

Pharmacological Identification of Two Types of Presynaptic Voltage-Dependent Calcium Channels at CA3–CA1 Synapses of the Hippocampus

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The effects of voltage-dependent Ca channel (VDCC) antagonists on synaptic transmission were investigated at CA3–CA1 synapses of guinea pig hippocampal slices. After selectively loading presynaptic structures in area CA1 with the calcium indicator fura-2, we simultaneously recorded a presynaptic calcium transient ($[Ca]_i$) and the corresponding field excitatory postsynaptic potential (fEPSP) evoked by a single stimulus given to the Schaffer collateral–commissural (SCC) pathway. Application of nifedipine did not reduce either the $[Ca]_i$ or the fEPSP, suggesting that nifedipine-sensitive Ca channels do not significantly contribute to evoked synaptic transmission at low stimulation frequency. Application of ω -conotoxin GVIA (ω -CgTX) or ω -agatoxin-IVA (ω -Aga-IVA) dose-dependently blocked both the $[Ca]_i$ and the fEPSP. The time course of the block of the $[Ca]_i$ was similar to that of the fEPSP. About 40% of the total $[Ca]_i$ was ω -CgTX sensitive, and more than 20% was ω -Aga-IVA sensitive. Combined application of these two blockers showed no overlap of the ω -CgTX-sensitive with the ω -Aga-IVA-sensitive $[Ca]_i$. These results suggest that there are at least two types of presynaptic VDCCs at CA3–CA1 synapses of the hippocampus: ω -CgTX-sensitive and ω -Aga-IVA-sensitive Ca channels. Our results also suggest that these two types of Ca channels are colocalized at a single presynaptic terminal.

During application of ω -CgTX or ω -Aga-IVA, the initial slope of the fEPSP varied approximately as the fourth power of the amplitude of the $[Ca]_i$, suggesting that ω -CgTX-sensitive and ω -Aga-IVA-sensitive Ca channels have about equal efficacy in triggering transmitter release. These results in combination with similar findings at the squid giant synapse suggest that the nonlinear relationship between transmitter release and the Ca influx is well conserved from the molluscan to the mammalian nervous system.

[Key words: hippocampus, presynaptic calcium, calcium channel, ω -conotoxin GVIA, ω -agatoxin-IVA, nifedipine, phenytoin, synaptic transmission, fura-2, guinea pig]

VDCCs play an important role in triggering transmitter release (Llinás, 1991; Zucker, 1993). Discrimination between these channel types has been based on several criteria, including gat-

ing, ionic conductance, and pharmacology. So far, at least four distinct types of VDCCs have been found: L-, N-, P-, and T-type channels (Fox et al., 1987a,b; Llinás et al., 1989). Many investigations have been carried out to classify the types of Ca channels in neuronal cell bodies of various species, and the recording of Ca currents at the soma of many types of neurons has become a routine practice (Tsien et al., 1988, 1991; Miller, 1992). However, except for few preparations such as the squid giant synapse (Llinás et al., 1976; Augustine et al., 1985a), the recording of Ca currents in presynaptic terminals, especially in the mammalian CNS, is very difficult and often impractical. To identify the types of VDCCs contributing to transmitter release in the mammalian CNS, pharmacological tools are often used.

Studies of the effects of specific Ca channel blockers on the postsynaptic potential in PNS and CNS have suggested that ω -CgTX-sensitive N-type Ca channels are involved in triggering transmitter release (Kerr and Yoshikami, 1984; Kamiya et al., 1988; Dutar et al., 1989; Horne and Kemp, 1991; Protti et al., 1991), but dihydropyridine-sensitive L-type Ca channels in most cases are not (Jones and Heinemann, 1987; Kamiya et al., 1988; Atchison, 1989; Hamilton and Smith, 1992). Recently, it has been found that funnel-web spider toxin (FTX), a P-type VDCC blocker (Llinás et al., 1989), inhibited synaptic transmission at squid giant synapse, neuromuscular junction, and parallel and climbing fiber synapses in cerebellum (Llinás et al., 1989; Uchitel et al., 1992; Sugimori et al., 1993); and ω -Aga-IVA, another P-type VDCC blocker (Mintz et al., 1992b), reduced synaptic transmission at CA3–CA1 and mossy fiber–CA3 synapses of the hippocampus (Hirning et al., 1992; Ahlijanian et al., 1993; Castillo et al., 1993; Luebke et al., 1993; Takahashi and Momiya, 1993). These findings suggest that P-type Ca channels are present at presynaptic terminals.

The pharmacological effects of specific Ca channel blockers on high potassium-induced Ca uptake into synaptosomes, and high potassium-induced transmitter release from synaptosomes, brain slices, and PNS, have been extensively studied. Results from mammalian brain synaptosomes and brain slices have suggested in general that ω -Aga-IVA-sensitive or FTX-sensitive P-type Ca channels constitute the majority of VDCCs involved in transmitter release (Llinás et al., 1989; Mintz et al., 1992b; Turner et al., 1992; Uchitel et al., 1992; Burke et al., 1993; Geer et al., 1993), that ω -CgTX-sensitive N-type Ca channels constitute a smaller fraction (Reynolds et al., 1986; Dooley et al., 1987; Suszkiw et al., 1989; Lundy et al., 1990; Scheer, 1990; Pocock et al., 1992; Burke et al., 1993), and that L-type Ca channels are largely not involved in transmitter release (Middlemiss and Spedding, 1985; Reynolds et al., 1986; Suszkiw et al., 1986; Scheer, 1990; Pocock et al., 1992; Geer et al., 1993).

Received Oct. 21, 1993; revised Feb. 16, 1994; accepted Mar. 24, 1994.

We thank Drs. R. Gray and D. Johnston for kindly allowing us to use some of their equipment. We also thank S. Sinha, B. Avery, and Drs. O. Delbono, R. Gray, and P. E. Schulz for critical and helpful comments on the manuscript. The computer software for data acquisition and analysis was developed by S. S. Patel.

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Optical methods with Ca indicators have been successfully used in monitoring the Ca transient in presynaptic terminals of a few preparations, including squid giant synapse (Charlton et al., 1982; Swandulla et al., 1991), crayfish neuromuscular junction (Delaney et al., 1989), and hippocampal mossy fiber synapses (Regehr and Tank, 1991). Recently, we have been able to use optical techniques to record simultaneously the presynaptic $[Ca]_i$ and the fEPSP induced by a single electrical stimulation of the SCC pathway in hippocampal area CA1 (Wu and Saggau, 1994). Here we describe an application of this technique to study directly the effects of several VDCC blockers, including nifedipine, ω -CgTX, and ω -Aga-IVA, on both the presynaptic $[Ca]_i$ and the fEPSP in a population of hippocampal CA3–CA1 synapses. We confirmed the earlier suggestion by demonstrating that ω -CgTX and ω -Aga-IVA block synaptic transmission by inhibiting the presynaptic $[Ca]_i$ at CA3–CA1 synapses (Dutar et al., 1989; Horne and Kemp, 1991; Hirning et al., 1992). We also found that the ω -CgTX-sensitive $[Ca]_i$ constitutes about 40% of the total $[Ca]_i$ evoked by single action potentials, and the ω -Aga-IVA-sensitive $[Ca]_i$ constitutes more than 20%.

