Changes in Action Potential Duration Alter Reliance of Excitatory Synaptic Transmission on Multiple Types of Ca^{2+} Channels in Rat Hippocampus

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It has been established that multiple types of Ca^{2+} channels participate in triggering neurotransmitter release at central synapses, but there is uncertainty about the nature of their combined actions. We investigated synaptic transmission at CA3–CA1 synapses of rat hippocampal slices and asked whether the dependence on ω -CTx-GVIA-sensitive N-type channels and ω -Aga-IVA-sensitive P/Q-type Ca^{2+} channels can be altered by physiological mechanisms. The reliance on multiple types of Ca^{2+} channels was not absolute but depended strongly on the amount of Ca^{2+} influx through individual channels, which was manipulated by prolonging the presynaptic action potential with the K^+ channel blocker 4-aminopyridine (4-AP) and by varying the extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$). We quantified the influence of spike broadening on Ca^{2+} influx through various Ca^{2+} channels by imposing mock action potentials on voltage-clamped cerebellar granule neurons. In field recordings of the EPSP in hippocampal slices, action potential prolongation increased the EPSP slope by 2-fold and de-

creased its reliance on either N-type or P/Q-type Ca^{2+} channels. The inhibition of synaptic transmission by N-type channel blockade was virtually eliminated in the presence of 4-AP, but it could be restored by lowering $[\text{Ca}^{2+}]_o$. These results rule out a scenario in which a significant fraction of presynaptic terminals rely solely on N-type channels to trigger transmission. The change in sensitivity to the neurotoxins with 4-AP could be explained in terms of a nonlinear relationship between Ca^{2+} entry and synaptic strength, which rises steeply at low $[\text{Ca}^{2+}]_o$, but approaches saturation at high $[\text{Ca}^{2+}]_o$. This relationship was evaluated experimentally by varying $[\text{Ca}^{2+}]_o$ in the absence and presence of 4-AP. One consequence of this relationship is that down-modulation of presynaptic Ca^{2+} channels by various modulators would increase the relative impact of spike broadening greatly.

Key words: 4-aminopyridine; synaptic transmission; rat hippocampus; Ca^{2+} channel; ω -conotoxin-GVIA; ω -agatoxin-IVA

Voltage-gated Ca^{2+} entry provides an essential link between membrane depolarization and exocytosis at nerve terminals (Katz, 1969; Llinás et al., 1976; Augustine et al., 1987). Accordingly, there has been considerable interest in identifying Ca^{2+} channels that are critical for presynaptic Ca^{2+} entry and the release of neurotransmitter (for reviews, see Miller, 1990; Dunlap et al., 1995; Tsien et al., 1995). The existence of several types of neuronal Ca^{2+} channels has been established through a combination of electrophysiology and molecular biology (Tsien et al., 1988; Perez-Reyes et al., 1992; Snutch and Reiner, 1992; Zhang et al., 1993; Birnbaumer et al., 1994). As many as five or six different types of these channels have been found in an individual neuron (Mintz et al., 1992; Randall and Tsien, 1995).

Several recent papers have reported that neurotransmission at

specific synapses in the CNS depends on the concerted actions of more than one type of Ca^{2+} channel (Luebke et al., 1993; Takahashi and Momiyama, 1993; Castillo et al., 1994; Regehr and Mintz, 1994; Wheeler et al., 1994b; Mintz et al., 1995). The different channel types have been distinguished pharmacologically by selective blockade with the toxins ω -CTx-GVIA and ω -Aga-IVA. These studies are consistent with a scenario in which a mixed population of Ca^{2+} channels coexist at single release sites and contribute jointly to the local Ca^{2+} transient that triggers vesicular fusion. However, several important questions remain open.

Is there a fixed combination of Ca^{2+} channels required for neurotransmitter release, or do they change under different physiological circumstances? Under what conditions is the number of channels at a release site sufficient to require that *both* types be blocked to inhibit transmission strongly (“spare channels” scenario proposed by Dunlap et al., 1995), and when is block of *either* type of Ca^{2+} channel sufficient to diminish transmitter release significantly? Is there a significant population of excitatory synapses in which only one type of Ca^{2+} channel is involved in triggering release, as might be inferred from a study of exocytosis in hippocampal cultures (Reuter, 1995)? Under what circumstances might transmission be triggered by opening of just one channel molecule (Yoshikami et al., 1988; Stanley, 1993)?

To begin to address these questions, we sought to determine whether blockade of one type of Ca^{2+} channel at a release site can be overcome by increasing the Ca^{2+} influx through channels of

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the other class. This would constitute strong evidence for the colocalization of both types of channels at the same site of exocytosis. To put this formally, the instantaneous flux of Ca^{2+} through the overall population of Ca^{2+} channels (F) can be represented as:

$$F = N_{\text{tot}} \cdot P_o \cdot i_{\text{Ca}}, \quad (1)$$

where N_{tot} is the total number of channels, P_o is the probability of Ca^{2+} channel opening, and i_{Ca} is the flux of Ca^{2+} through a single, fully open channel. For simplicity, let us suppose that the total pool of channels at a release site consists of N_A of the P/Q-type channels, blocked by ω -Aga-IVA and N_B of the ω -CTx-GVIA-sensitive N-type channels, and that both channel types can be treated as identical with respect to P_o and i_{Ca} . Because N_{tot} , P_o , and i_{Ca} fundamentally are multiplicative, increases in the time-averaged values of P_o and i_{Ca} should be able to compensate for decreases in N_{tot} after reductions in N_A with ω -Aga-IVA or in N_B with ω -CTx-GVIA.

We have manipulated both P_o by increasing action potential duration, and i_{Ca} by changing $[\text{Ca}^{2+}]_o$. By combining these maneuvers with selective blockade of N- or Q-type Ca^{2+} channels in various ways, we have uncovered a substantial amount of information about how different Ca^{2+} channels work in concert to support neurotransmitter release. Our findings also provide clues about how modulation of Ca^{2+} channels would alter the impact of spike broadening, as occurs under physiological settings (Kaczmarek and Levitan, 1987; Schwartz and Kandel, 1991).

Some of these results have appeared previously in abstract form (Wheeler et al., 1994a, 1995).

MATERIALS AND METHODS

Hippocampal slice preparation. Transverse hippocampal slices (400 μm) were obtained by standard methods (Alger et al., 1984) from 3- to 4-week-old Sprague–Dawley rats of either sex. Rats were anesthetized with halothane and decapitated, and the hippocampi were dissected quickly in cold Krebs buffer containing (in mM): NaCl 130, KCl 2.5, MgCl_2 1.3, CaCl_2 2.0, NaH_2PO_4 1.0, NaHCO_3 26.2, and glucose 11.0, and gassed with 95% O_2 and 5% CO_2 . Slices were prepared using a McIlwain Tissue Chopper (Mickle Laboratory Engineering, Surrey, UK). Slices were placed in a humidified holding chamber that was gassed continually with the O_2/CO_2 mixture on a piece of filter paper subfused with Krebs and allowed to recover at least 60 min before attempting recording. All slice recordings were made at room temperature (24°C) with slices submerged and superfused continuously (~1 ml/min), with Krebs pre-gassed with the O_2/CO_2 mixture. Drugs were applied by addition to the superfusion reservoir, and the time delay resulting from dead volume was accounted for in all figures. Experiments with ω -CTx-GVIA or ω -Aga-IVA were performed in the presence of cytochrome C (0.1 mg/ml) to saturate nonspecific peptide binding sites. Responses were acquired and analyzed using lab-specific programs written in Axobasic (Axon Instruments, Foster City, CA).

