Recruitment of GABAergic Inhibition and Synchronization of Inhibitory Interneurons in Rat Neocortex

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Benardo, Larry S. Recruitment of GABAergic inhibition and synchronization of inhibitory interneurons in rat neocortex. J. Neurophysiol. 77: 3134–3144, 1997. Intracellular recordings were obtained from pyramidal and interneuronal cells in rat neocortical slices to examine the recruitment of GABAergic inhibition and inhibitory interneurons. In the presence of the convulsant agent 4-aminopyridine (4-AP), after excitatory amino acid (EAA) irotropic transmission was blocked, large-amplitude triphasic inhibitory postsynaptic potentials (IPSPs) occurred rhythmically (every 10–40 s) and synchronously in pyramidal neurons. After exposure to the γ-aminobutyric acid-A (GABA_A) receptor antagonist picrotoxin, large-amplitude monophasic slow IPSPs persisted in these cells. In the presence of 4-AP and EAA blockers, interneurons showed periodic spike firing. Although some spikes rode on an underlying synaptic depolarization, much of the rhythmic firing consisted of spikes having highly variable amplitudes, arising abruptly from baseline, even during hyperpolarization. The spike firing and depolarizing synaptic potentials were completely suppressed by picrotoxin exposure, although monophasic slow IPSPs persisted in interneurons. This suggests that this subset of interneurons may participate in generating fast GABA_A, IPSPs, but not slow GABA_B IPSPs. Cell morphology was confirmed by intracellular injection of neurobiotin or the fluorescent dye Lucifer yellow CH. Dye injection into interneurons often (>70%) resulted in the labeling of two to six cells (dye coupling). These findings suggest that GABA_Aergic neurons may be synchronized via recurrent collateral firing and the depolarizing action of synaptically activated GABA_A receptors and a mechanism involving electrotonic coupling. Although inhibitory neurons mediating GABA_A IPSPs may be entrained by the excitatory GABA_A mechanism, they appear to be a separate subset of GABAergic neurons capable of functioning independently with autonomous pacing.

INTRODUCTION

Inhibition in neocortex is mediated primarily by γ-aminobutyric acid (GABA), and is composed of two types of inhibitory postsynaptic potentials (IPSPs), designated fast and slow (Avoli 1986; Benardo 1994; Connors et al. 1982, 1988; Deisz and Prince 1989; Howe et al. 1987). The pharmacologies of these two forms of inhibition are now well known (Benardo 1994; Connors et al. 1988; Deisz and Prince 1989). Fast IPSPs are mediated by GABA_A receptors and slow IPSPs are mediated by GABA_B receptors. The GABA-containing neurons in cortex that presumably mediate these responses are composed of interneurons (Houser et al. 1983).

Although these interneurons are morphologically diverse (Houser et al. 1983; Jones 1993), past physiological considerations of inhibitory neurons tended to group them all into a single class of neuron that was GABAergic and had a distinct physiology (Knowles and Schwartzkroin 1981; McCormick et al. 1985). However, results from more recent studies suggest this cell class can be divided into physiologically definable subclasses (Connors and Gutnick 1990; Kawaguchi 1993, 1995). The precise roles of different interneuronal populations are not completely known.

There is evidence in hippocampus (Lacaille and Schwartzkroin 1988; Lacaille et al. 1987; Miles and Wong 1984), lateral amygdala, and ventral tegmental area (Sugita et al. 1992), and thalamus (Crunelli and Leresche 1991; Steriade et al. 1985) that different populations of interneurons account for separate activation of fast and slow inhibition. In neocortex, glutamate microapplications onto inhibitory interneurons led to the separate (never mixed) activation of fast and slow IPSPs, suggesting that there are two populations of inhibitory neurons that individually give rise to fast GABA_A or slow GABA_B responses (Benardo 1994). Additional studies suggest that the inhibitory cells that provide for fast and slow inhibition may be further distinguished on the basis of the presynaptic glutamate receptors they possess (Benardo 1993; Ling and Benardo 1995a).

To examine this functional heterogeneity in more detail, the recruitment of inhibition and interneurons was investigated when somatosensory neocortical slices were exposed to 4-aminopyridine (4-AP) in the absence of excitatory amino acid (EAA) transmission. This resulted in the appearance of synchronized fast and slow IPSPs in pyramidal neurons, and spike firing in interneurons. GABA_A receptor antagonism blocked excitatory responses in interneurons, and synchronized fast IPSPs in pyramidal cells, but left synchronized slow GABA_B IPSPs. This suggests that the interneurons recorded did not mediate these GABA_B IPSPs, supporting the contention that separate classes of interneurons participate to give rise to fast and slow IPSPs.