In squid giant synapses the relationship between presynaptic Ca current and postsynaptic potential is nonlinear and follows a third-power function (Augustine et al., 1985b; Augustine and Charlton, 1986). This finding suggests the “cooperative” action of Ca in triggering transmitter release. By applying the nonspecific VDCC blocker cadmium, we obtained a similar result (Wu and Saggau, 1994). Transmitter release in area CA1 of hippocampus, as judged by the initial slope of the fEPSP, varied approximately with the fourth power of the presynaptic Ca influx, the volume average Ca current (Wu and Saggau, 1994). The presynaptic Ca channel in the squid giant synapse has been identified as P type using FTX (Llinás et al., 1989). It is important to know whether the relationship between transmitter release and the Ca influx through a particular type of VDCCs holds in the presynaptic terminals of the mammalian CNS. Our present results suggest that this power relationship is well conserved from the molluscan to the mammalian nervous system.

Materials and Methods

Brain slice preparation and electrical recording. Guinea pigs about 1–2 months old were anesthetized (methoxyflurane) and quickly decapitated. The brains were immediately removed and 400 μ m transverse hippocampal brain slices were prepared on a vibrating tissue cutter and stored in artificial cerebrospinal fluid containing (in mM) NaCl, 124; KCl, 5; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 22; and D-glucose, 10. The artificial cerebrospinal fluid was temperature controlled at 28–30°C and gassed with 95% O_2 and 5% CO_2 , maintaining a constant pH of 7.4. After a resting period of at least 1 hr, the slices were transferred to the recording chamber. Evoked fEPSPs (about 0.8–1.2 mV in amplitude) were recorded from stratum radiatum with glass microelectrodes (2 M NaCl, 1–5 M Ω). Before calculating the initial slopes of the fEPSPs, the signals were corrected for contamination by the presynaptic volleys by subtracting responses obtained during routine application of the glutamate antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μ M) and aminophosphonovalerate (APV; 50 μ M; see Wu and Saggau, 1994). Data are expressed as mean \pm SEM.

Optical recording of the presynaptic Ca transient. The technique for selectively loading presynaptic structures including terminals of CA3–CA1 synapses with the Ca indicator fura-2 has been described in detail elsewhere (Wu and Saggau, 1994). In brief, a small amount of fura-2 AM (Molecular Probes) solution (0.9 mM fura-2 AM, 10% DMSO, and 1% pluronic acid) in a pipette with a tip diameter of about 2 μ m was pressure injected under visual control into stratum radiatum of area CA1 of the hippocampal slice (Fig. 1A). This resulted in loading and subsequent diffusion of the indicator via the SCC pathway to presynaptic terminals. About 1 hr after injection, fluorescence emerging from an

area with a diameter of about 150 μ m in stratum radiatum (Fig. 1A, shaded region), approximately 500 μ m from the injection site, was detected by a single photodiode, connected to a low-noise, current-to-voltage converter and amplifier. The optical recording system for the Ca signals includes an inverted microscope (Axiovert 10, Achroplan 50 \times , 0.9 NA, Zeiss) equipped with a Xenon burner, an electromechanical shutter (Uniblitz, Vincent), and a quartz illuminator, as well as matched epifluorescence filter sets for ratio measurements (360:380 nm; Omega). Calibration of Ca signals was obtained by forming the ratio between two continuous recordings (100 msec) of fura-2 fluorescence intensity at two excitation wavelengths with an interval of 2–5 sec (Grynkiewicz et al., 1985). The fluorescence intensities did not change significantly within this interval. The background fluorescence of the slice measured before the injection of fura-2 AM was subtracted. A viscosity correction was not made since its exact value is unknown. This factor could cause an error in our calculations of absolute Ca concentration, but should not interfere with the more important relative values. The amplitude of the $[Ca]_i$ was measured as the difference between the maximal concentration and the resting level. Averaging techniques ($n \leq 4$; interval, 10 sec) were used to improve the signal-to-noise ratio. Data in each experiment were normalized to baseline, then pooled together and expressed as mean \pm SEM. At the end of each experiment, the application of the glutamate antagonist (CNQX and APV) verified the presynaptic origin of the observed $[Ca]_i$ by blocking the fEPSP but not the $[Ca]_i$ (Fig. 1 in Wu and Saggau, 1994).

Drugs. Nifedipine (Sigma) was dissolved in DMSO and diluted in artificial cerebrospinal fluid. Since nifedipine is known to be photolabile (O’Regan et al., 1991), the drug solution was made up in the dark and used within 2 hr. Additional precautions were taken during optical recording (see Results). ω -CgTX (fraction GVIA) was purchased from Sigma. ω -Aga-IVA was obtained from Peptides International (Mintz and Bean, 1993).

Results

Nifedipine reduces neither the $[Ca]_i$ nor the fEPSP

Following a selective loading of presynaptic structures of the CA3–CA1 synapses with the Ca indicator fura-2 (Wu and Saggau, 1994), we simultaneously recorded the presynaptic $[Ca]_i$ and the fEPSP evoked by single electrical stimulation of the SCC pathway in the stratum radiatum of area CA1 of the hippocampal slice (Fig. 1A). Bath application of the L-type Ca channel blocker nifedipine (10 μ M) for 30 min did not affect either the $[Ca]_i$ ($101 \pm 2\%$) or the corresponding fEPSP ($104 \pm 4\%$, $n = 5$). Figure 1, B and C, shows a typical experiment. Since nifedipine is light sensitive (O’Regan et al., 1991), we did not record the Ca transient during the first 20–30 min of application of this drug (Fig. 1B).

