Modulation of Transmitter Release by Action Potential Duration at the Hippocampal CA3-CA1 Synapse

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Qian, Jing and Peter Saggau. Modulation of transmitter release by action potential duration at the hippocampal CA3-CA1 synapse. J. Neurophysiol. 81: 288–298, 1999. Presynaptic Ca\textsuperscript{2+} influx through voltage-dependent Ca\textsuperscript{2+} channels triggers neurotransmitter release. Action potential duration plays a determinant role in the dynamics of presynaptic Ca\textsuperscript{2+} influx. In this study, the presynaptic Ca\textsuperscript{2+} influx was optically measured with a low-affinity Ca\textsuperscript{2+} indicator (Pura). The effect of action potential duration on Ca\textsuperscript{2+} influx and transmitter release was investigated. The K\textsuperscript{+} channel blocker 4-aminopyridine (4-AP) was applied to broaden the action potential and thereby increase presynaptic Ca\textsuperscript{2+} influx. This increase of Ca\textsuperscript{2+} influx appeared to be much less effective in enhancing transmitter release than raising the extracellular Ca\textsuperscript{2+} concentration. 4-AP did not change the Ca\textsuperscript{2+} dependence of transmitter release but instead shifted the synaptic transmission curve toward larger total Ca\textsuperscript{2+} influx. These results suggest that changing the duration of Ca\textsuperscript{2+} influx is not equivalent to changing its amplitude in locally building up an effective Ca\textsuperscript{2+} concentration near the Ca\textsuperscript{2+} sensor of the release machinery. Furthermore, in the presence of 4-AP, the N-type Ca\textsuperscript{2+} channel blocker ω-CgTx GVIA was much less effective in blocking transmitter release. This phenomenon was not simply due to a saturation of the release machinery by the increased overall Ca\textsuperscript{2+} influx because a similar reduction of Ca\textsuperscript{2+} influx by application of the nonspecific Ca\textsuperscript{2+} channel blocker Cd\textsuperscript{2+} resulted in much more inhibition of transmitter release. Rather, the different potencies of ω-CgTx GVIA and Cd\textsuperscript{2+} in inhibiting transmitter release suggest that the Ca\textsuperscript{2+} sensor is possibly located at a distance from a cluster of Ca\textsuperscript{2+} channels such that it is sensitive to the location of Ca\textsuperscript{2+} channels within the cluster.

INTRODUCTION

Synaptic transmission at fast synapses is a rapid process. The short latency between Ca\textsuperscript{2+} influx and transmitter release suggests a close association between presynaptic Ca\textsuperscript{2+} channels and the Ca\textsuperscript{2+} sensor of the release machinery. This Ca\textsuperscript{2+} sensor was proposed to be located within a fraction of a micrometer from a cluster of Ca\textsuperscript{2+} channels (Llinas et al. 1981). Therefore the release machinery might sense a Ca\textsuperscript{2+} microdomain that is formed by diffusion of ions from a cluster of opening Ca\textsuperscript{2+} channels rather than from a single opening channel. In other words, Ca\textsuperscript{2+} nanodomains, formed around individual channels, are overlapped in terms of their involvement in triggering the fusion of a synaptic vesicle.

Several lines of evidence are consistent with this hypothesis. An intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) profile with the size of an active zone was visualized by using a low-sensitivity Ca\textsuperscript{2+}-dependent photoprotein at the squid giant synapse (Llinas et al. 1992). At mammalian central synapses such as hippocampal and cerebellar synapses, the involvement of different types of Ca\textsuperscript{2+} channels and their supraadditive contribution to neurotransmitter release suggest that a microdomain composed of different types of Ca\textsuperscript{2+} channels is required to trigger transmitter release (Mintz et al. 1995; Wu and Saggau 1994b). At the giant calyx synapse in the rat brain stem, the relationship between presynaptic Ca\textsuperscript{2+} current and transmitter release was recently studied with patch-clamp recording techniques. The results of this study suggest that the release of a single synaptic vesicle requires multiple Ca\textsuperscript{2+} channels to open simultaneously (Borst and Sakmann 1996).

In this microdomain model, local [Ca\textsuperscript{2+}] near the Ca\textsuperscript{2+} sensor is built up by diffusion of ions from a cluster of simultaneously opening Ca\textsuperscript{2+} channels. In addition to the arrangement of Ca\textsuperscript{2+} channels within active zones, amplitude and duration of Ca\textsuperscript{2+} influx are other key factors in the process of Ca\textsuperscript{2+} diffusion. Simulation studies were undertaken to estimate the role of Ca\textsuperscript{2+} diffusion in the buildup of high Ca\textsuperscript{2+} concentrations near the release site (Chad and Eckert 1984; Fogelson and Zucker 1985; Roberts 1994; Simon and Llinas 1985). In general, high [Ca\textsuperscript{2+}] is restricted to locations immediately adjacent to Ca\textsuperscript{2+} channels and quickly reaches a steady-level after Ca\textsuperscript{2+} channels are opened. In early experiments conducted at the squid giant synapse, very different power functions, which represent the relationship between presynaptic Ca\textsuperscript{2+} influx and neurotransmitter release, were obtained when presynaptic Ca\textsuperscript{2+} influx was manipulated. A high opening probability and recruitment of Ca\textsuperscript{2+} channels rather than from a single channel blocker (Furaptra) was applied to broaden the action potential duration on Ca\textsuperscript{2+} currents or the duration of action potentials (Augustine 1990). Such a discrepancy was contributed to low channel opening probability and recruitment of Ca\textsuperscript{2+} channels by prolonged action potentials at this synapse (Augustine et al. 1991). In contrast, at a mammalian central synapse in rat cerebellum, Sabatini and Regehr (1997) observed similar power relationships when amplitude or duration of Ca\textsuperscript{2+} influx was manipulated. A high opening probability of Ca\textsuperscript{2+} channels at this cerebellar synapse was proposed to interpret the observed independence of the power relationship of action potential duration. However, preliminary data from the CA3-CA1 synapse of guinea pig hippocampus indicated rather different efficiencies in regulation of transmitter release between modulation of Ca\textsuperscript{2+} and K\textsuperscript{+} channels (Sinha et al. 1996). Therefore, the effect of action potential duration on presynaptic Ca\textsuperscript{2+} influx and transmitter release at the rat hippocampal CA3-CA1 synapse was systematically investigated.
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METHODOLOGICAL

