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N- and P/Q-Type Ca^{2+} Channels Mediate Transmitter Release with a Similar Cooperativity at Rat Hippocampal Autapses

Christopher A. Reid, John M. Bekkers, and John D. Clements

Division of Neuroscience, John Curtin School of Medical Research, Australian National University, Canberra ACT 0200, Australia

The relationship between extracellular Ca^{2+} concentration and EPSC amplitude was investigated at excitatory autapses on cultured hippocampal neurons. This relationship was steeply nonlinear, implicating the cooperative involvement of several Ca^{2+} ions in the release of each vesicle of transmitter. The cooperativity was estimated to be 3.1 using a power function fit and 3.3 using a Hill equation fit. However, simulations suggest that these values underestimate the true cooperativity. The role of different Ca^{2+} channel subtypes in shaping the Ca^{2+} dose–response relationship was studied using the selective Ca^{2+} channel blockers ω-agatoxin GIVA (ω-Aga), which blocks P/Q-type channels, and ω-conotoxin GVIA (ω-CTx), which blocks N-type channels. Both blockers broadened the dose–response relationship, and the Hill coefficient was reduced to 2.5 by ω-Aga and to 2.6 by ω-CTx. This broadening is consistent with a nonuniform distribution of Ca^{2+} channel subtypes across presynaptic terminals. The similar Hill coefficients in ω-Aga or ω-CTx suggest that there was no difference in the degree of cooperativity for transmitter release mediated via N- or P/Q-type Ca^{2+} channels. A model of the role of calcium in transmitter release is developed. It is based on a modified Dodge–Rahamimoff equation that includes a nonlinear relationship between extracellular and intracellular Ca^{2+} concentration, has a cooperativity of 4, and incorporates a nonuniform distribution of Ca^{2+} channel subtypes across presynaptic terminals. The model predictions are consistent with all of the results reported in this study.

Key words: calcium channel; synaptic transmission; cooperativity; ω-agatoxin GIVA; ω-conotoxin GVIA; synaptic terminal; vesicle release complex

The nonlinear relationship between extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) and transmitter release was first characterized at the frog neuromuscular junction (NMJ) by Dodge and Rahamimoff (1967). In this preparation, the excitatory junction potential amplitude varies as the 4th power of $[Ca^{2+}]_o$ at low concentrations. This implicates the cooperative involvement of four Ca^{2+} ions in the release of each vesicle of transmitter (Dodge and Rahamimoff, 1967). The relationship between transmitter release and $[Ca^{2+}]_o$ is also steeply nonlinear at central synapses. However, it is more difficult to measure the degree of cooperativity in CNS preparations, and it is estimated that between two and four Ca^{2+} ions are involved in the release of each vesicle (Wu and Saggau, 1994; Mintz et al., 1995; Borst and Sakmann, 1996; Takahashi et al., 1996). It has been suggested that different Ca^{2+} channel subtypes mediate transmitter release with different cooperativities (Mintz et al., 1995), but this finding remains controversial (Wu and Saggau, 1994).

Several Ca^{2+} channels subtypes, including the N and P/Q types, support the release of neurotransmitter at many central synapses (Luebke et al., 1993; Wheeler et al., 1994; Wu and Saggau, 1994; Mintz et al., 1995; Scholz and Miller, 1995). Recent evidence suggests that release is more steeply dependent on intraterminal Ca^{2+} concentration ($[Ca^{2+}]_{it}$) for P/Q- than for N-type Ca^{2+} channels at synapses in the cerebellum (Mintz et al., 1995). Cooperativity was estimated at 4 for P/Q-type Ca^{2+} channels and 2.5 for N-type channels. This study used Ca^{2+}-sensitive dyes to measure $[Ca^{2+}]_{it}$ before and after selective block of each Ca^{2+} channel subtype. Cooperativity was estimated from two data points, one recorded before and one after selective block, and these points spanned a different $[Ca^{2+}]_{it}$ range for each channel subtype. A study using a similar technique in the hippocampus, and exploring the $[Ca^{2+}]_{it}$ range more thoroughly, found no significant difference in the cooperativity associated with different Ca^{2+} channel subtypes (Wu and Saggau, 1994).

In the present study we used selective Ca^{2+} channel blockers and traditional dose–response analysis over a wide range of Ca^{2+} concentrations to investigate the cooperativity associated with different Ca^{2+} channel subtypes at a central synapse. The results support earlier evidence for a nonuniform distribution of Ca^{2+} channel subtypes across presynaptic terminals. However, there was no significant difference in cooperativity for transmitter release mediated via N- or P/Q-type Ca^{2+} channels.

MATERIALS AND METHODS

Cell culture. Single, isolated hippocampal neurons were grown on "microdots" as described previously (Bekkers and Stevens, 1991; Segal, 1991). Cells were used after 10–16 d in culture.

Electrophysiology. Whole-cell patch-clamp recordings were obtained from isolated excitatory neurons that formed autaptic synapses with abundant terminals. Patch electrodes contained (in mM): $KMeSO_4$ 125, KCl 5, EGTA 10, HEPES 10, Na_2ATP 2, $MgCl_2$ 2, and GTP 0.4, pH 7.3, with osmolarity adjusted to 290 mOsm with sorbitol. The bath solution contained (in mM): NaCl 135, KCl 5, $MgCl_2$ 10, glucose 10, and HEPES 10, pH 7.3, with osmolarity adjusted to 310 mOsm with sorbitol. $CaCl_2$ was added from stock to a final concentration of between 0.4 and 10 mM. Salts were from Johnson Matthey (Karlsruhe, Germany) or Sigma (St. Louis, MO).

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Correspondence should be addressed to John Clements at the above address.