ω -CgTX partially blocks both the $[Ca]_i$ and the fEPSP

Addition of 1 μ M ω -CgTX, an N-type Ca channel blocker (Olivera et al., 1985, 1990), partially suppressed both the $[Ca]_i$ and the fEPSP (Fig. 2A,B). This suppression was not accompanied by a significant change of either the presynaptic resting Ca level (Fig. 2A) or the presynaptic volley (Fig. 2B), and was often irreversible. In some slices, the suppression partially recovered following a 30 min washout of ω -CgTX (Fig. 2A). Figure 2C summarizes the dose-dependent suppression after 45 min of exposure to ω -CgTX at different concentrations (0.01–1 μ M). The lowest concentration of ω -CgTX we used (10 nM) weakly suppressed the $[Ca]_i$ to $90 \pm 3\%$ and the fEPSP to $63 \pm 8\%$ of baseline ($n = 4$). The effects of ω -CgTX were saturating at concentrations higher than 100 nM. At 1 μ M, ω -CgTX reduced the $[Ca]_i$ to $59 \pm 8\%$ and the corresponding fEPSP to $24 \pm 11\%$ of baseline ($n = 4$). The time courses of suppression of the $[Ca]_i$ and the fEPSP were similar (Fig. 2A), and were faster with higher concentrations of ω -CgTX. The percentage reductions of the fEPSP by ω -CgTX are similar to other reports at this synapse

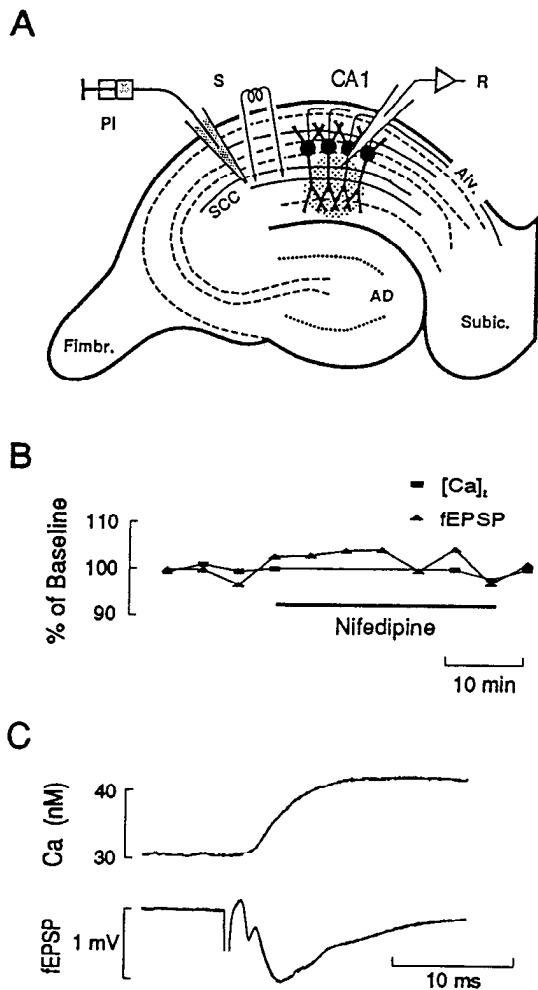


Figure 1. Nifedipine does not change either the $[Ca]_i$ or the fEPSP. *A*, Experimental arrangement. The membrane-permeant calcium indicator (fura-2 AM, 0.9 mM) was pressure injected (PI) under visual control into stratum radiatum (SR). Fluorescence emerging from an area with a diameter of about 150 μm in SR (shaded area), 500 μm away from the injection site, was recorded by a single photodiode. The preparation was electrically stimulated by a bipolar electrode (S) positioned in SR. An extracellular recording electrode (R) was placed in about the center of the shaded region to record the fEPSP. *B*, No significant change of the $[Ca]_i$ and the fEPSP during bath application of nifedipine (10 μM). *C*, Sampled recordings of the $[Ca]_i$ and the fEPSP before and during the application of nifedipine are superimposed.

(Dutar et al., 1989; Horne and Kemp, 1991) and at the mossy fiber-CA3 synapse (Kamiya et al., 1988).

The summarized data in Figure 2C show that the relationship between the mean of the initial slope of the fEPSP (Y) and the mean of the amplitude of the $[Ca]_i$ (X) at each concentration was nonlinear. This relationship could be approximated by a power function,

$$Y = X^m, \quad (1)$$

where $m = 3.5$, and X and Y are normalized to their respective baselines. By substituting X in Equation 1 with the measured mean $[Ca]_i$ (normalized) at each concentration, different traces of the calculated fEPSP were obtained by varying the value of the exponent m . This exponent m ($m = 3.5$) was determined as the best fit of the calculated (Fig. 2C, \square) with the measured mean fEPSP (Fig. 2C, \blacktriangle) as guessed by eye. The exponent (m

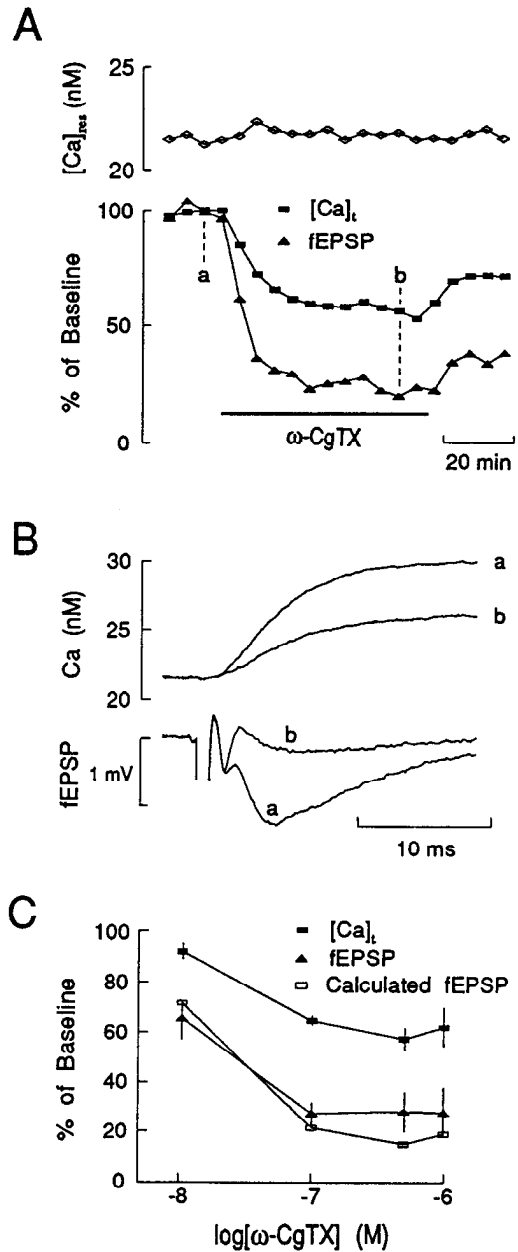


Figure 2. ω -CgTX decreases both the $[Ca]_i$ and the fEPSP in area CA1 of hippocampus. *A*, A typical experiment showing the reduction of the $[Ca]_i$ and the fEPSP during bath application of 1 μM ω -CgTX (bottom graph). This reduction was not accompanied by a significant change of the resting Ca level ($[Ca]_{rest}$, top graph). *B*, Sampled recordings of the $[Ca]_i$ and the fEPSP taken at the times indicated in *A*. *C*, Dose response of ω -CgTX block of the amplitude of the $[Ca]_i$ (\blacksquare) and the initial slope of the fEPSP (\blacktriangle). Observations (mean \pm SEM) from three or four slices for each concentration are normalized. The blocking effect was determined 45 min after application of ω -CgTX. \square represent the calculated slope of the fEPSP based on Equation 1, where $m = 3.5$, and X are taken from the measured $[Ca]_i$ at each concentration.