METHODS

Preparation of slices and recording techniques

The methods used in these experiments were similar to those described in previous reports (Benardo 1993, 1994). After halothane anesthesia, Sprague-Dawley rats (80–100 g) were decapitated, and brains were rapidly removed and placed in ice-cold physiological solution. Coronal somatosensory cortical slices 400 µm thick were prepared with the use of a tissue chopper.

Slices were placed in a recording chamber (Fine Science Tools, Foster City, CA) and maintained at 35.5 ± 1°C (SD), at a pH of 7.4, exposed to a warmed, humidified atmosphere of 5% CO₂ in
O2. Slices were bathed from below with a solution composed of (in mM) 124 NaCl, 5 KCl, 1.6 MgCl2, 2 CaCl2, 26 NaHCO3, and 10 d-glucose. 4-AP (50–75 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), 3–3/2-carboxypiperazine-4-yl-propyl-1-phosphonate (CPP; 10 μM), picrotoxin (50 μM), and the GABAA antagonist CGP 35348 (200 μM) were added to the solution in some experiments. CPP and CNQX were obtained from Tocris Cookson (St. Louis, MO), and CGP 35348 was obtained from from Ciba-Geigy (Basel, Switzerland). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Recordings were made with microelectrodes pulled from 1-mm thin-walled, fiber-filled capillaries to a resistance of 20–60 MΩ routinely filled with 2 M potassium acetate. In experiments in which cells were prepared for fluorescence imaging, the tips of the recording electrodes were filled with a 5% Lucifer yellow CH dilithium salt dissolved in distilled water, and backfilled with 1 M lithium acetate. In other experiments in which cells were visualized with the use of light microscopy, the tips of the recording electrodes were filled with 2% neurobiotin (Vector Laboratories, Burlingame, CA) in 2 M potassium acetate, and backfilled with 2 M potassium acetate. Simultaneous penetrations were made with separately manipulated electrodes.

Membrane potentials were measured with the use of high-impedance amplifiers (Axoclamp 2A, Axon Instruments, Foster City, CA) operating in current-clamp mode. Slices were stimulated with the use of a sharpened monopolar, coated tungsten electrode placed in the deep gray matter lateral to the recording sites. Single cathodal shocks 200 μs in duration were delivered through a digitally controlled stimulus isolation unit (WPI, Sarasota, FL). Voltage and current signals were displayed on a digital oscilloscope (Hitachi Densihi, Woodbury, NY) and stored digitally on videotape for later off-line analysis. Rest potential was determined with respect to the bath potential on withdrawal of the intracellular electrode.

Staining and histological methods

Neurons were injected with Lucifer yellow CH with the use of hyperpolarizing pulses (850 ms at 1 Hz) of 1–3 nA for 5–15 min. After injection, the slices remained in the chamber for 30–90 min. Slices were removed and fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. Slices were then dehydrated in a series of alcohol rinses and cleared in methylsalicylate and mounted with Vectashield (Vector Laboratories), to retard fluorescence quenching before being examined with an epifluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with appropriate filters.

Neurobiotin was injected into cells with the use of depolarizing pulses (150 ms at 3.3 Hz) of 1–3 nA for 5–15 min. Slices containing filled cells were fixed overnight by immersion in 4% paraformaldehyde as above. Whole slices were rinsed in phosphate-buffered saline (PBS), then incubated in PBS containing 0.5% H2O2 for 1 h to suppress endogenous peroxidase activity and washed in PBS for 45 min. Slices were then incubated overnight at room temperature in PBS containing avidin-biotin-peroxidase complex (ABC solution, Vector Laboratories) at a dilution of 1:200 and 0.5% Triton X-100. The slices were rinsed and reacted with 3,3’-diaminobenzidine tetrahydrochloride (0.05%) and H2O2 (0.003%) in PBS, then cleared and mounted on slides for microscopic viewing. Given the problems of focusing through the thickness of whole mounted slices, photomicrography of filled neurons provided limited morphological information. Therefore the dendrites, axonal processes, and somata of the labeled potential. A typical example of this is shown in Fig. 1A.

The mean reversal potential was +32.0 ± 13.8 mV, and the mean resting membrane potential was −68.8 ± 6.7 mV. Cells were exposed to solutions containing 4-AP (50–75 μM) and the EAA receptor antagonists CNQX (10 μM) and CPP (10 μM). Although excitatory synaptic transmission was blocked in these neurons, two types of spontaneous synaptic events persisted. These consisted of large-amplitude inhibitory events and smaller-amplitude IPSPs (e.g., Fig. 1C), as has been previously reported (Aram et al. 1991).