Currents were recorded by a patch-clamp amplifier (Axon Instruments, Foster City, CA), low-pass-filtered at 5 kHz, and digitally sampled at 10 kHz. Patch electrodes had resistances ranging from 2.0 to 3.5 MΩ. Series resistance was typically 5 MΩ (range, 4–7 MΩ), and compensation was set at 80–90%. Errors associated with uncompensated series resistance were small under our recording conditions (see Results). Neurons were voltage-clamped at −60 mV, and a 1–2 msec voltage step to 0 mV was applied at 6 sec intervals, evoking an AMPA receptor-mediated autaptic EPSC. AMPA EPSCs were measured by averaging the amplitude over a 5–10 msec range around the peak of the current. Residual non-AMPA current, measured in the same way in the presence of 10 μM 6-cyano-7-nitroquinoxaline-2–3-dione (CNQX; Research Biochemicals, Natick, MA) during each experiment, was subtracted from all EPSC measurements. Solutions were applied via a series of glass flow pipes through which solutions flowed continuously at ~0.1 ml/min. The internal diameters of the pipes (500 μm) were larger than the diameters of the microdots, which ensured a uniform drug concentration at all autaptic contacts. Solution exchanges were made by quickly moving the flow pipes between autaptic stimuli. ω-Conotoxin GVIA (ω-CTx) was obtained from Alomone Labs (Jerusalem, Israel), and ω-agatoxin GIVA (ω-Aga) was a gift from Pfizer (Groton, CT). ω-Aga experiments were done in bath solutions containing cytochrome *c* (Sigma) at 1 mg/ml to reduce nonspecific binding of the toxin. Control experiments showed that cytochrome *c* alone had no effect on EPSC amplitude (98 ± 4%; $n = 3$). All experiments were performed at room temperature (20–24 °C).

Analysis. All analysis was done using AxoGraph (Axon Instruments). Four different equations were used to fit the observed Ca^{2+} dose–response curve: the Hill equation, two forms of the Dodge–Rahamimoff equation, and a power function.

The Hill equation provides a useful empirical description of the dose–response relationship, although it reflects a physically unrealistic reaction scheme (Weiss, 1997). The Hill equation is:

$$E = S[Ca^{2+}]_o^{N_H}/(EC_{50}^{N_H} + [Ca^{2+}]_o^{N_H}),$$

where E is the EPSC amplitude; S is a scaling factor; EC_{50} is the Ca^{2+} concentration giving half of the maximal synaptic response; and N_H is the Hill coefficient, an empirical value related to the cooperativity underlying the dose–response relationship.

In contrast, the Dodge–Rahamimoff equation is based on physically plausible assumptions. It requires that Ca^{2+} ions bind to several independent sites on a presynaptic protein complex to promote the release of a transmitter vesicle (Dodge and Rahamimoff, 1967). It also assumes that Mg^{2+} ions can bind to the same sites but do not promote vesicle release. The standard Dodge–Rahamimoff equation is:

$$E = S(([Ca^{2+}]_o/K_1)/(1 + [Ca^{2+}]_o/K_1 + [Mg^{2+}]_o/K_2))^{N_D},$$

where K_1 is the affinity for Ca^{2+} binding to the vesicle release complex; K_2 is the affinity for Mg^{2+} binding to the vesicle release complex; and N_D is the number of Ca^{2+} ion binding sites that must be occupied to trigger the release of a transmitter vesicle:

The two affinity parameters, K_1 and K_2, are expressed in terms of extracellular Ca^{2+} and Mg^{2+} concentrations. The effective intraterminal Ca^{2+} and Mg^{2+} concentrations are assumed to be linearly related to the extracellular concentrations. However, this relationship was recently shown to be sublinear at $[Ca^{2+}]_o > 1$ mM (Mintz et al., 1995; Borst and Sakmann, 1996). To address this problem, we modified the Dodge–Rahamimoff equation by explicitly incorporating a sublinear relationship between $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_o$. The extracellular calcium concentration term was replaced by an empirical expression for the effective intraterminal concentration in the modified equation:

$$[Ca^{2+}]_{it} = [Ca^{2+}]_o/(1 + ([Ca^{2+}]_o/K_s)^{N_s})^{1/N_s},$$

where K_s is the $[Ca^{2+}]_o$ where flux into the terminal is reduced by $(1/2)^{1/N_s}$; and N_s is the degree of cooperativity for Ca^{2+} inhibition of Ca^{2+} flux.

This expression was chosen for two reasons: (1) the relationship between $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_o$ is linear at low extracellular concentrations but is sublinear at higher concentrations; and (2) when $N_s = 1$, the expression reduces to the form predicted by the Michaelis–Menten expression for Ca^{2+} flux through a channel pore with a single rate-limiting Ca^{2+} binding site (Hille, 1992; Church and Stanley, 1996). The modified Dodge–Rahamimoff equation could not be used to estimate cooperativity. The cooperativity parameters, N_D and N_s, can interact, leading to a nonunique solution. For this reason, both parameters should be fixed when fitting this equation to dose–response data.

In the limit as $[Ca^{2+}]_o \rightarrow 0$, both the Hill and Dodge–Rahamimoff equations reduce to a power function form. The power function is:

$$E = S[Ca^{2+}]_o^{N_P},$$

where N_P is an empirical parameter indicating the degree of cooperativity of the dose–response relationship.

This function is only valid in the low concentration limit, so it was fit over the three lowest Ca^{2+} concentration points of each dose–response curve. All theoretical curves were fit to the data by minimizing χ^2. In 3 of 20 cells a single outlier point was deleted before performing the Dodge–Rahamimoff fit to the full dose–response curve. The quality of the fit for the Hill and Dodge–Rahamimoff equations was determined using a χ^2 test. Other statistical comparisons were made using Student's unpaired t test.