$= 3.5$) in Equation 1 is similar to that ($m = 3.6$) obtained by applying the nonspecific VDCC blocker cadmium (Wu and Saggau, 1994), suggesting that reduction of the $[Ca]_i$ is sufficient to account for the inhibition of synaptic transmission by ω -CgTX.

Augustine et al. (1985a) showed in the squid giant synapse that the integral over time of the presynaptic Ca current corresponds excellently to absorption signals obtained with arsen-

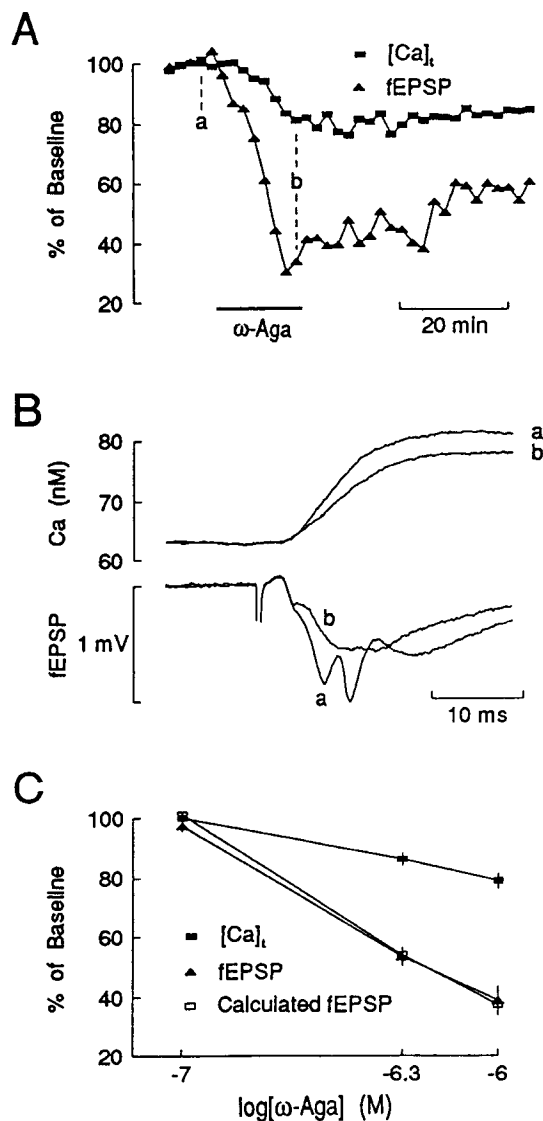


Figure 3. ω -Aga-IVA decreases both the $[Ca]_i$ and the fEPSP. *A*, A typical experiment showing the decrease of the $[Ca]_i$ and the fEPSP during bath application of ω -Aga-IVA (ω -Aga, 1 μ M). *B*, Sampled recordings of the $[Ca]_i$ and the fEPSP taken at the times indicated in *A*. *C*, Dose response of ω -Aga-IVA block of the amplitude of the $[Ca]_i$ (■) and the initial slope of the fEPSP (▲). Observations (mean \pm SEM) from three or four slices for each concentration are normalized. Block was determined 15 min after application of ω -Aga-IVA. □ represent the calculated slope of the fEPSP based on Equation 1, where $m = 4.1$, and X are taken from the measured $[Ca]_i$ at each concentration.

azo III, another Ca indicator. Our Ca signals showed very little noise, which allowed us to take the reverse approach and differentiate the Ca transient to obtain a volume-averaged Ca influx, which is presumably proportional to the average presynaptic Ca current. As little is known about the endogenous Ca buffers in the presynaptic terminals, we made the assumption that the fura-2 signal we recorded is proportional to the total amount of Ca that enters following an action potential. Thus, a curve proportional to the volume-averaged presynaptic Ca influx was obtained by taking the first derivative of the Ca transient over time (see Fig. 3 in Wu and Saggau, 1994). The time course of the volume-averaged Ca influx rather than that of the Ca transient was close to that of the fEPSP, suggesting

that the volume-averaged Ca influx may reflect the time course of evoked transmitter release (Wu and Saggau, 1994). The amplitude of the volume-averaged Ca influx (normalized to baseline) was shown to be linearly related to the amplitude of the $[Ca]_i$ (normalized) during application of cadmium at the same CA3-CA1 synapses (Wu and Saggau, 1994). This linear relationship was confirmed during application of ω -CgTX (data not shown), suggesting that the amplitude of the $[Ca]_i$ is proportional to the amplitude of the volume-averaged Ca influx or Ca current.

ω -Aga-IVA partially blocks both the $[Ca]_i$ and the fEPSP

Addition of 1 μ M ω -Aga-IVA partially reduced both the $[Ca]_i$ and the fEPSP (Fig. 3*A,B*). The presynaptic volley (Fig. 3*B*; see also Fig. 4*B*) and the presynaptic resting Ca level (data not shown) did not change significantly during application of this drug. The reduction was irreversible or only slightly reversible after 45 min of washout (Fig. 3*A*). Figure 3*C* summarizes the dose-dependent reductions by 15 min exposure to ω -Aga-IVA at different concentrations (0.1–1 μ M). At 100 nM, ω -Aga-IVA had no significant effect on either the $[Ca]_i$ ($101 \pm 1\%$) or the fEPSP ($98 \pm 2\%$, $n = 3$). At 500 nM, ω -Aga-IVA reduced the $[Ca]_i$ to $86 \pm 2\%$ and the fEPSP to $53 \pm 3\%$ of baseline ($n = 3$). At 1 μ M, ω -Aga-IVA reduced the $[Ca]_i$ to $79 \pm 2\%$ and the corresponding fEPSP to $39 \pm 5\%$ of baseline ($n = 4$). Suppressions of the $[Ca]_i$ and the fEPSP were of similar time courses (Fig. 3*A*), and were faster with higher concentrations of ω -Aga-IVA.

The summarized data in Figure 3*C* show the nonlinear relationship between the mean of the initial slope of the fEPSP and the mean of the amplitude of the $[Ca]_i$ at different concentrations. This relationship could also be approximated by Equation 1 with $m = 4.1$. By substituting X in Equation 1 with the measured mean $[Ca]_i$ (normalized to baseline) at each tested concentration, the calculated fEPSP was obtained (Fig. 3*C*, □), which closely resembled the measured fEPSP (Fig. 3*C*, ▲). The exponent ($m = 4.1$) in this equation is close to those obtained by applying either cadmium (Wu and Saggau, 1994) or ω -CgTX (Fig. 2*C*), suggesting that ω -Aga-IVA inhibits synaptic transmission by reducing the presynaptic $[Ca]_i$. The relationship between the amplitude of the $[Ca]_i$ and the volume-averaged Ca influx (the first derivative over time of the $[Ca]_i$) during application of ω -Aga-IVA was linear (data not shown).

