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Either N- or P-type Calcium Channels Mediate GABA Release at Distinct Hippocampal Inhibitory Synapses

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Summary

Transmitter release at most central synapses depends on multiple types of calcium channels. Identification of the channels mediating GABA release in hippocampus is complicated by the heterogeneity of interneurons. Unitary IPSPs were recorded from pairs of inhibitory and pyramidal cells in hippocampal slice cultures. The N-type channel antagonist ω-conotoxin MVIIA abolished IPSPs generated by interneurons in st. radiatum, whereas the P/Q-type antagonist ω-agatoxin IVA had no effect. In contrast, ω-agatoxin IVA abolished IPSPs generated by st. lucidum and st. oriens interneurons, but ω-conotoxin MVIIA had no effect. After unitary IPSPs were blocked by toxin, transmission could not be restored by increasing presynaptic calcium entry. The axons of the two types of interneurons terminated within distinct strata of area CA3. Thus, GABA release onto pyramidal cells, unlike glutamate release, is mediated entirely by either N- or P-type calcium channels, depending on the presynaptic cell and the postsynaptic location of the synapse.

Introduction

Neurons express a variety of high voltage–activated calcium currents with distinctive biophysical and pharmacological properties. At least six molecular species have been identified, defined primarily by the identity of their α1 subunits (see Birnbaumer et al., 1994). These channels are subject to inhibition by numerous neurotransmitters, acting through G-protein coupled receptors (Hille, 1994). Direct inhibition by G-protein subunits produces a shift in the voltage dependence of channel gating, resulting in a reduced calcium influx (Bean, 1989).

At synaptic terminals, high voltage–activated calcium channels play a pivotal role in coupling the presynaptic action potential to the transmitter release process. The properties of the presynaptic calcium channels are thus likely to greatly influence synaptic function and modulation. Much attention has been focused on the identification of calcium channels present at synaptic terminals in a variety of preparations. The emerging consensus from these studies is that several subtypes of high voltage–activated calcium currents act together to initiate transmitter release at most central synapses (Luebke et al., 1993; Takahashi and Momiyama, 1993; Castillo et al., 1994; Wheeler et al., 1994; Wu and Saggau, 1994; Mintz et al., 1995; Reuter, 1995; Taschenberger and Grantyn, 1995). Very few synapses allow direct access to presynaptic elements (Takahashi et al., 1996). The responses of cells to stimulation of a population of nerve terminals, using either an extracellular electrode or an elevation of the external K^+ concentration, have therefore usually been examined. Such techniques activate large numbers of synapses of undetermined origin. These stimulation techniques are probably adequate when synapses are formed by a homogeneous population of neurons (e.g., Mintz et al., 1995), but they may lead to misleading results when a heterogeneous population of presynaptic cells is involved.

Hippocampal pyramidal cells receive inhibitory input from a diverse population of interneurons (Buckmaster and Soltesz, 1996; Freund and Buzsáki, 1996), and this is reflected in the variable properties of inhibitory synaptic responses. Spontaneous, action potential–independent GABA release occurs primarily at perisomatic rather than dendritic synapses, suggesting release probability differs at these synapses (Soltesz et al., 1995). In addition, GABA release from some but not all inhibitory synapses is inhibited by presynaptic $GABA_B$ autoreceptors in the hippocampus (Lambert and Wilson, 1993; Pearce et al., 1995), in part via an inhibition of presynaptic calcium channels (Doze et al., 1995). Finally, evoked inhibitory synaptic responses are highly sensitive to the N-type calcium current antagonist ω-conotoxin GVIA (Horne and Kemp, 1991; Potier et al., 1993), whereas K^+-evoked GABA release is predominantly suppressed by the selective P/Q-type antagonist ω-agatoxin IVA (Doze et al., 1995). These observations suggest that different calcium channel subtypes may be present at distinct inhibitory synapses.

We have used paired recordings from visually identified inhibitory and pyramidal cells in hippocampal slice cultures to examine the effect of calcium channel antagonists on GABA release at synapses originating from single interneurons. We show that, in contrast to excitatory synapses, hippocampal inhibitory synapses apparently express only one subtype of calcium current (either N- or P-type). By filling inhibitory neurons with biocytin, we correlate their morphological characteristics with the pharmacological profile of GABA release at their terminals.

Results

Paired Recordings from Visually Identified Inhibitory and Pyramidal Cells

Paired recordings were obtained from inhibitory cells whose somata were located in either st. oriens, lucidum, or radiatum, and from pyramidal cells located 50–200 μm away (Figure 1A). The interneuron was voltage-clamped at −60 mV in order to prevent fluctuations of the membrane potential during the course of the experiment. Under these conditions, a brief voltage step (1–10

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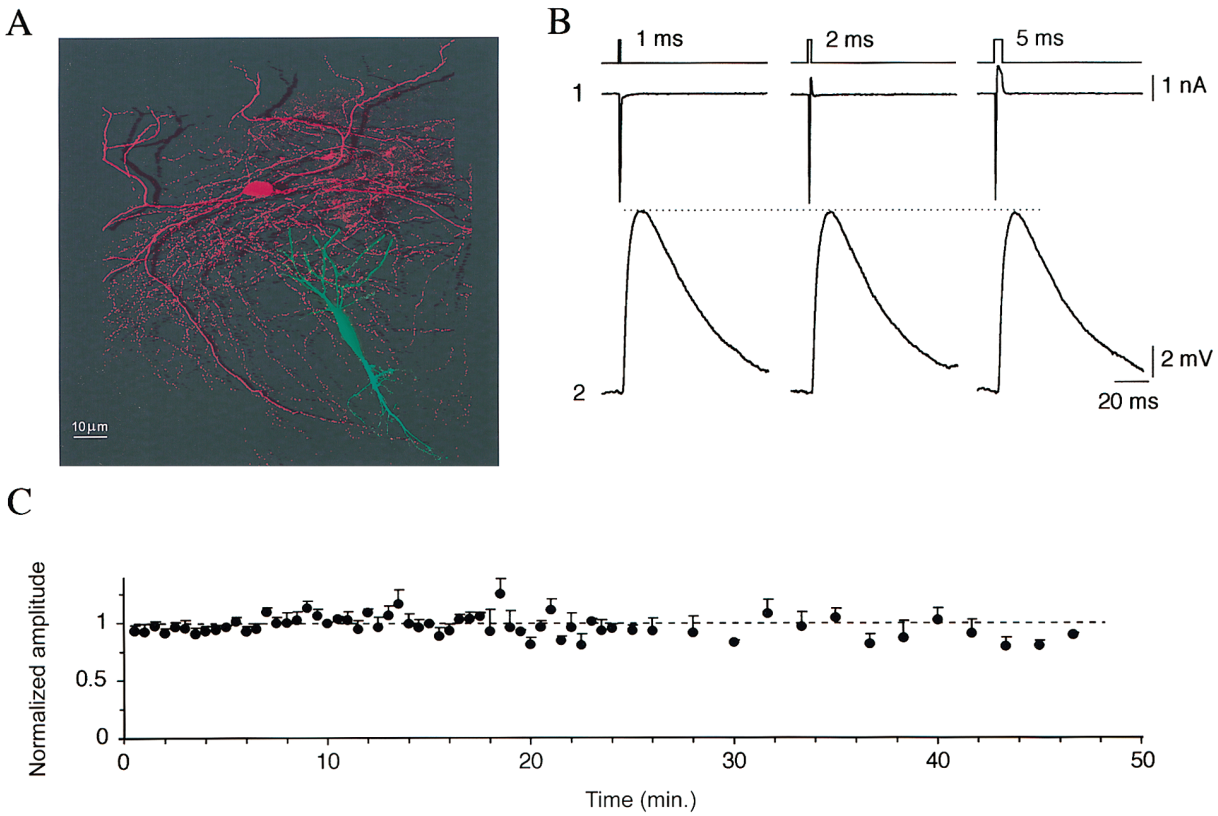