The large-amplitude events occurred spontaneously and synchronously (demonstrated by simultaneous intracellular or extracellular recordings; see also Aram et al. 1991 and Fig. 5) at frequencies that were highly variable within individual cells (i.e., occurring every 10–40 s; mean ± SD throughout. Student’s t-test was applied to determine statistical significance.

RESULTS

Properties of synchronized IPSPs

Intracellular recordings were obtained from 57 pyramidal cells primarily in layer V, but also from layers II–III and VI. The mean input resistance was 32.0 ± 13.8 MΩ, and the mean resting membrane potential was −68.8 ± 6.7 mV. Interestingly, many cells would occasionally fire a series of decrementing large potentials having interevent intervals of 0.5–1.0 s. In the present study solitary large-amplitude events were triphasic in appearance when observed at −60 mV, having a mean peak amplitude of −9.3 ± 3.2 mV (n = 38 cells), a time-to-peak of 29.5 ± 15.7 ms, and an overall duration of 1,192 ± 1,023 ms (as determined from an average of 4 events in each cell). Similar events could be evoked by extracellular stimulation within the slice.

Reversal potentials for the triphasic response were estimated by setting the membrane potential to different levels by varying the amount of steady-state current injected into the cell. In the 15 cells tested, varying the membrane potential led to corresponding changes in the amplitude of each component of the triphasic response (Fig. 1A). Equilibrium potentials for each phase of the triphasic response were then obtained from plots of IPSP amplitude versus membrane potential. A typical example of this is shown in Fig. 1B. The mean reversal potential was −72.1 ± 5.4 mV for the early component, −60.4 ± 8.7 mV for the middle component, and −82.5 ± 6.7 mV for the late component.

Effects of picrotoxin on the triphasic IPSP

The addition of picrotoxin modified several aspects of the spontaneous synaptic activity recorded in 4-AP and EAA
blocks (Fig. 1C). Within 10–15 min the smaller-amplitude IPSPs were abolished (Fig. 1D). With further exposure the middle depolarizing component of the response became attenuated (cf. responses noted by * in Fig. 1, C and D), and it was completely blocked after ~30 min. Concomitantly, the early fast component was replaced by a potential having a significantly slower time-to-peak (Fig. 1D; mean 104.7 ± 26.6 ms; n = 20). The mean amplitude of this

**FIG. 1.** Effects of picrotoxin and CGP 35348 on the synchronized inhibitory postsynaptic potential (IPSP) in a layer V pyramidal cell. A: in presence of 4-aminopyridine (4-AP) and excitatory amino acid (EAA) blockers, the synchronized IPSP was composed of 3 components corresponding to the fast ɣ-aminobutyric acid-A (GABA_A) response, the depolarizing GABA_A response, and the slow GABA_B response. Reversal potentials for the 3 components were estimated by varying membrane potential (Vm) by injection of direct current. Measurements were made at indicated points. B: values obtained were plotted against membrane potential under control conditions. Lines were fit by linear regression analysis and crossed the 0 line at -71, -76, and -82.5 mV, respectively. C: control trace showing large triphasic IPSP with prominent depolarizing component (*) and baseline spontaneous smaller IPSPs. D: in same cell, 15 min after addition of picrotoxin, components are altered as shown and background small IPSPs are abolished. With additional exposure to picrotoxin, early hyperpolarizing and depolarizing GABA_A responses were completely blocked such that synchronized event became slower and monophasic. E: exposure to the GABA_B antagonist CGP 35348 (200 µM) abolished this slow monophasic event. Resting potential: -69 mV. Calibration in D: 5 mV, 200 ms (applies to C–E).
synchronized event was $-8.6 \pm 3.3$ mV, and the mean duration was $1.077 \pm 283$ ms (determined from 4 averaged traces). The frequency of this remaining event remained highly variable in individual cells (occurring every 20–60 s). When analyzed in detail ($n = 5$), the average frequency was found to be significantly decreased ($40.6 \pm 8.4\%$; $P < 0.05$) versus those recorded in the absence of picrotoxin (i.e., decreased from a mean of 3.3 events per min to 2.0 events per min), suggesting different pacemakers. In addition, the amplitudes of spontaneous events were somewhat variable from one potential to the next.

The large IPSP (spontaneous or evoked; shown in Fig. 2) that persisted in the presence of picrotoxin was monophasic and reversed monotonically. The mean reversal potential was $-87.8 \pm 3.1$ mV ($n = 4$). This reversal potential corresponds to that of the third component of the triphasic synchronized IPSP generated before the addition of picrotoxin. This, plus the observation that this event was blocked by the GABA$_B$ antagonist CGP 35348 (Fig. 1E), strongly suggested that this large slow IPSP results from the synchronized activation of postsynaptic GABA$_B$ receptors.