Model synapse. A model synapse was constructed to investigate the role of different Ca^{2+} channel subtypes, and their distribution across synaptic terminals, in synaptic function. The model predicts EPSC amplitude as a function of $[Ca^{2+}]_o$ in the presence of Ca^{2+} channel blockers. Three classes of terminal were included in the model: one class with only P/Q-type channels (QQ), one class with only N-type channels (NN), and one class with both channel subtypes (NQ). The postsynaptic response of the three classes was summed to produce the model synaptic response. It was assumed that at an individual terminal, Ca^{2+} entering through different channels combined to act on the same vesicle release site or sites. At NQ terminals, N-type channels were assumed to contribute one-half of the effective intraterminal Ca^{2+} and P/Q-type channels the other half. The two channel subtypes were assumed to have similar activation and Ca^{2+} flux properties. Each class of terminal generated a dose–response curve based on a modified Dodge–Rahamimoff equation with parameter values derived from the fits to experimental data recorded in the absence of Ca^{2+} channel blockers. The effects of Cd^{2+} were modeled by reducing Ca^{2+} influx uniformly at each class of terminal. ω-CTx blocked influx at NN terminals and reduced influx by half at NQ terminals, whereas ω-Aga blocked influx at QQ terminals and reduced it by half at NQ terminals. The proportion of model terminals in the three classes were set at 45% QQ, 45% NQ, and 10% NN, based on results from a previous study (Reid et al., 1997).

RESULTS

Ca^{2+} dose–response curve for autaptic EPSCs

The amplitude of the AMPA EPSC was measured as a function of $[Ca^{2+}]_o$ at autaptic synapses on cultured hippocampal neurons (Fig. 1*A*). EPSC amplitude measurements at each $[Ca^{2+}]_o$ were bracketed with measurements at 2 mM $[Ca^{2+}]_o$ to ensure the stability of the recording (Fig. 1*B*). The relationship between EPSC amplitude and $[Ca^{2+}]_o$ was highly nonlinear (Fig. 1*C*), consistent with the cooperative involvement of several Ca^{2+} ions in transmitter release.

One potential source of nonlinearity is reduced driving force caused by inadequate voltage clamp of larger synaptic currents. To test for this possibility, we applied CNQX (0.5 μM) at low and high $[Ca^{2+}]_o$ (1.2 and 10 mM). If clamp error is a significant problem, then the larger EPSC recorded in high $[Ca^{2+}]_o$ should be less sensitive to an antagonist. CNQX reduced EPSC amplitude by 80 ± 6% in low $[Ca^{2+}]_o$ and by 75 ± 5% in high $[Ca^{2+}]_o$ ($n = 5$; results not shown). Thus, clamp error generally is small under our recording conditions.

We estimated the number of Ca^{2+} ions that cooperate to trigger the release of a vesicle (the degree of cooperativity) using several approaches. These were based on fitting an equation to the EPSC amplitude versus $[Ca^{2+}]_o$ dose–response curve. The Dodge–Rahamimoff equation, the Hill equation, and the power function were used. Each equation contains a parameter related to cooperativity (see Materials and Methods). The standard Dodge–Rahamimoff equation is:

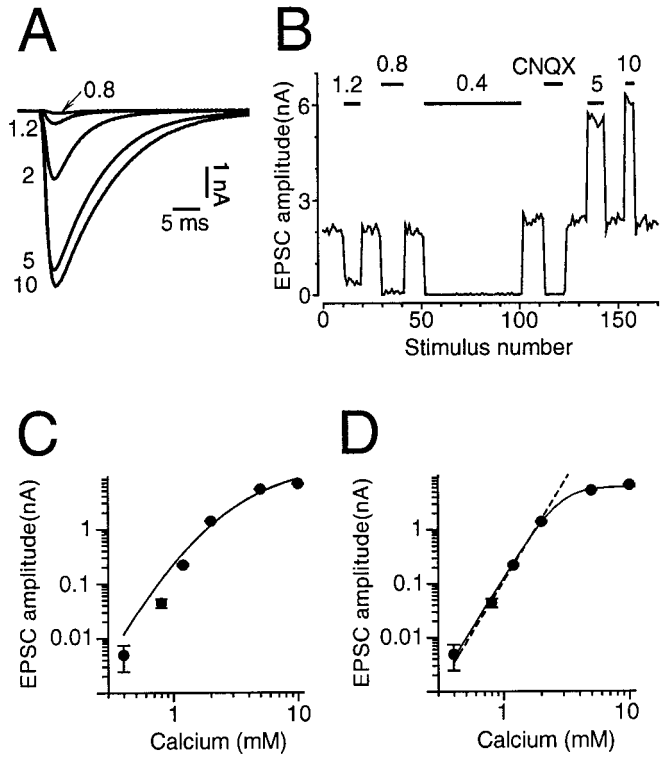

Figure 1. Nonlinear relationship between EPSC amplitude and $[Ca^{2+}]_o$. The cooperative involvement of several Ca^{2+} ions in the release of each vesicle of neurotransmitter is demonstrated by the nonlinear relationship between $[Ca^{2+}]_o$ and EPSC amplitude. *A*, AMPA EPSCs recorded at several different $[Ca^{2+}]_o$. Each *trace* is the average of 10–50 EPSCs. *B*, Amplitude of individual EPSCs (same cell as in *A*) plotted against stimulus number. Each $[Ca^{2+}]_o$ is bracketed by an epoch in 2 mM $[Ca^{2+}]_o$ to ensure recording stability. *C*, Average EPSC amplitude plotted against $[Ca^{2+}]_o$ on log–log axes. Error bars indicate ±1 SD. The *solid line* is the optimally fitted Dodge–Rahamimoff equation. The fit was poor and could be rejected ($p > 0.05$). *D*, Same data as in *C*, showing the optimally fitted Hill equation (*solid line*) with a Hill coefficient of 3.7. This equation provided an adequate fit ($p < 0.05$). The degree of cooperativity was independently estimated at 3.8 by fitting a power function over the 0.4–1.2 $[Ca^{2+}]_o$ range (*dashed line*).

$$E = S(([Ca^{2+}]_o/K_1)/(1 + [Ca^{2+}]_o/K_1 + [Mg^{2+}]_o/K_2))^{N_D},$$

and the free parameters in the fit were the cooperativity, N_D, and the scaling factor, S. The Ca^{2+} and Mg^{2+} affinities, K_1 and K_2, were fixed to 2.7 and 4.8 mM, respectively (Dodge and Rahamimoff, 1967; Donaldson and Stricker, 1996). This equation did not provide a good description of the data, and the fit could be rejected in every case ($p < 0.05$; $n = 9$; Fig. 1*C*). The fit could also be rejected when either K_1 or K_2 or both were made free parameters ($p < 0.05$; $n = 9$). The most likely explanation for the failure of this approach is that the standard Dodge–Rahamimoff equation assumes a linear relationship between $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_o$, whereas it is now known to be sublinear at higher $[Ca^{2+}]_o$ (Mintz et al., 1995; Borst and Sakmann, 1996). This possibility is investigated below using the modified Dodge–Rahamimoff equation, which incorporates a sublinear relationship between $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_o$.