Transverse hippocampal slices (300–350 μm) were prepared from male Sprague-Dawley rats (four weeks of age) and incubated at 30°C in artificial cerebrospinal fluid containing (in mM): 124 NaCl, 3 KCl, 2.5 CaCl2, 2 MgCl2, 22 NaHCO3, 1.25 NaH2PO4, 10 Dglucose, gassed with 95% O2/5% CO2 to maintain a constant pH of 7.4. The dentate gyrus and part of CA3 were routinely removed to prevent epileptiform activity during application of 4-AP. The presynaptic Ca2+ influx was measured with the low-affinity Ca2+ indicator Furaptra in all experiments, except where the use of Fura-2 is explicitly stated. The procedure for loading Ca2+ indicator into presynaptic terminals of CA3–CA1 synapses was described in detail elsewhere (Wu and Saggau 1994a). Briefly, a small amount of 1 mM Furaptra-AM or Fura-2 AM (Molecular Probes, Eugene, OR) dissolved in dimethyl sulfoxide (DMSO) solution (80% DMSO + 20% pluronic acid) was pressure injected into stratum radiatum (SR) of area CA1, where it was locally taken up into CA3 axons. Approximately 2 h after injection, an area with a diameter of 200 μm in SR, ~800 μm away from the injection site, was illuminated at a single excitation wavelength (380 nm). Fluorescence was collected by a ×50 objective lens (N. A. 0.9), filtered by a long-pass filter (495 nm), and converted into an electrical signal by a single photodiode.

A bipolar tungsten electrode was positioned in SR of area CA1 to stimulate afferent inputs to CA1 neurons. An extracellular glass microelectrode (1–5 MΩ, filled with 2 M NaCl) was used to record field excitatory postsynaptic potentials (fEPSPs) in SR of area CA1. Slices were stimulated every 20 s to elicit a submaximal response; stimulation-induced presynaptic Ca2+ transients ([Ca2+]p) and fEPSPs were simultaneously sampled. Three successive traces were averaged to improve signal-to-noise ratio. The amplitude of Ca2+ fluorescence signal (∆F) was measured as the difference between peak (F + ∆F) and resting fluorescence (F). Signals were corrected for dye bleaching by forming the ratio ∆F/F. [Ca2+]p is approximately proportional to the normalized change in fluorescence of the Ca2+ indicator, ∆F/F. Autofluorescence of the brain slices was measured and subtracted from the total fluorescence signal. The maximal slope of the fEPSP was taken as the measure of synaptic transmission.

Data in each experiment were normalized to baseline before any drug application unless otherwise stated and then pooled together and expressed as means ± SD.

DRUGS

4-Aminopyridine (4-AP) was purchased from Sigma. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) and d-2-amino-5-phosphonovaleric acid (d-APV) were purchased from Tocris Cookson; ω-CgTx GVIA was purchased from Bachem. Adenosine was bought from RBI. All other chemicals were from Fisher.

RESULTS

Effect of 4-AP on the relationship between [Ca2+]p, and fEPSP

The K+ channel blocker 4-AP has been shown to prolong somatic action potentials in hippocampal pyramidal neurons (Storm 1987). Such broadening of presynaptic action potential by 4-AP prolongs Ca2+ influx and thereby enhances synaptic transmission at the CA3–CA1 synapse (Wheeler et al. 1996). The effect of 4-AP on the relationship between [Ca2+]p, and fEPSP was tested first.

REDUCED POWER RELATIONSHIP DURING APPLICATION OF 4-AP. As expected, application of 4-AP increased both transmitter release and Ca2+ influx-induced fluorescence signal (∆F/F), which was measured with the low-affinity Ca2+ indicator Furaptra. Figure 1A shows the time course of normalized [Ca2+]p, and fEPSP in a typical experiment with 40 μM 4-AP. Application of 4-AP increased ∆F/F and corresponding fEPSP in a dose-dependent manner as summarized in Fig. 1B. The close to fourth power relationship between [Ca2+]p, and fEPSP, which was found at this synapse (Qian et al. 1997), does not describe the action of 4-AP. Instead, an approximately linear relationship between fEPSP and [Ca2+]p, was found (Fig. 1C). The average estimated power number was m ~ 0.94. A concentration of 40 μM 4-AP was used throughout the following experiments unless otherwise stated.

PPRESYNAPTIC CA2+ INFLUX IS ENHANCED DURING APPLICATION OF 4-AP. During the application of 4-AP, the estimated power number (~1.0) was much lower than that obtained by manipulation of [Ca2+]p, (m = 3.8) (Qian et al. 1997). Such a reduced power number could be due to an increase in the recruitment of presynaptic fibers. As both the measured ∆F/F and the fEPSP represent population signals, such a recruitment of presynaptic fibers would linearly increase the fEPSP slope and the fluorescence signal ∆F/F, resulting in a power number of 1. To test if the excitability of presynaptic fibers was altered during application of 4-AP, the presynaptic fiber volley was measured after blocking postsynaptic responses with the ionotropic glutamate receptor antagonists CNQX (10 μM) and d-APV (25 μM). During application of 4-AP, the amplitude of the fiber volley was not significantly different from control, but the fiber volley was much broader than under control conditions as shown in Fig. 2A.