The concentration of ω -Aga-IVA we used (0.5–1 μ M) to block the presynaptic $[Ca]_i$ in the hippocampal slice is higher than that used (up to 200 nM) to block P-type VDCCs in dissociated neurons and brain synaptosomes (Mintz et al., 1992a,b). The possibility that ω -Aga-IVA may also block ω -CgTX-sensitive $[Ca]_i$ was eliminated by coapplication of both blockers. In the presence of ω -CgTX, ω -Aga-IVA still blocked both the $[Ca]_i$ and the fEPSP; Figure 4, *A* and *B*, shows a typical experiment. Preexposing the hippocampal slices to 1 μ M ω -CgTX for 15–20 min reduced the $[Ca]_i$ to $71 \pm 3\%$ and the fEPSP to $38 \pm 4\%$ of baseline ($n = 3$). Adding 1 μ M ω -Aga-IVA for 15 min further reduced the $[Ca]_i$ to $47 \pm 6\%$ and the fEPSP to $3 \pm 3\%$ of the baseline ($n = 3$) (Fig. 4*C*). After 15–20 min exposure, 1 μ M ω -CgTX usually achieved more than 80% of its maximal effect (as measured after 45 min exposure) and the remaining effect developed very slowly (Fig. 2*A*). Therefore, the measured additional inhibitory effects of ω -Aga-IVA in the presence of ω -CgTX are mostly from ω -Aga-IVA. The similar percentage decreases (relative to the controls before any drug application) of $[Ca]_i$ caused by ω -Aga-IVA in the presence ($25 \pm 3\%$, $n = 3$)

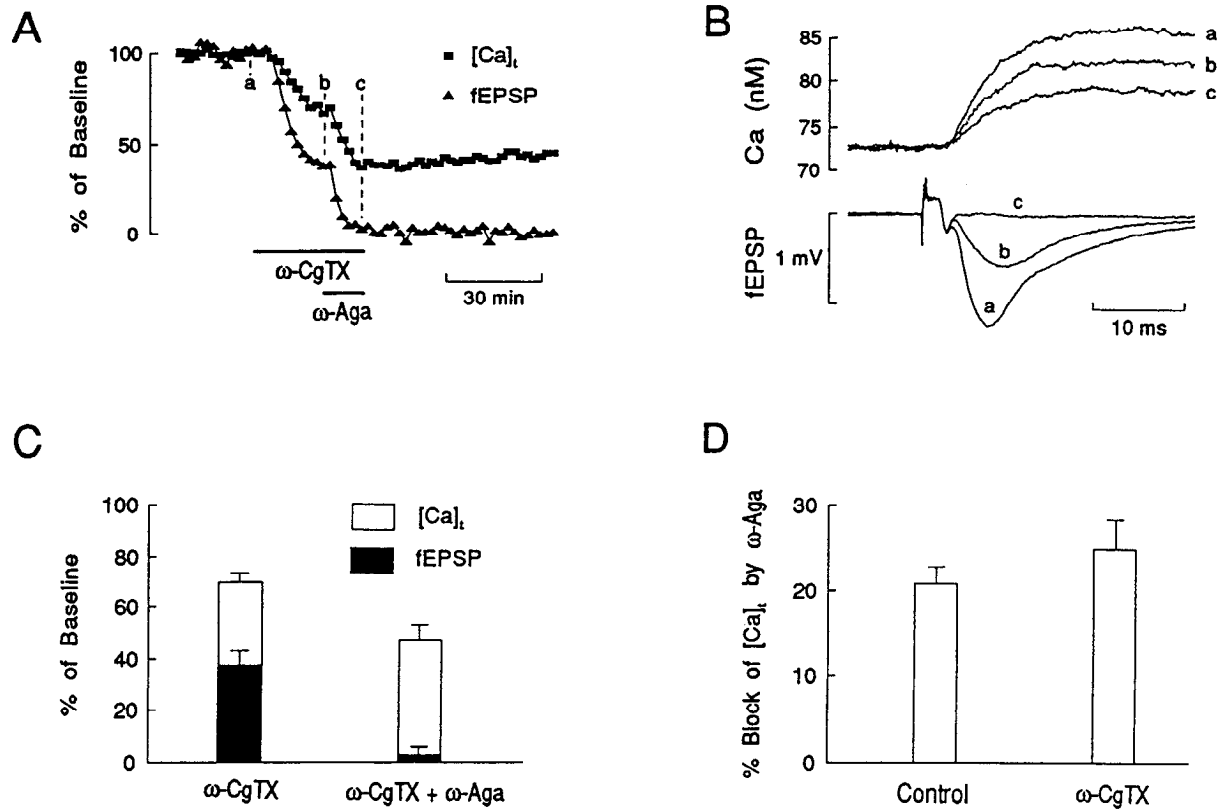


Figure 4. The blocking effect of ω -Aga-IVA is not occluded by ω -CgTX. *A*, A typical experiment showing the changes of the $[Ca]_i$ and the fEPSP during application of $1 \mu M$ ω -CgTX and $1 \mu M$ ω -Aga-IVA (ω -Aga). *B*, Sampled recordings of the $[Ca]_i$ and the fEPSP taken at the times indicated in *A*. *C*, Inhibition of both the $[Ca]_i$ and the fEPSP during the application of $1 \mu M$ ω -CgTX and coapplication of both $1 \mu M$ ω -CgTX and $1 \mu M$ ω -Aga-IVA. The block for ω -CgTX was determined 15–20 min after its application. The block for combined application (ω -CgTX + ω -Aga) was determined 15 min after the coapplication, which followed a preexposure to ω -CgTX for 15–20 min. Observations (mean \pm SEM) from three slices were normalized to the baseline before application of ω -CgTX. *D*, Comparison of the blocking effect of $[Ca]_i$ by ω -Aga-IVA in the absence (Control, $n = 4$) and the presence of ω -CgTX ($n = 3$). Data were normalized to the baseline before application of both toxins.

and absence ($21 \pm 2\%$, $n = 4$) of ω -CgTX suggest that ω -Aga-IVA blocks a separate population of VDCCs without affecting ω -CgTX-sensitive Ca channels (Fig. 4D).

Discussion

Using a combination of optical and electrophysiological techniques, we found two pharmacologically distinct presynaptic VDCCs, that is, ω -CgTX-sensitive and ω -Aga-IVA-sensitive Ca channels, which contribute to transmitter release evoked by single presynaptic action potentials at CA3–CA1 synapses in guinea pig hippocampal slices. We also compared the efficacy of these two types of presynaptic VDCCs in controlling transmitter release by determining the relationship between the fEPSP and the presynaptic $[Ca]_i$ during application of either of the blockers.