**Intracellular recordings from neocortical interneurons**

To examine the origin of the synchronized triphasic IPSPs, I attempted to make intracellular recordings in presumptive inhibitory GABAergic interneurons in the neocortex. Immunocytochemical studies show that such interneurons are unevenly distributed across the cortical layers (Jones 1993; Peters and Kara 1985). In layer I, 95–100% of cells are GABAergic and constitute an important source of inhibition to layer V pyramidal neurons (Salin and Prince 1996). Likewise, there is a high proportion of GABAergic neurons (relative to pyramidal neurons) in layer VI. By confining impalements primarily to these regions, I hoped to increase the probability of recording from these interneurons.

Intracellular recordings were obtained from 23 morphologically identified interneurons. Of these cells, 15 were recorded in layer I, 4 in layer V, and 4 in layer VI. The cells ($n = 23$) had a mean input resistance of $37.5 \pm 21.2$ MΩ (range 8.0–94.0 MΩ) and a mean resting potential of $-63.6 \pm 7.3$ mV. The mean spike height was $67.7 \pm 12.8$ mV, and the mean spike duration at spike half-amplitude was $1.6 \pm 1.4$ ms (range 0.4–4.0 ms). Except for input resistance, these characteristics, which are derived from morphologically heterogeneous cells, overlap with those previously observed for interneurons (Hestrin and Armstrong 1996; Kawaguchi 1993, 1995; Zhou and Hablitz 1996). The higher input resistances reported in these previous studies may be due to the use by those researchers of whole cell methods that yield higher resistance measurements versus current clamp (cf. McCormick et al. 1985). The longer spike duration of some cells probably reflects 4-AP actions on spike repolarization in some cells (Zhou and Hablitz 1996).

In the presence of 4-AP and EAA blockers, all confirmed interneurons showed periodic spontaneous spike firing. The behaviors displayed by these cells could be empirically divided into three types. One cell class ($n = 7$) showed prolonged rhythmic bursting, which lasted seconds and consisted of trains of action potentials (Fig. 3A, expanded in Fig. 3B). At rest potential these bursts of spikes rode on waves of depolarization (Fig. 3A, bottom). However, depolarizing the cell with injected current could reveal a prominent initial hyperpolarizing response, along with some smaller late hyperpolarizing phases (Fig. 3, A, top and B, top). The other striking observation was that the spikes triggered during the rhythmic trains had highly variable amplitudes. Moreover, these action potentials arose abruptly from

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**FIG. 2.** Reversal potential estimations for monophasic IPSP in a layer V pyramidal cell after addition of picrotoxin to bath. 4-AP and EAA blockers were also present. A: membrane potential was altered by current injection into cell and large event was evoked (stimulus artifact) via a stimulating electrode placed in layer VI. Stimuli were delivered every 15 s to avoid depression or failure of event. B: plot relating amplitude of evoked event to membrane potential, with linear regression line through data points. Reversal potential: $-85$ mV (rest potential: $-72$ mV).
baseline, even occurring during the initial hyperpolarizing phase of the event (Fig. 3, A, top and B, top). Hyperpolarizing cells as negative as −90 mV led to larger slow depolarizing waves, and did not abort all spike firing, although the spikes triggered were of somewhat lower amplitudes.

A second category of neurons (*n* = 8) seemed less excitable (Fig. 4, A and B), firing fewer spikes for brief periods (Fig. 4, A, bottom and B, bottom), although rhythmic sub-threshold activity could persist for several seconds (Fig. 4A, bottom). Depolarizing current injection into this cell showed its response to be somewhat similar to that of the cell in Fig. 3, A–C; this maneuver likewise uncovered a superimposed hyperpolarization (Fig. 4, A, top and B, top). Note again the sudden occurrence of spikes from a variety of voltage levels (Fig. 4, A and B), and their various spike heights.

The third group of interneurons (*n* = 8) was seen to fire one or more action potentials initially (Fig. 4, D and E), having amplitudes that were less than full spike height (cf. amplitude of Fig. 4E with that of Fig. 4F, inset). At more negative potentials (Fig. 4, D, bottom and E, bottom) this was followed by a depolarization. At less negative voltage levels (Fig. 4, D, top and E, top), a triphasic response could be seen.