In addition to monitoring the fiber volley, comparison of measurements with indicators of very different Ca2+ affinity, Fura-2 and Furaptra, during application of 4-AP was used to rule out the possibility that the increase of ∆F/F was just an artifact of fiber recruitment. In these experiments, the stimulation-evoked fluorescence signal (∆F/F) was composed of the combined fluorescence response from all activated presynaptic terminals within the recording area. With the low-affinity indicator Furaptra, ∆F/F is linearly proportional to Ca2+ influx and to the number of activated presynaptic fibers (N), i.e., ∆F/F ∝ N · Ca2+ influx. Therefore either changes in the Ca2+ influx or the recruitment of presynaptic fibers would result in a linear change of ∆F/F and be indistinguishable. In contrast, when the high-affinity indicator Fura-2 is used as a Ca2+ indicator, the change of ∆F/F is still linearly proportional to the number of presynaptic fibers (N) but nonlinearly related to the change of Ca2+ influx caused by saturation of this indicator, i.e., ∆F/F ∝ N · f(Ca2+ influx) (Fig. 2B). Ca2+ influx-induced ∆F/F is related to the saturation of the indicator, which is dependent on the dye concentration inside terminals. In measurements with a paired-pulse stimulation paradigm (30-ms interval between 2 stimuli), the ratio of the second response to the first is a measure of the relative dye concentration inside terminals. The lower the Fura-2 concentration inside terminals, the less increase of ∆F/F by 4-AP would be expected. Alternatively, if the increase of ∆F/F is due to the recruitment of presynaptic fibers, a simi-
4-Aminopyridine (4-AP) increases Ca\textsuperscript{2+} indicator signal and transmitter release. A: time course of normalized ΔF/F and slope of record field excitatory postsynaptic potential (fEPSP) for a typical experiment. Application of 4-AP (40 μM) increased the Ca\textsuperscript{2+} indicator signal and enhanced transmitter release. B: dose response of 4-AP on single stimulus-induced Ca\textsuperscript{2+} indicator signal and transmitter release. Application of 4-AP dose-dependently increased ΔF/F to 123 ± 8% (2 μM, n = 4), 145 ± 5% (5 μM, n = 2), 176 ± 3% (10 μM, n = 4), 231 ± 4% (30 μM, n = 3), 255 ± 31% (40 μM, n = 29), and 263 ± 21% (100 μM, n = 4). The corresponding fEPSPs were increased to 121 ± 6% (2 μM), 162 ± 23% (5 μM), 182 ± 25% (10 μM), 217 ± 14% (30 μM), 215 ± 47% (40 μM), and 228 ± 29% (100 μM). C: relationship between [Ca\textsubscript{pre}] and fEPSP during application of 4-AP. During prolongation of action potential, a decreased power relationship (m ~ 0.94) between [Ca\textsubscript{pre}] and transmitter release was found.

Consistent with the hypothesis that 4-AP enhances Ca\textsuperscript{2+} influx in individual terminals, 4-AP-induced increase of ΔF/F was dependent on the ratio of paired responses tested under control conditions, as shown in Fig. 2C. The smallest increase of ΔF/F by 4-AP was ~30% with a ratio of paired responses about 0.4 in control. The largest increase was ~100% with a ratio of ~0.8. With increase of dye concentra-
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A possible recruitment of presynaptic fibers cannot be completely ruled out. However, the smallest increase of \( \Delta F/F \) measured with Fura-2 was \( \sim 30\% \), which could be contributed by both an increase of presynaptic Ca\(^{2+}\) influx and a possible recruitment of additional presynaptic fibers. This value sets the upper limit for the recruitment of presynaptic fibers by 4AP. Thus, for the 4AP-induced increase of \( \Delta F/F \) measured with Furaptra (155%), the possible increase in the recruitment of presynaptic fibers should be much <30% because the enhanced Ca\(^{2+}\) influx also contributes to the increase of \( \Delta F/F \). Therefore, the observed increase of \( \Delta F/F \) after application of 4-AP was mainly due to an enhanced Ca\(^{2+}\) influx rather than recruitment of presynaptic fibers. During the measurement with Fura-2, the resting fluorescence was not changed during application of 4-AP, indicating that 4-AP did not alter the resting Ca\(^{2+}\) concentration.

\( Ca^{2+} \) dependency of transmitter release in the presence of 4-AP

PARADOXICAL RELATIONSHIP BETWEEN \([Ca_{pre}]\), and fEPSP IN THE PRESENCE OF 4-AP. To test if enhanced transmitter release caused by prolonged Ca\(^{2+}\) influx could be compensated by reducing the amplitude of Ca\(^{2+}\) influx, the nonspecific Ca\(^{2+}\) channel blocker Cd\(^{2+}\) was applied during transmitter release was increased by application of 4-AP. Figure 3A shows the time course of a typical experiment. Application of 30 \( \mu \)M Cd\(^{2+}\) reduced synaptic transmission to much below control level; however, the \([Ca_{pre}]\), stayed above baseline. Thus a paradoxical relationship between \([Ca_{pre}]\), and fEPSP was obtained when \([Ca_{pre}]\), and fEPSP were normalized to control. If normalized to the value in the presence of 4-AP, application of 10, 20, and 30 \( \mu \)M Cd\(^{2+}\) reduced \([Ca_{pre}]\), by 21.6 \( \pm \) 2.1% (\( n = 9 \)), 43.1 \( \pm \) 6.7% (\( n = 9 \)), and 58.7 \( \pm \) 6.5% (\( n = 7 \)), respectively, and the corresponding fEPSP was inhibited by 39.4 \( \pm \) 7.6%, 75.0 \( \pm \) 4.9% and 92.8 \( \pm \) 2.4%, respectively. As revealed by a double-log plot (Fig. 3B), the estimated apparent power numbers in the presence of 4-AP were \( m = 2.1 \pm 0.5 \) (10 \( \mu \)M Cd\(^{2+}\)), \( m = 2.5 \pm 0.5 \) (20 \( \mu \)M Cd\(^{2+}\)), and \( m = 3.0 \pm 0.5 \) (30 \( \mu \)M Cd\(^{2+}\)), respectively. This paradoxical relationship between \([Ca_{pre}]\), and fEPSP was also observed when \([Ca_{pre}]\), was reduced from 2.5 to 0.5 mM. In the presence of 4-AP, \([Ca_{pre}]\), was reduced by 12.9 \( \pm \) 2.8% (1.5 mM, \( n = 9 \)), 25.8 \( \pm \) 4.1% (1.0 mM, \( n = 9 \)), and 45.2 \( \pm \) 3.4% (0.5 mM, \( n = 9 \)), and the corresponding fEPSP was decreased by 10.2 \( \pm \) 4.6%, 30.2 \( \pm \) 10.7%, and 63.9 \( \pm \) 13.9% as shown in Fig. 3B. The estimated apparent power numbers were \( m = 0.9 \pm 0.5 \), \( m = 1.3 \pm 0.5 \), and \( m = 1.9 \pm 0.5 \), respectively. This paradoxical relationship further indicates that varying the duration of Ca\(^{2+}\) influx is not equivalent to changing amplitude in the buildup of Ca\(^{2+}\) concentration near the Ca\(^{2+}\) sensor of release machinery, consistent with the much-reduced apparent numbers observed during application of 4-AP (Fig. 1).