Fluorometric recording of the presynaptic Ca transient

The technique to load selectively the presynaptic terminals with the Ca indicator fura-2, and the recording of the presynaptic $[Ca]_i$, have been discussed in detail in a previous article (Wu and Saggau, 1994). Our data showed that cadmium (Wu and Saggau, 1994), ω -CgTX, and ω -Aga-IVA dose-dependently blocked both the presynaptic $[Ca]_i$ and the fEPSP. During application of each of these blockers, the changes of the amplitude of the $[Ca]_i$ and the initial slope of the fEPSP were of similar time courses, and the initial slope of the fEPSP varied approximately as the fourth

power of the amplitude of the $[Ca]_i$. A dose-dependent block of the $[Ca]_i$ and the fEPSP, similar time courses of the changes of the $[Ca]_i$ and the fEPSP, and an approximately fourth power nonlinear relationship between the fEPSP and the $[Ca]_i$ were also found during application of adenosine and the GABA_B receptor agonist baclofen (Saggau et al., 1993; Wu and Saggau, 1993). Application of the potassium channel blocker 4-aminopyridine (L. G. Wu and P. Saggau, unpublished observations) or the adenosine A₁ antagonist 8-cyclopentyl-1,3-dipropylxanthine (Wu and Saggau, 1993) increased both the $[Ca]_i$ and the fEPSP with a similar time course and a similar power relationship. So far, we have not found a manipulation that can modulate the presynaptic $[Ca]_i$ without changing the fEPSP with a similar time course and with a roughly fourth power relationship. This evidence suggests that the recorded presynaptic $[Ca]_i$ is an indicator of the local Ca that triggers transmitter release. Since the optical recording area may cover presynaptic terminals, where transmitter release occurs, as well as axons, our data imply that the $[Ca]_i$ may be primarily from presynaptic terminals or from both terminals and the axons having VDCCs with similar pharmacological properties as terminals. In either case, we can study quantitatively the relative contribution of different types of presynaptic Ca channels to the $[Ca]_i$ evoked by single action potentials. The presynaptic Ca accumulation evoked by tetanic stimulation at the crayfish neuromuscular junction has been shown to occur locally in boutons and small processes

where synaptic vesicles and synaptic "active zones" are located (Zucker et al., 1991). This further supports the suggestion that the $[Ca]_i$ is primarily from presynaptic terminals.

It is unlikely that a detectable fraction of the recorded presynaptic $[Ca]_i$ is contributed by interneurons, because the number of inhibitory interneurons in a hippocampal slice is small and their processes are mostly not parallel to the SCC pathway (Brown and Zador, 1990). If a significant portion of the $[Ca]_i$ is from interneurons, application of CNQX (10 μ M) and APV (50 μ M) should block the Ca signals from interneurons that are indirectly activated by CA3 axonal terminals; and antidromic stimulation of CA1 pyramidal neurons should elicit a $[Ca]_i$ due to indirect activation of interneurons. However, we failed to observe these phenomena (Fig. 1 in Wu and Saggau, 1994), suggesting that the interneurons indirectly activated by stimulation of CA3 axons were not loaded with fura-2. This makes it unlikely that a significant portion of the Ca signal is from interneurons directly activated by stimulation.

Sharp spatial Ca gradients have been demonstrated in presynaptic terminals during synaptic transmission (Swandulla et al., 1991; Llinás et al., 1992). The fura-2 signals in our experiments provide a measure of the averaged Ca concentration in the optical recording area covering terminals as well as axons, and as such may greatly underestimate the Ca concentration near the inner surface of the membrane where transmitter is released. This may result in a reduced amplitude of the $[Ca]_i$, which was usually in the range of 10–20 nM in our experiments. Another factor contributing to the reduced amplitude of the $[Ca]_i$ is that the measured $[Ca]_i$ is the averaged Ca concentration for all the structures loaded with fura-2 in the recording area, but not all of the loaded structures are stimulated. However, the amplitude of the $[Ca]_i$ evoked by a stimulus with maximal intensity was less than 30–40 nM (Wu and Saggau, unpublished observations), which is only about two to four times higher than that during routine experiments. Similar observations have recently been reported in single hippocampal mossy fiber terminals (Regehr et al., 1994) and the giant presynaptic terminals of the chick ciliary ganglion (Yawo and Chuhma, 1993); in both cases, a 10–30 nM increase of the Ca concentration was evoked by single nerve stimulus. Due to the above considerations, the amplitude change of the $[Ca]_i$ during application of VDCC blockers in each experiment was expressed as the percentage of the $[Ca]_i$ before the blocker application, and as such the absolute change of the $[Ca]_i$ was normalized. The data were then pooled after such a normalization in each experiment.

Since the average Ca level may not be linearly related to the local Ca level, the power relationship we measured between the fEPSP and the $[Ca]_i$ may not well reflect the relationship between the transmitter release and the local Ca level responsible for this release. Nevertheless, the measured power relationship is useful in comparing the relative efficacy of each type of presynaptic VDCCs in triggering transmitter release.

Dihydropyridine-sensitive L-type Ca channels do not significantly participate in normal synaptic transmission

The dihydropyridine antagonist nifedipine had no effect on either the presynaptic $[Ca]_i$ or the fEPSP in area CA1 of the hippocampus, suggesting that L-type Ca channels do not significantly participate in triggering transmitter release evoked by a single presynaptic action potential. Although the potency of dihydropyridines is voltage dependent (Docherty and Brown, 1986; Fox et al., 1987; Meyers and Barker, 1989; Ozawa et al.,

1989), at holding potentials around resting potential (–60––80 mV) nifedipine (0.5–1 μ M) still blocked 30–60% of L-type Ca current in cultured hippocampal neurons (Meyers and Barker, 1989). Other dihydropyridine antagonists, such as nifedipine, nitrendipine, and nimodipine, were also shown to block a significant portion of the total high-threshold Ca current in hippocampal neurons activated from around resting potential (Ozawa et al., 1989; Mintz et al., 1992a). Assuming that at resting potential nifedipine can block 30–60% of the total L-type Ca current activated by an action potential, and having shown that a 5% decrease of the $[Ca]_i$ is well detectable (Wu and Saggau, 1994), the lack of effect of nifedipine on the $[Ca]_i$ suggests that L-type Ca channels contribute to less than 8–16% of the $[Ca]_i$. Assuming further a fourth power relationship between the fEPSP and the $[Ca]_i$ (Wu and Saggau, 1994), and knowing a 10% decrease of the fEPSP is well detectable, then a 2.5% decrease of the $[Ca]_i$ is indirectly detectable. Taken together, the lack of effect of nifedipine on the fEPSP would suggest that L-type Ca channels contribute to less than 4–8% of the $[Ca]_i$. Our results are consistent with other reports that high potassium-induced Ca influx in brain synaptosomes (Reynolds et al., 1986; Suszkiw et al., 1986; Scheer, 1990; Pocock et al., 1992) and synaptic transmission in hippocampal slices (Jones and Heinemann, 1987; Kamiya et al., 1988) are usually insensitive to dihydropyridine antagonists. Both the L-type Ca channel blocker nifedipine and the activator BAY-K 8644 have also been shown to have no significant effect on presynaptic Ca influx induced by a train of stimulation (5–20 Hz, 5–10 sec) as recorded with an extracellular ion-sensitive electrode in stratum radiatum of hippocampal area CA1 (Jones and Heinemann, 1987). This evidence corroborates our results very well. However, the possibility that presynaptic L-type Ca channels may be activated by other patterns of stimulation is not ruled out.