Figure 1. Paired Recording from Monosynaptically Connected Inhibitory and Pyramidal Cells

(A) Pseudo-colored, three-dimensional reconstruction of a cell pair using confocal microscopy. The inhibitory neuron (red) was located in st. oriens and was filled with 1% biocytin. The postsynaptic CA3 pyramidal cell (green) was injected with Lucifer Yellow.

(B) A voltage pulse to 0 mV triggered an action current in the inhibitory neuron (1). This action current elicited monosynaptic unitary IPSPs in the pyramidal cell (2) in the presence of NBQX (20 μM) and CPP (10 μM). The pyramidal cell was filled with 1 M KCl, resulting in a depolarizing IPSP. The amplitude and shape of the IPSP were unaffected by increasing the duration of the voltage pulse applied to the presynaptic neuron. Each trace represents the average of 16 consecutive IPSPs. Same cell pair as in (A).

(C) No washout of synaptic transmission was apparent within 50 min of recording. IPSP amplitudes were normalized to their initial value (first 2.5 min). Averages and S. E. M. were derived from 4–13 IPSPs.

ms to 0 mV) applied to the interneuron elicited an action current of several nA. Such action currents triggered unitary inhibitory postsynaptic potentials (IPSPs) in 50–80% of neighboring pyramidal cells. The monosynaptic nature of these IPSPs was ascertained by their short and constant latency (3.1 ± 0.2 ms, n = 18) and the total absence of transmission failures. In addition, all recordings of IPSPs were made in the presence of the ionotropic glutamate receptor antagonists 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX, 20 μM), and either 3-((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP, 10 μM) or D-2-amino-5-phosphonovalerate (AP5, 50 μM). Unitary IPSPs elicited in this way were fully blocked by the $GABA_A$ receptor antagonist bicuculline methochloride (40 μM).

The amplitude and shape of IPSPs were independent of the duration of the voltage pulse applied to the interneuron (Figure 1B). In addition, unitary IPSPs were eliminated by tetrodotoxin (1 μM, not shown). These observations indicate that changes in the somatic membrane potential of the interneuron were not electrotonically conducted to the nerve terminal, but rather that GABA release was triggered by action potentials actively propagated along the axon.

Whole-cell recording from presynaptic neurons has been reported to lead to a rapid washout of synaptic transmission in some preparations (Barbour, 1993; Ohno-Shosaku et al., 1994), perhaps owing to the dialysis of essential presynaptic factors. Under our recording conditions, however, no significant decrement in the amplitude of unitary IPSPs was apparent during the course of >50 min recording, provided ATP (4 mM) and GTP (0.4 mM) were included in the internal solution (Figure 1C).

Selective Blockade of Unitary IPSPs by Calcium Channel Subtype–Specific Toxins

The calcium channels mediating unitary IPSPs were identified pharmacologically. We first examined the effect of synthetic ω-conotoxin MVIIA (CmTx), an N-type-specific calcium channel antagonist (Olivera et al., 1987), on synaptic inhibition originating from st. radiatum interneurons. The toxin was applied by bath perfusion in the presence of either bovine albumin or cytochrome C (1 mg/ml), in order to prevent unspecific binding of the toxin. Application of 1 μM CmTx completely suppressed unitary IPSPs in 11 pairs recorded with the interneuron

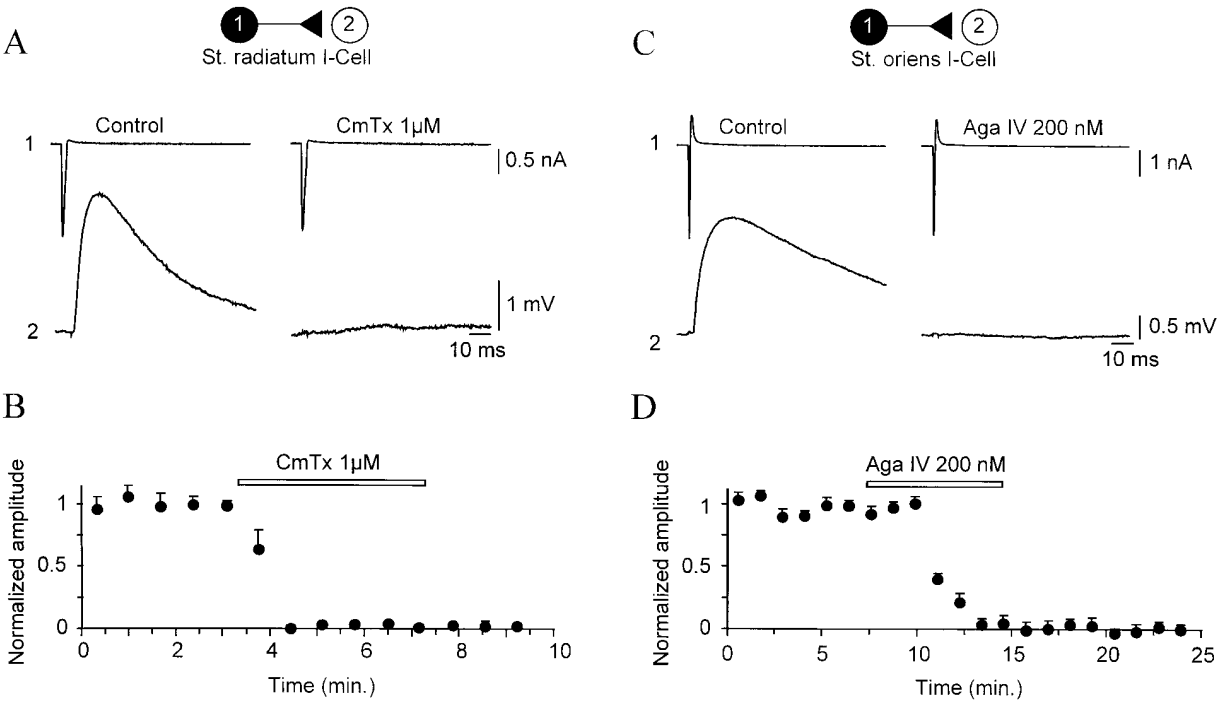

Figure 2. Distinct Ca^{2+} Channels Mediate GABA Release at Terminals of Two Types of Interneuron