SYNAPTIC TRANSMISSION CURVE IS SHIFTED DURING APPLICATION OF 4-AP. To test how an increase of \([Ca_{pre}]\), by a means other than by application of 4-AP affects transmitter release, \([Ca_{pre}]\), was raised. When the \([Ca_{pre}]\), was raised from 2.5 to 5 mM, the size of the presynaptic volley decreased significantly. In some cases, this change could not

FIG. 2. 4-AP enhances Ca\(^{2+}\) influx rather than increasing recruitment of axons. A: presynaptic fiber volleys in control and during application of 4-AP. There was no significant change in the amplitude of the fiber volley; however, it was broader. B: Fura-2 and Furaptra responses to paired-pulse stimulation under control conditions and during application of 4-AP. Fura-2 was saturated as indicated by the smaller second response. The degree of the saturation depends on [Fura-2] in the terminals; thus, the relative size of the 2nd response will increase with [Fura-2]. C: plot of 4-AP-induced increases in \( \Delta F/F \) vs. ratio of responses to paired stimuli under control conditions. 4-AP-induced increase of \( \Delta F/F \) measured with Fura-2 was dependent on the concentration of the indicator within the terminals. The observed nonlinear response of Fura-2 signals indicates that the increased \( \Delta F/F \) during application of 4-AP was mainly due to an enhanced Ca\(^{2+}\) influx.
be completely compensated by removing Mg$^{2+}$ from the bath. This significant change in the presynaptic fiber volley under high [Ca$^{2+}$]$_o$ makes it difficult to interpret the measured [Ca$^{2+}$]$_{pre}$. Therefore, instead of raising [Ca$^{2+}$]$_o$, [Mg$^{2+}$]$_o$ was reduced to increase [Ca$^{2+}$]$_{pre}$. In contrast to raising [Ca$^{2+}$]$_o$, reducing [Mg$^{2+}$]$_o$ did not significantly affect the size of presynaptic fiber volley. When [Mg$^{2+}$]$_o$ was decreased from 2.0 to 0 mM, transmitter release was greatly enhanced. d-APV (25 μM) was routinely used to prevent the activation of N-methyl-d-aspartate (NMDA) receptors caused by the removal of their Mg$^{2+}$ block. Before decreasing [Mg$^{2+}$]$_o$, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor currents were partially blocked by application of 0.5–0.8 μM CNQX to reduce the fEPSP to 30–40% of control. On average, 0 Mg$^{2+}$ increased [Ca$^{2+}$]$_{pre}$, and fEPSP by 34.1 ± 6.8 and 95.3 ± 11.8% (n = 8), respectively. The enhanced fEPSP returned to baseline after the Mg$^{2+}$ concentration was restored, indicating that there was no long-term alternation of synaptic transmission during such manipulation. The average power number for 0 Mg$^{2+}$ was $m = 2.1 ± 0.5$ (10 μM Cd$^{2+}$), $m = 2.5 ± 0.5$ (20 μM Cd$^{2+}$), and $m = 3.0 ± 0.5$ (30 μM Cd$^{2+}$). This paradoxical relationship between [Ca$^{2+}$]$_{pre}$ and fEPSP was also observed when [Ca$^{2+}$]$_o$ was manipulated. In the presence of 4-AP, [Ca$^{2+}$]$_{pre}$ was reduced by 12.9 ± 2.8% (1.5 mM [Ca$^{2+}$]$_o$, n = 9), 25.8 ± 4.1% (1.0 mM [Ca$^{2+}$]$_o$, n = 9), and 45.2 ± 3.4% (0.5 mM [Ca$^{2+}$]$_o$, n = 9), whereas the corresponding fEPSP was decreased by 10.2 ± 4.6, 30.2 ± 10.7, and 63.9 ± 13.9%, respectively. The estimated apparent power numbers were $m = 0.9 ± 0.3$, $m = 1.3 ± 0.5$, and $m = 1.9 ± 0.5$ for 1.5, 1.0, and 0.5 mM [Ca$^{2+}$]$_o$, respectively (dashed lines correspond to power numbers $m = 1, 2$ and 3).

Figure 3. Paradoxical relationship between [Ca$^{2+}$]$_{pre}$ and fEPSP. A: time course of a typical experiment illustrating the paradoxical relationship between [Ca$^{2+}$]$_{pre}$ and fEPSP. In the presence of 4-AP, transmitter release was reduced by Cd$^{2+}$ to a level below control, whereas the [Ca$^{2+}$]$_{pre}$ stayed above control. B: summary data for application of Cd$^{2+}$ or low [Ca$^{2+}$]$_o$, in the presence of 4-AP. To estimate the apparent power in the presence of 4-AP, data were normalized to the value after application of 4-AP. Double-log plot of normalized [Ca$^{2+}$]$_{pre}$ and fEPSP shows a decrease in apparent power numbers during the application of Cd$^{2+}$ (squares) or low [Ca$^{2+}$]$_o$, (circles). In the presence of 4-AP, [Ca$^{2+}$]$_{pre}$ was reduced by 21.6 ± 2.1% (n = 9), 43.1 ± 6.7% (n = 9), and 58.7 ± 6.5% (n = 7) during application of 10, 20, and 30 μM Cd$^{2+}$, respectively, whereas synaptic transmission was inhibited by 39.4 ± 7.6, 75.0 ± 4.9, and 92.8 ± 2.4%, respectively. The estimated apparent power numbers were $m = 2.1 ± 0.5$ (10 μM Cd$^{2+}$), $m = 2.5 ± 0.5$ (20 μM Cd$^{2+}$), and $m = 3.0 ± 0.5$ (30 μM Cd$^{2+}$). The relation between the total Ca$^{2+}$ concentration near the Ca$^{2+}$ sensor can be represented by a
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Two important situations such as prolongation of Ca\textsuperscript{2+} influx are manipulated. However, this factor might be altered under some conditions. The relationship between the total Ca\textsuperscript{2+} influx and the local Ca\textsuperscript{2+} concentration near the Ca\textsuperscript{2+} sensor of release machinery. Both curves can be fitted by Hill equations with similar Hill coefficients (n = 4.4, control; n = 4.1, 4-AP; K_d = 118% of Ca\textsuperscript{2+} concentration near the Ca\textsuperscript{2+} sensor under control conditions, f_EPSP_max = 300%), indicating that the Ca\textsuperscript{2+} dependence of the release machinery was not altered during application of 4-AP.

\[ \% \text{fEPSP} = \frac{f_{\text{EPSP}_{\text{max}}}}{1 + \left( \frac{K_d}{[\text{Ca}_{\text{pre}]}_o} \right)^n} \]

On average, the effect of 4-AP was equivalently to increasing the amplitude of Ca\textsuperscript{2+} influx by 86, 60, 54, and 45% for 0.5, 1, 1.5, and 2.5 mM of [Ca\textsuperscript{2+} ], respectively. Thus the average increase was 2.1 ± 1%. The equation shows the effect of different values of f_EPSP_max on the estimation of other parameters. Varying f_EPSP_max from 300% to 400% did not substantially change the n, K_d, and \( \alpha \).

