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Whole-Cell Voltage-Clamp Study of the Fading of GABA-Activated Currents in Acutely Dissociated Hippocampal Neurons

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SUMMARY AND CONCLUSIONS

1. The lability of the responses of mammalian central neurons to γ-aminobutyric acid (GABA) was studied using neurons acutely dissociated from the CA1 region of the adult guinea pig hippocampus as a model system. GABA was applied to the neuronal somata by pressure ejection and the resulting current (I_{GABA}) recorded under whole-cell voltage clamp.

2. In initial experiments we examined several basic properties of cells in this preparation. Our data confirm that passive and active membrane properties are similar to those which characterize cells in other preparations. In addition, GABA-dependent conductance (g_{GABA}), reversal potential (E_{GABA}), and the interaction of GABA with pentobarbital and bicuculline all appeared to be normal. Dendritic GABA application could cause depolarizing GABA responses, and somatic GABA application caused hyperpolarizations due to chloride (Cl^-) movements.

3. Repetitive brief applications (5–15 ms) of GABA (10^{-5} to 10^{-3} M) at a frequency of 0.5 Hz led to fading of successive peaks of I_{GABA} until, at a given holding potential, a steady state was reached in which I_{GABA} no longer changed. Imposing voltage steps lasting seconds during a train of steady-state GABA responses led initially to increased I_{GABA} that then diminished with maintenance of the step voltage.

4. The rate of decrease of I_{GABA} at each new holding potential was independent of the polarity of the step in holding potential but was highly dependent on the rate of GABA application. Application rates as low as 0.05 Hz led to fading of I_{GABA}, even with activation of relatively small conductances (5–15 nS).

5. Since I_{GABA} evoked by somatic GABA application in these cells is carried by Cl^-, the Cl^- equilibrium potential (E_{Cl}) is equal to the reversal potential for I_{GABA}, i.e., to E_{GABA}. The fading of I_{GABA} with changes in holding potential can be almost entirely accounted for by a shift in E_{Cl} resulting from transmembrane flux of Cl^- through the GABA-activated conductance.

6. Maneuvers that prevent changes in the intracellular concentration of Cl^- ions, $[Cl^-]_i$, including holding the membrane potential at E_{GABA} during repetitive GABA application or buffering $[Cl^-]_i$ with high pipette $[Cl^-]$, prevent changes in E_{GABA}.

7. Desensitization of the GABA response (an actual decrease in g_{GABA}) occurs in these neurons during prolonged application of GABA (>1 s) but with a slower time course than changes in E_{GABA}.

8. Whole-cell voltage-clamp techniques applied to tissue-cultured spinal cord neurons indicated that rapid shifts in E_{GABA} result from repetitive GABA application in these cells as well.

9. Shifts in E_{GABA} combine with desensitization to reduce the size of I_{GABA}. These changes would act in concert in vivo to decrease the amplitude of inhibitory postsynaptic potentials during repetitive activation of inhibitory interneurons.

INTRODUCTION

Factors affecting the efficacy of inhibitory postsynaptic potentials (IPSPs) are very im-

portant in the regulation of neuronal excitability in mammalian central nervous system (CNS). Removal or blockade of inhibition mediated by γ-aminobutyric acid (GABA) leads to abnormal neuronal activity, including epileptiform discharges (see Ref. 2 for review). CNS IPSPs are usually assumed to be inhibitory by virtue of both the membrane conductance increase and the consequent hyperpolarization caused by the inhibitory neurotransmitter (e.g., Refs. 17, 25). Decreases in IPSP driving force, and hence the membrane hyperpolarization, decrease inhibition. Indeed, a dramatic decrease in inhibition occurs when ammonia causes the collapse of the Cl^- gradient, perhaps by blocking an outwardly directed "Cl^- pump," in certain CNS neurons (29, 39).

The major inhibitory neurotransmitter in the vertebrate CNS is GABA (e.g., Ref. 27). Repetitive activation of afferent pathways in CNS results in depression of IPSPs (18, 29, 31, 43), and this may account in part for the marked increase in neuronal excitability caused by repetitive stimulation (8). Studies of alterations in the efficacy of GABA-ergic IPSPs, or of the action of directly applied GABA, have implicated several factors: the desensitization process (1, 10, 14, 36, 47), the overlapping of hyperpolarizing and depolarizing GABA responses (3, 4, 47), shifts in E_{GABA} (1, 9, 16, 31), and block of the IPSP neuronal pathway per se (31). Investigations on neurons in vivo or in the in vitro slice preparation are thus complicated, and many details of the mechanism of IPSP depression remain unexplored. Ideally, study of the postsynaptic factors in IPSP depression would involve directly applying GABA to the postsynaptic membrane. However, use of repetitive GABA application to mimic the repetitive elicitation of IPSPs is unlikely to produce readily interpretable results in the slice preparation, chiefly because of the multiplicity of GABA receptors typically activated by GABA application in the slice (4, 7, 15, 44, 47), the presence of GABA uptake systems (28), difficulty in applying GABA reliably to a particular site on the neuron, and so forth.

