}]$  was reduced to  $44 \pm 2\%$  ( $n = 9$ ) and the EPSC was reduced to  $3 \pm 1\%$  ( $n = 4$ ). At 30 nM, Aga reduced  $\Delta[\text{Ca}^{2+}]$  to  $71 \pm 3\%$  ( $n = 3$ ; Fig. 1*E*) and the EPSC to  $26 \pm 5\%$  ( $n = 6$ ), yielding the value  $m = 3.9$  ( $\log(0.71)/\log(0.26)$ ). Similar values (3.3–4.2) of  $m$  were obtained at concentrations of 60–200 nM (from data summarized in Fig. 1*E*). These results suggest that the value of  $m$  is independent of the concentration of Aga. The  $K_d$  for the block by Aga was  $\sim 30$  nM, because at this concentration Aga reached about half-maximal block of the  $\Delta[\text{Ca}^{2+}]$  (Fig. 1*E*).

### N-type channels control release less effectively

Addition of the N-type channel blocker  $\omega$ -conotoxin-GVIA (Ctx) reduced both the  $\Delta[\text{Ca}^{2+}]$  and the EPSC simultaneously (Fig. 2*A*). The membrane potential, the action potential, and the resting intracellular  $\text{Ca}^{2+}$  concentration in the terminal did not change significantly (Fig. 2*B*). The relation between the EPSC and the  $\Delta[\text{Ca}^{2+}]$  could be fit with relation (2) with  $m = 1.3 \pm 0.1$





**Figure 2.** Effect of Ctx on presynaptic  $\Delta[\text{Ca}^{2+}]$  and EPSC. *A*, Ctx ( $1 \mu\text{M}$ ) reduced both the  $\Delta[\text{Ca}^{2+}]$  and the EPSC evoked by single presynaptic action potentials with a similar time course. Amplitudes of both the  $\Delta[\text{Ca}^{2+}]$  and the EPSC were normalized to the values during the control period. *B*, Sample traces of the presynaptic  $\text{Ca}^{2+}$  influx (left), the presynaptic action potential (top right), and the EPSC (bottom right) before (*a*) and after (*b*, as indicated in *A*) Ctx application. The stimulus was given at time 0. *C*, EPSCs plotted against the  $\Delta[\text{Ca}^{2+}]$  from *A* on double logarithmic scales. The slope of the linear regression line was 1.3. *D*, Summary of the effect of Ctx on the  $\Delta[\text{Ca}^{2+}]$  and the EPSC. Each value was obtained from 3–11 cells. The effects of Ctx at 1 and 3  $\mu\text{M}$  were not significantly different ( $p > 0.5$ ; *t* test).

(seven pairs of presynaptic and postsynaptic recordings, Fig. 2*C*). The value of *m* obtained with application of Ctx was significantly lower than that with application of Aga ( $p < 0.01$ ; *t* test). At  $1 \mu\text{M}$ , Ctx reduced the  $\Delta[\text{Ca}^{2+}]$  to  $73 \pm 2\%$  ( $n = 11$ ) and the EPSC to  $64 \pm 2\%$  ( $n = 10$ , Fig. 2*D*). The effect of Ctx was saturated at  $1 \mu\text{M}$ , since no additional block on either the  $\Delta[\text{Ca}^{2+}]$  ( $n = 3$ ) or the EPSC ( $n = 3$ ) was observed at  $3 \mu\text{M}$  (Fig. 2*D*).

We previously observed that combined application of saturating concentrations of  $\omega$ -conotoxin-MVIIIC ( $8 \mu\text{M}$ ), a blocker of P/Q- and N-type channels (Hillyard et al., 1992; McDonough et al., 1996), Aga ( $100 \text{ nM}$ ), and Ctx ( $1 \mu\text{M}$ ) reduces the  $\Delta[\text{Ca}^{2+}]$  to  $26 \pm 2\%$  ( $n = 8$ ) and the EPSC to  $0.9 \pm 0.2\%$  ( $n = 4$ ; Wu et al., 1998). Similar results were obtained when Aga and Ctx were applied together in the absence of  $\omega$ -conotoxin-MVIIIC (Wu et al., 1998, see also Fig. 4). The block of  $\Delta[\text{Ca}^{2+}]$  by the combined toxin application was similar to the sum of the block by applying either Aga or Ctx alone, suggesting that Aga and Ctx block separate populations of  $\text{Ca}^{2+}$  channels. The resistant  $\Delta[\text{Ca}^{2+}]$  is mediated via R-type channels, which have  $m = 1.4 \pm 0.1$  ( $n = 4$  synapses; Wu et al., 1998). Reducing the extracellular  $\text{Ca}^{2+}$  concentration from 2 to 1 mM results in  $n = 2.7 \pm 0.2$  ( $n = 5$  synapses; Wu et al., 1998). Table 1 shows the average percentages of the  $\Delta[\text{Ca}^{2+}]$  evoked by single action potentials from three

subtypes of  $\text{Ca}^{2+}$  channels and their values of *m*. The sum of the three components was slightly  $>100\%$ , probably because of measurement error.

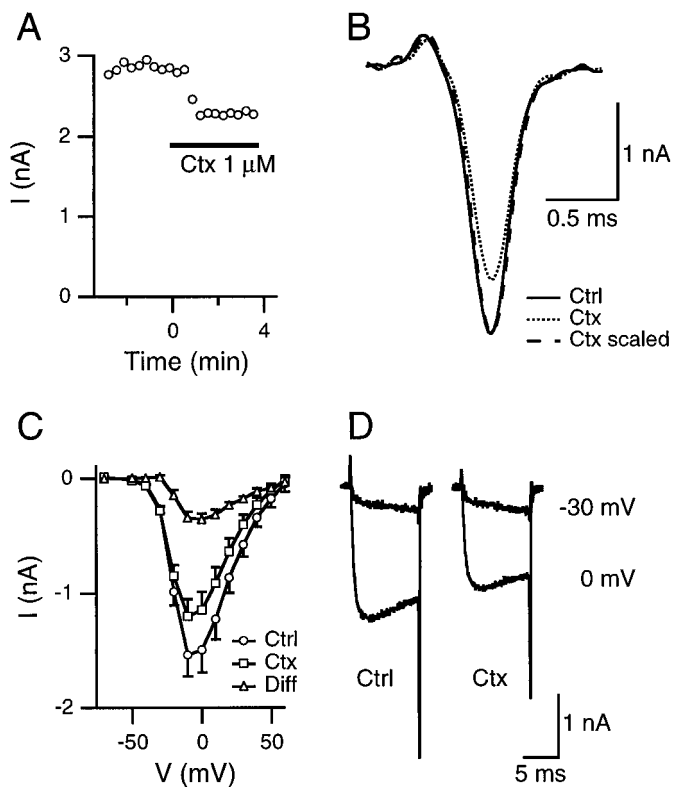
#### The difference in *m* is not caused by different $\text{Ca}^{2+}$ current kinetics