A recent modeling study suggests that the local Ca\textsuperscript{2+} concentration is proportional to the amplitude of Ca\textsuperscript{2+} influx because the predominant endogenous Ca\textsuperscript{2+} buffer in the terminals such as the hippocampal synapse is likely to be relatively slow and weak (Sinha et al. 1997). This means that \( \alpha \) would hold constant if the amplitude of Ca\textsuperscript{2+} influx is manipulated. However, this factor might be altered under some situations such as prolongation of Ca\textsuperscript{2+} influx. A Hill equation was used to describe the synaptic transmission curve under control conditions. The curve was fitted by a Hill function with the Hill coefficient of n = 4.4; f_EPSP_max was set as 300% of control. To simplify the equation, \( \alpha \) is normalized to control (\( \alpha = 1 \)). Thus the K_d is 118% of Ca\textsuperscript{2+} concentration near the Ca\textsuperscript{2+} sensor under control conditions. The justification for the value of f_EPSP_max is supported by a study of the release probability at a single synapse. Although there is a wide range of single synapse release probabilities, this study showed that the chance for release a single vesicle for most synapses is quite low and is ~35% with 2.5 mM Ca\textsuperscript{2+} and 1.5 mM Mg\textsuperscript{2+} and room temperature (Dobrunz and Stevens 1997). In other words, if the release probability reaches to 100%, post synaptic responses as measured by f_EPSP will be roughly three times larger than under control conditions. Varying f_EPSP_max would slightly affect the estimation of K_d and Hill coefficient n; however, it would not change the following main conclusion.

In the presence of 4-AP, the synaptic transmission curve can be described by a similar equation except for a reduced factor \( \alpha \). The curve is fitted by n = 4.1, K_d = 118%, and \( \alpha = 0.58 \). The reduction of \( \alpha \) leads to a shift of synaptic transmission curve toward higher [Ca\textsuperscript{2+}]. Such a shift is unlikely due to a reduced sensitivity of the release machinery because the Ca\textsuperscript{2+} cooperativity for triggering transmitter release, which is indicated by the Hill coefficient n, was not significantly changed. Furthermore, there was no evidence to indicate that 4-AP changed the resting intracellular Ca\textsuperscript{2+} concentration. The table in Fig. 4 shows the effects of f_EPSP_max values on estimates of other parameters. When f_EPSP_max is varied from 300 to 400%, the resulting error of estimating n in the presence of 4-AP is ~10% of the Hill coefficient under control conditions, whereas \( \alpha \) shows only a minor change. Therefore application of 4-AP did not significantly change the Ca\textsuperscript{2+} dependency of transmitter release but shifted the synaptic transmission curve. The shift of synaptic transmission curve suggests reduced coupling strength between the total Ca\textsuperscript{2+} influx and the local Ca\textsuperscript{2+} concentration near the Ca\textsuperscript{2+} sensor.

4-AP reduces the reliance of transmitter release on N-type channels

N-type channels were found to be involved in transmitter release at the studied synapse (Qian et al. 1997; Wheeler et al. 1994; Wu and Saggau 1994b). However, in the presence of 4-AP, synaptic transmission is much less sensitive to the Ca\textsuperscript{2+} channel toxin \( \omega \)-CgTx GVIA (Wheeler et al. 1996). This lack of effect of \( \omega \)-CgTx GVIA in blocking transmitter release was thought to be due to saturation of the release machinery by increased Ca\textsuperscript{2+} influx. It was concluded that even the reduction of Ca\textsuperscript{2+} influx by blockade of N-type channels could not overcome the saturation (Wheeler et al. 1996). To test this, the fraction of the [Ca\textsuperscript{2+}] sensitive to \( \omega \)-CgTx GVIA was examined in the presence of 4-AP. The corresponding reduction of f_EPSP was compared with that by either application of Cd\textsuperscript{2+} or reduction of [Ca\textsuperscript{2+}], to determine if the lack of effect of \( \omega \)-CgTx GVIA was entirely due to excessive Ca\textsuperscript{2+} influx. If blockade of N-type Ca\textsuperscript{2+} channels by this toxin would inhibit the same amount of synaptic transmission as applying Cd\textsuperscript{2+} to reduce Ca\textsuperscript{2+} influx to a similar level, this would indicate that the lack of effect of \( \omega \)-CgTx GVIA in the presence of 4-AP was indeed the consequence of saturation of release machinery. Because Cd\textsuperscript{2+} nonspecifically blocks Ca\textsuperscript{2+} channels, the same effectiveness in blocking transmitter release between application of \( \omega \)-CgTx GVIA and Cd\textsuperscript{2+} would strongly suggest that N-type channels have the same efficacy as other types of Ca\textsuperscript{2+} channels in controlling transmitter release. Figure 5A shows the time course of normalized [Ca\textsuperscript{2+}], and f_EPSP of a typical:  

\[ \% \text{fEPSP} = \frac{f_{\text{EPSP}_{\text{max}}}}{1 + \left( \frac{K_d}{[\text{Ca}_{\text{pre}]}_o} \right)^n} \]
4-AP reduces the reliance of transmitter release on N-type channels. A: time course of normalized [Ca<sub>pre</sub>]<sub>t</sub> and fEPSP in control, in the presence of 4-AP, and after application of ω-CgTx GVIA. In the presence of 4-AP, ω-CgTx GVIA was less effective in inhibiting transmitter release, although it greatly reduced [Ca<sub>pre</sub>]<sub>t</sub>. Traces in the presence of 4-AP and during application of ω-CgTx GVIA are shown in inset. B: summary data for the effects of ω-CgTx GVIA on [Ca<sub>pre</sub>]<sub>t</sub> and fEPSP in the presence of 4-AP compared with the application of Cd<sup>2+</sup>. A similar amount of reduction in [Ca<sub>pre</sub>]<sub>t</sub> by Cd<sup>2+</sup> inhibited significantly more transmitter release.