(A) Unitary IPSPs in a pyramidal cell (2) triggered by activation of an inhibitory cell (1) located in st. radiatum were entirely and irreversibly abolished upon application of 1 μM ω-conotoxin MVIIA.

(B) The time course of this effect.

(C) In contrast, unitary IPSPs originating from a st. oriens inhibitory cell could be entirely suppressed by 200 nM ω-agatoxin IVA.

(D) The time course of this effect. Each trace (A and C) represents the average of 20–24 consecutive IPSPs, and each point (B and D) the mean amplitude and S. E. M. of 4 consecutive IPSPs.

somata located in st. radiatum (Figure 2A; Table 1). IPSP amplitude declined exponentially with a time constant of 52 ± 1 s (n = 4) and was not reversed within 10–30 min of toxin removal. The toxin produced no significant change in the input resistance of the pyramidal cell (−4.3 ± 4.1%, P > 0.1, n = 5). Similar results were obtained with ω-conotoxin GVIA (n = 3 pairs).

Even when unitary IPSPs were suppressed by the toxin, large spontaneous IPSPs could still be recorded from the postsynaptic pyramidal cell (not shown). These spontaneous IPSPs were probably action potential–dependent because their amplitudes reached several mV. They may have originated from either synapses that were not accessible to the toxin or synapses that were not sensitive to CmTx.

We examined this question by performing recordings from other interneurons within the CA3 region. In 11 of 13 pairs in which the interneuron was located within st. oriens, CmTx had little or no effect on the amplitude of unitary IPSPs (Table 1). In the 2 other pairs, however, the toxin entirely suppressed unitary IPSPs (to 2.3% of control amplitude). In contrast, in 7 of 7 pairs tested, the specific P/Q-type calcium channel antagonist ω-agatoxin IVA (Aga IV, 200 nM) abolished transmission at synapses formed by st. oriens interneurons (Figure 2B; Table 1). This block was also irreversible and was slower than the effect of CmTx (time constant = 100 ± 24 s, n = 4). Similar results were obtained from 4 pairs in which the interneuron somata were located within st. lucidum, near the border of st. pyramidale (Table 1).

At the concentrations used in these experiments, Aga IV may not discriminate between P- and Q-type calcium channels (Zhang et al., 1993; Olivera et al., 1994). In order to clarify this issue, we examined the effect of

Table 1. Effects of Calcium Channel Antagonists on Unitary IPSPs

Interneuron soma in	ω-CTx MVIIA (1 μM)	ω-Aga IVA (200 nM)	ω-CTx MVIIA + ω-Aga IVA*	ω-CTx MVIIC (2 μM)
St. radiatum	98.6 ± 1.0 (11)	3.9 ± 1.7 (3)	100.5 ± 1.8 (3)	n.d.
St. lucidum/pyramidale	3.2 ± 0.7 (2)	97.9 ± 1.3 (4)	n.d.	n.d.
St. oriens	6.2 ± 1.5 (11)	99.8 ± 0.4 (7)	98.2 ± 0.5 (8)	7.9 ± 5.9 (5)

The table indicates the percentage reduction in amplitude (mean ± S.E.M.) of unitary IPSPs initated by interneurons located within distinct strata of the CA3 region. Number of observations are given in parentheses. n.d.: not determined.

* Effect of ω-CTx MVIIA after application of ω-Aga IVA (st. radiatum interneurons) or vice-versa (st. oriens interneurons).

ω-conotoxin MVIIC, which blocks N- and Q-type channels faster and more efficiently than P-type (Hillyard et al., 1992; Sather et al., 1993; McDonough et al., 1996) on unitary IPSPs originating from st. oriens interneurons. In 5 pairs, ω-conotoxin MVIIC (2 μM for ~3 min) reduced unitary IPSP amplitude only weakly (-7.9 ± 5.9%), suggesting that P- rather than Q-type calcium channels mediate GABA release at these synapses.

Finally, a contribution of L-type calcium channels to transmitter release has been reported under certain circumstances (Huston et al., 1995; Reuter, 1995). We found no effect of isradipine (2 μM), an L-type calcium channel antagonist, on unitary IPSPs generated by either st. radiatum or st. oriens interneurons (-2.7 ± 3.5%, n = 4 pairs).

We conclude that different subtypes of calcium channels are used to trigger GABA release at hippocampal inhibitory synapses. The pharmacological properties of unitary IPSPs suggest that either N- or P-type calcium channels mediate GABA release at distinct synapses.

Morphological Characterization of Interneuron Subtypes

Distinct inhibitory neurons may differentially influence postsynaptic pyramidal cells, for instance owing to differences in the somato-dendritic location of the synapses they form (e.g., Miles et al., 1996). We therefore examined the possibility that synapses operating with distinct calcium channel subtypes target different regions of pyramidal cell membrane. Paired recordings were combined with biocytin injection of the presynaptic inhibitory cell for camera lucida reconstruction of their entire axonal and dendritic arbors.

For 16 of 27 interneurons successfully filled and recorded (6 in st. radiatum, 8 in st. oriens, and 2 at the lucidum border of st. pyramidale), we could simultaneously record a postsynaptic pyramidal cell and establish the pharmacology of synaptic transmission. Two representative neurons are shown in Figure 3. The dendritic arbor of all interneurons was highly variable in shape. Dendritic spines were sometimes apparent at low density, regardless of the location of the cell soma. The axons of st. radiatum interneurons, generating CmTx-sensitive IPSPs, were primarily confined to st. radiatum and only very rarely entered or crossed st. pyramidale (Figure 3A). In contrast, st. oriens interneurons, generating Aga IV-sensitive IPSPs, established synaptic contacts primarily within st. pyramidale and st. oriens. Occasionally, their axons crossed st. pyramidale and ramified within st. lucidum, presumably making contacts onto the proximal apical dendrites of pyramidal cells. Two of these neurons were clearly displaced basket cells with a very dense axonal arborization restricted to st. pyramidale.