Another method for measuring the degree of cooperativity is to fit a Hill equation:

$$E = S[Ca^{2+}]_o{}^{N_H}/(EC_{50}{}^{N_H} + [Ca^{2+}]_o{}^{N_H}).$$

The fit provides an estimate of both cooperativity, N_H, and affinity, EC_{50}, of Ca^{2+} binding to the vesicle release complex. It permits empirical comparison between dose–response curves recorded under different conditions (Weiss, 1997). The Hill equation gave a good fit to the data in every case ($p > 0.05$; $n = 9$). The Hill constant, N_H, was 3.3 ± 0.1, and EC_{50} was 2.3 ± 0.2 mM ($n = 9$; Fig. 1*D, solid line*).

The shape of the dose–response curve at lower Ca^{2+} concentrations contains the most information about cooperativity. It is in this region that the curve should follow approximately a power function form. A power function fit restricted to this region may therefore provide a more reliable estimate of cooperativity than the Hill equation fit to the entire dose–response curve. A fit at lower Ca^{2+} concentrations would also be less affected by inadequate voltage clamp. The power function:

$$E = S[Ca^{2+}]_o{}^{N_P},$$

forms a straight line when plotted in log–log coordinates, and the slope of the line is equal to the cooperativity parameter, N_P. The degree of cooperativity was estimated at 3.1 ± 0.2 ($n = 9$; Fig. 1*D, dashed line*) by fitting the power function over the $[Ca^{2+}]_o$ range from 0.4 to 1.2 mM.

Ca^{2+} cooperativity in the presence of Cd^{2+}

We explored the effect of nonselective blockade of Ca^{2+} channels on cooperativity. Cd^{2+} is a competitive blocker of Ca^{2+} channels but is not selective for different Ca^{2+} channel subtypes (Sather et al., 1993; Zhang et al., 1993; Reid et al., 1997). As expected, Cd^{2+} increased the Ca^{2+} EC_{50} in a dose-dependent manner (Fig. 2*A,B*). However, Cd^{2+} produced no change in the degree of cooperativity, N_P (Fig. 2*C,D*). In summary, Cd^{2+} shifted the dose–response curve to the right without changing its steepness at lower Ca^{2+} concentrations.

Ca^{2+} cooperativity in the presence of ω-CTx and ω-Aga

We next explored the effect of selective blockade of Ca^{2+} channel subtypes on cooperativity. ω-CTx and ω-Aga were used to block N- and P/Q-type channels, respectively (Williams et al., 1992; Fujita et al., 1993; Wheeler et al., 1994; Scholz and Miller, 1995). Coapplication of ω-CTx (1 μM) and ω-Aga (0.5 μM) completely blocked the EPSC (>98%), suggesting that N- and P/Q-type Ca^{2+} channels predominantly mediate excitatory synaptic transmission (Wheeler et al., 1996; Reid et al., 1997). N-type Ca^{2+} channels were blocked by ω-CTx (1 μM) in an irreversible manner, and this reduced the EPSC amplitude by 46.6 ± 4% ($n = 7$) in 2 mM Ca^{2+} (Fig. 3*A,B*). ω-Aga at concentrations of >100 nM blocks both P- and Q-type Ca^{2+} channels. The EPSC amplitude reduction produced by ω-Aga was partially reversible in the autaptic culture preparation, so the toxin had to be present throughout the experiment. ω-Aga (0.5 μM) reduced the EPSC by 94 ± 0.4% ($n = 4$) in 2 mM Ca^{2+} (Fig. 3*C,D*).

There was no detectable difference between the degree of cooperativity in the presence of ω-Aga, ($N_P = 2.2 \pm 0.4$; $n = 4$) or ω-CTx ($N_P = 2.4 \pm 0.2$; $n = 7$) (unpaired t test, $p > 0.05$; Fig. 4*A,C*). These estimates are based on a power function fit restricted to the $[Ca^{2+}]_o$ range from 0.8 to 2 mM. Similarly, no difference in cooperativity could be detected when the Hill equation was fit over the entire $[Ca^{2+}]_o$ range. In the presence of ω-Aga, N_H was 2.5 ± 0.4 ($n = 4$), and in ω-CTx, N_H was 2.6 ± 0.2 ($n = 7$).