**Effect of 4-AP on the inhibition of presynaptic Ca<sup>2+</sup> influx by adenosine**

Inhibition of presynaptic Ca<sup>2+</sup> influx has been shown to be the main contributor to the presynaptic inhibition of transmitter release by various neuromodulators (Wu and Saggau 1997). Whether such modulation is exerted on Ca<sup>2+</sup> channels or K<sup>+</sup> channels is at debate. The K<sup>+</sup> channel hypothesis proposes that reduction of Ca<sup>2+</sup> influx is due to the narrowing of the action potential by G-protein–activated K<sup>+</sup> channels. This hypotheses was tested by measuring adenosine (AD)–mediated inhibition of [Ca<sub>pre</sub>]<sub>t</sub> in the presence of 4-AP. Figure 6A shows the time course of a typical experiment comparing the inhibition of [Ca<sub>pre</sub>]<sub>t</sub> by AD with and without 4-AP. The relative percentage inhibition of [Ca<sub>pre</sub>]<sub>t</sub> by 100 μM AD in the presence of 4-AP was slightly less than under control conditions. In the absence of 4-AP, the relative percentage inhibition of [Ca<sub>pre</sub>]<sub>t</sub> was 17.9 ± 4.2% (n = 9), 31.1 ± 1.8% (n = 4), and 50.3 ± 3.8% (n = 9) with 10, 30, and 100 μM AD, respectively. In the presence of 4AP, the same concentrations of AD inhibited [Ca<sub>pre</sub>]<sub>t</sub> by 12.0 ± 3.7% (n = 12), 23.6 ± 4.3% (n = 4), and 38.4 ± 4.7% (n = 12), respectively. As summarized in Fig. 6B, the relative percentage inhibition of [Ca<sub>pre</sub>]<sub>t</sub> by AD in the presence of 4-AP was ~25% smaller than control as indicated by the slope of the regression line (0.75). Therefore 4-AP did not greatly alter the AD-mediated inhibition of [Ca<sub>pre</sub>]<sub>t</sub>.
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FIG. 6. 4-AP does not block the inhibition of \([Ca_{\text{pre}}]\) mediated by adenosine (AD). A: time course of normalized \([Ca_{\text{pre}}]\) and fEPSP in control, in the presence of 4-AP, and during application of AD. In the presence of 4-AP, the inhibition of \([Ca_{\text{pre}}]\) by AD was slightly reduced. Traces for the application of AD under control conditions and in the presence of 4-AP are shown in inset. B: summary data comparing the inhibition of \([Ca_{\text{pre}}]\) in control and in the presence of 4-AP. Under control conditions, the relative percentage inhibition of \([Ca_{\text{pre}}]\) were 17.9 ± 4.2% \((n = 9)\), 31.1 ± 1.8% \((n = 4)\), and 50.3 ± 3.8% \((n = 9)\) for 10, 30, and 100 μM AD, respectively. In the presence of 4-AP, the same concentrations of AD inhibited \([Ca_{\text{pre}}]\) by 12.0 ± 3.7% \((n = 12)\), 23.6 ± 4.3% \((n = 4)\), and 38.4 ± 4.7% \((n = 12)\), respectively. The relative percentage inhibition of \([Ca_{\text{pre}}]\) by AD in the presence of 4-AP was ~25% smaller than control, as indicated by the slope of the regression line \((0.75)\).

DISCUSSION

Measurement of \(Ca^{2+}\) cooperativity in the transmitter release

\(Ca^{2+}\) cooperativity of transmitter release was studied for several decades in a wide range of preparations. There are some variations in the estimated apparent power number across different species and synapses. The interpretation of the apparent power number as the number of \(Ca^{2+}\) binding sites in the release machinery depends on how precisely the measured \(Ca^{2+}\) influx reflects the \(Ca^{2+}\) concentration near the \(Ca^{2+}\) sensor. However, several factors could influence the measurement and interpretation of apparent power numbers.

Apparently, the AD receptor does not mainly act on 4-AP–sensitive \(K^+\) channels; otherwise, 4-AP should block the inhibition of \([Ca_{\text{pre}}]\) by AD. If AD receptors act on the other types of \(K^+\) channels, the percentage inhibition of \([Ca_{\text{pre}}]\) by AD in the presence of 4-AP should be larger than in the absence of the drug because the ratio of presumable AD-modulated \(K^+\) channels would become larger after blocking 4-AP-sensitive \(K^+\) channels. Our experimental results suggest that \(K^+\) channels are not the main target for activation of presynaptic AD receptors. Similar conclusions can be extended to the inhibition of presynaptic \(Ca^{2+}\) influx by neuropeptide Y and muscarinic receptors because they have been shown to share the pathway with AD receptors (Qian and Saggau 1997; Qian et al. 1997).
First, the relative location of Ca\(^{2+}\) channels to the Ca\(^{2+}\) sensor of the release machinery may greatly influence the apparent power number. Even if all Ca\(^{2+}\) sources were assumed to be the same, different diffusion distances from these Ca\(^{2+}\) sources to the Ca\(^{2+}\) sensor of the release machinery would produce a different Ca\(^{2+}\) concentration profile near the release site. Such a difference in the buildup of effective Ca\(^{2+}\) concentration is undetectable by the techniques currently used but would affect transmitter release greatly. This might be the reason for the reported difference in power numbers between N- and P/Q-type channels in the cerebellum (Mintz et al. 1995). A similar observation was made at the hippocampal CA3–CA1 synapse. A slight difference in the estimated power numbers for N- and P/Q-type channels was obtained in the rat (Qian and Saggu 1997; Qian et al. 1997). The difference in coupling strength for different types of Ca\(^{2+}\) channels to the Ca\(^{2+}\) sensor was more obvious during application of 4-AP (Fig. 5). In these experiments, the N-type channel blocker \(\omega\)-CGTX GVIA had little effect on blocking transmitter release, whereas the nonspecific Ca\(^{2+}\) channel blocker Cd\(^{2+}\) substantially reduced synaptic transmission, although both agents resulted in similar reductions of the overall Ca\(^{2+}\) influx. Recently, R-type channels were found to contribute to the release of neurotransmitter but with a much lower efficacy compared with other types of Ca\(^{2+}\) channels at the giant calyx synapse of the medial nucleus in the trapezoid body (MNTB) in the rat brain stem (Wu et al. 1998).

Second, a different temporal relationship between overall Ca\(^{2+}\) influx and the Ca\(^{2+}\) concentration near the Ca\(^{2+}\) sensor could be another reason for the discrepancy in the measurement of apparent power numbers. Extremely low apparent power numbers (\(~1.0\) ) were observed at the squid giant synapse (Augustine 1990) and crayfish neuromuscular junction (Zucker et al. 1991) when the presynaptic action potential was prolonged. Similar results were also obtained in this study (Fig. 1C). The coupling strength between the total Ca\(^{2+}\) influx and effective Ca\(^{2+}\) concentration during prolonged action potential was weakened by a factor of 0.58 (Fig. 4). Diffusion of Ca\(^{2+}\) ions could contribute to this reduced coupling. As Ca\(^{2+}\) ions diffuse away, Ca\(^{2+}\) influx with a longer duration may be less effective in building up the local high Ca\(^{2+}\) concentration near the Ca\(^{2+}\) sensor compared with Ca\(^{2+}\) influx with higher amplitude but shorter duration.