In the squid giant synapse, broadening presynaptic action potentials increases EPSCs (Augustine et al., 1991). The relation between the EPSC and the presynaptic  $\text{Ca}^{2+}$  influx was estimated to be approximately linear from this study, suggesting that during an action potential  $\text{Ca}^{2+}$  channel domains do not overlap at this synapse (Augustine et al., 1991). If one subtype of  $\text{Ca}^{2+}$  channels opens much more briefly than others, its  $\text{Ca}^{2+}$  domains are less likely to overlap. We wondered whether the difference in the values of *m* at the MNTB synapse could be caused by a difference in the time course of  $\text{Ca}^{2+}$  currents. Presynaptic  $\text{Ca}^{2+}$  currents elicited by an action potential waveform command ( $I_{\text{Ca}(\text{AP})}$ ) were recorded in the presence of  $\text{Na}^+$  and  $\text{K}^+$  channel blockers (Borst and Sakmann, 1996). Application of Ctx ( $1 \mu\text{M}$ ) reduced the amplitude of the  $I_{\text{Ca}(\text{AP})}$  to  $79 \pm 1\%$  ( $n = 6$ ; Fig. 3*A*), similar to its inhibitory effect on the  $\Delta[\text{Ca}^{2+}]$  (Fig. 2). This effect was not accompanied by a significant change in the waveform of the current measured at half width ( $p > 0.5$ ; paired *t* test;  $n = 5$ ; Fig.

**Table 1.** Contribution of presynaptic  $\text{Ca}^{2+}$  channel subtypes to  $\text{Ca}^{2+}$  influx and release

	P/Q-type	N-type	R-type	Cooperativity ( <i>n</i> )
%	$56 \pm (9)$	$27 \pm 2 (11)$	$26 \pm 2 (8)$	
<i>m</i> or <i>n</i>	$3.7 \pm 0.2 (5)$	$1.3 \pm 0.1 (7)$	$1.4 \pm 0.1 (4)$	$2.7 \pm 0.2 (5)$

The percentage refers to the presynaptic  $\Delta[\text{Ca}^{2+}]$  evoked by single action potentials. Data are expressed as mean  $\pm$  SEM (number of synapses measured). The *m* for different subtypes of  $\text{Ca}^{2+}$  channels was measured based on relation (2). The *n* for the cooperativity of the  $\text{Ca}^{2+}$  in triggering release was measured by lowering the extracellular  $\text{Ca}^{2+}$  concentration (Wu et al., 1998).



**Figure 3.** Presynaptic Ca<sup>2+</sup> currents elicited by an action potential waveform command ( $I_{Ca(AP)}$ ) before and after Ctx application. *A*, Application of Ctx (1  $\mu$ M) reduced the  $I_{Ca(AP)}$ . *B*, Sample traces of the  $I_{Ca(AP)}$  before (Ctrl) and after Ctx application, the latter of which was also scaled (Ctx scaled) for comparison with the Ctrl trace. *C*, The  $I$ - $V$  relation before (Ctrl) and after Ctx application. The difference between these two  $I$ - $V$  curves yielded the Ctx-sensitive  $I$ - $V$  curve (Diff), which activated at potentials more positive than  $-30$  mV. In contrast, the threshold for activation of Ctx-insensitive currents (Ctx) was at approximately  $-40$  mV. *D*, Sample Ca<sup>2+</sup> currents elicited by a 10 msec step pulse to  $-30$  and  $0$  mV before (left) and after (right) Ctx application. Note that currents elicited at  $-30$  mV did not change significantly after Ctx application.

3B). Similarly, application of  $\omega$ -conotoxin-MVIIIC, a blocker for N- and P/Q-type Ca<sup>2+</sup> channels, revealed no significant difference in the time course of the  $I_{Ca(AP)}$  between the R-type and the total current (Wu et al., 1998). These results suggest that the lower value of  $m$  for N- and R-type channels is not caused by a difference in the kinetics of the  $I_{Ca(AP)}$ .

In addition to studying the time course of the  $I_{Ca(AP)}$ , we also examined the currents elicited by 10 msec depolarizing steps from a holding potential of  $-80$  mV. The peak current-voltage ( $I$ - $V$ ) curves were obtained before and after Ctx application ( $n = 6$ ; Fig. 3C,D). The difference between these two  $I$ - $V$  curves is the Ctx-sensitive N-type current, which activated at potentials more positive than  $-30$  mV (Fig. 3C,D), at least 10 mV more positive than the other two subtypes of Ca<sup>2+</sup> currents. Ctx (1  $\mu$ M) inhibited the current elicited at  $0$  mV to  $76 \pm 3\%$  ( $n = 6$ ) of control. A similar block was observed at  $-10$  to  $30$  mV. These results were similar to the effect of Ctx on  $\Delta[Ca^{2+}]$  or  $I_{Ca(AP)}$  (Figs. 2, 3).