Another difference between these cells was the size and density of their axonal varicosities (Figure 3C). The axons of st. radiatum interneurons had few varicosities; they were of small size and irregularly distributed. In contrast, the density of varicosities was much higher along axons formed by st. oriens and st. lucidum/pyramidale interneurons, and the varicosities were consistently larger in size.

These data suggest N- or P-type calcium channels are present at inhibitory terminals having distinct morphological features and forming synapses with different regions of the somato-dendritic membrane of CA3 pyramidal cells.

Manipulations of Presynaptic Calcium Entry after Block of Unitary IPSPs

The complete blockade of unitary IPSPs by either Aga IV or CmTx suggests that only one type of channel mediates transmitter release at these synapses. However, unitary IPSPs may be effectively suppressed without a complete blockade of calcium influx into presynaptic terminals. Owing to the supralinear relationship between the presynaptic calcium concentration and transmitter release, a small reduction of calcium influx may lead to a large decrease in the amplitude of the postsynaptic response. Assuming a 4th power relationship (Dodge and Rahamimoff, 1967), the fraction of calcium current blocked by the toxin (ΔI_{Ca}) could be estimated as: $\Delta I_{Ca} = 1-(\Delta IPSP)^{1/4}$, where ΔIPSP represents the fraction of synaptic response remaining after application of the toxin. As illustrated in Figure 4A, a complete block of the synaptic response may occur as a consequence of a reduction in presynaptic calcium influx of only 61%. This calculation suggests that a significant fraction of calcium influx in the synaptic terminals may not have been blocked by the toxin even though the IPSP was entirely suppressed.

We therefore attempted to increase presynaptic calcium influx, to determine if a fraction of the calcium channels remained unblocked after unitary IPSPs were abolished. Our first approach was to increase the driving force for calcium ions by elevating the extracellular calcium concentration while keeping the magnesium concentration constant. In the absence of toxins, increasing the Ca^{2+}/Mg^{2+} ratio from 1.5 to 5 led to a marked increase in the mean amplitude of unitary IPSPs (Figure 4B). A reduction in the peak amplitude of sodium current in the presynaptic neuron was also apparent, probably owing to an elevation of the activation threshold of sodium channels by extracellular calcium ions (Hille, 1968). After unitary IPSPs had been totally suppressed at a given connection by either CmTx (for st. radiatum interneurons) or Aga IV (for st. oriens interneurons), however, the same increase in the Ca^{2+}/Mg^{2+} ratio did not restore an IPSP (Figure 4B; −98.0 ± 1.0% of control IPSP, n = 8 pairs).

If a fraction of presynaptic calcium channels were still functional after blockade of synaptic transmission, then calcium influx through these channels, although insufficient to elicit transmitter release in response to single action potentials, might accumulate in the presynaptic terminal during the repeptitive discharge of the presynaptic neuron. Such 'residual calcium' (Katz and Miledi, 1968) might then reach the threshold for transmitter release. In 7 paired recordings with the interneuron located in st. oriens (n = 3), st. lucidum/pyramidale (n = 1), or st. radiatum (n = 3), a burst of action potentials was generated in the presynaptic cell while recording in current-clamp mode (Figure 5). Before toxin application, repetitive presynaptic firing (50–80 Hz) led to a summation of IPSPs in the pyramidal cell. After transmission

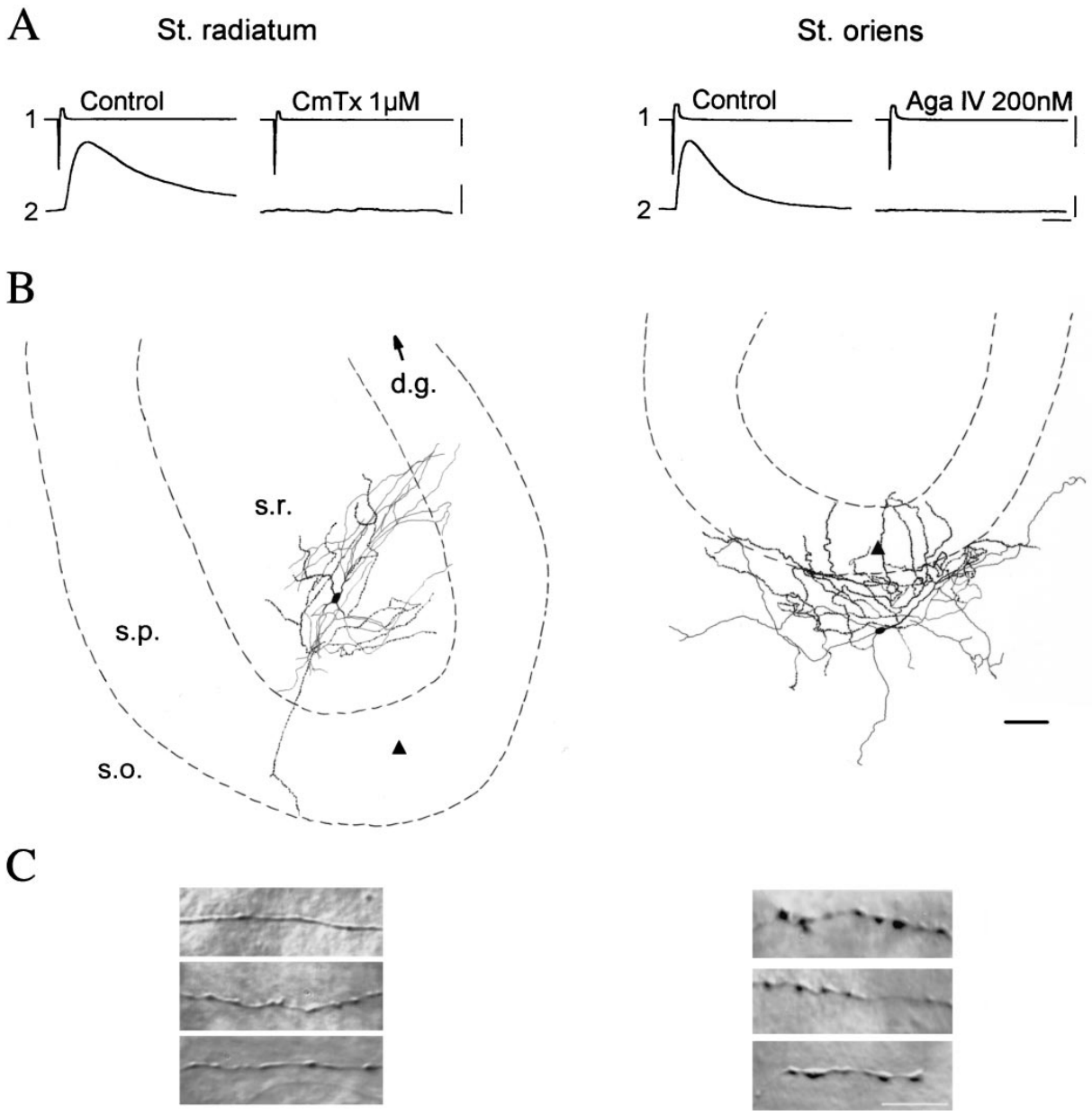

Figure 3. Correlated Physiology and Morphology of Distinct Interneurons in the CA3 Region

(A) Unitary IPSPs produced by interneurons in st. radiatum were entirely abolished upon application of 1 μM ω-conotoxin MVIIA. In contrast, IPSPs originating from st. oriens interneurons were blocked by 200 nM ω-agatoxin IVA.