In nine cells the reversal potentials for hyperpolarizing and depolarizing events were analyzed. This consideration yielded a mean value of −68.8 ± 5.4 mV for the initial fast hyperpolarization and −55.0 ± 8.6 mV for the later, depolarizing phase of the potential. In most cases a third reversal potential was impossible to reliably detect. This could be due to the simple absence of a true GABA<sub>A</sub>-mediated slow IPSP in the observed events (i.e., the depolarizing phase was superimposed on a GABA<sub>A</sub>-mediated hyperpolarization), or because the large depolarizing component simply obscured a small late hyperpolarization. In three cases the reversal potential of the late hyperpolarization could be assessed, yielding a mean of −79.0 ± 3.6 mV.

**Interneuron firing coincides with IPSPs generated in pyramidal neurons**

As might be expected, paired recordings of interneurons and pyramidal cells (*n* = 9) showed that firing in interneurons occurred in phase with synchronized IPSPs recorded in pyramidal neurons (Fig. 5). In all cases initial spikes seen in interneurons coincided with the peak of the fast component of the large-amplitude IPSPs seen in pyramidal neurons (Fig. 5, A and B). Interestingly, barrages of smaller events following the initial giant potentials, when present (Fig. 5A), were likewise synchronous, although they were depolarizing in interneurons and hyperpolarizing in pyramidal cells. Thus neurons of both cell classes are capable of oscillating in synchrony for a period following a synchronous IPSP. The frequency of this rhythmic activity was 5–7 Hz.

Addition of picrotoxin to the bath blocked all spontaneous interneuronal spiking as well as the early hyperpolarizing and late depolarizing events in the group of interneurons recorded in this study, but left spontaneous slow IPSPs (Fig. 5B; *n* = 5). In interneuron-pyramidal pairs (*n* = 4) these slow spontaneous hyperpolarizing events occurred coincidentally in both cells (e.g., Fig. 5B). In other neuronal pairs this activity could desynchronize somewhat, so that events occurred in each cell, but with a temporal separation of up to 100–200 ms. This appeared to be dependent, at least in part, on the distance between the pair of neurons recorded (i.e., greater with increasing distance between cells), which ranged up to several hundred micrometers. This may indicate that for each cell there are multiple paths or perhaps multiple sites for slow IPSP generation that are connected through a more loosely organized network than that responsible for more highly synchronized fast IPSPs.

**Interneuronal morphology and dye coupling**

Interneurons were filled with either the fluorescent dye Lucifer yellow CH or neurobiotin to confirm their nonpyramidal morphology. Only cells with well-filled somata and dendrites were utilized. As can be seen in the examples in Figs. 3–5, the cells stained had varied features. Cells were characterized as having aspiny, multipolar morphology, with one notable exception (i.e., the inverted pyramidal cell in Fig. 5B). A very high proportion of multipolar cells has been demonstrated to be GABAergic (Houser et al. 1983; Jones 1993), and chandelier (e.g., Fig. 3, C and D) and inverted pyramidal cells (Fig. 5B) are known to be GABAergic (Cobas et al. 1987; Prieto et al. 1994; Somogyi 1989.). By design, many of the cells in this study were located very superficially in the cortex, where well over 90% of cells are GABAergic (Jones 1993; Peters and Kara 1985). In addition, the behavior of these interneurons in the presence of 4-AP and EAA antagonists was quite distinct from that of pyramidal neurons. Therefore it seems reasonable to suggest that the interneurons recorded were in fact inhibitory GABAergic interneurons.

As seen in Figs. 3–5, many interneuronal impalements resulted in more than one cell staining. This occurred regardless of the dye [Lucifer yellow CH (*n* = 11; Fig. 6, filled bars) or neurobiotin (*n* = 17; Fig. 6, open bars)] used to mark cells. In 20 cases more than one cell was stained by a single dye injection. The distribution of the number of cells filled with each injection appears in Fig. 6A. In the eight interneuronal impalements yielding single cell fills, neurons were sufficiently well filled to allow identification of cell morphology; however, for some cells the staining tended to be lighter than those in which multiple fills resulted. It is unclear whether insufficient dye injection was a limiting factor for those impalements resulting in single cell fills. Regardless, it is clear that a majority of these interneurons appear to be dye coupled. This is in contrast to pyramidal neurons, which were found to be coupled to one or two other pyramids in ~25% of dye fills (*n* = 31; Fig. 6B). This is in good agreement with previous work showing that pyramidal cells were coupled in ~20% of dye injections from single impalements in mature rat neocortex (Connors et al. 1983).