#### Multiple subtypes of Ca<sup>2+</sup> channels control release at single release sites

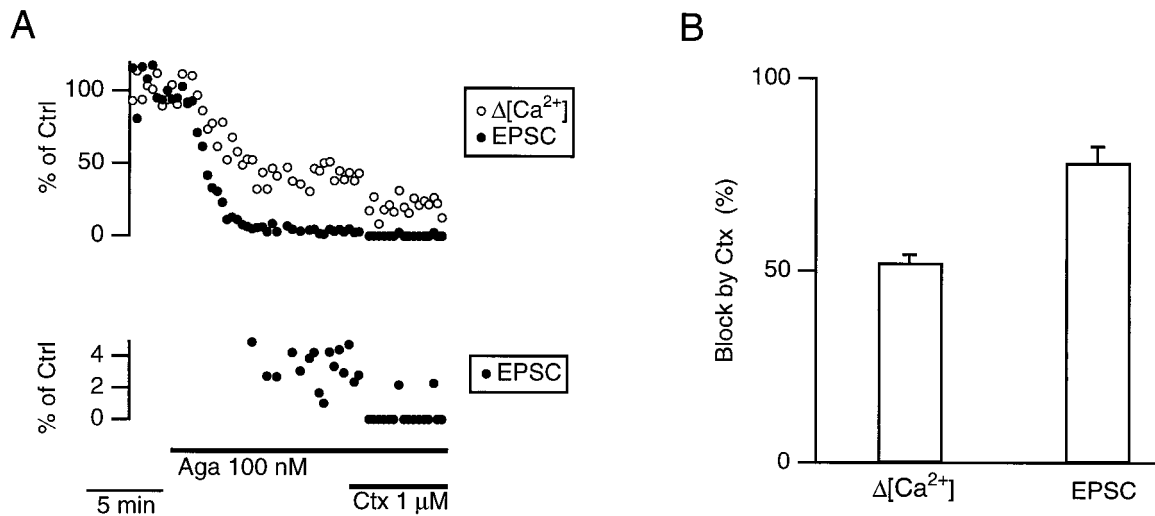
Next, we investigated whether the difference in the value of  $m$  could be accounted for by differential distribution of Ca<sup>2+</sup> chan-

nel subtypes at different release sites. Because Aga blocked  $97 \pm 1\%$  of the EPSC (Fig. 1E), we assumed that P/Q-type channels participate in controlling release at all release sites. In a calyx, about half of the Ca<sup>2+</sup> influx is via P/Q-type channels, and the other half is approximately equally divided between N- and R-type channels (Table 1). We investigated three hypotheses that may account for the lower values of  $m$  for N- and R-type channels (see introductory remarks). First, N- and R-type channels may be associated with different release sites. Second, N- and R-type channels may be located at every release site, but they may be further from the Ca<sup>2+</sup> sensor than P/Q-type channels. Third, there may be distant Ca<sup>2+</sup> channels, which are located so far away from release sites that they do not contribute to release during a single action potential. The fraction of distant N- and R-type channels may be larger than that of distant P/Q-type channels.

If N- and R-type channels do not control release at the same release site, the Ca<sup>2+</sup> influx at each release site should be contributed about half by P/Q-type channels and half by either N- or R-type channels. This arrangement of Ca<sup>2+</sup> channels would result in a lower value of  $m$  for N- and R-type channels (Reid et al., 1998; see also Appendix for details). It predicts that when P/Q-type channels are completely blocked, blocking N-type channels will reduce release by  $\leq 50\%$ , because only half of release sites are associated with N-type channels. In the presence of Aga (100 nM) to completely block P/Q-type channels, application of Ctx (1  $\mu$ M) reduced the  $\Delta[Ca^{2+}]$  by  $52 \pm 2\%$  ( $n = 5$ ) of the value immediately before Ctx application (Fig. 4), as expected from Table 1. However, Ctx reduced the EPSC by  $78 \pm 4\%$  ( $n = 4$ ) of the value immediately before Ctx application, which is clearly higher than the predicted 50%. Thus, the hypothesis that N- and R-type channels are associated with different release sites is not supported. N-type channels are present in at least 78% of release sites. These results suggest that transmitter release at most release sites is controlled by three subtypes of Ca<sup>2+</sup> channels, P/Q-, N-, and R-type. As an approximation, we assumed that all three subtypes of channels control release at every release site.

#### EGTA does not preferentially block release mediated by N-type Ca<sup>2+</sup> channels

If N- and R-type channels near release sites were located further from the Ca<sup>2+</sup> sensor than P/Q-type channels, the Ca<sup>2+</sup> entering via N- or R-type channels is less likely to bind to the Ca<sup>2+</sup> sensor than that entering the terminal via P/Q-type channels. This will result in a lower value of  $m$  for N- and R-type channels. One prediction of this hypothesis is that the Ca<sup>2+</sup> entering via N- or R-type channels will diffuse across a longer distance to reach the Ca<sup>2+</sup> sensor, and thus will be more likely to be bound by an exogenous Ca<sup>2+</sup> buffer. We tested whether release controlled by the Ca<sup>2+</sup> influx through N-type channels is more susceptible to the block by a high concentration of the slow Ca<sup>2+</sup> buffer EGTA. EPSCs were recorded while a pipette containing 10 mM EGTA was sealed to the corresponding terminal in the cell-attached configuration. After obtaining a baseline value for the EPSC amplitude, the whole-cell configuration was established in the presynaptic recording. The EPSC decreased to a new steady-state level in  $\sim 3$  min (Fig. 5A), probably reflecting diffusion of EGTA from the presynaptic pipette into the terminal (Borst and Sakmann, 1996). Application of Ctx (1  $\mu$ M) further reduced the EPSC by  $38 \pm 5\%$  ( $n = 5$ ) of the value at the new steady-state level (Fig. 5A). Ctx blocked a similar fraction of the EPSC in the presence or absence of EGTA (Fig. 5B). These results suggest



**Figure 4.** Effect of Ctx on  $\Delta[\text{Ca}^{2+}]$  and EPSC in the presence of Aga. *A*, An experiment showing that Ctx ( $1 \mu\text{M}$ ) reduced both the  $\Delta[\text{Ca}^{2+}]$  and the EPSC in the presence of Aga ( $100 \text{ nM}$ ). In the *bottom* panel, the EPSC is plotted on a different scale. If the maximum amplitude of the postsynaptic current was less than twice the SD of the baseline noise, the response was classified as a failure. *B*, In the presence of Aga ( $100 \text{ nM}$ ), the block (%) of the  $\Delta[\text{Ca}^{2+}]$  and of the EPSC by Ctx ( $1 \mu\text{M}$ ) were significantly different ( $p < 0.01$ ; paired *t* test). The percentages were normalized to the values immediately before Ctx application.

that calcium ions that reach the  $\text{Ca}^{2+}$  sensor diffuse approximately the same distance, irrespective of the subtype of channels through which they enter.