Intracellular recordings in the slice. Intracellular recordings from CA3 somata were made using 1.2 mm outer diameter (OD) borosilicate glass pipettes (Clark Electromedical, Reading, UK) pulled to tip sizes (Flaming Brown micropipette puller, Sutter, Novato, CA) that resulted in a tip resistance of ~90 M Ω on filling with K-acetate (3 M). Sufficient current was injected to maintain a resting membrane potential of ~75 mV. Somatic action potentials were elicited every 5 sec by injecting 0.3 nA of current via the intracellular electrode for 40–80 msec. The electrodes were controlled with an Axoclamp 2-A amplifier (Axon) in bridge mode, amplified and filtered at 3 kHz (Brownlee Precision Amplifier, San Jose, CA), and digitized at 10 kHz (TL-1 DMA interface, Axon).

Extracellular recordings in the slice. Synaptic transmission was elicited every 30 sec using bipolar, twisted gold wire electrodes. EPSPs were recorded with glass pipettes (1.0 mm OD aluminosilicate, Sutter), pulled to resistances of ~1 M Ω , and filled with NaCl (3 M). Synaptic potentials were monitored with an Axoclamp 2-A amplifier, amplified and filtered at 3 kHz (Brownlee Precision Amplifier), and digitized at 20 kHz. The

strength of synaptic transmission was quantified by measuring the initial slope of the EPSP. Averaged data are reported as mean $\pm$ SEM and n represents the number of independent pathways (usually two per slice).

Preparation of cerebellar granule cells. Cerebellar neurons were cultured as described previously (Randall and Tsien, 1995) from 2- to 5-d-old Sprague–Dawley rats of either sex. After removal of the dura, the cerebellum was cut into small (~1 mm³) pieces and digested with trypsin (type XI, 10 mg/ml, Sigma, St. Louis, MO) for 4 min at 24°C in a Ca^{2+} - and Mg^{2+} -containing, HEPES-buffered salt solution supplemented with 0.5 mg/ml DNase (type IV, Sigma). This treatment was followed by trituration with fire-polished Pasteur pipettes in a high Mg^{2+} , 0 Ca^{2+} , bicarbonate-buffered saline. After three centrifugation/resuspension steps, the cells were plated on square #1 coverslips (VWR), coated previously with Matrigel (Collaborative Research, Bedford, MA). The cells were maintained in culture under 5% CO_2 at 37°C, in minimal essential medium (5.3 mM K⁺) (Gibco, Gaithersburg, MD), supplemented with glucose 5 gm/l, transferrin 100 mg/l, insulin 25 mg/l, glutamine 300 mg/l, cytosine arabinoside 2–5 μM , and 10% fetal calf serum (HyClone, Logan, UT). Cultures were fed after 2 d by replacement of 50–70% of the culture medium.

Whole-cell recordings in cultured granule cells. Before recording, dishes containing a single coverslip were removed from the incubator and the culture medium being replaced with Tyrode solution containing (in mM): NaCl 119, KCl 5, CaCl_2 2, MgCl_2 1, Glucose 30, HEPES-NaOH 25, pH 7.3, 313 mOsm. The coverslip was broken into numerous pieces and a single shard placed in the recording chamber. Standard patch-clamp recordings (Hamill et al., 1981) (Axopatch 1D amplifier, Axon) were made with fire-polished patch-clamp pipettes, fabricated from 1.2 mm OD borosilicate glass. The pipettes were filled with (in mM): CsMeSO₄ 108, MgCl_2 4.5, EGTA 9, HEPES 24, ATP 4, GTP 0.3 and showed resistances of 2–5 M Ω . For recordings of Ba^{2+} currents, the external solution contained TEA-Cl 160, BaCl_2 5, HEPES-CsOH 10, pH 7.3, 313 mOsm. Giga-seals (>5 G Ω) were formed by gentle mouth suction, after which fast capacitance transients were neutralized. The whole-cell configuration was achieved using a combination of additional suction and application of ~200 μsec , 2V DC pulses. Whole-cell capacitance was neutralized, and series resistances of ~8 M Ω were compensated ~80%.

Ba^{2+} currents activated by mock action potentials. Voltage-activated Ba^{2+} currents were dissected pharmacologically, as described by Randall and Tsien (1995), and elicited by a series of voltage ramps similar to an action potential. All recordings were made in the presence of 10 μM nimodipine to block L-type Ca^{2+} channels. N-type currents were isolated by the addition of 1 μM ω -CTx-GVIA followed by subtraction of the resultant current from the control current. Similarly, P/Q-type currents were identified by the addition of 500 nM ω -Aga-IVA. The current remaining, which was insensitive to nimodipine, ω -CTx-GVIA, and ω -Aga-IVA, but was potently blocked by Cd^{2+} or Ni^{2+} , was defined as the R-type current (Zhang et al., 1993; Randall and Tsien, 1995). Charge transfer was calculated by integrating to find the area of the divalent current waveform. Mock action potentials consisted of an initial voltage ramp from –70 to +50 mV over 1 msec, followed by a repolarization to –90 mV over a period ranging from 2 to 9 msec, ending with a depolarization to –70 mV over 10 msec.

Application of drugs to granule cells. Drug-containing solutions were delivered by gravity feed from a linear array of glass capillaries positioned near the recorded cell. In addition, the recording chamber was perfused continuously at ~1 ml/min with a peristaltic pump connected to a small antechamber. The bath solution was removed by continuous suction. Cytochrome c (0.1 mg/ml) was included in all recording solutions to block nonspecific peptide binding sites.

Fine chemicals. Nimodipine was provided by Dr. A. Scriabine (Miles Pharmaceuticals, West Haven, CT) and FPL 64176 by Dr. A.J.G. Baxter (Fisons PLC, Loughborough, UK). ω -Aga-IVA was purchased from Peptides International (Louisville, KY), and ω -CTx-GVIA was obtained from Peninsula Laboratories (Belmont, CA). Cytochrome C (from bovine heart), Tyrode reagents, and 4-AP were purchased from Sigma (St. Louis). HEPES and tetrodotoxin were obtained from Calbiochem (La Jolla, CA). ATP and GTP were purchased from Boehringer Mannheim (Indianapolis, IN). D(–)-2-Amino-5-phosphonopentanoic acid (D-AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and (R)-3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP) were purchased from Tocris-Cookson (St. Louis, MO). Krebs reagents (biochemika grade) were purchased from Fluka (Ronkonkoma, NY).

Figure 1. 4-AP causes a large increase in the strength of synaptic transmission and broadens action potentials measured intra- or extracellularly. *A*, Typical example of EPSPs recorded in the same slice before (dotted line) and after (solid line) application of 4-AP. Traces are averages of five consecutive stimulations, and the stimulus artifacts were deleted for clarity. *B*, *Top*, Ensemble average showing the time course and effect of 4-AP on the strength of synaptic transmission ($n = 31$). All values were normalized to the average slope during the 5 min before application of 4-AP. *Bottom*, Ensemble average of the time between stimulation and detection of maximum slope of the EPSP showing increased synaptic delay with the addition of 4-AP to the perfusion medium ($n = 31$). *C*, Isolated fiber volleys recorded extracellularly in area CA1 before (dotted line) and after (solid line) application of 4-AP. Recordings were made in the presence of CNQX (10 μM) and CPP (20 μM) to block glutamatergic transmission. Stimulating electrodes were placed closer than usual to the recording electrode to facilitate fiber volley recordings. These data were filtered at 10 kHz and digitized at 50 kHz. Traces are averages of five consecutive stimulations. *D*, Intracellular recording from area CA3 of action potentials elicited before (dotted line) and after (solid line) application of 4-AP. Traces are averages of five consecutive stimulations.