(B) Schematic camera lucida reconstruction of the biocytin-filled interneurons recorded from (A). Note the high density of varicosities (filled dots) on axons of st. oriens interneurons. Filled triangles represent the position of the recorded postsynaptic pyramidal cells. St. radiatum interneurons had axons primarily confined to st. radiatum. In contrast, axons of st. oriens interneurons showed dense arborizations within or close to st. pyramidale. s.o., st. oriens; s.p., st. pyramidale; s.r., st. radiatum; d.g., dentate gyrus.

(C) Three representative sections of the axonal arbor of each cell, visualized with a 63x oil immersion objective. The axon of the st. radiatum interneuron had sparse varicosities of small size. In contrast, varicosities were more numerous and larger along axons of st. oriens interneurons. Scale bars: (A) 2 nA (upper traces), 2 mV (lower traces), 20 ms; (B) 100 μm; (C) 10 μm.

had been blocked at the same synapses by either CmTx or Aga IV, however, IPSPs were never detected during repetitive firing of the interneuron. We therefore conclude that only one class of calcium channel is able to initiate GABA release from the terminals of these interneurons.

Difference between Excitatory and Inhibitory Synapses onto CA3 Pyramidal Cells

In contrast to our results at hippocampal inhibitory synapses, most evidence indicates transmitter release at hippocampal excitatory synapses is initiated by multiple types of calcium channels (Luebke et al., 1993; Taka-

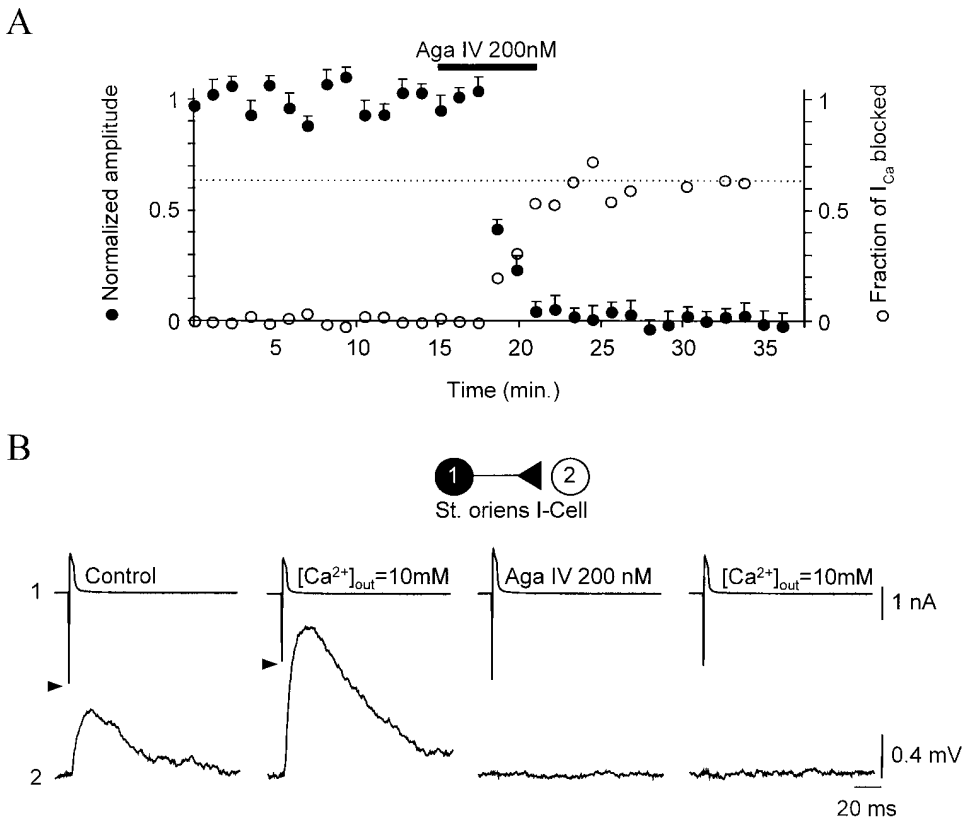

Figure 4. GABA Release from Inhibitory Synapses Is Mediated by Only One Ca^{2+} Channel Type

(A) Estimation of the fraction of presynaptic Ca^{2+} current blocked by ω-agatoxin IVA. Dual recordings were made from a st. oriens interneuron and a postsynaptic CA3 pyramidal cell. Open circles: normalized amplitude of the unitary IPSP. Each point represents mean amplitude ± S. E. M. of 4 consecutive IPSPs. The fraction of initial Ca^{2+} influx blocked by the toxin, ΔI_{Ca}, is estimated to be $1-(\Delta IPSP)^{1/4}$, where ΔIPSP represents the remaining fraction of IPSP amplitude (see text). Points with negative ΔIPSP were not used for the estimation of ΔI_{Ca}.

(B) Elevation of the external Ca^{2+} concentration from 3 to 10 mM led to a 128% increase in IPSP amplitude in this cell pair. Note the reduction of the Na^{+} current in the inhibitory cell (arrowheads), probably reflecting a shift in the activation threshold. The same increase in external Ca^{2+} concentration did not restore any transmission at this synapse after application of 200 nM ω-agatoxin IVA. Each trace represents the average of 20 consecutive IPSPs.

hashi and Momiyama, 1993; Wheeler et al, 1994; Reuter, 1995). Does this difference stem from the difference in the number of synapses sampled with the various recording techniques, or are hippocampal excitatory and inhibitory synapses fundamentally different? In order to answer this question, we recorded from pairs of monosynaptically coupled CA3 pyramidal cells using the same configuration as that employed for studying unitary IPSPs. The monosynaptic nature of unitary excitatory postsynaptic potentials (EPSPs) was established by the short and constant latency of the EPSP and the reliability of transmission during repetitive firing of the presynaptic neuron.