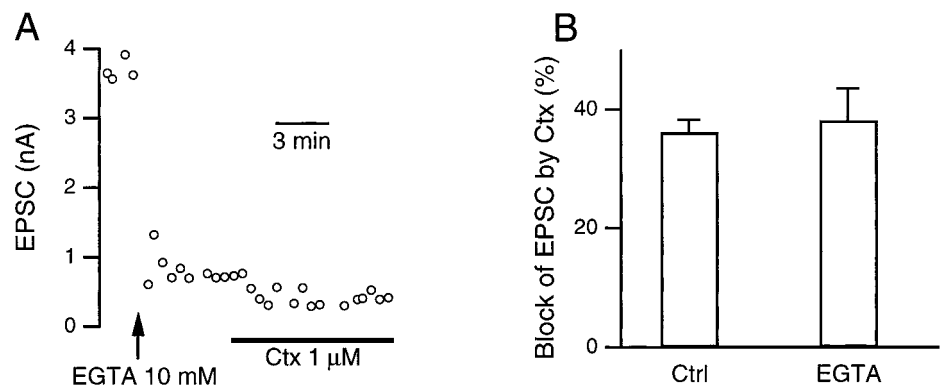
#### Double labeling with antibodies to $\text{Ca}^{2+}$ channels and synaptotagmin

If a larger fraction of N- and R-type channels than P/Q-type channels were located far from release sites, from which  $\text{Ca}^{2+}$  entering will be less likely to bind to the  $\text{Ca}^{2+}$  sensor that triggers release, the value of *m* for N- or R-type will be lower than that for P/Q-type channels. To address this hypothesis, we examined the distribution of  $\text{Ca}^{2+}$  channels in the terminal by double labeling with antibodies to  $\text{Ca}^{2+}$  channels (Westenbroek et al., 1992, 1995; Yokoyama et al., 1995) and to synaptotagmin, a synaptic vesicle protein (Brose et al., 1992). Confocal microscopic examination of tissue sections labeled with monoclonal antibodies against synaptotagmin (Yoshida et al., 1992) revealed staining that was restricted to the presynaptic calyx terminal enveloping the cell body of MNTB neurons (Fig. 6*B*). In contrast, immunostaining for the  $\alpha_{1A}$  subunit of P/Q-type  $\text{Ca}^{2+}$  channels (Westenbroek et al., 1995) was localized in punctate clusters in the release face of the calyx-type synapse along the surface of the cell, as well as scattered throughout the postsynaptic cell body (Fig. 6*A*). Staining in

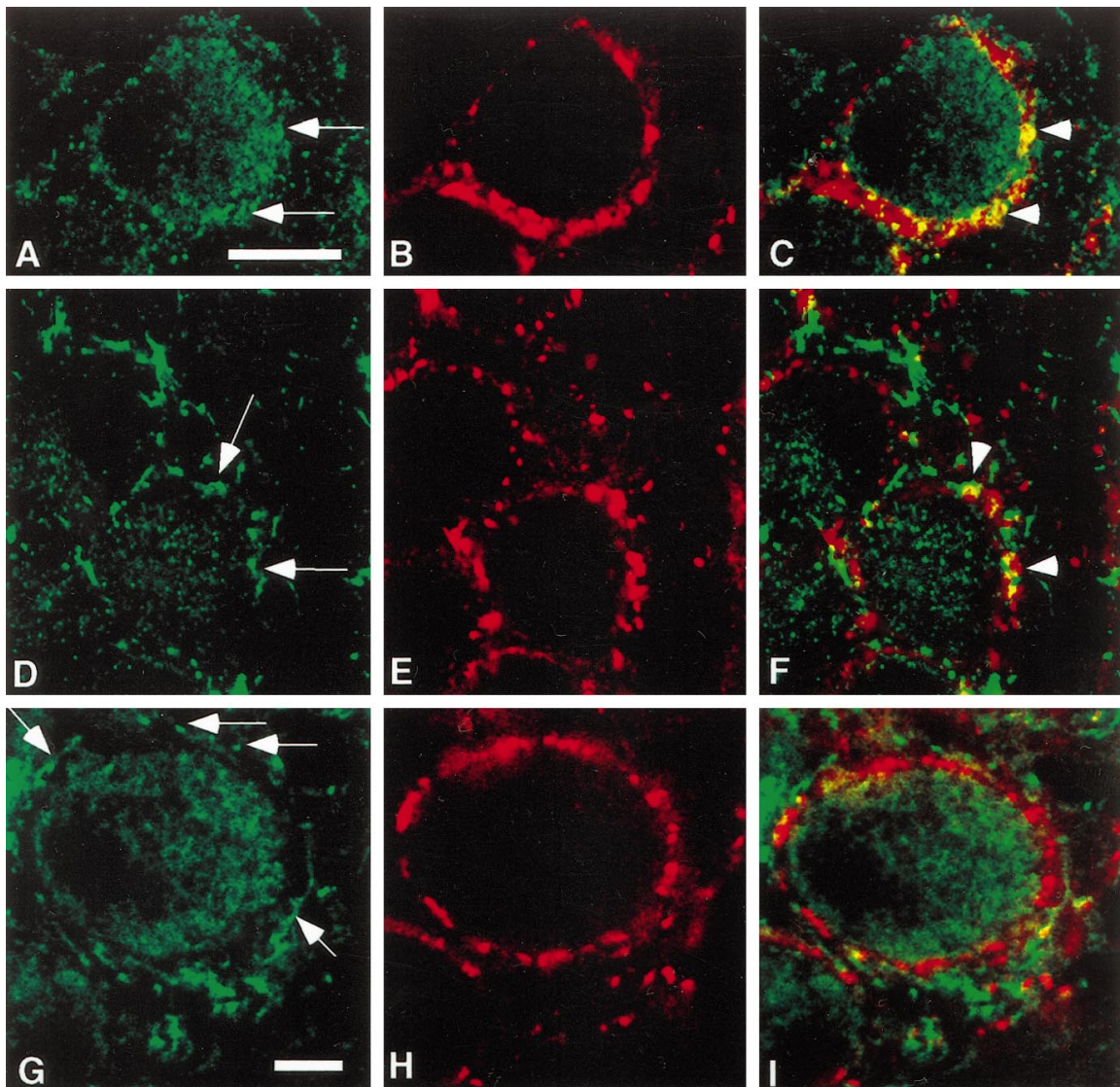
the soma presumably represents newly synthesized P/Q-type channels in the endoplasmic reticulum and Golgi complex, which were more prominent in these neurons in young rats than we previously observed in adults (Westenbroek et al., 1995). Merged images showed that staining for  $\alpha_{1A}$  was colocalized with staining for synaptotagmin at sites along the release face of the calyx (Fig. 6*C*, *yellow–orange* regions). Although some punctate staining of  $\alpha_{1A}$  appeared to be in the cytosol of the terminal in a single optical section (about  $1 \mu\text{m}$ ), inspection of adjacent sections suggested that it originated from the irregularly shaped release face as well. This staining was carried out using anti-CNA5 antibodies, which recognize the rB isoform of  $\alpha_{1A}$  (Starr et al., 1991; Sakurai et al., 1996). A similar pattern of staining was observed with the anti-CNA6 antibodies against the BI isoform (Mori et al., 1991; Sakurai et al., 1996). Altogether, these results show that many areas of the calyx synapse that were labeled for synaptotagmin were also labeled for both isoforms of the  $\alpha_{1A}$  subunits of P/Q-type  $\text{Ca}^{2+}$  channels.