RESULTS

Effects of K^+ channel blockade on action potentials and synaptic transmission

We tested a number of compounds, including 4-AP (10–300 μM), tetraethylammonium chloride (3–30 mM), Iberitoxin (10 nM), *Leiurus quinquestriatus* venom (50–500 ng/mL), *Stichodactyla* toxin (30–100 nM), α -dendrotoxin (20–1000 nM), and veratridine (10–1000 μM) for their ability to alter action potential duration or synaptic transmission. *Leiurus* venom and veratridine blocked transmission and were not studied further. Some of the other agents enhanced transmission in an inconsistent way. 4-AP proved to be a reliable and readily reversible effector for prolonging the spike (Storm, 1987) and strengthening synaptic transmission (Harvey and Marshall, 1977). Figure 1 illustrates the effect of 100 μM 4-AP, a dose sufficient to produce a maximal effect on synaptic

transmission, without giving appreciable rundown, as was observed with 300 μM 4-AP. Recordings of field potentials displayed a clear effect of 4-AP on the initial slope and peak amplitude of the EPSP (Fig. 1*A*). The synaptic delay as measured by latency to onset of the EPSP also was increased significantly (Kumamoto and Kuba, 1985; Yawo and Chuhma, 1994). Pooled results from a set of such experiments showed that the initial EPSP slope increased by 2-fold on average (Fig. 1*B*). The increase was stable in the presence of 4-AP and was completely reversible after washout of the drug (Fig. 3*E*). Also noted was a dramatic increase in postsynaptic excitability, as indicated by the appearance of highly synchronized, multiple population spikes after application of 4-AP (Fig. 1*A*, solid trace).

Other aspects of the response to 4-AP were consistent with the known effects of the drug on repolarizing K^+ current and

action potential duration in the presynaptic cell (Gustafsson et al., 1982; Segal and Barker, 1984; Storm, 1987). As illustrated in Figure 1C, the expected decrease in i_K and prolonged action potential were reflected in a broadened fiber volley (Johnston and Wu, 1995). This extracellular potential reflects synchronized action potentials in presynaptic axons arising from CA3 neurons, elicited by the field stimulation. It is clear that the application of 4-AP widens the fiber volley and decreases the slope of its descending phase. Prolongation of action potential duration in response to 4-AP also was registered in direct intracellular recordings from cell bodies of the presynaptic CA3 pyramidal cells (Fig. 1D). The rate of repolarization in the CA3 somata was reduced by ~20%. There also was a modest decrease in the absolute amplitude of the action potential spike, as has been reported previously (Storm, 1987). Although the action potential in the cell body may not be identical to that of the nerve terminal, the inaccessibility of the bouton to electrophysiological recording makes the somatic recording the best possible compromise. Furthermore, it seems likely that the relatively larger axons would tend to have a dominant effect on the waveform produced by the electrically compact terminals.

The 4-AP-induced changes in action potential duration in the presynaptic cell seem consistent qualitatively with the increased synaptic delay, which proceeds in parallel with the enhanced synaptic transmission (Fig. 1B). If anything, the increase in axonal/terminal action potential duration (1.9 msec as measured by the increased time to maximum initial slope of the field EPSP) seemed to be substantially greater than the prolongation of the somatic action potential (0.5 msec). Quantitative differences in the degree to which action potentials are prolonged might be indicative of regional variations in the contribution of K^+ channel subtypes to action potential repolarization (Sheng et al., 1992).

Dependence on action potential duration of Ca^{2+} influx through identified channel types

To explore the impact of action potential broadening on various types of Ca^{2+} channels, we measured Ba^{2+} influx through pharmacologically identified Ca^{2+} channels in response to mock action potentials (Fig. 2). Cultured cerebellar granule cells were used for these experiments, because they are electrotonically compact cells for which Ca^{2+} channel pharmacology has been well characterized (Randall and Tsien, 1995). Whole-cell patch-clamp recordings of Ba^{2+} currents were made in the presence of nimodipine to eliminate currents carried by L-type Ca^{2+} channels. Application of ω -CTx-GVIA (1 μM) reduced the current elicited by the mock action potentials, and the difference between the initial and resultant currents was defined as N type. Likewise, the current removed by application of ω -Aga-IVA (0.5 μM) was defined as P/Q type (P-type and Q-type channels both being largely blocked at the high dose of ω -Aga-IVA [Randall and Tsien, 1995]). The remaining current, which was resistant to both toxins, but was eliminated by Cd^{2+} (100 μM), was called R type. The waveforms of the mock action potentials and the consequent influx through N- and P/Q-type Ca^{2+} are shown in Figure 2A. Consistent with earlier studies (Simon and Llinás, 1985; McCobb and Beam, 1991; Scroggs and Fox, 1992), charge entry through Ca^{2+} channels was found to occur predominantly during the repolarization phase of the mock action potential.

To mimic the effects of 4-AP, we applied mock action potentials of increasing width. The amount of charge transfer through all Ca^{2+} channel types increased by approximately threefold as the repolarization times were varied from 2 to 9 msec. Thus, divalent

Figure 2. Mock action potentials show that charge transfer through N- and P/Q-type Ca^{2+} channels is increased but delayed as the spike is broadened. *A*, Mock action potentials of various widths shown in the top were used to elicit Ba^{2+} currents through N- (middle) and P/Q-type (bottom) Ca^{2+} channels. Ba^{2+} currents are averages of six cells that were treated sequentially with ω -CTx-GVIA (1 μM) and ω -Aga-IVA (500 nM) in the continuing presence nimodipine (10 μM). The current components sensitive to ω -CTx-GVIA (I_N) and ω -Aga-IVA ($I_{P/Q}$) were derived by subtraction and normalized to the peak of the current evoked by the shortest mock action potential. *B*, The average charge transfer, calculated by integrating the current trace, through N-, P/Q-, and R-type Ca^{2+} channels during mock action potentials with repolarization times of 2–9 msec. The R-type charge transfer represents the current that was blocked by 100 μM Cd^{2+} , but was insensitive to nimodipine, ω -CTx-GVIA, and ω -Aga-IVA. *C*, The cumulative charge transfer through N-type channels is shown as a function of the elapsed time of the mock action potential (AP). The data are from the same six cells presented above. *D*, The same analysis is shown for charge transfer through the P/Q-type channel. *E*, The delay in charge transfer through N- (open circles) and P/Q-type (open squares) Ca^{2+} channels in the same six cells as a function of mock action potential duration is quantified by showing the time to half charge transfer during mock action potentials of various widths.