**Discussion**

GABA-mediated events have been described as prominent features of 4-AP-induced abnormal activity in cortical structures (Avoli et al. 1993, 1994; Barkai et al. 1995; Fueta and Avoli 1992). Moreover, prior studies have demonstrated large-amplitude synchronous IPSPs in neocortical pyramidal cells in the presence of 4-AP and EAA receptor blockers.
FIG. 3. Cortical interneurons may show burst firing in the presence of 4-AP and EAA blockers. A: some cells fired in bursts at rest (bottom) with spikes riding on waves of depolarization. At slow sweep speeds some spikes were attenuated because of insufficient sampling. However, when traces were expanded (in B) it was clear that spikes were indeed of varying amplitudes. C, inset: response to an intracellular current pulse. At more depolarized levels (top traces) an initial hyperpolarization was revealed. Resting potential: -57 mV; maximum spike height: 75 mV. C: photomicrograph of layer I cell recorded in A and B that had been filled with Lucifer yellow. Three cells were filled by the single injection [1 central cell (lower boundary drawn), 1 behind and below this cell, and 1 cell to the left and above the central neuron]. Because of thickness of whole mounted slices, photomicrographs of labeled cells did not allow complete resolution of cellular anatomy. Accordingly, camera lucida drawings like that in D (and subsequent figures) were made to capture multiplanar elements. Calibration in B: 12.5 mV, 1 s (A); 12.5 mV, 100 ms (B). Calibration in C, inset: 12.5 mV, 100 ms. For filled neuron, calibration bar = 100 μm.
FIG. 4. Two additional firing patterns of cortical interneurons in the presence of 4-AP and EAA blockers. Traces illustrate additional types of spontaneous activity encountered in interneurons. A and B: other cells were less excitable, firing an initial action potential at $-73 \text{ mV}$ (bottom) followed by a depolarization on which spikes of different amplitudes occurred, followed by several subthreshold depolarizations (cf. Fig. 3). Depolarization revealed an early hyperpolarization (top). C: layer V cell was confirmed to be an interneuron. C and F, insets: responses to an intracellular current pulse. Resting potential: $-59 \text{ mV}$; maximum spike height: 63 mV. D and E: other cells fired a few initial spikes followed by a depolarization at $-75 \text{ mV}$ (bottom). These spikes were of different amplitudes. At more depolarized levels a triphasic potential could be revealed (top). F: this layer I cell was confirmed to be an interneuron. Here 2 cells were filled with a single injection. Resting potential: $-63 \text{ mV}$; maximum spike height: 67 mV. Calibration in B: 12.5 mV, 1 s (A); 12.5 mV, 100 ms (B). Time calibration in B: 400 ms (D); 40 ms (E). Voltage calibration in D: 12.5 mV (applies to D and E). Calibration in C, inset: 12.5 mV, 50 ms (also applies to F). For filled neurons, calibration bar = 100 μm.

(Aram et al. 1991; Van Brederode and Spain 1995). The present study confirms and extends these previous findings. One notable difference is that in the present study the IPSPs recorded in pyramidal neurons were triphasic. These potentials consisted of fast hyperpolarizing and depolarizing components that were GABA<sub>A</sub> mediated, and a slower component that was GABA<sub>B</sub> mediated. Second, whereas the behaviors of neocortical interneurons during such events were not previously explored, these activities were focused on here. The early GABA<sub>A</sub>-mediated phases of these IPSPs appeared to be generated by the simultaneous firing of presumed GABA<sub>A</sub>ergic interneurons. These inhibitory cells were paced by GABA<sub>A</sub>-mediated synaptic excitation, as is the case in hippocampus (Michelson and Wong 1991), perhaps aided by another mechanism that is independent of chemical synaptic transmission.

The recruitment of inhibitory circuitry was promoted by exposure to the convulsant 4-AP. This agent is rather unique (but see Zhou and Habilitz 1993) in that it acts to enhance inhibitory as well as excitatory synaptic output. There are
FIG. 5. Interneuronal firing coincides with fast IPSPs and both are blocked by picrotoxin application. Traces illustrate simultaneous intracellular recordings of confirmed interneurons (I cells) and pyramidal cells (P cells). A: firing in layer I interneuron occurred coincident with fast hyperpolarizing IPSP recorded in the layer V pyramidal cell. Rhythmic depolarizations in interneurons coincided with repetitive hyperpolarizations in pyramidal cell. Both cells were held at −62 mV. A, right: camera lucida drawing of neurobiotin-filled cells. Interneuronal injection yielded a cluster of 3 filled cells and the pyramidal injection gave only a single filled cell. Interneuron rest potential: −68 mV; maximum spike height: 78 mV. Pyramidal cell rest potential: −64 mV; at −62 mV, presumed I cell in layer VI fired a few spikes at beginning of a triphasic response. A simultaneous IPSP was seen in layer II/III P cell (also at −62 mV). After exposure to picrotoxin, the interneuron became silent. Spontaneous monophasic slow IPSPs were then seen in both interneuronal and pyramidal cell records. In this case, the interneuronal fill revealed a single inverted pyramidal cell and the pyramidal injection revealed a single filled cell (B, right). Interneuron rest potential: −68 mV; maximum spike height: 72 mV. Pyramidal cell rest potential: −71 mV.