Examination of tissue sections labeled with antibodies to  $\alpha_{1B}$  subunit of N-type  $\text{Ca}^{2+}$  channels revealed staining of this channel along both the release face and the nonrelease face of the calyx, as well as substantial intracellular staining in the cell body of

**Figure 5.** Effect of Ctx on EPSC after EGTA was dialyzed into the terminal. *A*, While the EPSC was recorded, a cell-attached recording was made on the presynaptic terminal with a pipette containing  $10 \text{ mM}$  EGTA. A suction pulse (*arrow*) was applied to establish the whole-cell configuration, allowing EGTA to diffuse from the pipette into the terminal. The EPSC decreased rapidly and reached a new baseline value. Ctx ( $1 \mu\text{M}$ ) was then applied to the bath, which further decreased the EPSC. *B*, Comparison of the block of the EPSC by Ctx ( $1 \mu\text{M}$ ) in the control (*Ctrl*;  $n = 10$ ) and after EGTA ( $n = 5$ ) was dialyzed into the presynaptic terminal. In both cases, the block was normalized to the value immediately before Ctx application.







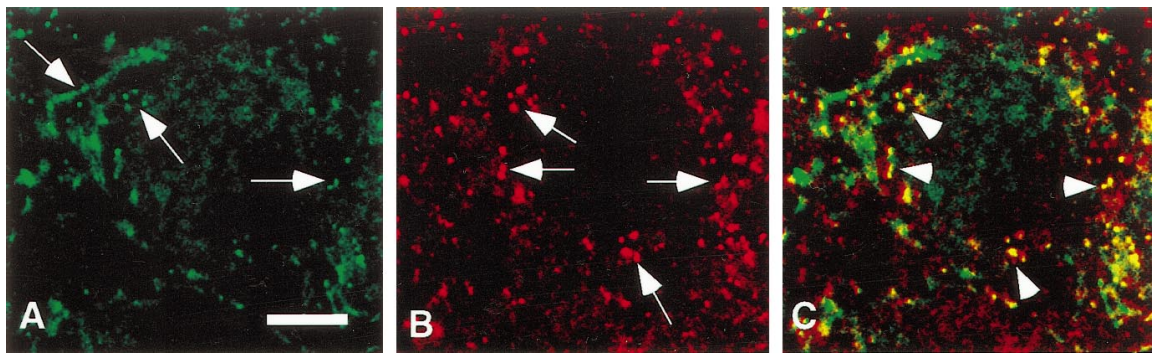
**Figure 6.** Distribution of the  $\alpha_1$  subunit of classes A, B, and E  $\text{Ca}^{2+}$  channels in synapses in the MNTB. *A, B*, Tissue section double-labeled using antibodies to the  $\alpha_1$  subunit of class A  $\text{Ca}^{2+}$  channels (*A*, arrows) and anti-synaptotagmin (*B*), illustrating their distribution in the synapse surrounding neurons located in the MNTB. *C*, Merged image of *A* and *B* illustrating regions of colocalization (yellow; arrowheads) between the  $\alpha_1$  subunit of class A  $\text{Ca}^{2+}$  channels (green) and synaptotagmin (red), demonstrating the presence of these channels at the release face of the synapse. *D, E*, Tissue section double-labeled using antibodies to the  $\alpha_1$  subunit of class B  $\text{Ca}^{2+}$  channels (*D*, arrows) and anti-synaptotagmin (*E*), illustrating their distribution in the synapse surrounding MNTB neurons. *F*, Merged image of the staining observed in *D* and *E*, illustrating few regions of colocalization (yellow, arrowheads) between class B  $\text{Ca}^{2+}$  channels and synaptotagmin in the synapse and the presence of class B  $\text{Ca}^{2+}$  channels in other regions of the synapse. *G, H*, Tissue section double-labeled with antibodies to the  $\alpha_1$  subunit of class E  $\text{Ca}^{2+}$  channels (*G*, arrows) and anti-synaptotagmin antibodies (*H*), illustrating the presence of synaptotagmin at the release face of the synapse and the distribution of class E channels at other sites in the synapse. *I*, Merged image of the staining shown in *G* and *H*, illustrating very few sites of colocalization (yellow) of these two proteins. Arrows illustrate sites of class E  $\text{Ca}^{2+}$  channels outside the release face of the synapse. Scale bars: *A–F*, 10  $\mu\text{m}$ ; *G–I*, 5  $\mu\text{m}$ .