cation entry increased linearly with prolonged action potential duration (Fig. 2B). We also found that the relative increase in charge transfer with increasing repolarization time was the same for all channel types. Whereas broadening the mock action potential led to an increase in charge transfer, it also slowed the development of the current through N-type (Fig. 2C) and P/Q-type (Fig. 2D) Ca^{2+} channels. Figure 2E shows that the increase in latency with slowing mock action potential repolarization time was linear and similar for both ω -CTx-GVIA- and ω -Aga-IVA-sensitive channels. These results agree qualitatively with those presented in Figure 1. Taken together, they show that broadening

Figure 3. 4-AP reduces the dependence of synaptic transmission on N- and P/Q-type Ca^{2+} channels but does not enhance contribution of L-type channels. *A*, Ensemble averages showing block by 4-AP of inhibition by ω -CTx-GVIA. Control graph (open symbols) shows partial inhibition of basal transmission by a 10 min application of 1 μM ω -CTx-GVIA ($n = 30$). 4-AP graph (solid symbols) shows a dramatic reduction in the effect of an identical treatment with ω -CTx-GVIA at least 30 min after application of 4-AP ($n = 12$). Error bars are smaller than symbols. *B*, Ensemble averages showing block by 4-AP of inhibition by ω -Aga-IVA. Control graph (open symbols) shows inhibition of basal transmission by a 20 min application of 1 μM ω -Aga-IVA ($n = 8$). 4-AP graph (solid symbols) shows a reduction in the effect of an identical treatment with ω -Aga-IVA at least 30 min after application of 4-AP ($n = 12$). Error bars are smaller than symbols. *C*, Ensemble average showing that application of the L-type Ca^{2+} channel antagonist nimodipine has no effect on synaptic transmission in the presence of 4-AP ($n = 4$). *D*, Ensemble average showing that application of the L-type Ca^{2+} channel agonist FPL 64176 has no effect on synaptic transmission in the presence of 4-AP ($n = 2$). *E*, Example of experiments in which simultaneous blockade of N-type and P/Q-type Ca^{2+} channels with ω -CTx-GVIA and ω -Aga-IVA, respectively, abolished transmission completely in the presence of 4-AP. Washout of 4-AP shows that inhibition of N-type channels is not reversed during the time course of this experiment.

the action potential increases Ca^{2+} entry and, thus, leads to enhanced synaptic strength, and that retardation of action potential repolarization delays Ca^{2+} entry and results in postponed release of neurotransmitter.

Broadening the action potential reduces the responsiveness of transmission to Ca^{2+} channel blockers

Figure 3 shows the effect of action potential prolongation on the pharmacology of synaptic transmission. Under control conditions, application of a saturating dose of ω -CTx-GVIA blocked $46 \pm 1\%$ of synaptic transmission at steady state ($n = 30$; Fig. 3*A*), as shown

previously (Wheeler et al., 1994b). In the presence of 4-AP, however, complete blockade of N-type Ca^{2+} channels reduced the strength of synaptic transmission by only $9 \pm 2\%$ ($n = 6$; Fig. 3*A*). Qualitatively similar findings were obtained with ω -Aga-IVA at 1 μM , nearly 10 times the IC_{50} for blockade of Q-type Ca^{2+} channels (Randall and Tsien, 1995) and more than 500 times the IC_{50} for P-type channels (Mintz and Bean, 1993). ω -Aga-IVA reduced EPSP slope by $95 \pm 1\%$ under control conditions ($n = 8$), but only by $74 \pm 2\%$ in the presence of 4-AP ($n = 12$; Fig. 3*B*). Thus, the reliance of synaptic transmission on either class of Ca^{2+} channel was attenuated strikingly by the presence of 4-AP.

One possible interpretation of these results is that the prolonged action potentials activate yet another type of Ca^{2+} channel that normally contributes very little to Ca^{2+} influx. Because the various dihydropyridine (DHP)-insensitive channels show very similar kinetics of recruitment by mock action potential broadening (Fig. 2*B*), the most obvious candidate is the DHP-sensitive L-type Ca^{2+} channel, which is known to activate more slowly than DHP-insensitive channels (Sather et al., 1993). However, application of nimodipine (5 μM), a blocker of L-type Ca^{2+} channels, did not reduce the field EPSP slope (Fig. 3*C*). In addition, exposure to FPL 64176 (1 μM), a powerful stimulator of these channels (McKechnie et al., 1989), failed to enhance synaptic transmission (Fig. 3*D*). Thus, the lessened impact of the neurotoxins in the presence of 4-AP (Fig. 3*A,B*) cannot be explained by a recruitment of L-type channels.

A more general test for the possible involvement of channels other than those sensitive to ω -CTx-GVIA and ω -Aga-IVA was carried out by examining the combined effects of these neurotoxins. As illustrated in Figure 3*E*, we used a protocol that capitalized on the largely irreversible blocking action of ω -CTx-GVIA on N-type channels (McCleskey et al., 1987; Williams et al., 1992) and provided internal checks for the efficacy of the toxin action. ω -CTx-GVIA was administered in the presence of 4-AP and, accordingly, produced little immediate inhibition of transmission. However, after the 4-AP was washed out, the EPSP slope returned to about half the value seen before application of 4-AP. This degree of inhibition was consistent with the effect of ω -CTx-GVIA on control transmission in the absence of 4-AP (Wheeler et al., 1994b) and established that ω -CTx-GVIA had been fully effective despite its limited ability to affect the 4-AP-potentiated transmission. Reapplication of 4-AP returned the slope to near its previously potentiated value, whereupon addition of ω -Aga-IVA caused complete abolition of the field EPSP. Thus, even in the presence of 4-AP, the combined action of both Ca^{2+} channel blockers is to eliminate the synaptic transmission completely. There is no clear indication of participation of channels other than those blocked by ω -CTx-GVIA and ω -Aga-IVA, despite the favorable condition of the broadened spike. This restricts the possible degree of involvement of other channel types (e.g., R-type) (Wu and Saggau, 1995) but does not rule them out entirely. The most stringent tests must await development of pharmacological agents specific for ω -CTx-GVIA- and ω -Aga-IVA-insensitive channels.

4-AP effects are explained by prolongation of action potentials leading to enhanced Ca^{2+} influx

It is worth noting that whether or not 4-AP was present, neither ω -CTx-GVIA nor ω -Aga-IVA changed the fiber volley (data not shown). Hence, the effect of 4-AP cannot be explained by the unmasking of an effect of either toxin on presynaptic excitability. Instead, the 4-AP-induced change in toxin responsiveness must be

Figure 4. Decreasing $[\text{Ca}^{2+}]_o$ reverses the effect of 4-AP on Ca^{2+} channel blockade. *A*, Example of experiments in which reducing $[\text{Ca}^{2+}]_o$ from 2.0 to 0.5 mM lead to recovery of the effect of a 10 min application of 1 μM ω -CTx-GVIA, despite the continued presence of 4-AP. *B*, Ensemble averages showing the effect of a 10 min application of ω -CTx-GVIA on basal transmission (triangles; $n = 30$), transmission potentiated by 4-AP (inverted triangles; $n = 31$), and transmission in the presence of 4-AP and 0.5 mM $[\text{Ca}^{2+}]_o$ (open circles; $n = 6$). *C*, Graph shows the percent inhibition caused by a 10 min application of 1 μM ω -CTx-GVIA in the presence of 4-AP, 4-AP and 0.75 mM $[\text{Ca}^{2+}]_o$ ($n = 4$), 4-AP and 0.5 mM $[\text{Ca}^{2+}]_o$, and control conditions.