Under these conditions, CmTx (1 μM) reduced unitary EPSP amplitude by 47.5 ± 5.7% (n = 7 pairs) (Figure 6). Inhibition of unitary EPSPs by Aga IV (200 nM) was more pronounced (-64.3 ± 5.1%, n = 3 pairs). When both CmTx and Aga IV were applied successively, however, suppression of unitary EPSPs was not always complete (-91.5 ± 6.6% of control, n = 4 pairs), consistent with the suggestion that another type of channel, pharmacologically distinct from the N- and P-types, contributes to glutamate release at excitatory synapses (Wheeler et al., 1994; Dunlap et al., 1995). Finally, in two cell pairs, ω-conotoxin MVIIC (2 μM for ~3 min), reduced unitary EPSP amplitude by 36.7 and 42.8 %, respectively, consistent with the rapid block of N-type channels by this toxin (McDonough et al., 1996).

A contribution of multiple calcium channel subtypes to transmitter release can thus be demonstrated by paired recordings at another synapse terminating on the same postsynaptic cells. Thus, the complete blockade of unitary IPSPs by a single toxin cannot be attributed to our recording conditions. We conclude that a single class of calcium channel mediates GABA release from hippocampal inhibitory synapses, whereas multiple classes contribute to glutamate release at excitatory synapses.

Discussion

We have identified the calcium channels mediating GABA release at various hippocampal inhibitory synapses. Our results show that at least two subpopulations of hippocampal interneurons can be distinguished according to the calcium channel subtypes they express at their synaptic terminals. Reconstruction of the axonal

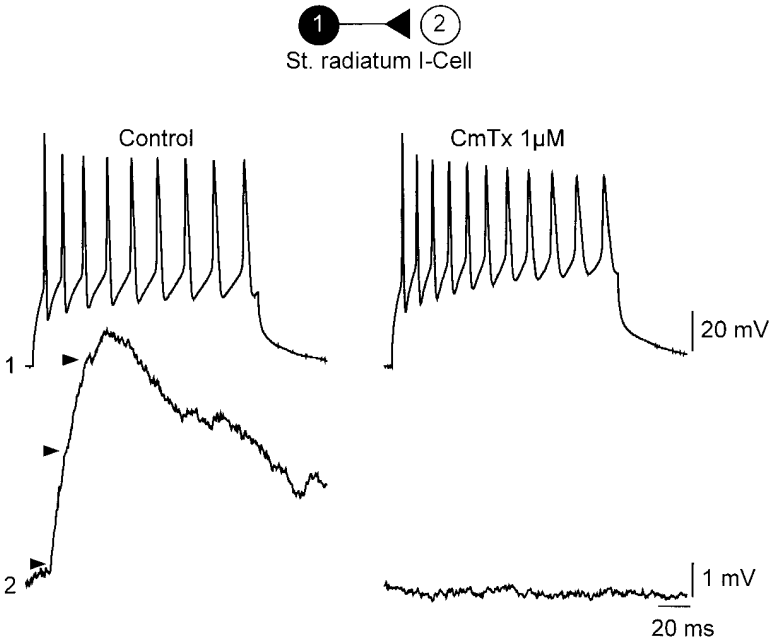

Figure 5. GABA Release Cannot Be Recovered with High-Frequency Discharge of the Interneuron

A st. radiatum inhibitory cell (1) was recorded in current-clamp mode at a membrane potential of about −70 mV. A 2 nA depolarizing current step elicited a burst of action potentials at 60–75 Hz. Summated IPSPs were detected in a monosynaptically connected pyramidal cell (2). The three first IPSPs occurred with similar latencies (arrowheads). After application of 1 μM ω-conotoxin MVIIA, no IPSP was triggered by any presynaptic action potential during the burst. Each trace represents a single episode.

arborization of the various interneurons indicates that the type of calcium channel present at the terminals is correlated with the region of the postsynaptic membrane they target. Finally, we conclude that release at inhibitory synapses is initiated by only one pharmacologically unique class of channel, whereas excitatory synapses terminating on CA3 pyramidal cells use multiple calcium channel subtypes.

GABA release from synapses formed by interneurons located within st. radiatum was blocked by ω-conotoxin MVIIA, whereas IPSPs produced by most interneurons located within st. oriens or at the border of st. pyramidale and st. lucidum were blocked by ω-agatoxin IVA but not ω-conotoxin MVIIA. These results suggest that class B and class A calcium channels play a predominant role in mediating GABA release at synapses originating from these two populations of interneurons, respectively. ω-conotoxin MVIIA-sensitive, N-type calcium currents are thought to represent the only functional correlate of class B channels (Birnbaumer et al., 1994). Subtypes of class A channels may not be discerned unambiguously using ω-agatoxin IVA because both P- and Q-type currents are blocked by the toxin, albeit with different affinities (Mintz et al., 1992; Sather et al., 1993; Wheeler et al., 1994). Because of difficulties in accurately controlling toxin concentration at all synapses in complex tissue (Dunlap et al., 1995), we did not attempt to discriminate P- and Q-type currents according to their sensitivity

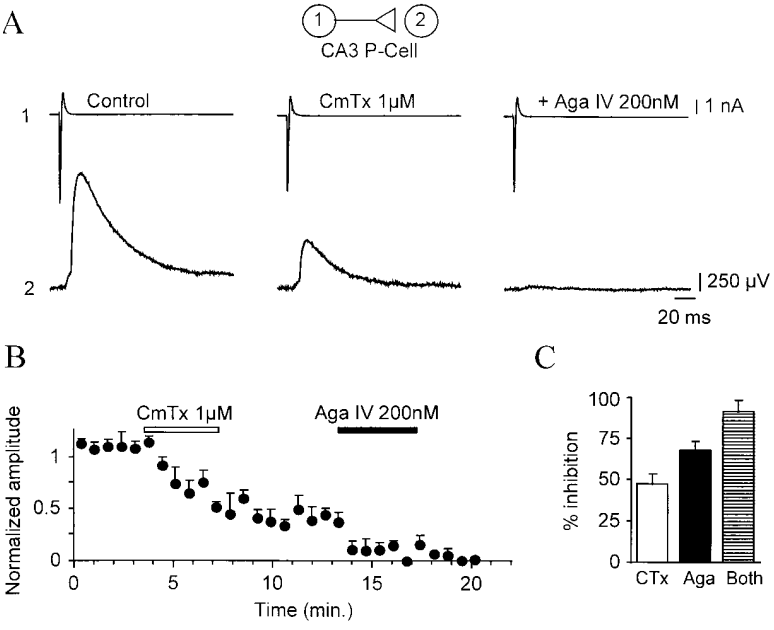

Figure 6. Multiple Ca^{2+} Channel Types Mediate Unitary EPSPs between CA3 Pyramidal Cells

A presynaptic CA3 pyramidal cell (1) was recorded in the whole-cell voltage-clamp mode. A postsynaptic pyramidal neuron (2) was impaled with a 2M KCH3SO4-filled sharp microelectrode. The monosynaptic nature of the connection was assessed from the fixed latency of the EPSP and the reliability of transmission during repetitive firing of cell (1).