postsynaptic neurons (Fig. 6*D*). N-type  $\text{Ca}^{2+}$  channel staining of the calyx was often adjacent to or surrounding the staining observed with anti-synaptotagmin antibodies rather than superimposed on it (Fig. 6, compare *D, E*). Merged images showed areas of superimposition of staining for synaptotagmin and N-type  $\text{Ca}^{2+}$  channels (Fig. 6*F*, yellow–orange staining), but many areas of synaptotagmin staining did not have colocalized N-type  $\text{Ca}^{2+}$  channels (Fig. 6*F*, red staining), and many areas of staining for N-type  $\text{Ca}^{2+}$  channels were not stained for synaptotagmin (Fig. 6*F*, bright green staining). Immunostaining for N-type  $\text{Ca}^{2+}$  channels was also present along the outer surface of the calyx and appeared to extend along the surface membrane of calyx to areas

adjacent to the axon, where no synaptotagmin staining was observed (data not shown).

Antibodies against the  $\alpha_{1E}$  subunits of R-type  $\text{Ca}^{2+}$  channels were mostly not colocalized with synaptotagmin antibodies (Fig. 6, compare *G, H*). Merged images show few areas of superimposition of staining for  $\alpha_{1E}$  and synaptotagmin (Fig. 6*I*). These R-type channels appeared present primarily along the outer surface of the calyx and only rarely on the inner surface that surrounds the neurons and contains the sites of neurotransmitter release. Staining for  $\alpha_{1E}$  also extended to areas adjacent to the axon where no staining for synaptotagmin was observed.

Double-labeling studies using antibodies to N-type and P/Q-



**Figure 7.** Double labeling of class A and class B Ca<sup>2+</sup> channels in the MNTB. *A, B*, Double labeling of a tissue section from the MNTB using antibodies to the class B (*A*, arrows) and class A (*B*, arrows)  $\alpha_1$  subunits of Ca<sup>2+</sup> channels. *C*, Merged image of staining shown in *A* and *B* to illustrate regions of only class B staining (green), or regions of only class A staining (red) and regions of colocalization (yellow, arrowheads) of these two channels in the synapses and neurons located in the MNTB. Scale bar: *A–C*, 10  $\mu$ m.

type Ca<sup>2+</sup> channels revealed distinct distributions of these channels. P/Q-type Ca<sup>2+</sup> channels are located in punctate clusters in the calyx membrane (Fig. 7*B*), whereas N-type channels are more diffusely distributed throughout the calyx (Fig. 7*A*). Merging these images shows that N-type Ca<sup>2+</sup> channels often are colocalized with P/Q-type channels (Fig. 7*C*, yellow–orange staining) but also are often located elsewhere in the calyx (Fig. 7*C*, green staining). The double-labeled images are consistent with the conclusion that N-type Ca<sup>2+</sup> channels are colocalized with P/Q-type Ca<sup>2+</sup> channels in release sites, but are also localized elsewhere in the calyx terminals.

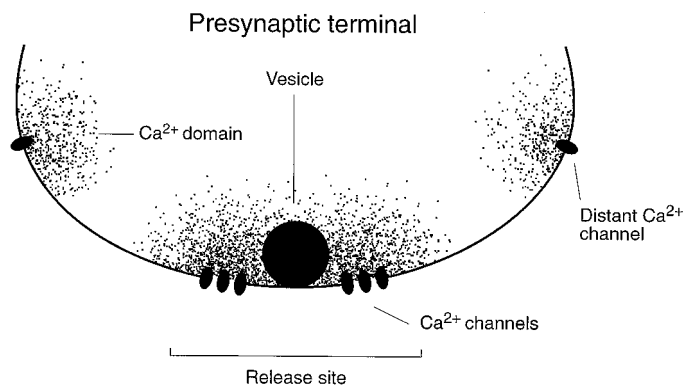
## DISCUSSION

We have demonstrated that three classes of Ca<sup>2+</sup> channels, R- (Wu et al., 1998), P/Q-, and N-type control transmitter release at single release sites in single calyx-type terminals in the rat MNTB. The value of *m* for P/Q-type channels was higher than for N- and R-type channels, meaning that P/Q-type channels trigger release more effectively than N- and R-type. This difference was not caused by a difference in their kinetic properties or by a greater diffusional distance between the N-type or R-type Ca<sup>2+</sup> channels that contributed to transmitter release and the

Ca<sup>2+</sup> sensor that triggered release. Double immunocytochemical labeling showed that P/Q-type Ca<sup>2+</sup> channels containing  $\alpha_{1A}$  subunits were colocalized mostly with synaptic vesicle clusters, whereas N-type channels containing  $\alpha_{1B}$  and R-type channels containing  $\alpha_{1E}$  were only partly colocalized with vesicle clusters. These results suggest that the subcellular distribution of Ca<sup>2+</sup> channels within a terminal is one of the factors that determines their effectiveness in triggering release. As summarized in Figure 8, our results are best explained by assuming that a larger fraction of N- and R-type channels are localized distant from release sites. However, within a release site, all three subtypes of channels are localized at a similar distance from the Ca<sup>2+</sup> sensor.