attributed to events downstream of the presynaptic action potential. The results in Figure 3*E* provide reassurance that the influence of 4-AP cannot be attributed to a mere pharmacological side effect such as rendering Ca^{2+} channels insensitive to neurotoxins. Instead, we hypothesize that the effect of 4-AP is mediated by its well-known action on K^+ channels (Gustafsson et al., 1982) and action potential prolongation (Storm, 1987), which lead to increased open probability (P_o) of presynaptic Ca^{2+} channels (Fig. 2) (see also Jackson et al., 1991; Basavappa et al., 1994). This interpretation leads to a strong prediction; it should be possible to counteract the effects of 4-AP on the pharmacology of transmission simply by reduction of $[\text{Ca}^{2+}]_o$. To put this more formally, because P_o and unitary Ca^{2+} influx (i_{Ca}) are multiplicative factors in controlling the total flux of Ca^{2+} (F) (Eq. 1), decreases in i_{Ca} might be expected to compensate for increases in P_o . If the product $P_o i_{\text{Ca}}$ is held fixed, the additional impact of pharmacologically induced changes in N_{tot} on synaptic transmission also should remain very much the same.

This prediction was tested in experiments of the kind illustrated in Figure 4*A*. A more than 2-fold increase in the EPSP slope was observed after application of 4-AP but was reversed on reducing

$[\text{Ca}^{2+}]_o$ from 2 to 0.5 mM. Subsequent application of ω -CTx-GVIA caused a reduction in the strength of transmission of $36 \pm 3\%$ ($n = 8$). This degree of inhibition was seen consistently, as illustrated by pooled results from multiple experiments (open circles in Fig. 4*B*). Thus, the toxin effect in 4-AP, 0.5 mM Ca^{2+} , was far in excess of the $9 \pm 1\%$ reduction seen in 4-AP, 2 mM Ca^{2+} , but comparable to that seen under control conditions (no 4-AP, 2 mM Ca^{2+}). This contrast was emphasized further at the end of the experiment shown in Figure 4*A* by returning $[\text{Ca}^{2+}]_o$ to 2 mM in the continued presence of 4-AP. In this case, the EPSP slope responded by increasing to a level $\sim 10\%$ lower than its value before application of ω -CTx-GVIA. This verifies that at 2 mM Ca^{2+} , the effect of the toxin is relatively small. The recovery of responsiveness to ω -CTx-GVIA in the presence of 4-AP varied in extent with the external Ca^{2+} concentration (Fig. 4*C*). When $[\text{Ca}^{2+}]_o$ was lowered sufficiently to reduce the slope of the EPSP to control levels, the sensitivity of synaptic transmission to ω -CTx-GVIA was recovered almost completely. Thus, the effects of 4-AP on P_o were largely reversed by reducing i_{Ca} , just as predicted from Equation 1.

These observations lend additional support to the idea that

modification of the pharmacological profile of transmission by 4-AP involves changes in action potential duration rather than any pharmacological side effect. Like the results presented in Figure 3E, these data demonstrate that ω -CTx-GVIA is fully effective in blocking N-type channels in the presence of 4-AP. In an additional check, we asked whether the effects of 4-AP on synaptic transmission might have been the result of blocking K^+ channels on the postsynaptic cell. We found that even in the presence of 4-AP, field EPSPs were unaffected by D-AP5 ($25 \mu\text{M}$) but were abolished completely by CNQX ($10 \mu\text{M}$) (data not shown), indicating that synaptic transmission was supported entirely by AMPA and not NMDA receptor channels. Hence, any postsynaptic depolarization induced by 4-AP blockade of K^+ efflux would tend to diminish the driving force and thereby lessen the synaptic current, in contrast to what actually was observed. Our overall conclusion is that the alterations in synaptic pharmacology induced by 4-AP cannot be explained readily by interference with toxin binding or by nonspecific depolarization of the postsynaptic cell.

Increased action potential duration also changes the dependence of transmission in extracellular Ca^{2+}

The results thus far not only illuminate the role of various types of Ca^{2+} channels in synaptic transmission, but they also raise interesting questions about the underlying relationship between Ca^{2+} influx and transmitter release. Indeed, a general question is whether effects of action potential broadening, selective neurotoxins, and external Ca^{2+} concentration can be accommodated within a single quantitative framework. To pursue this question, we sought additional information about how synaptic strength changes as Ca^{2+} entry varies over a broad range. Increasing $[\text{Ca}^{2+}]_o$ is a conceptually simple maneuver for augmenting the Ca^{2+} influx, but there are practical limits to how much influx can be increased. The unitary Ca^{2+} current i_{Ca} increases in a roughly hyperbolic fashion with external Ca^{2+} , rising in proportion to $[\text{Ca}^{2+}]_o$ over a limited range of concentrations, but approaching a limiting value at higher $[\text{Ca}^{2+}]_o$ (Hess et al., 1986). In addition, long-lasting changes in synaptic strength similar to long-term potentiation are induced at levels of $[\text{Ca}^{2+}]_o$ above 6–8 mM, presumably via Ca^{2+} influx into postsynaptic spines (Bliss et al., 1987; Wheeler and Tsien, unpublished observation) (see also Wang and Kelly, 1995). Together, these considerations restrict the experimentally useful range of $[\text{Ca}^{2+}]_o$ in hippocampal slice experiments.

We turned to action potential broadening as a complementary approach for increasing Ca^{2+} influx. Because changes in action potential duration are felt by the voltage sensors of voltage-gated Ca^{2+} channels and have no direct effect on the permeation pathway, such changes bypass the limitations on raising $[\text{Ca}^{2+}]_o$. Based on the results in Figure 2, we would expect that the P_o of various channel types should increase to the same degree as the spike is prolonged. The elevation of P_o would be expected to act in a multiplicative fashion to amplify the impact of variations in $[\text{Ca}^{2+}]_o$ (Eq. 1). Spike broadening and elevated Ca^{2+} would combine in achieving a higher level of Ca^{2+} influx than could be reached with either maneuver alone. In this way, Ca^{2+} entry might increase to the point at which the release machinery begins to saturate.

Figure 5A shows an example of experiments designed to look for saturation. At the times indicated, $[\text{Ca}^{2+}]_o$ was changed from the control value of 2 mM to the test value indicated in parentheses. Because changes in response to variations $[\text{Ca}^{2+}]_o$ were near maximal in about 6–8 min, 10 min exposures to test concentra-

Figure 5. The sensitivity of synaptic transmission to changes in $[\text{Ca}^{2+}]_o$ is decreased in the presence of 4-AP. *A*, Example of experiments in which $[\text{Ca}^{2+}]_o$ was raised ($\uparrow$) or lowered ($\downarrow$) from a control concentration of 2 mM to a concentration shown in parentheses before and after application of 4-AP. *B*, Averages from experiments shown in the previous panel ($n = 4$ –12). All data were normalized to the EPSP slope seen in 2 mM $[\text{Ca}^{2+}]_o$ before application of 4-AP. Thus, each point represents the slope of the EPSP in the given condition as a percentage of the slope in the control condition.

tions were used in all cases. Several such maneuvers were carried out in each slice in the presence and absence of 4-AP. The average field EPSP slope measured during the last 2 min of exposure to a test $[\text{Ca}^{2+}]_o$ was represented as a percentage of the average baseline slope. Baseline data were taken during the last 5 min before the first change in $[\text{Ca}^{2+}]_o$ for control changes or during the last 5 min before application of 4-AP for data recorded in the presence of 4-AP. The normalized data were pooled and the results plotted in Figure 5B. The experimental results show that the dose-response relationship between $[\text{Ca}^{2+}]_o$ and EPSP slope undergoes a marked change after exposure to 4-AP. In 4-AP, the external Ca^{2+} concentration needed to achieve a given level of synaptic strength was about three- to fourfold lower than in the absence of the K^+ channel inhibitor.