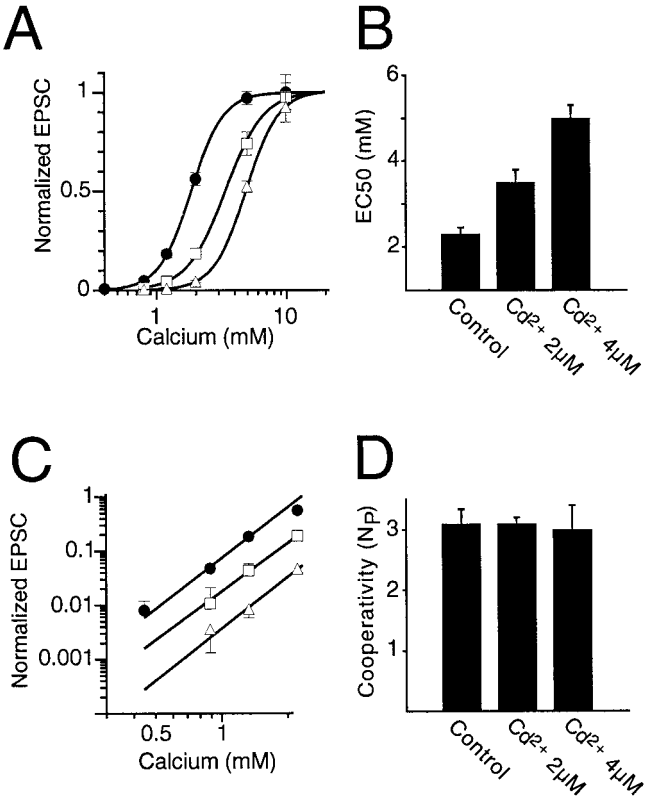

Figure 2. Cd^{2+} does not change the steepness of the Ca^{2+} dose–response curve. The competitive Ca^{2+} channel antagonist Cd^{2+} shifts the Ca^{2+} dose–response curve to the right in a dose-dependent manner. *A*, Linear log plot of the normalized EPSC amplitude versus $[Ca^{2+}]_o$ for three individual cells recorded in normal bath solution (*filled circles*) and in the presence of 2 μM Cd^{2+} (*open squares*) and 4 μM Cd^{2+} (*open triangles*). Each *point* is the ensemble average amplitude ± 1 SD. The *solid lines* are Hill equation fits to the data. *B*, Cd^{2+} increased the EC_{50} for Ca^{2+}. Average EC_{50} values are shown in control solution ($n = 9$) and in the presence of Cd^{2+} (2 and 4 μM; $n = 5$). Error bars indicate SEM. *C*, Cd^{2+} did not broaden or change the steepness of the dose–response curve. Log–log plot of normalized EPSC amplitude versus $[Ca^{2+}]_o$ is shown for the same three cells as in *A*. *Solid lines* show power function fits to the data. *D*, Cd^{2+} had no effect on cooperativity. Average cooperativity (N_P) over the 0.4–1.2 mM $[Ca^{2+}]_o$ range in control solution ($n = 9$) and 0.8–2 mM $[Ca^{2+}]_o$ range in the presence of Cd^{2+} (2 and 4 μM; $n = 5$). Error bars indicate SEM.

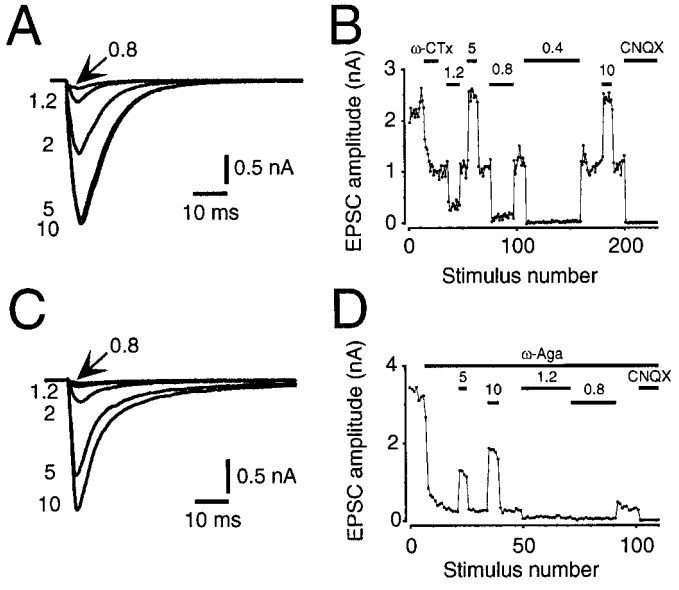

Figure 3. Dose–response relationships for EPSCs recorded in the presence of ω-CTx and ω-Aga. *A*, AMPA EPSCs recorded at several different $[Ca^{2+}]_o$ in the presence of ω-CTx. Each *trace* is the average of 5–20 EPSCs. *B*, Amplitude of individual EPSCs (same cell as in *A*) plotted against stimulus number. Each $[Ca^{2+}]_o$ is bracketed by an epoch in 2 mM $[Ca^{2+}]_o$ to ensure recording stability. *C*, AMPA EPSCs recorded at several different $[Ca^{2+}]_o$ in the presence of ω-Aga. Each *trace* is the average of 5–20 EPSCs. *D*, Amplitude of individual EPSCs (same cell as in C) plotted against stimulus number.

Nonuniform distribution of Ca^{2+} channel subtypes

If both N- and P/Q-type Ca^{2+} channels are present in the same ratio on all presynaptic terminals (uniform distribution) and are functionally equivalent, then selective block of one subtype will produce a uniform shift in EC_{50} at all terminals. The dose–response curve will shift to the right with little change in its shape or steepness. In contrast, if the distribution of Ca^{2+} channel subtypes across synaptic terminals is nonuniform (Reuter, 1995; Reid et al., 1997) then selective block will only shift the EC_{50} at the subset of terminals that possess both Ca^{2+} channel subtypes. Other terminals either will be completely blocked or will have no shift in their EC_{50}. The resulting mixture of terminals with different EC_{50} values will broaden the dose–response relationship and reduce its overall steepness. Thus, a reduction in steepness of the dose–response curve in the presence of a selective Ca^{2+} channel blocker would imply a nonuniform distribution of Ca^{2+} channel subtypes. In contrast, a nonselective Ca^{2+} channel blocker should not alter the overall steepness of the dose–response curve. The Hill equation was fitted over the entire dose–response curve, so the Hill coefficient, N_H, provides an empirical measure of its overall steepness. The selective Ca^{2+} channel blocker ω-CTx reduced N_H to 2.6 ± 0.2 ($n = 7$), and ω-Aga reduced N_H to 2.5 ± 0.4 ($n = 4$) (cf. 3.3 in control). Both reductions were significant (unpaired *t* test, $p < 0.05$). In contrast, the nonselective blocker Cd^{2+} (4 μM) increased N_H to 3.6 ± 0.3 ($n = 5$), but this increase was not significant ($p > 0.05$). These results are consistent with a nonuniform distribution of the Ca^{2+} channel subtypes across presynaptic terminals.