Third, a saturation of the release machinery by excessive Ca\(^{2+}\) influx could also reduce the apparent power numbers, as shown in Fig. 3B. It is conceivable that apparent power numbers would be reduced when excessive Ca\(^{2+}\) influx moves transmitter release machinery toward saturation. The apparent power number reflects the Hill coefficient only when local Ca\(^{2+}\) concentration is small compared with the \(K_d\) (Fig. 4). However, these discrepancies can be reconciled by a model of four Ca\(^{2+}\) binding sites with a coupling factor to represent the relation between the total Ca\(^{2+}\) influx and the local Ca\(^{2+}\) concentration. As shown in Fig. 4, the Hill coefficient is similar under both control conditions and prolonged action potentials, although apparent power numbers were significantly different in each case.

**Effects of 4-AP on the presynaptic Ca\(^{2+}\) influx and transmitter release**

Application of 4-AP increased the Ca\(^{2+}\) fluorescence signals (\(\Delta F/F\)) and enhanced transmitter release (Fig. 1). As demonstrated by experiments shown in Fig. 2, such an increase of Ca\(^{2+}\) signal is mainly due to an enhanced presynaptic Ca\(^{2+}\) influx at individual terminals rather than an artifact of fiber recruitment. Broadening the action potential has been shown to mainly prolong the duration of Ca\(^{2+}\) influx with minor effects on the peak of Ca\(^{2+}\) current when a series of mock action potentials was tested at the cell body (Toth and Miller 1995; Wheeler et al. 1996). A similar situation is likely to occur at the hippocampal CA3–CA1 synapse.

In contrast to the higher power number obtained by changing Ca\(^{2+}\) current through individual Ca\(^{2+}\) channels (manipulation of [Ca\(^{2+}\)]\(_o\), or application of Cd\(^{2+}\)), much lower apparent power numbers were measured when the duration of presynaptic action potentials was prolonged by 4-AP (Fig. 1C). Clearly, the power law obtained by manipulating the amplitude of the Ca\(^{2+}\) influx does not explain the effect of prolonged Ca\(^{2+}\) influx on the relationship between [Ca\(_{\text{rev}}\)] and transmitter release. More convincing evidence for the differential modulation of transmitter release by amplitude and duration of Ca\(^{2+}\) influx comes from paradoxical results obtained during application of Cd\(^{2+}\) in the presence of 4-AP. Enhancement of transmitter release, as a result of prolonged Ca\(^{2+}\) influx, could not be compensated by reducing the amplitude of Ca\(^{2+}\) influx (Fig. 3A). These results indicate that, for a given total Ca\(^{2+}\) influx, buildup of local effective Ca\(^{2+}\) concentration near the release site is more sensitive to the amplitude of Ca\(^{2+}\) influx than to the duration.

**Prolonged action potential shifts synaptic transmission curve**

The difference in the modulation of transmitter release by amplitude and duration of Ca\(^{2+}\) influx is clearly illustrated when the synaptic transmission curve is constructed. A Hill equation with a Hill coefficient of \(~4\) describes the synaptic transmission curve when the amplitude of Ca\(^{2+}\) influx was manipulated (Fig. 4). A similar number of Ca\(^{2+}\) binding sites in the release machinery was estimated from studies of transmitter release in other synapses. By homogeneously raising presynaptic Ca\(^{2+}\) concentration with photolabile Ca\(^{2+}\) chelators, the rate of transmitter release is best described by a model of three to four Ca\(^{2+}\) binding sites at the bipolar cell terminal in goldfish retina (Heidelberger et al. 1994) and crayfish neuromuscular junction (Lando and Zucker 1994). In this model, local Ca\(^{2+}\) concentration near the Ca\(^{2+}\) sensor is assumed to be linearly proportional to the amplitude of the Ca\(^{2+}\) influx. This property is well predicted by equations for free diffusion (Crank 1978). However, endogenous Ca\(^{2+}\) buffers may cause a slight deviation from this simple relationship. Thus the actual number of Ca\(^{2+}\) binding sites could be different from the Hill coefficient used in the current model.

Because of the limitation of the technique used in this study, the optical signal represents the change of volume average Ca\(^{2+}\) concentration rather than the change of local Ca\(^{2+}\) concentration near the release site. Thus a coupling factor \(\alpha\) was introduced into this model to account for the relationship between the total Ca\(^{2+}\) influx and the local Ca\(^{2+}\) concentration. In this model, synaptic transmission curves under control conditions and during application of 4-AP can be reconciled with one Hill equation. The major differences...
between these two curves are different values of the coupling factor $\alpha$. When action potentials are prolonged, there is a reduction in this factor. Mathematically, an increase of $K_g$ could be alternatively used to reconcile the two synaptic transmission curves. However, there is no obvious mechanism by which prolonging the presynaptic action potential would alter the $Ca^{2+}$ affinity of the $Ca^{2+}$ sensor.

Several factors could contribute to the reduction of the coupling factor $\alpha$. The first interpretation is the diffusion of $Ca^{2+}$ after entering into presynaptic terminals. Prolonged action potential duration primarily opens $Ca^{2+}$ channels during a longer period without increasing the amplitude of $Ca^{2+}$ influx. Because of $Ca^{2+}$ diffusion, the high $Ca^{2+}$ concentration near a $Ca^{2+}$ sensor will not be built up as effectively by prolonging the duration of $Ca^{2+}$ influx as by increasing the amplitude. Thus the measured overall $Ca^{2+}$ influx is less weighted when converted into $Ca^{2+}$ concentration near the $Ca^{2+}$ sensor, resulting in a reduction of this coupling factor. This interpretation is supported by modeling studies of $Ca^{2+}$ time course near the membrane after $Ca^{2+}$ influx (Nowycky and Pinter 1993; Roberts 1994). For the first millisecond of the $Ca^{2+}$ entry, the submembrane $[Ca^{2+}]$ increases rapidly. For the next few milliseconds, the rate of increase quickly declines as $Ca^{2+}$ entry is balanced by radial diffusion and binding by mobile or fixed $Ca^{2+}$ buffers. An extreme case was observed in the neuromuscular junction of jellyfish, where short action potentials produce larger and briefer $Ca^{2+}$ influx and result in large excitatory junction potentials and prolonged action potentials elicit slow $Ca^{2+}$ entry and small synaptic transmission, although there is more total $Ca^{2+}$ influx in the latter case (Spencer et al. 1989).