Implications for the relationship between Ca^{2+} influx and transmitter release

Figure 6A pools experimental results on the effect of varying the external Ca^{2+} concentration in the absence and presence of 4-AP in an initial attempt at describing a “dose-response relationship” between Ca^{2+} influx and transmitter release at hippocampal syn-

Figure 6. Quantitative summary of principal findings and explanation of how action potential prolongation lessened the reliance of synaptic transmission on individual Ca^{2+} channel types. *A*, Relationship between $[\text{Ca}^{2+}]_o$ and EPSP slope in the absence (*open circles*) or presence of 4-AP (*solid circles*). The data are the same as those in Figure 5*B* except that the 4-AP data have been shifted to 3.3-fold higher $[\text{Ca}^{2+}]_o$ (as shown by the *upper x-axis*) to align the points taken in the absence and presence of 4-AP along a single monotonic relationship, and an extra point representing the response to 5 mM Ca^{2+} in the absence of 4-AP has been added. The *smooth curve* was generated according to the equation derived by Dodge and Rahamimoff (1967):

$$\text{EPSP slope (\%)} = \{ (W[\text{Ca}^{2+}]_o / (1 + [\text{Ca}^{2+}]_o/K_1 + [\text{Mg}^{2+}]_o/K_2))^n \} + Y. \quad (1)$$

The exponent n was set to 3.7 in accordance with the measured relationship between $d[\text{Ca}^{2+}]_o/dt$ and EPSP slope, as reported by Wu and Saggau (1994b). The best fit ($R^2 = 0.979$) was obtained when $W = 26.7\% \text{ mM}^{-1}$, $K_1 = 0.171 \text{ mM}$, $K_2 = 0.396 \text{ mM}$, and $Y = 6.19\%$. *B*, *Smooth curve* from *A* was used to estimate the change in relative Ca^{2+} influx caused by the Ca^{2+} channel blockade. Ordinate represents values of EPSP slope under various conditions (indicated in the *panel*) shown as a percentage of the EPSP slope in control (no 4-AP, no toxin) (Fig. 3*A,B*). Relative Ca^{2+} influx, plotted on the *abscissa*, is taken as proportional to $[\text{Ca}^{2+}]_o$, with influx at $[\text{Ca}^{2+}]_o = 2 \text{ mM}$ defined as 100%. The 4-AP data again are shifted to 3.3-fold higher values of relative Ca^{2+} influx in accord with the results in *A*. The effects of ω -CTx-GVIA and ω -Aga-IVA on Ca^{2+} influx were modeled by assuming that the corresponding toxin-sensitive currents were carried by N- and P/Q-type Ca^{2+} channels, respectively, two nonoverlapping subsets of Ca^{2+} channels that together accounted for the total Ca^{2+} influx supporting transmission. Thus, if the fraction of Ca^{2+} influx supported by Q-type Ca^{2+} channel is a , the fraction generated by N-type channels is $1 - a$. In all cases, the SEM is smaller than the symbol size.

apses. The multiplicative effect of action potential prolongation (that is, increased P_o) was represented by sliding the data points obtained with 4-AP to the right along the log Ca^{2+} scale. The interchangeability of variations in action potential duration and extracellular Ca^{2+} concentration is based on Equation 1. The best alignment of the two dose-response relationships was observed when the rightward displacement was a factor of 3.3-fold, the effect of 100 μM 4-AP being roughly equivalent to raising $[\text{Ca}^{2+}]_o$ three- to fourfold (see Fig. 5*B*). With this alignment, the collective set of data conformed to a single trajectory that could be described by a smooth curve derived from the Dodge-Rahamimoff equation (Dodge and Rahamimoff, 1967), with the exponent n set to 3.7 (Wu and Saggau, 1994b). It is evident that at low levels of Ca^{2+} entry, the data fall reasonably close to a power-law dependence on Ca^{2+} influx, as demonstrated at hippocampal synapses by Wu and Saggau (1994b) and at cerebellar synapses by Mintz et al. (1995). However, as the Ca^{2+} influx increased, the enhancement in synaptic strength deviated further and further from a strict power-law dependence and seemed to approach saturation. The observed leveling off is consistent with a saturation of some Ca^{2+} -dependent mechanism(s) upstream to the release event, possibly including those controlling exocytosis.

Figure 6*B* extends the analysis to collected data for the relative strength of synaptic transmission under the influence of ω -CTx-GVIA or ω -Aga-IVA in control (*open symbols*) and in the presence of 4-AP (*solid symbols*). The data points for synaptic transmission in the absence of toxins (*open and solid circles*) and the *smooth curve* are the same as in Figure 6*A*, whereas the *horizontal axis* is given as Ca^{2+} influx rather than $[\text{Ca}^{2+}]_o$. [The parallelism between $[\text{Ca}^{2+}]_o$ (Fig. 6*A*) and Ca^{2+} influx (Fig. 6*B*) is justified by the roughly linear dependence of i_{Ca} with $[\text{Ca}^{2+}]_o$ over the limited concentration range examined here (Hess et al., 1986)]. The data points in the presence of the Ca^{2+} channel blockers were plotted according to the simplifying assumption that the total Ca^{2+} influx could be subdivided into complementary fractions, respectively supported by P/Q-type channels (a) and N-type channels ($1-a$). This seems reasonable, because there is strong evidence that ω -CTx-GVIA-sensitive and ω -Aga-IVA-sensitive channels provide nonoverlapping contributions to the overall Ca^{2+} influx at neuronal cell bodies (Mintz and Bean, 1993; Randall and Tsien, 1995), hippocampal nerve terminals (Wu and Saggau, 1994a), and parallel fiber synapses (Mintz et al., 1995). It also seems likely that N- and P/Q-type Ca^{2+} channels account for most if not all of the Ca^{2+} entry supporting synaptic transmission (Wheeler et al., 1994b), even after the action potential has been prolonged with 4-AP (Fig. 3*E*). For simplicity, we ignored the possible contributions of R-type Ca^{2+} currents to excitation-secretion coupling (see Wu and Saggau, 1995) pending direct evidence from a specific inhibitor of this pathway.

The theoretical relationship derived from the $[\text{Ca}^{2+}]_o$ data (Fig. 6*A*) was used to estimate the relative contributions of N- and P/Q-type channels to Ca^{2+} influx. In Figure 6*B*, a was varied systematically between 0.6 and 0.95, and the goodness of fit between the toxin data and the theoretical curve was checked by R^2 analysis (Fig. 6*B*, *inset*). The best fit was found when a was set to 0.81, corresponding to P/Q- and N-type Ca^{2+} influxes in an

The optimal value of a was determined by trial and error: by varying this parameter from 0.6 to 0.95 and testing the quality of fit between the data and the solid curve derived in *A*. The *inset* shows how the goodness of fit (as reflected by R^2 values) varied with a and indicates that the best fit ($R^2 = 0.964$) was obtained when $a = 0.81$.