The modified Dodge–Rahamimoff equation

An underlying assumption used to derive the standard Dodge–Rahamimoff equation was that $[Ca^{2+}]_{it}$ varies linearly with $[Ca^{2+}]_o$, but this relationship was recently found to be sublinear at central synapses for $[Ca^{2+}]_o$ of >1 mM (Mintz et al., 1995; Borst and Sakmann, 1996). This sublinearity implies that experimental dose–response curves will reach saturation more rapidly than predicted by the standard Dodge–Rahamimoff equation. Rapid saturation was seen consistently in the present study and was responsible for the poor quality of the fit with this equation (Fig. 1*C*). (Note that the discrepancies between the fit and the data appear larger at low $[Ca^{2+}]_o$ because of the log–log coordinates.) Similar behavior has been reported previously (Wheeler et al., 1996) (but see Allen and Stevens, 1994). To address this problem, we incorporated a sublinear relationship between $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_o$ into a modified Dodge–Rahamimoff equation (see Materials and Methods). The modified equation was fit to individual dose–response curves, and it produced a good fit in eight of nine cases ($p > 0.05$; Fig. 5*A*). The parameters K_1 and K_2 were fixed at previously reported values for excitatory hippocampal synapses (2.7

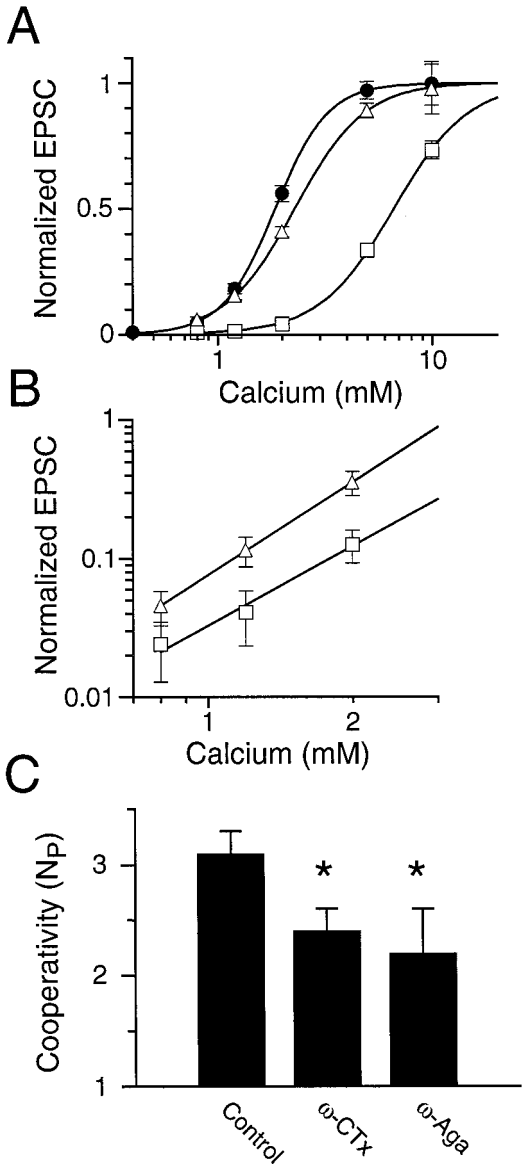

Figure 4. ω-CTx and ω-Aga broaden the Ca^{2+} dose–response curve. Selective Ca^{2+} channel blockers shift the Ca^{2+} dose–response curve to the right and broaden it. *A*, Linear log plot of the normalized EPSC amplitude versus $[Ca^{2+}]_o$ for three individual cells in normal bath solution (*filled circles*) and in the presence of ω-CTx (*open triangles*) and ω-Aga (*open squares*). Each *point* is the ensemble average amplitude ± 1 SD. The *solid lines* are Hill equation fits to the data. *B*, Log–log plot of normalized EPSC amplitude versus $[Ca^{2+}]_o$ for two individual cells recorded in the presence of ω-CTx (*open triangles*) or ω-Aga (*open squares*). Each *point* is the ensemble average amplitude ± 1 SD. The *solid lines* are power function fits over the $[Ca^{2+}]_o$ range from 0.8 to 2 mM. *C*, The broadening of the dose–response curve by selective toxins reduces the cooperativity (N_P). Average N_P is shown in control ($n = 9$) and in the presence of either ω-CTx ($n = 7$) or ω-Aga ($n = 4$). The significant reduction in N_P in the presence of selective toxin is consistent with a nonuniform distribution of Ca^{2+} channel subtypes across presynaptic terminals. Error bars indicate SEM. *Statistical significance ($p < 0.05$).

and 4.8 mM, respectively) (Donaldson and Stricker, 1996), N_D was fixed at 4 (Dodge and Rahamimoff, 1967), and N_s was fixed at 2. Only the scaling factor, *S*, and the calcium block affinity, K_s, were free parameters, and the optimum value of K_s was 2.1 ± 0.2 mM ($n = 9$).