several mechanisms that may provide for this enhanced synaptic transmission: the compound enhances release by a presynaptic effect on action potential width (Otis and Mody 1992; Zhou and Hablitz 1996), it produces tetrodotoxin-sensitive repetitive firing in nerve terminals (Llinas et al. 1976; Yeh et al. 1976), and it enhances release of transmitter from synaptosomes (Tibbs et al. 1989). 4-AP–induced enhancement of synaptic neurotransmission by any of the above mechanisms may have facilitated the sympathetically mediated depolarizing GABA response observed in pyramidal cells, and especially in interneurons, where the response was more prominent. Previously, depolarizing GABA responses were observed in pyramidal cells mainly following exogenous applications of GABA (Connors et al. 1988; McCormick 1989). The membrane depolarization in pyramidal cells (and perhaps in interneurons) that is mediated by GABA_A receptors appears to result from an activity-dependent shift in the concentration gradient for chloride (Ling and Benardo 1995b; Staley et al. 1995), and converts the response to one of excitation.

Spike firing in interneurons coincided with the fast component of the synchronized IPSPs recorded in pyramidal cells. Interneurons often fired action potentials that appeared to be initiated by the underlying (GABA_A-mediated) depolarizing envelopes, but these were often intermingled with spontaneous spikes of different amplitudes, and in other cases spikes were seen to arise from baseline without clear generator potentials (Figs. 3–5). These action potential firing patterns suggest that many spikes from interneurons were probably generated from ectopic initiation sites. Although cell firing was blocked in the presence of picrotoxin, the characteristics of the spiking suggested that its occurrence was not depen-
dent on an underlying chemical synaptic event in the cell recorded. It is postulated that the attenuated spikes observed (e.g., the response in Fig. 3B, and the early phase of the responses shown in Fig. 4, B and E) were triggered independently from chemical transmission. These spikes could arise from coupled interneurons that were transmitted electrotonically to the cell recorded.

Dye injection into interneurons frequently resulted in multiple-cell staining. Both Lucifer yellow CH and neurobiotin, which are low-molecular-weight intracellular tracers, appear capable of traversing gap junctions. Dye coupling has been taken as presumptive evidence for electrotonic coupling via gap junctions (Benardo and Foster 1986; Connors et al. 1983; Dudek et al. 1983; Stewart 1978). Moreover, maneuvers aimed at uncoupling cells prevent the passage of both Lucifer yellow (e.g., see Connors et al. 1984; Gutnick and Lobel-Yaakov 1983) and neurobiotin (Peinado et al. 1993; Rorig et al. 1996) in neocortical neurons. However, the relationship between dye coupling and electrotonic coupling remains controversial. Nonetheless, it is tempting to suggest that the subset of interneurons recorded was electrotonically coupled via gap junctions, and that this is the source of the ectopic, attenuated action potentials recorded in these cells. Of course, this property would strongly contribute to the generation of the large, fast, synchronized IPSPs in pyramidal cells seen following 4-AP exposure. But it has been suggested that injury may induce dye coupling (Gutnick et al. 1985). Three arguments against this possibility are the following: 1) pyramidal cells, which have larger dendritic arbors than do many interneurons and thus would be more likely to be damaged in slicing, showed significantly less dye coupling; 2) thin slices, which should include a greater proportion of cells having injured dendrites, did not result in large numbers of dye coupled interneurons (e.g., Kawaguchi 1993, 1995); and 3) purposely injuring cells by severing processes does not result in greater frequencies of dye coupling (unpublished observations).

Dye coupling among interneurons has been noted previously in rat neocortex (Connors et al. 1983), yet Kawaguchi (1993, 1995) did not report multiple fills in studies of interneurons in thin slices maintained at lower tempera-

tures with the use of patch electrodes. Nevertheless, gap junctions have been seen among human and rodent neocortical interneurons (Mollgard 1975; Mollgard and Moller 1975; Peters 1980; Sloper 1972; Sloper and Powell 1978; Smith and Moskovitz 1979). Some of these neurons were found to correspond to basket cells and therefore were postulated to be inhibitory (Sloper and Powell 1978). This hypothesis is supported by a recent study showing that neocortical basket cells (and chandelier cells) (Huntley et al. 1994) may actually define a subpopulation of GABAergic cells (Jones 1993). Gap junctions have also been demonstrated in interneurons in cerebellum (Sotelo and Llinas 1972), hippocampus (Kosaka 1983; Kosaka and Hama 1985), dorsal cochlear nucleus (Mugnaini 1985), and olfactory bulb (Reyher et al. 1991), suggesting that they may be a common feature among central interneurons. Considering the available evidence, it seems reasonable to suggest that gap junctions exist between cortical interneurons and provide for electrotonic coupling between these cells, thereby contributing to the synchronization of inhibition following 4-AP exposure, and perhaps under other conditions. Alternatively, it is possible for functional coupling to persist in cellular systems devoid of organized gap junctions (e.g., Williams and De Haan 1981).