ω -CgTX-sensitive N-type Ca channels are present in presynaptic terminals

We showed that ω -CgTX, but not nifedipine, inhibits synaptic transmission by reducing the presynaptic Ca influx without affecting the presynaptic volley at CA3–CA1 synapses of the hippocampus. These results are consistent with other findings that ω -CgTX inhibits synaptic transmission in area CA1 and CA3 of the hippocampus (Kamiya et al., 1988; Dutar et al., 1989; Horne and Kemp, 1991), suggesting that N-type Ca channels are present in presynaptic terminals of CA3–CA1 synapses. As can be seen in the dose–response curve (Fig. 2C), about 40% of the $[Ca]_i$ can be blocked by ω -CgTX. In rat brain synaptosomes or tissue minces, it has been reported that high potassium-induced Ca influx was either insensitive (Mintz et al., 1992b; Geer et al., 1993) or only partially sensitive to ω -CgTX (Reynolds et al., 1986; Suszkiw et al., 1989; Lundy et al., 1990; Scheer, 1990; Pocock et al., 1992). The latter finding is in accordance with our results. However, the heterogeneity of the brain synaptosomes or tissue minces and the long duration of the high potassium-induced depolarization (compared to the duration of a single action potential) make it difficult to compare our data to those from synaptosomes or tissue minces.

In several slices, the blocking effect of ω -CgTX was slightly reversible. The reason is unclear. It has been reported that ω -CgTX weakly and reversibly blocks T-type Ca channels in chick dorsal root ganglion neurons (McCleskey et al., 1987). Whether a small fraction of T-type Ca channels is blocked by

ω -CgTX in those slices showing slightly reversible effects of ω -CgTX remains to be determined.

ω -Aga-IVA-sensitive Ca channels are present in presynaptic terminals

Our data showed that ω -Aga-IVA inhibits synaptic transmission by reducing the presynaptic Ca transient without affecting the presynaptic volley in area CA1 of the hippocampus. The ω -Aga-IVA-sensitive fraction of the $[Ca]_i$ did not overlap with the fraction blocked by ω -CgTX, and no nifedipine-sensitive fraction of the $[Ca]_i$ was found. These findings suggest that ω -Aga-IVA blocks a population of Ca channels other than N or L type in the investigated presynaptic terminals. This ω -Aga-IVA-sensitive population of Ca channels in presynaptic terminals may have properties similar to the P-type Ca channels found in somata of many types of dissociated neurons (Mintz et al., 1992a,b; Mintz and Bean, 1993). Our results corroborate the recent evidence that ω -Aga-IVA blocks synaptic transmission at CA3-CA1 and mossy fiber-CA3 synapses (Hirning et al., 1992; Ahlijanian et al., 1993; Castillo et al., 1993; Luebke et al., 1993; Takahashi and Momiyama, 1993), reduces high potassium-induced Ca influx into brain synaptosomes and tissue minces (Mintz et al., 1992b; Geer et al., 1993), and inhibits glutamate release from rat brain synaptosomes and hippocampal slice in CA1 region (Turner et al., 1992; Burke et al., 1993).

The minimal effective concentration of ω -Aga-IVA in our experiments was between 100 and 500 nM, which was higher than that used to block the P-type Ca current in somata of dissociated neurons (Mintz et al., 1992a,b; Mintz and Bean, 1993). One explanation may be that in our recording chamber the hippocampal slices were sitting on a glass coverslip, which may limit the fluid exchange at the bottom of the preparation, and the high molecular weight of ω -Aga-IVA may hinder its diffusion into synaptic clefts in brain slices much more severely than onto somata of dissociated neurons. However, the glass coverslip in our recording chamber did not affect the blocking effect of ω -CgTX, as the effect of ω -CgTX on the fEPSP in our experiments is similar to those reported by other groups (Kammiya et al., 1988; Dutar et al., 1989; Horne and Kemp, 1991). Furthermore, the requirement for a high concentration (0.5–3 μ M) of ω -Aga-IVA to block synaptic transmission in the hippocampal slice was also reported by several other groups (Hirning et al., 1992; Ahlijanian et al., 1993; Castillo et al., 1993), but a relatively low concentration (100–200 nM) of ω -Aga-IVA was reported to inhibit the majority of the EPSP at CA3-CA1 synapses of the hippocampal slice (Luebke et al., 1993; Takahashi and Momiyama, 1993). The reason for this difference is unknown.

A recent report showed that high concentrations (200–800 nM) of ω -Aga-IVA do not block T-, L-, or N-type Ca channels in sympathetic neurons (Mintz and Bean, 1993). Our results suggest that 1 μ M ω -Aga-IVA does not block N-type Ca channels, and that L-type Ca channels are not activated by single stimulation in the investigated presynaptic terminals. Taken together, these results suggest that ω -Aga-IVA at high concentrations does not block T-, L-, or N-type Ca channels at presynaptic terminals of hippocampal CA3-CA1 synapses. However, high concentrations of ω -Aga-IVA have recently been found to partially block Q-type Ca channels, a new type of Ca channels defined as sensitive to ω -conotoxin MVIIC but insensitive to nimodipine, ω -CgTX, and low concentrations of ω -Aga-IVA (Randall et al., 1993; Sather et al., 1993). Q-type channels have

also been proposed to be localized in the presynaptic terminals of rat hippocampal area CA1 (Randall et al., 1993). Therefore, it is possible that ω -Aga-IVA at 1 μ M may also inhibit Q-type Ca channels. Currently, we do not know whether ω -Aga-IVA reduces the presynaptic $[Ca]_i$ by inhibiting P-, Q-, or both types of Ca channels. Due to this consideration, we refer to the Ca channels blocked by ω -Aga-IVA as ω -Aga-IVA-sensitive type. Further classification of ω -Aga-IVA-sensitive channels requires examination of the occlusion between the blocking effects of ω -Aga-IVA and ω -conotoxin MVIIC on the $[Ca]_i$.