~4:1 ratio. In addition, four individual estimates of the fraction of P/Q-type channels were made by solving for the value of a , which fit the theoretical curve given the relative EPSP slopes measured in the presence or absence of toxins and 4-AP. In this manner, we found that $a = 0.70 \pm 0.08$, well within the acceptable range predicted from the R^2 analysis.

The overall conclusion is that the experiments studying effects of ω -CTx-GVIA and ω -Aga-IVA and of varying $[\text{Ca}^{2+}]_o$ were mutually consistent with each other and conform reasonably closely to the Dodge–Rahamimoff relationship between Ca^{2+} influx and transmitter release (Dodge and Rahamimoff, 1967; Augustine et al., 1985; Thomas et al., 1993; Lando and Zucker, 1994). There is no need for invoking any effect of the toxins other than blocking their known channel targets. These results are consistent with and complementary to those of Mintz et al. (1995) who show that various maneuvers that reduce i_{Ca} through various Ca^{2+} channel types also reduce the strength of synaptic transmission according to a power-law relationship.

Ca^{2+} channel blockade enhances effectiveness of modulation via changes in action potential duration

The existence of multiple channel types at presynaptic release sites increases the potential for precise modulation of Ca^{2+} entry and transmitter release (Tsien et al., 1988). We were interested in the question of how substances that modulate one or more types of Ca^{2+} channel might shape the response of synaptic transmission to physiological mechanisms that alter action potential duration. An entree to this field is provided in Figure 7. Previous blockade of either N-type Ca^{2+} channels (*A*) or P/Q-type Ca^{2+} channels (*B*) led to a significant enhancement of the degree of potentiation of transmission seen with application of 4-AP. After exposure to ω -CTx-GVIA, prolonging the action potential with 4-AP increased the strength of transmission to 350% of control. Similarly, treatment with 4-AP in the continued presence of ω -Aga-IVA boosted the slope of the EPSP to more than 500% of control. It is worth noting that the theoretical relationship shown in Figure 6 would have predicted 2.7-fold increase when 4-AP was applied in the presence of the N-type Ca^{2+} channel blockers and a 7.7-fold increase when P/Q-type channels were blocked. In other words, reducing Ca^{2+} influx by inhibiting Ca^{2+} channels increased dramatically the relative effectiveness of alterations in action potential duration in modulating synaptic strength.

DISCUSSION

We found that the Ca^{2+} channel requirements for fast transmitter release were not immutable but varied markedly as the duration of the presynaptic spike was altered. Action potential prolongation by 4-AP greatly reduced the reliance of synaptic transmission on either N- or P/Q-type Ca^{2+} channels, as determined by application of neurotoxins. ω -CTx-GVIA reduced the measured synaptic strength by only 9% in the presence of 4-AP, in sharp contrast to the 46% reduction under normal conditions. The impact of blocking P/Q-type channels with ω -Aga-IVA also was attenuated significantly, but inhibition of both P/Q-type and N-type Ca^{2+} channels still resulted in the complete abolition of synaptic transmission. As we discuss below, these findings can be accounted for readily by consideration of factors that control Ca^{2+} movements through presynaptic channels and the likely relationship between Ca^{2+} influx and secretion. Our observations help reconcile two apparently opposing views of the operation of Ca^{2+} channels at central synapses, the “power-law” hypothesis (Luebke et al., 1993; Takahashi and Momiyama, 1993; Castillo et

Figure 7. Previous blockade of N- or P/Q-type Ca^{2+} channels facilitates the effect of 4-AP on synaptic transmission. *A*, Ensemble averages showing increase in the relative potentiation caused by application of 4-AP after selective blockade of N-type Ca^{2+} channels with 1 μM ω -CTx-GVIA (solid symbols; $n = 5$). Control graph (open symbols) is taken from Figure 1C and is reproduced for comparison. *B*, Same as previous panel except that the effects of selective blockade of P/Q-type Ca^{2+} channels with ω -Aga-IVA is shown ($n = 2$).

al., 1994; Regehr and Mintz, 1994; Wheeler et al., 1994b; Wu and Saggau, 1994a; Mintz et al., 1995) and the “spare channels” hypothesis (Dunlap et al., 1994; Wheeler et al., 1994b; Wheeler et al., 1994c; Dunlap et al., 1995; Turner and Dunlap, 1995b). Although illuminating biophysical constraints on models of excitation–secretion coupling at hippocampal synapses, our results also carry interesting implications for the physiological regulation of synaptic strength under various stimulation conditions.

Reconciliation of supra-additive and subadditive effects of Ca^{2+} channel blockade

Figure 6 presents an overview of our experiments. Summarized are observations on the different effects of blocking N- or P/Q-type channels or varying $[\text{Ca}^{2+}]_o$ in the absence and presence of 4-AP. All of the data can be accommodated by a simple description of the properties of excitatory synaptic transmission, conforming to the Dodge–Rahamimoff equation. We used this quantitative framework to account for the effects of Ca^{2+} channel modulation and of variations in spike width on the pharmacology of synaptic transmission. The form of the overall dose–response relationship in Figure 6A is central to understanding how distinct channels may contribute jointly to transmitter release. Depending

on whether or not the release machinery was operating near saturation, the same physiological or pharmacological interventions might have very different effects. Because the dose–response relationship follows a power-law relationship at low levels of Ca^{2+} influx and release, the separate effects of blocking N-type and P/Q-type channels would be expected to add up to more than 100% for synaptic transmission evoked by normal action potentials. This prediction agrees well with findings of supra-additive block by ω -CTx-GVIA and ω -Aga-IVA (46 and 95%, respectively, in Fig. 3*A,B*) (see also Luebke et al., 1993; Takahashi and Momiyama, 1993; Castillo et al., 1994; Regehr and Mintz, 1994; Wheeler et al., 1994b; Wu and Saggau, 1994a; Mintz et al., 1995).

A very different set of predictions emerges from consideration of the synaptic machinery when it operates closer to saturation. In this case, the impact of inhibiting a fixed minority fraction of Ca^{2+} influx would be very much lessened as the relationship between synaptic strength and Ca^{2+} influx begins to level off. The response of synaptic transmission to blockade of the complementary majority fraction of Ca^{2+} influx still would remain dramatic. However, summing up the separate effects no longer would produce supra-additive block. This pattern of behavior is similar to what we have seen with 4-AP-enhanced synaptic transmission. In Figure 3*A,B*, for example, the fractional blockade is 9% with ω -CTx-GVIA and 74% with ω -Aga-IVA, which add up to less than the nearly 100% block these toxins cause when applied together. It is striking that even mild changes in action potential duration can make such a difference in converting between supra-additive and subadditive behavior.