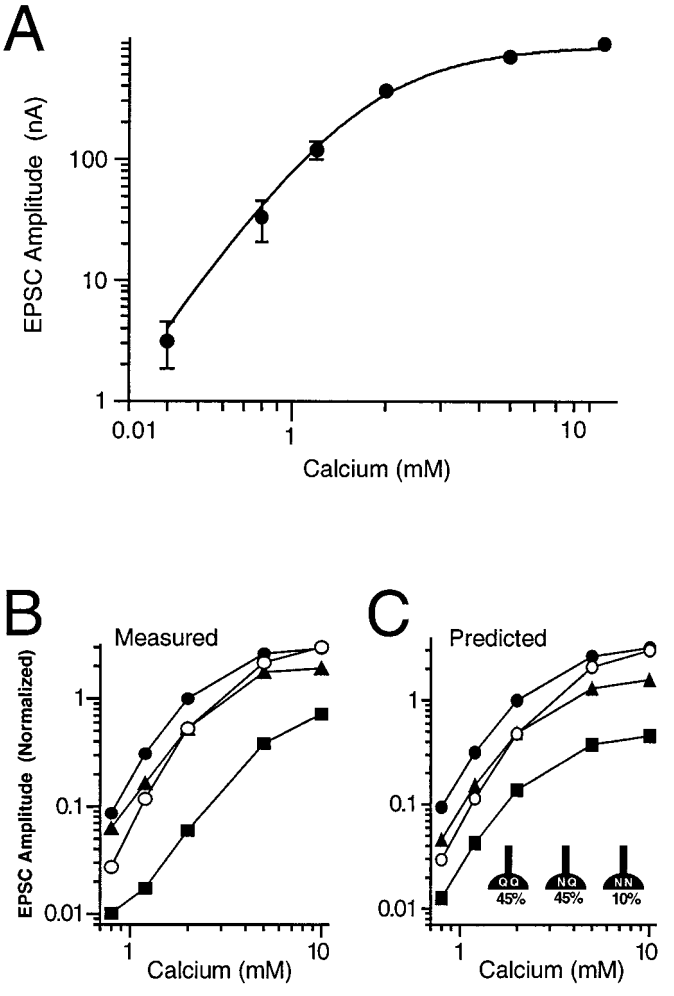

Figure 5. A synaptic model predicts the Ca^{2+} dose–response curve observed in the presence of ω-CTx, ω-Aga, and Cd^{2+}. The modified Dodge–Rahamimoff equation, which incorporates a sublinear relationship between $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_o$, provides an accurate description of the Ca^{2+} dose–response curve. *A*, Log–log plot of EPSC amplitude versus $[Ca^{2+}]_o$ is shown for an individual cell. The *solid line* is the optimally fitted modified Dodge–Rahamimoff equation, which represents a good fit ($p < 0.05$). *B*, Log–log plot of average normalized EPSC amplitude versus $[Ca^{2+}]_o$ under four different experimental conditions: control (*filled circles*; $n = 9$), ω-Aga (0.5 μM; *filled squares*; $n = 5$), ω-CTx (1 μM; *filled triangles*; $n = 5$), and Cd^{2+} (2 μM; *open circles*, $n = 7$). EPSC amplitudes from individual cells were normalized to the amplitude recorded at 2 mM $[Ca^{2+}]_o$ in the absence of blockers. *C*, A model based on the modified Dodge–Rahamimoff equation (see *inset* and Results) accurately predicted the observed dose–response curves in *B*. Theoretical dose–response curves are shown in control and in the presence of Ca^{2+} channel blockers. *Inset*, Schematic overview of the model. Presynaptic terminals were divided into three classes: one with only P/Q-type Ca^{2+} channels (*QQ*), one with only N-type Ca^{2+} channels (*NN*), and one with both channel subtypes (*NQ*).

A model synapse

The modified Dodge–Rahamimoff equation was incorporated into a model synapse with a nonuniform distribution of Ca^{2+} channel subtypes across its terminals (see Materials and Methods). Dose–response curves were generated by the model synapse in the presence and absence of Ca^{2+} channel blockers. The model predicted all the main features of our data, and the experimental dose–response curves (Fig. 5*B*) are very similar to the model curves (Fig. 5*C*). In constructing Figure 5, *B* and *C*, all

experimental and theoretical EPSC amplitudes were normalized to the response in 2 mM Ca^{2+} with no blockers. The cooperativity parameter, N_P, was calculated from power function fits to the theoretical dose–response data using the same $[Ca^{2+}]_o$ range and the same number of data points that were used when fitting the experimental data. The calculated value of N_P was 3.0 in the absence of blockers, 3.0 in Cd^{2+}, and 2.6 in ω-CTx or ω-Aga. These values, calculated from the theoretical curves, all fell within the 95% confidence intervals for experimental estimates of N_P.

The estimated cooperativity, N_P, obtained by fitting a power function to the theoretical dose–response curves was <4, although these curves were constructed from a model with a cooperativity, N_D, of 4. This suggests that power function fits systematically underestimate cooperativity under our experimental conditions. This is because the experimental data could not be extended to sufficiently low $[Ca^{2+}]_o$. When the power function fit was applied to the extrapolated theoretical dose–response curves over a concentration range from 0.005 to 0.1 mM, all values of N_P converged to 4, as expected.

DISCUSSION

We used dose–response analysis to investigate Ca^{2+} cooperativity at a central synapse and found no difference between the degree of cooperativity for transmitter release mediated via N- or P/Q-type Ca^{2+} channels. This result is consistent with the findings of one recent study in the hippocampus (Wu and Saggau, 1994) but contrasts with results from the cerebellum (Mintz et al., 1995). Selective blockers of N- or P/Q-type Ca^{2+} channels broadened the dose–response relationship, thereby implying a nonuniform distribution of Ca^{2+} channel subtypes across synaptic terminals. This finding is consistent with previous reports (Reuter, 1995; Reid et al., 1997) and was incorporated into a model of the role of calcium in synaptic transmission that predicted all the main features of our data. Experimental estimates of Ca^{2+} cooperativity fell in the range of 2–3, under a variety of recording conditions and using several standard analytical approaches. However, the model results suggest that these values systematically underestimate the true cooperativity.

Dose–response analysis in the presence of Ca^{2+} channel nonlinearity

In traditional dose–response studies it is assumed that the effective Ca^{2+} concentration attained at release sites in presynaptic terminals during synaptic activation varies linearly with $[Ca^{2+}]_o$. On the time scale of synaptic transmission, the effective intraterminal Ca^{2+} concentration is approximately proportional to the Ca^{2+} flux into the terminal during the presynaptic action potential and to the duration of the action potential. Studies of Ca^{2+} channel properties suggest that Ca^{2+} flux through a channel varies linearly with $[Ca^{2+}]_o$ at low concentrations but is sublinear at higher concentrations because of transient Ca^{2+}-dependent block of the channel pore (Church and Stanley, 1996). Another potential source of nonlinearity is the membrane potential shielding effect produced by divalent cations, which becomes significant at concentrations above ~2 mM (Hille, 1992). This may reduce the presynaptic action potential amplitude and duration, thereby reducing net Ca^{2+} influx and contributing to sublinearity. The predicted nonlinearity has been directly confirmed using Ca^{2+} imaging techniques (Mintz et al., 1995) and by patch clamping a large presynaptic terminal (Borst and Sakmann, 1996). The relationship between peak Ca^{2+} concentration in the terminal during synaptic activation and $[Ca^{2+}]_o$ was approximately linear for $[Ca^{2+}]_o \leq 1$ mM but was significantly sublinear at higher concentrations. This finding indicates that power function fits to the dose–response curve should be restricted to the concentration range below ~1 mM or corrected for the observed sublinearity at higher concentrations (Borst and Sakmann, 1996). In the present study, the dose–response curves recorded in the presence of Ca^{2+} channel blockers were not corrected and were fit from 0.8 to 2 mM. Cooperativity may be systematically underestimated under these conditions. However, this does not preclude a useful comparison between the degree of cooperativity in ω-CTx and in ω-Aga, because both values were estimated over the same $[Ca^{2+}]_o$ range, and any systematic error should be similar.