(A) Unitary EPSPs were partially blocked by 1 μM ω-conotoxin MVIIA (-58.6% of control amplitude). Further block was obtained upon application of 200 nM ω-agatoxin IVA. Each trace represents the average of 20 EPSPs.

(B) The time course of the effect of both toxins.

(C) Pooled data from 10 cell pairs. ω-conotoxin MVIIA (1 μM) reduced unitary EPSP amplitude by 47.5 ± 5.7% and ω-agatoxin IVA (200 nM) by 64.3 ± 5.1%. When both toxins were applied, unitary EPSPs were not always fully blocked (% inhibition = 91.2 ± 6.6%).

to ω-agatoxin IVA. ω-conotoxin MVIIC, however, which reportedly blocks N- and Q- with much faster kinetics than P-type currents (Hillyard et al., 1992; Sather et al., 1993; McDonough et al., 1996), partially suppressed unitary EPSPs but did not affect GABA release at synapses formed by st. oriens interneurons. We therefore suggest that P- rather than Q-type calcium currents predominantly mediate GABA release at these synapses. Distinct CA3 interneurons thus express different calcium channels at their synaptic terminals. The lack of effect of ω-agatoxin IVA at terminals originating from st. radiatum interneurons is consistent with the absence of P/Q-type calcium channels in the somato-dendritic membrane of these cells (Lambert and Wilson, 1996). Using paired recordings from unidentified inhibitory and pyramidal cells in culture, Ohno-Shosaku et al. (1994) reported a similar distinction between two populations of interneurons. However, some unitary IPSCs were not entirely blocked by either toxin, suggesting the possible existence of a third population of synapses expressing both N- and P/Q-type calcium channels.

Inhibitory synapses exert different postsynaptic effects upon hippocampal cells, depending on their somato-dendritic location. Synapses impinging on distal dendrites may primarily influence the generation of calcium spikes and propagation of local EPSPs, whereas perisomatic contacts mostly control repetitive spike generation (Miles et al., 1996). We restricted our recordings to interneurons with a clearly visible soma located in st. oriens or st. radiatum. Other classes of interneuron were not examined in our experiments; for instance, those with somata of very small size or located within st. pyramidale, as well as interneurons that did not generate a detectable IPSP in neighboring pyramidal cells. We show that st. radiatum interneurons, generating CmTx-sensitive IPSPs, had axons mostly confined to st. radiatum, suggesting they may primarily contact distal apical dendrites of pyramidal cells. In contrast, most interneurons in st. oriens, making Aga IV-sensitive IPSPs, extended their axonal arbor close to or within st. pyramidale (Figure 3), suggesting that they established mostly perisomatic and proximal dendritic contacts. Consistent with this result, IPSPs evoked by extracellular stimulation in st. radiatum in both the CA1 and CA3 areas in hippocampal slices have been reported to be largely suppressed by the N-type calcium channel antagonist ω-conotoxin GVIA (Horne and Kemp, 1991; Potier et al., 1993). Likewise, spontaneous IPSPs in principal cells have been shown to originate primarily from synapses formed onto the perisomatic membrane of a postsynaptic cell (Soltesz et al., 1995; Miles et al., 1996). Our results predict that these events should be more sensitive to P-type than N-type calcium channel antagonists. Indeed, a selective suppression of K^+-evoked IPSCs in pyramidal cells in hippocampal slices by high concentrations of ω-agatoxin IVA has been reported (Doze et al., 1995).

The size and density of putative presynaptic terminals were also different at CmTx- and Aga IV-sensitive synapses. Electron microscopy is needed to ascertain the identity and quantitate the properties of the varicosities found along inhibitory cell axons. Nevertheless, the size of presynaptic terminals has been shown to be different for perisomatic versus dendritic inhibitory synapses (Halasy et al., 1996; Miles et al., 1996). Terminal size also correlates with the number of active zones (Yeow and Peterson, 1991; Pierce and Lewin, 1994) and is therefore likely to influence the potency of those synapses.

Experiments using extracellularly evoked synaptic currents indicate that multiple calcium channels participate in transmitter release at central synapses (Luebke et al., 1993; Takahashi and Momiyama, 1993; Wheeler et al., 1994). When presynaptic calcium influx was measured by optical means at several glutamatergic synaptic terminals in hippocampal cell cultures, however, a remarkable variability in their sensitivity to ω-conotoxin GVIA was apparent (Reuter, 1995). We found that unitary EPSPs were only partially blocked by either CmTx or Aga IV, and that a significant fraction of the EPSP was still detected when both toxins were present. We therefore conclude that at least three types of calcium channels participate in glutamate release, even at synapses originating from a single presynaptic neuron.

In contrast, unitary IPSPs originating from a given interneuron could be entirely suppressed by a single, subtype-specific toxin. IPSPs generated by st. radiatum interneurons were only very weakly affected by Aga IV. Likewise, IPSPs generated by st. oriens and lucidum/pyramidale interneurons were very weakly affected by CmTx. This small reduction (3–6%) may reflect either 1) a lack of absolute selectivity of the toxins, 2) some small spontaneous rundown in IPSP amplitude, or 3) the presence of another, minor fraction of toxin-resistant channels. In the latter case, we can estimate this secondary fraction would not produce more than 0.7–1.5% of the total influx, assuming a 4th power relationship between presynaptic calcium influx and the amplitude of the IPSP (Dodge and Rahamimoff, 1967). Increasing presynaptic calcium influx, by either raising calcium driving force or by repetitively activating the presynaptic neuron, did not restore any transmission after block with either toxin. This makes the existence of "spare calcium channels" of a different subtype at these synapses improbable, in contrast to recent suggestions for hippocampal glutamatergic synapses (Dunlap et al., 1995). Optical measurements of calcium influx may be needed to rule out a contribution of a secondary fraction of channels to release at these synapses.