The same reasoning provides a way of resolving different points of view based on recordings of action-potential-driven transmission in brain slices and studies of K^+ -evoked neurotransmitter release from synaptosomes. Working with cortical synaptosomes depolarized for tens of milliseconds, Turner et al. (1993) found that ω -CTx-GVIA and ω -Aga-IVA produced only marginal reductions of glutamate release when applied individually but gave a dramatic block when administered together. These results were interpreted in terms of a hypothesis of “spare channels,” which postulates that ω -CTx-GVIA- and ω -Aga-IVA-sensitive channels are abundant enough in combination to provide far more Ca^{2+} influx than the bare minimum needed to trigger transmitter release (Dunlap et al., 1994; Dunlap et al., 1995). The concept of spare channels also can be expressed in terms of the relationship between Ca^{2+} influx and the secretory response; as this relationship saturates, block of a fixed fraction of the Ca^{2+} influx will have only marginal effects on transmitter release. A combination of toxins then would be required to reduce Ca^{2+} influx to the degree necessary to inhibit transmission severely. Indeed, one would expect a continuum of behavior, ranging from the supra-additivity of individual blocking effects for conventional synaptic transmission (Luebke et al., 1993; Takahashi and Momiyama, 1993; Castillo et al., 1994; Regehr and Mintz, 1994; Wheeler et al., 1994b; Mintz et al., 1995), to the subadditivity we found for 4-AP-enhanced transmission (Fig. 3), to the strikingly subadditive effects of toxins reported by Turner et al. (1993). Their experimental system would be geared particularly to produce saturation, because the K^+ -induced depolarizations are much longer than even 4-AP prolonged action potentials and would be expected to produce a much larger Ca^{2+} influx per stimulus. The underlying basis of saturation in their synaptosomes may involve the Ca^{2+} responsiveness of the secretory apparatus itself or additional processes, such as vesicular depletion during the prolonged depolarization. Interestingly, Turner and Dunlap (1995a) report that lowering $[\text{Ca}^{2+}]_o$ from 2 mM to <0.1 mM can compensate for the length of the K^+ depolarizations, converting the

combined effects of ω -CTx-GVIA and ω -Aga-IVA from subadditive to supra-additive. This is in good agreement with theoretical predictions based on Figure 6.

How many Ca^{2+} channels does it take to trigger neurotransmission?

This work has some additional implications beyond issues of the specific kinds of Ca^{2+} channels under study. If synaptic transmission depends strongly on the concerted activity of multiple types of channels, an obvious inference is that any one channel molecule generally would not provide enough Ca^{2+} influx to support transmitter release with maximal efficiency. At first sight, this conclusion seems at variance with that reached by Stanley (1993) in his study of Ca^{2+} channel activity and acetylcholine release at calyx synapses from chick ciliary ganglion. Stanley found localized secretory events in the wake of openings of single Ca^{2+} channels and proposed that activity of an individual channel would be sufficient to trigger exocytosis (see also Yoshikami et al., 1988). Some experimental and conceptual differences are worth pointing out and may help settle the apparent conflict. Stanley mainly focused on release events after low levels of channel activity, but he noted also that a large number of such events were associated with simultaneous openings of several Ca^{2+} channels. Thus, his results do not exclude the possibility that openings of multiple Ca^{2+} channels are more effective than a single channel opening in triggering release, just as our results do not imply an absolute requirement for multiple channel openings, only a quantitative advantage.

Another difference in emphasis arises from the treatment of synaptic failures. In Stanley's study, trials in which depolarization failed to produce acetylcholine release had no impact, because his analysis was contingent on detection of an exocytotic event. In our experimental paradigm, failures of transmission at individual synapses will have profound impact on the overall measurement of synaptic strength. Stanley (1993) deliberately chose conditions to enhance his chances of identifying clear-cut unitary Ca^{2+} channel openings; the external Ca^{2+} concentration was 6 mM, and the test potential was -35 to -30 mV, near the level of depolarization at which channel activity first is recruited. These factors would tend to increase the Ca^{2+} influx per channel opening while minimizing the likelihood of observing overlapping openings of multiple channels. In contrast, action potentials under normal ionic conditions are more likely to produce activation of several Ca^{2+} channels but less Ca^{2+} entry through any particular channel. The synaptic transmission evoked by 4-AP-prolonged action potentials may represent an intermediate case in which the contributions of individual channels are augmented and the functional dependence on Ca^{2+} entry through multiple channels is attenuated accordingly. In conclusion, there is no direct conflict between Stanley's findings and our results, even though the experimental systems lend themselves to emphasis of different aspects of excitation–secretion coupling.

Setting limits on possible heterogeneity of synapses

Because field EPSP recordings represent the averaged activity of many synapses, one must consider the possibility of heterogeneity among various nerve terminals in the nature of the Ca^{2+} channels that trigger transmitter release (Wheeler et al., 1994b). This issue was brought to the fore in a recent study of single presynaptic nerve terminals in hippocampal cultures (Reuter, 1995). Using

the fluorescent dye FM1–43 to monitor exocytosis (Ryan et al., 1993), Reuter found significant variability among individual presynaptic boutons in their response to ω -CTx-GVIA; loss of dye fluorescence was blocked completely in a substantial fraction (~45%) of boutons but reduced only partially (~40% inhibition) in the remainder. Given the limitations of existing culture methods (see Malgaroli and Tsien, 1992; Ryan et al., 1993), one possible interpretation of these findings, discussed by Reuter (1995), is that the synapses in the hippocampal cultures are immature developmentally and/or heterogeneous anatomically and, therefore, vary in their pharmacological responsiveness (see also Horne and Kemp, 1991; Scholz and Miller, 1995). An alternative proposal is that substantial functional heterogeneity exists even at specific anatomical locations, e.g., at CA3–CA1 synapses in hippocampal slices. At the extreme, one might imagine that a significant subpopulation of nerve terminals relies solely on N-type Ca^{2+} channels, without any participation of P/Q-type channels. This particular scenario is unlikely in Reuter's system, because he found that almost all boutons showed a significant decrease in exocytosis after exposure to ω -Aga-IVA. The extreme hypothesis also can be excluded for CA3–CA1 synapses in hippocampal slices by the results in this study. If a significant fraction of these synapses were to depend entirely on N-type channels for Ca^{2+} influx, the response to ω -CTx-GVIA should have remained substantial even after action potential prolongation with 4-AP, contrary to our data (Fig. 3). The present data seem more in line with our earlier evidence for the coexistence of N- and P/Q-type Ca^{2+} channels at individual nerve terminals, based on the supra-additive effects of toxins and their enhancement of paired-pulse facilitation (Wheeler et al., 1994b). However, moderate variations in the quantitative contributions of these channel subtypes cannot be ruled out.

General implications for synaptic function

A synaptic arrangement in which several Ca^{2+} channels act conjointly to help trigger transmitter release may offer inherent functional advantages. There may be important restrictions on the amount of Ca^{2+} influx that can be provided by any one channel, as set by the limited probability of channel activation during a normal presynaptic spike, the brevity of channel openness during action potential repolarization, and the small amplitude of i_{Ca} . The unitary Ca^{2+} flux may be constrained by the intrinsic properties of a Ca^{2+} -selective pore (Tsien et al., 1987). The use of multiple channels at a single release site may overcome these limitations, thereby increasing the likelihood of vesicle fusion after the arrival of the presynaptic action potential. The pooling of Ca^{2+} influx through multiple channels would tend to smooth out fluctuations arising from the stochastic behavior of the individual channels.

Additional functional advantages may be derived from the involvement of more than a single Ca^{2+} channel type. The most obvious possibility is that of differential modulation by neurohumoral factors (Tsien et al., 1988; Mogul et al., 1993; Swartz et al., 1993; Mynlieff and Beam, 1994). Selective modulation of one channel type would provide a reliable means for reducing Ca^{2+} entry and synaptic transmission in a very controlled manner. Depending on the circumstances, such changes might alter severely the responsiveness of synaptic strength to changes in stimulus frequency and spike broadening.

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