Presynaptic Ca channels resistant to nifedipine, ω -CgTX, and ω -Aga-IVA

In the presence of high concentrations of both ω -CgTX and ω -Aga-IVA, more than 40% of the $[Ca]_i$ still remained (Fig. 4), and this component was not sensitive to nifedipine (Fig. 1). Although there may in principle exist N- and P-type Ca channels insensitive to ω -CgTX (Plummer et al., 1989) and ω -Aga-IVA, it seems unlikely that unblocked N- and P-type Ca channels could account for more than 40% of the $[Ca]_i$ remaining in the presence of these two blockers. It is also highly unlikely that the reversal of Na/Ca exchange could explain the remaining $[Ca]_i$ (Zucker, 1993), because high concentrations of the nonspecific VDCC blocker cadmium (1–5 mM) completely blocked the $[Ca]_i$ (Wu and Saggau, unpublished observations), and the short rise time of the $[Ca]_i$ did not change significantly after application of any blocker we used (Figs. 2–4). It is more likely that other types of Ca channels resistant to N-, P-, and L-type Ca channel blockers, such as T type and/or some unidentified type, are present in presynaptic terminals. Interestingly, a component of Ca current that remains unblocked in the combined presence of N-, P-, and L-type Ca channel blockers has been found in the soma of several types of neurons, including CA3 pyramidal neurons of the hippocampus (Mintz et al., 1992a; Dolphin et al., 1993). Recently, we found that adenosine and the GABA_A agonist baclofen inhibit the fEPSP primarily by reducing the $[Ca]_i$ at the same synapses, and the reduced $[Ca]_i$ is partially due to inhibition of ω -CgTX-sensitive, ω -Aga-IVA-insensitive Ca channels (Saggau et al., 1993; Wu and Saggau, 1993 and unpublished observations). Following a preapplication of 1 μ M ω -CgTX, both adenosine and baclofen further reduced the $[Ca]_i$ and the fEPSP, suggesting that adenosine and baclofen also inhibit the fEPSP by reducing the ω -CgTX-resistant and ω -Aga-IVA-resistant $[Ca]_i$. Taken together, our data suggest that some presynaptic Ca channels resistant to P-, N-, and L-type Ca channel blockers may also be involved in triggering transmitter release at CA3-CA1 synapses.

Recently, based on the evidence that ω -conotoxin MVIIC potently blocks synaptic transmission, Q-type Ca channels have been proposed to be localized in presynaptic terminals of CA3-CA1 synapses of rat hippocampus (Wheeler et al., 1993). It is possible that the presynaptic $[Ca]_i$ insensitive to nifedipine, ω -CgTX, and ω -Aga-IVA is partly or completely due to Q-type Ca channels. Further classification of Q-type Ca channels may require examination of the blocking effect of ω -conotoxin MVIIC on the presynaptic $[Ca]_i$ in the presence of ω -CgTX and ω -Aga-IVA.

ω -CgTX-sensitive and ω -Aga-IVA-sensitive Ca channels trigger transmitter release with about the same efficacy

The initial slope of the fEPSP was found to be approximately proportional to the fourth power of the amplitude of the $[Ca]_i$,

during bath application of ω -CgTX ($m = 3.5$; see Eq. 1), ω -Aga-IVA ($m = 4.1$), or cadmium ($m = 3.6$) (Wu and Saggau, 1994). Since the amplitude of the $[Ca]$, is proportional to the volume-averaged Ca influx or Ca current (Wu and Saggau, 1994), the fEPSP is also approximately the fourth power of the volume-averaged N-type or ω -Aga-IVA-sensitive Ca influx or Ca current. This result is similar to the finding that the postsynaptic potential is about the third power of the presynaptic Ca current in the squid giant synapse (Augustine, 1985b, 1986). The presynaptic Ca current in the squid giant synapse has been identified as P-type Ca current using FTX (Llinás et al., 1989). Taken together, these data suggest that the nonlinear relationship between transmitter release and the Ca influx through N- or P-type Ca channel is well conserved from molluscan to the mammalian nervous system. Our results indicate that ω -CgTX-sensitive Ca channels, ω -Aga-IVA-sensitive Ca channels, or the sum of all types of VDCCs in the presynaptic terminals trigger transmitter release with about the same efficacy in the CA3-CA1 synapses.

The nonlinear relationship between transmitter release and the presynaptic Ca influx provides an efficient control of transmitter release by modifying presynaptic VDCCs. Many transmitters and modulators, such as glutamate, GABA, and adenosine, have been reported to modulate the VDCCs in the soma of neurons (e.g., Scholz and Miller, 1991; Swartz and Bean, 1992; see also Nicoll et al., 1990, for review) and to inhibit transmitter release at many synapses (e.g., Dutar and Nicoll, 1988; Baskys and Malenka, 1991; Scholz and Miller, 1991; Barrie and Nicholls, 1993). It would be quite efficient if these transmitters or modulators inhibit transmitter release by modulating the presynaptic Ca influx. Adenosine and baclofen have been shown to inhibit synaptic transmission primarily by reducing the presynaptic $[Ca]$, in area CA1 of the hippocampus (Saggau et al., 1993; Wu and Saggau, 1993), although it has also been suggested that these two substances inhibit spontaneous transmitter release by mechanisms independent of inhibition of presynaptic Ca influx (Scanziani et al., 1992; Scholz and Miller, 1992).

Colocalization of ω -CgTX- and ω -Aga-IVA-sensitive Ca channels at single presynaptic terminals

Our data show that ω -CgTX-sensitive and ω -Aga-IVA-sensitive Ca channels coexist in the same population of presynaptic terminals. Since the Ca signals we recorded are an average from a number of presynaptic terminals (Wu and Saggau, 1994), we do not have direct evidence that a single presynaptic terminal contains both ω -CgTX- and ω -Aga-IVA-sensitive Ca channels. Assuming that these two types of Ca channels are segregated into different terminals, then the fEPSP is contributed by at least these two types of synapses containing presynaptic ω -CgTX- or ω -Aga-IVA-sensitive Ca channels alternatively. The sum of the fEPSP contributed by these two types of synapses must not exceed 100%. As shown in Figure 2, a saturating concentration (1 μ M) of ω -CgTX inhibited the fEPSP by $76 \pm 11\%$ ($n = 4$). Under the above assumption, this would indicate that $76 \pm 11\%$ of the fEPSP was contributed by synapses containing presynaptic ω -CgTX-sensitive Ca channels. Similarly, ω -Aga-IVA at 1 μ M inhibited the fEPSP by $61 \pm 5\%$ ($n = 4$), indicating that $61 \pm 5\%$ of the fEPSP was contributed by synapses containing presynaptic ω -Aga-IVA-sensitive Ca channels. The sum of the fEPSP contributed by these two types of synapses is $137 \pm 7\%$. The 95% confidence interval for the mean of the sum of the fEPSP is $137 \pm 17\%$, which is much more than 100%. Therefore,

the above assumption is not accepted. It is highly unlikely that ω -CgTX- and ω -Aga-IVA-sensitive Ca channels are completely segregated into different presynaptic terminals.

The above analysis suggests that ω -CgTX- and ω -Aga-IVA-sensitive Ca channels are colocalized at the same presynaptic terminal. The colocalization of two types of Ca channels in the same presynaptic terminal is of biological significance for several reasons: (1) in case one type of Ca channel is not functioning, the other type of Ca channel may prevent complete block of synaptic transmission; (2) different types of Ca channels may be differentially modulated by patterns of stimulation or by endogenous substances (e.g., Mogul et al., 1993; Yawo and Chuhma, 1993; for review, see Nicoll et al., 1990), thus providing a variety of mechanisms for presynaptic modulation of transmitter release.

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