## Multiple subtypes of Ca<sup>2+</sup> channels in the calyx of Held

Takahashi et al. (1996) reported that the majority (96%) of the presynaptic Ca<sup>2+</sup> current at the rat MNTB synapse is Aga-sensitive P/Q-type and that Ctx has no effect on the presynaptic Ca<sup>2+</sup> current. This is not consistent with our results (Table 1). The rats used by Takahashi et al. (1996) were 8- to 18-d-old, whereas we used 8- to 10-d-old rats. A developmental change of presynaptic Ca<sup>2+</sup> channel subtypes in the MNTB (Iwasaki and Takahashi, 1998) might partly account for this discrepancy, although in contrast to our results, Iwasaki and Takahashi (1998) did not observe a contribution of N-type Ca<sup>2+</sup> channels to synaptic transmission in 10-d-old rats. Several lines of evidence support that three subtypes rather than a single subtype of Ca<sup>2+</sup> channels are present in 8- to 10-d-old rats. Aga blocked slightly more than half of the total presynaptic Ca<sup>2+</sup> current at 100 nM. Ctx blocked about a quarter of the presynaptic Ca<sup>2+</sup> current and ~35% of the EPSC at saturating concentrations. Ctx from two sources (Bachem and Research Biochemicals) was used, and no significant difference was noticed. The threshold for activation of the Ctx-sensitive current was at least 10 mV more positive than that of the Ctx-insensitive current, indicating that the biophysical properties of the N-type current are different from the Ctx-insensitive current. In addition, the N- and P/Q-type channel blocker  $\omega$ -conotoxin-MVIIC reaches its maximal effect at 8  $\mu$ M by blocking about three quarters of the Ca<sup>2+</sup> current (Wu et al., 1998), similar to the summed effect of coapplication of Aga and Ctx (Table 1). Coapplication of  $\omega$ -conotoxin-MVIIC, Aga, and Ctx at saturating concentrations causes a similar effect as applying only  $\omega$ -conotoxin-MVIIC (Wu et al., 1998). The  $\omega$ -conotoxin-MVIIC-sensitive current showed a slowly activating component



**Figure 8.** A schematic view of the distribution of Ca<sup>2+</sup> channels in the calyx of Held (not drawn to scale). Within a release site, three subtypes of channels, P/Q-, N-, and R-type channels are equally close to the docked vesicle. The local Ca<sup>2+</sup> domains created by the opening of channels overlap within the release site. Some Ca<sup>2+</sup> channels, labeled as “distant Ca<sup>2+</sup> channel”, are located so far from this or other release sites that their Ca<sup>2+</sup> domains do not contribute to release. A larger fraction of N- and R-type than P/Q-type channels are distant Ca<sup>2+</sup> channels.



when activated at  $-30$  to  $-20$  mV, whereas  $\omega$ -conotoxin-MV1IC-insensitive R-type current does not have this component (Wu et al., 1998). This result suggests that the R-type current is kinetically different from other subtypes of currents. Furthermore, immunostaining showed that  $\alpha_{1A}$  subunits of P/Q-type,  $\alpha_{1B}$  subunits of N-type, and  $\alpha_{1E}$  subunits of R-type Ca<sup>2+</sup> channels are mostly or partly localized at the presynaptic release sites. Taken together, the pharmacological evidence shows the presence of three subtypes of Ca<sup>2+</sup> channels in a single terminal, which differ in their threshold of activation, current kinetics, and the composition of their  $\alpha_1$  subunits.

In neuronal somata, the percentages of different Ca<sup>2+</sup> currents evoked by a depolarizing voltage step may be different from that evoked by an action potential (McCobb and Beam, 1991; Scroggs and Fox, 1992). Whether this is also the case in synaptic terminals had previously not been examined. Ctx and  $\omega$ -conotoxin-MV1IC (Wu et al., 1998) reduced the Ca<sup>2+</sup> influx by an action potential to a similar degree as the current evoked by a depolarizing voltage step. These results suggest that none of the three subtypes of Ca<sup>2+</sup> channels is preferentially activated by action potentials.

### Different effectiveness of Ca<sup>2+</sup> channel subtypes

If release rates are close to maximal, the relation between Ca<sup>2+</sup> influx and release becomes less steep. Blocking a small fraction of Ca<sup>2+</sup> channels would then cause a relatively small effect on release and thus cause an apparently smaller effectiveness of Ca<sup>2+</sup> channels in controlling release (Turner et al., 1993; Wheeler et al., 1996). Could the low value of  $m$  for the N-type or the R-type Ca<sup>2+</sup> channels observed in our experiments be caused by saturation of the release process? Because Aga, at 30 nM, gave a similar reduction in  $\Delta[Ca^{2+}]$ , but a much larger reduction in the EPSC than 1  $\mu$ M Ctx, this possibility is unlikely. When extracellular Ca<sup>2+</sup> concentration was reduced from 2 to 1 mM, a large effect on the release was also observed (Wu et al., 1998), again suggesting that at physiological extracellular Ca<sup>2+</sup> concentrations, release rates are submaximal.

The value of  $m$  for P/Q- and N-type channels has also been measured for populations of other synapses (Wu and Saggau, 1994; Mintz et al., 1995; Qian et al., 1997). The value of  $m$  is slightly lower for the N-type ( $m = 3.5$ ) than for the P/Q-type channels ( $m = 4.1$ – $4.2$ ) in guinea pig and rat hippocampal synapses (Wu and Saggau, 1994; Qian et al., 1997). This difference is more pronounced ( $m = 2.5$  for the N-type and 4.0 for the P-type) at cerebellar parallel fiber synapses (Mintz et al., 1995). At MNTB synapse the largest difference is in the values of  $m$  for P/Q- and N-type channels (Table 1). Consistent with results in cerebellar and hippocampal synapses, the  $m$  for the P/Q-type channels is also the highest in the MNTB synapse and it seems possible that the high  $m$  value observed by Mintz et al. (1995) for the P/Q-type channels is related to the presence of toxin-resistant channels with a relatively low effectiveness in the parallel fiber terminals, similar to the situation in the MNTB.

Ca<sup>2+</sup> domains caused by the opening of three subtypes of channels are likely to overlap at single release sites at MNTB synapses, consistent with our earlier results (Borst and Sakmann, 1996, 1998, 1999) and with results obtained in hippocampal (Wu and Saggau, 1994) and cerebellar (Mintz et al., 1995) synapses. At the MNTB synapse at least three Ca<sup>2+</sup> channels, each of a different subtype, can contribute to release of a vesicle in the calyx. Although probably  $>60$  Ca<sup>2+</sup> channels open for each vesicle that is released during an action potential (Borst and

Sakmann, 1996), a significant fraction of N- and R-type Ca<sup>2+</sup> channels may be distant channels that do not contribute to release evoked by a single action potential.