The modified Dodge–Rahamimoff equation consistent with cooperativity of 4

The sublinear relationship between $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_o$ was incorporated into the modified Dodge–Rahamimoff equation. This greatly improved the fit to the dose–response data, compared with the standard Dodge–Rahamimoff equation. The improvement was attributable to the faster saturation of the modified Dodge–Rahamimoff curve at higher $[Ca^{2+}]_o$. The fit had the same number of free parameters as the standard equation, so the improvement was not attributable to an increase in the degrees of freedom. This result is compatible with a Ca^{2+} cooperativity of 4 for transmitter release, as reported previously at the NMJ and at a central synapse (Dodge and Rahamimoff, 1967; Borst and Sakmann, 1996). In the present study, when the release cooperativity parameter, N_D, was reduced to 3, the quality of the fit was also reduced, and an adequate fit was obtained in only three of nine cells ($p > 0.05$). The fit was also sensitive to the setting of N_s. If this parameter was fixed at 1, thereby giving a Michaelis–Menten formulation for Ca^{2+} flux (Church and Stanley, 1996), the modified Dodge–Rahamimoff equation no longer fit any of the dose–response curves ($n = 9$; $p < 0.05$). Thus, the sublinearity in the relationship between $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_o$ is not simply attributable to Ca^{2+} binding at a single site in the channel (Church and Stanley, 1996). It is possible that there are multiple binding sites for divalent cations in N- and P/Q-type channels (Hille, 1992) or that an independent process, such as membrane potential shielding by Ca^{2+}, contributes to the sublinearity. Voltage-clamp error is another possible source of sublinearity. In summary, the cooperativity for transmitter release, N_D, must be at least 4, and the cooperativity for Ca^{2+} flux inhibition, N_s, must be at least 2, to obtain a good fit between the modified Dodge–Rahamimoff equation and the dose–response curve observed in the absence of blockers.

No difference between cooperativities for N- and P/Q-type channels

We have shown that cooperativity may be systematically underestimated by the power function fit in the experimental $[Ca^{2+}]_o$ range. Despite this, the comparison of the N_P values under different experimental conditions may still be valid, if the systematic error is similar. This possibility was confirmed by analyzing the model dose–response curves. The values obtained for N_P were nearly identical for release mediated by N- or P/Q-type channels (both 2.6). Our results were consistent with the model in which both channel subtypes were assumed to have a cooperativity of 4. To investigate the sensitivity of the power function fit to differences in cooperativity, the model was altered such that Ca^{2+} entering a terminal through N- and P/Q-type channels induced transmitter release with cooperativities of 2.5 and 4,

respectively (Mintz et al., 1995). A power function fit was performed over the same range used for the experimental data, and the results for N_P were 1.6 in the presence of ω-Aga and 2.6 in the presence of ω-CTx. Thus, the different cooperativities could easily be detected from the dose–response curves. This result implies that systematic errors do not preclude useful comparison between cooperativity estimates for N- and P/Q-type channels under our experimental conditions. Our finding that N_P was similar for release mediated by N- or P/Q-type channels is consistent with previous results in the hippocampus (Wu and Saggau, 1994) but contrasts with results obtained in the cerebellum. This may reflect differences between the functional organization of Ca^{2+} channel subtypes at different synapses.

The nonuniform distribution of Ca^{2+} channel subtypes

The model synapse predicts a broader dose–response relationship in the presence of selective Ca^{2+} channel antagonists than in the presence of a nonselective antagonist (Fig. 5*B*). This broadening is reflected in the reduced values for N_P in the presence of ω-CTx and ω-Aga. These results arise from the nonuniform distribution of Ca^{2+} channel subtypes across the model terminals and are in general agreement with the experimental dose–response data. This finding is consistent with previous reports of a nonuniform distribution of Ca^{2+} channel subtypes across synaptic terminals (Reuter, 1995; Reid et al., 1997). Ca^{2+} imaging studies measure the average Ca flux into many presynaptic terminals (Wu and Saggau, 1994; Mintz et al., 1995). Interpretation of results from these studies will be complicated by a nonuniform distribution of Ca^{2+} channel subtypes.

Conclusions

Dose–response analysis remains a useful tool for investigating Ca^{2+} cooperativity at central synapses, but it is important to incorporate the sublinear relationship between $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_o$ in the analysis. Ca^{2+} imaging or patch-clamp recording from presynaptic terminals can be used to investigate the details of this relationship. There was no difference between the degree of cooperativity for transmitter release mediated via N- or P/Q-type Ca^{2+} channels in our system. Selective blockers of N- or P/Q-type Ca^{2+} channels broadened the dose–response relationship, consistent with a nonuniform distribution of Ca^{2+} channel subtypes across synaptic terminals. Traditional dose–response analysis cannot be extended to sufficiently low $[Ca^{2+}]_o$ because of signal-to-noise limitations and therefore systematically underestimates Ca^{2+} cooperativity. Our results are consistent with the cooperative involvement of four Ca^{2+} ions for each vesicle of transmitter released at a central synapse and with a nonuniform distribution of Ca^{2+} channel subtypes across synaptic terminals.

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