What are the functional consequences of N- versus P-type channel expression? N- and P-type calcium channels have not only a distinct pharmacology but may also differ in their biophysical and kinetic properties. Compared to P-type channels, for example, N-type channels have been reported to be more sensitive to membrane potential and to become inactivated to a greater extent during a prolonged depolarization in rat motoneurons (Umemiya and Berger, 1995). Differences in inactivation rates are likely to influence transmitter release during repetitive firing of a presynaptic neuron. In addition, distinct calcium channels may be differentially modulated. GABA release from hippocampal inhibitory synapses is regulated by multiple neurotransmitters, including GABA itself (Lambert and Wilson, 1993), glutamate (Poncer et al., 1995), acetylcholine (Pitler and Alger, 1992; Behrends and Ten Bruggencate, 1993), and opioids (Cohen et al., 1992). Although some of these

modulators are known to directly interfere with spontaneous GABA exocytosis at some step subsequent to presynaptic calcium influx (see Thompson et al., 1993), a direct inhibition of presynaptic calcium channels has been shown to contribute to presynaptic inhibition of evoked transmitter release by these neurotransmitters at some synapses (Doze et al., 1995; Huston et al., 1995; Wu and Saggau, 1995; Takahashi et al., 1996). Interestingly, presynaptic inhibitory receptors may not be homogeneously distributed among inhibitory terminals (Lambert and Wilson, 1993). The differential expression of presynaptic calcium channel subtypes and of the presynaptic inhibitory receptors regulating these channels may thus permit various sources of synaptic inhibition to be differentially regulated, perhaps thereby altering qualitatively the function of the hippocampal network.

Experimental Procedures

Hippocampal slice cultures were prepared from 5-day-old rats as described previously (Gähwiler, 1981; Gähwiler et al., 1991). After 2–4 weeks in vitro, cultures were placed in a recording chamber mounted on an inverted microscope and were continuously superfused with saline containing (in mM) : NaCl, 137; KCl, 2.7; $NaHCO_3$, 11.6; NaH_2PO_4, 0.4; $MgCl_2$, 2; $CaCl_2$, 3; D-glucose, 5.6; and Phenol Red (10 mg/l) at a rate of 1–2 ml/min.

Paired recordings were made at room temperature (20–25°C) from visually identified CA3 interneurons and pyramidal cells. Whole-cell voltage-clamp recordings were obtained from interneurons with their cell bodies in either stratum oriens or stratum radiatum using an Axopatch 200A amplifier and pipettes (3–5 MΩ) containing (in mM): K-gluconate, 120; KCl, 10; HEPES, 10; EGTA, 1; Mg-ATP, 4; Na-GTP, 0.4; $CaCl_2$, 0.1; and $MgCl_2$, 0.5. Cells were voltage clamped at −60 mV. Series resistance and cell capacitance were regularly monitored and were 13.6 ± 0.8 MΩ and 17.5 ± 1.3 pF, respectively (n = 63 cells). Application of a 5 ms voltage step from −60 to 0 mV to the interneuron elicited an inward current of several nA with fixed latency, followed by a smaller outward current. Interneurons were made to fire such action currents every 8–12 s. A standard four-prepulse leak subtraction protocol, using 15 mV hyperpolarizing steps, was employed. A pyramidal cell was simultaneously recorded in current-clamp mode with an Axoclamp-2A amplifier using sharp microelectrodes filled with 1 M KCl and 10 mM HEPES. The cell was held at approximately −70 mV. Monosynaptic, unitary IPSPs could be detected in about half of all interneuron–pyramidal cell pairs. In some experiments, we recorded from pairs of monosynaptically connected CA3 pyramidal cells. In these cases, unitary EPSPs were recorded using a 1 M $KMeSO_4$ and 10 mM HEPES internal solution.

Membrane current from the presynaptic neuron was recorded at 1 mV/pA and filtered at 10 kHz. The voltage of the postsynaptic pyramidal cell was amplified 100x and filtered at 2 kHz. Both signals were acquired at 10 kHz using Clampex software and were analyzed off-line with Clampfit software (Axon Instruments, Foster City, CA).

In some recordings, 1% biocytin was included in the whole-cell recording solution. The K-gluconate concentration was then lowered so as to maintain appropriate osmolarity. Cells were filled for 20–45 min before the patch pipette was gently removed in order to form an outside-out patch. Slices were then fixed overnight at 4°C in 0.1 M phosphate buffer (PB) (pH 7.4) containing 4% paraformaldehyde and 2.5% glutaraldehyde. The samples were washed in PB for 3 hr prior to preincubation in PB containing 0.4% Triton X-100 for 4 hr. Endogenous peroxidase was removed subsequently by incubation in 0.1 M PB, 10% methanol and 0.15% hydrogen peroxide for 25 min at room temperature. After thorough washing, the cultures were processed within 24 hr using avidin–horseradish peroxidase histochemistry (ABC Kit, Vector Laboratories, Inc., Burlingame, CA) using diaminobenzidine as the chromogen and nickel sulfate intensification. The cultures were dehydrated and counterstained for Nissl substance before camera lucida-type drawings were made with a 100× objective (1.3 N.A., oil immersion) using Neurolucida software (MicroBrightField, Inc., Colchester, USA). Representative sections of the inhibitory cells axons were imaged using a 63 × 1.4 N.A. oil immersion lens.

When both the inhibitory and pyramidal cells were to be visualized (Figure 1A), CA3 pyramidal cells were impaled with microelectrodes containing 1% Lucifer Yellow in 1 M LiCl, and the inhibitory cells were recorded in the whole-cell mode with a 1% biocytin internal solution. The samples were fixed overnight in 0.1 M PB containing 4% paraformaldehyde. The preparations were washed in PB and then exposed to 0.4% Triton X-100 for 2 hr before revealing the biocytin-filled cell with NeuraLite avidin–Texas Red (Molecular Probes Inc., Portland, OR) (1:200). After mounting with Slowfade (Molecular Probes), the cells were visualized with a Zeiss LSM 410 confocal laser scanning microscope using lasers pretuned to 543 nm (TR) and 488 nm (LY) and a 63 × 1.4 N.A. oil immersion lens. Optical sections of 0.2 μm were selected, and the image was averaged to improve the signal-to-noise ratio. The images were then reconstructed in 3-D using a simulated fluorescence projection (Imaris Software, Bitplane AG, Zurich).

Data are given as mean ± SEM throughout. Statistical comparisons have been performed with the unpaired Student's *t*-test, unless otherwise noted. Drugs were purchased from the following sources: NBQX (Tocris Cookson, Bristol, UK), ω-conotoxin MVIIA (Latoxan, Rosans, France); ω-conotoxin MVIIC and ω-conotoxin GVIA (Bachem Feinchemikalien AG, Bubendorf, Switzerland). ω-agatoxin IVA was a gift of Pfizer Inc. (Groton, USA) and CPP was a gift from Sandoz, Ltd. (Basel, Switzerland).

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