### Different localization of Ca<sup>2+</sup> channel subtypes

Double labeling with antibodies to different Ca<sup>2+</sup> channel subtypes and to the vesicle protein synaptotagmin provided a direct comparison of localization of Ca<sup>2+</sup> channel subtypes and release sites at a nerve terminal. The  $\alpha_{1A}$  subunits of P/Q-type Ca<sup>2+</sup> channels are highly effective in triggering release and are colocalized in clusters with synaptotagmin, apparently at the release face of the calyx, whereas the  $\alpha_{1B}$  subunits of N-type and  $\alpha_{1E}$  subunits of R-type Ca<sup>2+</sup> channels are partly or mostly not colocalized at release sites and P/Q-type channels. The correlation between the effectiveness in triggering release and colocalization with synaptotagmin suggests that Ca<sup>2+</sup> channel localization can determine its effectiveness in triggering release.

Given a similar low value of  $m$  for N- and R-type channels (Table 1), why does it appear that a larger fraction of  $\alpha_{1E}$  subunits of R-type than  $\alpha_{1B}$  subunits of N-type Ca<sup>2+</sup> channels are not located at release sites? The  $m$  value for R-type Ca<sup>2+</sup> channels was measured using the blocker Ni<sup>2+</sup>, which reduced presynaptic Ca<sup>2+</sup> influx primarily, but not exclusively, by blocking R-type Ca<sup>2+</sup> currents (Wu et al., 1998). Thus, the  $m$  value measured this way could have been an overestimate. Alternatively, there may be two subtypes of R-type channels (Tottene et al., 1996; Wu et al., 1998), one of which is not recognized by the antibody against the  $\alpha_{1E}$  subunit and is located at the release site.

One mechanism contributing to localization of Ca<sup>2+</sup> channels at release sites is thought to be binding of the synaptic protein interaction (synprint) site on N- and P/Q-type Ca<sup>2+</sup> channels to SNARE proteins (Sheng et al., 1994; Rettig et al., 1996). Disruption of this interaction reduces the effectiveness of Ca<sup>2+</sup> entry through N-type channels in triggering transmitter release at cholinergic synapses between pairs of cultured sympathetic ganglion neurons and between cultured motor neurons and muscle cells (Mochida et al., 1996; Rettig et al., 1997). The different localization of Ca<sup>2+</sup> channels observed here could reflect competition among Ca<sup>2+</sup> channel subtypes for localization at release sites, with P/Q-type channels having a higher affinity than N-type or R-type channels for the SNARE complex in the calyx.

### Contribution of channel subtypes to transmitter release

This particular arrangement of Ca<sup>2+</sup> channel subtypes at terminals may have an important functional impact on various aspects of synaptic transmission. For example, distant Ca<sup>2+</sup> channels located far from release sites may contribute little to the local increase in Ca<sup>2+</sup> concentration that triggers transmitter release (Fig. 8), but may contribute to the residual Ca<sup>2+</sup> concentration following an action potential which influences different forms of short-term synaptic plasticity such as paired-pulse facilitation and post-tetanic potentiation (Zucker, 1996). Several neurotransmitters and neuromodulators inhibit transmitter release by modulation of presynaptic Ca<sup>2+</sup> channels (Wu and Saggau, 1997). Modulation of the highly effective Ca<sup>2+</sup> channel subtypes will have a larger effect on transmitter release, whereas modulation of the less effective channel subtypes will cause a smaller change of transmitter release. Modulation of distant Ca<sup>2+</sup> channels will only affect release during high-frequency signaling.

## APPENDIX

**Nonuniform distribution of different subtypes of Ca<sup>2+</sup> channels at different release sites leads to a lower value of *m***

Lowering the extracellular Ca<sup>2+</sup> concentration decreases the driving force for the Ca<sup>2+</sup> influx through every Ca<sup>2+</sup> channel, although there might be minor differences between Ca<sup>2+</sup> channel subtypes in their affinity for extracellular Ca<sup>2+</sup>. The *n* (= 2.7 ± 0.2; 5 synapses) measured this way (Wu et al., 1998) is likely to reflect the cooperativity of Ca<sup>2+</sup> in triggering release (Augustine et al., 1991; Mintz et al., 1995). Thus, we regarded the value of *n* in each release site as 2.7 (Table 1). Since Aga blocked 97 ± 1% of the EPSC, we assumed that P/Q-type channels contributed to release at all release sites. Table 1 shows the percentage of the presynaptic Ca<sup>2+</sup> influx contributed by each subtype of channels. By scaling the measured percentages down by 8%, the sum of three components (mean only) is exactly 100%, which simplified the calculations below. After scaling, P/Q-type channels contributed ~50% of the total Ca<sup>2+</sup> influx, whereas N- and R-type each contributed ~25%. Thus, 50% of the Ca<sup>2+</sup> influx at each release site is contributed by P/Q-type channels. N- and R-type channels share their targeted release sites with P/Q-type channels.

If N- and R-type channels are separated in every release site, P- and N-type channels control release in 50% of release sites, whereas P- and R-type channels control release in the other 50% of release sites. When N-type channels (~25% of total channels, Table 1) are blocked, at 50% of release sites Ca<sup>2+</sup> will enter exclusively via P/Q-type channels, and the remaining 50% of release sites will be unaffected. The remaining fraction of release (Release<sub>normalized</sub>) after the block of N-type channels is: Release<sub>normalized</sub> = 50%\*(0.5<sup>2.7</sup>) + 50%\*(1<sup>2.7</sup>) = 58%. The apparent power can be measured from relation (2), 58% = (1 - 0.25)<sup>*m*</sup>, *m* = 1.9, close to the measured value (*m* = 1.3 ± 0.1). Similar calculations were made for R-type channels, yielding similar results. This view could account at least partly for the lower values of *m* (see also Reid et al., 1998).

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