The recruitment of $Ca^{2+}$ channel during prolonged action potentials could also result in the reduction of the coupling factor. In this scenario, prolonged action potentials not only keep $Ca^{2+}$ channel open longer but also recruit $Ca^{2+}$ channels, which are not activated under control conditions. Presynaptic $Ca^{2+}$ channels were found to associate with synaptic vesicle docking proteins (Sheng et al. 1994). The impact of this interaction on the behavior of presynaptic $Ca^{2+}$ channels is not clear. Binding of syntaxin, a protein of the vesicle docking complex, to $Ca^{2+}$ channels was demonstrated to stabilize inactivation, thereby reducing the availability of presynaptic $Ca^{2+}$ channels (Bezprozvanny et al. 1995). The possible interaction between $Ca^{2+}$ channels and other synaptic vesicle docking proteins may also exert an inhibition on the activity of $Ca^{2+}$ channels. Prolonged action potentials could in principle reverse such inhibition on presynaptic $Ca^{2+}$ channels, resulting in recruitment of $Ca^{2+}$ channels. This situation was proposed at the squid giant synapse where only $\sim 10\%$ of $Ca^{2+}$ channels were estimated to be activated under physiological action potential, and prolonged action potentials mainly recruit more channels (Augustine et al. 1991). In the CNS, study of presynaptic $Ca^{2+}$ currents at the rat calyx of Held synapse suggests that the opening probability of $Ca^{2+}$ channels reaches 70\% of the maximum under physiological conditions (Borst and Sakmann 1998). Thus prolonged action potential could further increase the opening probability but not as much as at the squid synapse. If this is the case in the hippocampus, a less synchronized opening of $Ca^{2+}$ channels, as a result of channel recruitment, could contribute in part to a reduction of the coupling factor. Channel recruitment would also alter the spatial property of $Ca^{2+}$ influx. If prolonged action potentials mainly recruit $Ca^{2+}$ channels that are not located near the $Ca^{2+}$ sensor, this part of $Ca^{2+}$ influx would not contribute as much to the buildup of $Ca^{2+}$ concentration near $Ca^{2+}$ sensor as to the overall $[Ca^{2+}]_{\text{pre}}$. Consequently, a reduced coupling factor would be expected. The extent to which the recruitment of $Ca^{2+}$ channels contributes to the reduction of the coupling factor depends on the opening probability of presynaptic $Ca^{2+}$ channels and the location of $Ca^{2+}$ channels that are recruited by prolonged action potentials.

This model successfully accounts for several observations during 4-AP application. Consistent with a previous study (Wheeler et al. 1996), 4-AP did not alter the $Ca^{2+}$ dependency of transmitter release, although the measured apparent power numbers were largely different under control conditions and during application of 4-AP. The low apparent power number obtained by application of 4-AP is due to the reduction in the coupling strength between the total $Ca^{2+}$ influx and the local $Ca^{2+}$ concentration. The model predicts a reduction of apparent power numbers as the release machinery approaches saturation. This is confirmed by the smaller apparent power numbers obtained during manipulation of the amplitude of $Ca^{2+}$ currents in the presence of 4-AP.

**Degree in the overlap of presynaptic $Ca^{2+}$ domains**

At the hippocampal CA3–CA1 synapse, at least N- and P/Q-type $Ca^{2+}$ channels are involved in transmitter release in a supraadditive manner. This suggests that the multiple types of $Ca^{2+}$ channels that are required to simultaneously open and close their $Ca^{2+}$ nanodomains are overlapped to form a microdomain for the release of neurotransmitter (Wheeler et al. 1996; Wu and Saggau 1994b). However, previous experiments with $Ca^{2+}$ channel toxins did not provide enough information to reveal how many $Ca^{2+}$ channels are required to form such a $Ca^{2+}$ microdomain. In this study, comparing the coupling strength between different types of $Ca^{2+}$ channels does not support a large $Ca^{2+}$ microdomain. The main evidence is based on the different power numbers between N- and non-N-type $Ca^{2+}$ channels. Under control conditions, the power number for N- and P/Q-type channels was different. This difference in the coupling strength to the $Ca^{2+}$ sensor becomes more prominent during prolonged action potentials. In the presence of 4-AP, $\omega$-CgTx GV1A was found to have little effect on transmitter release (Fig. 5). This reduction in the reliance of transmitter release on N-type channels cannot be simply explained as a consequence of saturating the release machinery by excess $Ca^{2+}$ influx because application of Cd$^{2+}$ to nonspecifically reduce the overall $Ca^{2+}$ influx to similar levels resulted in substantially more inhibition of transmitter release (Fig. 5). This clear difference in coupling strength from different types of $Ca^{2+}$ channels suggests that the degree in the overlap of $Ca^{2+}$ nanodomains is not very large. Otherwise, the $Ca^{2+}$ sensor should be insensitive to the distribution of different types of $Ca^{2+}$ channels within its microdomain. This suggests that presynaptic $Ca^{2+}$ channels and the $Ca^{2+}$ sensor at the transmitter release face are organized in a way such that the $Ca^{2+}$ sensor of the release machinery is sensitive to the distribution of different presynaptic $Ca^{2+}$ channels.
These data provide helpful information about the organization of presynaptic Ca\textsuperscript{2+} channels at the transmitter release face of presynaptic terminals in the mammalian CNS. The extension of the Ca\textsuperscript{2+} microdomain is probably limited by the observed differential coupling strength of different Ca\textsuperscript{2+} channels to the Ca\textsuperscript{2+} sensor. To sense the difference in the location of each channel, the Ca\textsuperscript{2+} sensor is likely to be located close to a cluster of Ca\textsuperscript{2+} channels. On the other hand, the distance between Ca\textsuperscript{2+} sensor and Ca\textsuperscript{2+} channels may be within a certain range, such that the Ca\textsuperscript{2+} concentration near the sensor is contributed by multiple Ca\textsuperscript{2+} channels. In addition, the diffusion of Ca\textsuperscript{2+} must be fast enough to result in a differential buildup of local high Ca\textsuperscript{2+} concentration near the Ca\textsuperscript{2+} sensor, depending on amplitude and duration of Ca\textsuperscript{2+} influx.

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