Michelson and Wong (1994) found dye coupling among hilar type II interneurons and postulated that electrotonic coupling maintained the monophasic slow IPSPs recorded in hippocampus following exposure to 4-AP, EAA blockers, and picrotoxin. Type II hilar neurons continued to burst in the presence of these drugs, and this bursting correlated with synchronized slow IPSPs in CA3 pyramidal neurons. Staining of the type I hilar interneurons that form excitatory GABA_A-mediated recurrent connections apparently resulted in only single fills (Michelson and Wong 1994). Nevertheless, the interneurons recorded in the present study appeared to function like hilar type I interneurons, i.e., they were associated with GABA_A-mediated excitatory responses, because GABA_A blockade with picrotoxin suppressed their firing. The fact that a majority of these neocortical interneurons was also found to be dye coupled may reflect another difference in the organization and structure of inhibitory GABAergic neuronal networks in neocortex versus hippocampus (see Ling and Benardo 1995a).
It is difficult to identify the primary mechanism, i.e., GABA$_A$-mediated excitation versus possible electrotonic coupling, that synchronizes and paces interneurons following exposure to 4-AP and EAA antagonists. The two processes appear to function in concert, triggering inhibitory neurons yielding GABA$_A$ or GABA$_B$ IPSPs. However, it appears that as in hippocampus (Michelson and Wong 1994), the monophasic synchronized slow GABA$_A$-mediated IPSP may be entrained and paced by the excitatory GABA$_A$ mechanism, because, before the addition of picrotoxin: 1) synchronized IPSPs (in pyramidal cells) were all triphasic; 2) slower, monophasic IPSPs never appeared in isolation; and 3) synchronous IPSPs occurred at faster frequencies. Electrotonic coupling between interneurons giving rise to GABA$_A$ and GABA$_B$ IPSPs seems unlikely because the interneurons recorded here did not continue to fire spontaneously during or before slow IPSPs once GABA$_A$ receptors were blocked with picrotoxin. So, although interneuronal aggregates may be synaptically coupled via excitatory GABA$_A$ transmission, the populations of interneurons giving rise to GABA$_A$ and GABA$_B$ IPSPs appear to be distinct. Moreover, these cells are segregated into potentially separate networks, which under the proper set of circumstances appear capable of functioning independently, with autonomous pacing.

Unfortunately, cells whose firing correlated with synchronized slow IPSPs were not encountered in the present study. Two possible reasons for this failure to record from GABA$_A$ interneurons are that the cells giving rise to slow GABA$_A$ IPSPs appear to be sparser (Benardo 1994) and may be located at distinct anatomic sites (Benardo 1994; Kang et al. 1994) that were not probed in the present study. It is possible that the interneuronal population that gives rise to GABA$_A$ IPSPs is also coupled, like its analogues, the hilar type II cells in hippocampus, and that this property supports the occurrence of spontaneous synchronous slow IPSPs seen following exposure to picrotoxin. Alternatively, presynaptic effects of 4-AP may initiate other transmitter systems (e.g., noradrenergic, metabotropic glutamatergic, etc.), that in turn excite GABA$_B$ interneurons, triggering slow IPSPs, although the (muscarinic) cholinergic system does not appear to participate (unpublished observations). Resolution of this issue awaits recording from these elusive cells that give rise to slow IPSPs.

The findings of the present study underscore the structural and functional heterogeneity that has been suggested to exist within the population of neocortical inhibitory interneurons (Benardo 1993, 1994; Kagwaguchi 1993, 1995; Kagwaguchi and Kubota 1993; see Benardo and Wong 1995 for review). Thus it appears that interneurons giving rise to GABA$_A$ or GABA$_B$ IPSPs can be separately activated either singly (Benardo 1994) or synchronously, forming separate networks (this study). In addition, the interneurons may be differentially recruited synchronically (Benardo 1993), as a consequence of the types of presynaptic glutamate receptors they possess (Ling and Benardo 1995a). Further research will surely shed additional light on the structural and functional properties that differentiate inhibitory interneurons as GABA$_A$ or GABA$_B$ type.

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