The current report is a whole-cell voltage clamp study of pyramidal neurons acutely dissociated from the CA1 field of the adult guinea pig hippocampus (36). We addressed the following questions. *1*) What postsynaptic factors affect GABA responses? *2*) Can shifts in E_{GABA} be caused solely be repetitive application of small amounts of GABA? *3*) What are the relative contributions of desensitization and changes in driving force to the changing responses? Since these neurons are totally isolated and can be visualized directly, GABA application can be localized precisely to the neuronal somata. Under these conditions, it appears that the major factor affecting the response to repetitive or prolonged application of GABA is the lability of the intracellular concentration of Cl^-, although desensitization plays a role. A preliminary report of this work has appeared (24).

METHODS

Pyramidal cells were isolated from adult guinea pig hippocampus by the method of Numann and Wong (36). After decapitation of the animal, one hippocampus is removed from the brain and placed briefly in chilled (0°C) physiological saline. Slices, 700 μm thick, are then obtained by sectioning transverse to the longitudinal axis of the hippocampus with a tissue chopper. Each slice is further subdivided with a scalpel blade into $\sim$1-mm^3 chunks that contain the cell field of interest for this study, the CA1 area. The tissue chunks are then incubated in a gently stirred saline solution containing enzyme. The physiological saline consists of (in mM): NaCl, 138; $NaHCO_3$, 12; KCl, 5.0; $MgCl_2$, 2; $CaCl_2$, 2; and glucose, 10. We have used papain type IV (1.5 mg/ml, Sigma Chemical) or trypsin type IX (1 mg/ml, Sigma Chemical) in a balanced physiological saline solution. The enzymatic solution is oxygenated and warmed to 31°C.

After 1–3 h in the enzyme solution, one or two tissue chunks are removed, washed in fresh saline, and then triturated using fire-polished Pasteur pipettes, in a small volume ($\sim$1 ml) of saline. The cell suspension is transferred to a small tissue culture dish on the stage of an inverted microscope, and the cells are allowed to settle and adhere to the bottom for 8–12 min before constant superfusion is started. Experiments were performed at room temperature (24°C).

Viable cells appear bright under phase contrast and have a three-dimensional appearance under Hoffman interference contrast optics. Although the yield is variable, we typically obtained 20–100 viable cells per dissociated chunk of tissue. The cells adhere more or less firmly to the bottom of the culture dish. Most cells are pyramidal in shape (Fig. 1*D*), but many have nonpyramidal features such as small round soma (<10 μm) or lack of a major apical dendrite. Data reported in this study were obtained from 57 pyramidal shaped neurons.

The whole-cell clamp configuration of the patch-

clamp technology was used to record from these neurons (19, 23). Microelectrodes were pulled from 1.5-mm thin-wall borosilicate capillary glass (WPI) on a vertical electrode puller, using the double-pull method. Tip diameters ranged from 1 to 3 μm, and electrode resistance measured in the bath was 2–8 MΩ. A typical electrode-filling solution consisted of (in mM) potassium gluconate, 122; $MgCl_2$, 2; ethyleneglycol-bis(β-aminoethylether)-*N,N'*-tetra-acetic acid (EGTA), 11; $CaCl_2$, 1; and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10. The pH was adjusted to 7.3 with KOH. Pipette [Cl^-] was altered by substituting equimolar KCl for potassium gluconate.

Pyramidal cells in the guinea pig hippocampus are electrotonically compact; the entire cell in vitro has an electronic length of <1.0 (11, 26, 46). Since the acutely dissociated neurons are physically more compact and yet otherwise have similar membrane properties (see below), they are electrotonically shorter than this value. Combining this consideration with our ability to restrict GABA application to the soma immediately adjacent to the recording pipette means that voltage control of the responses was good.

The recording amplifier was a Dagan 8900 patch clamp/whole-cell clamp. Series resistance was compensated according to the electrode resistance in the bath. Liquid junction potentials between the microelectrodes and the bath solution were measured as described by Hagiwara and Ohmori (22) and ranged from −5 to −13 mV (inside of electrode negative). These values were added to the applied potential to obtain the actual clamp potential. Data were recorded on chart recorder (Gould 220) and on FM tape with a bandwidth of DC–1,250 Hz (Hewlett-Packard). Some records were digitized and analyzed by using an on-line data acquisition system (pClamp, Axon Instruments).

After forming a gigaohm seal (typically 2–10 GΩ) in the cell-attached configuration and adjusting the capacitance neutralization circuit, a whole-cell clamp was obtained by applying more suction to the pipette. Two methods were used to determine when access was gained to the inside of the cell. *1)* While in voltage clamp, small voltage steps were applied and the current record was monitored (23). Access to the cell interior was signified by a large increase in the capacitive transients at the onset and offset of the current responses. *2)* While in current clamp, small constant current pulses were delivered and the voltage record was monitored (Fig. 1*A*). Whole-cell recording was indicated by a sudden shift in the potential and decrease in input resistance (arrow, Fig. 1*A*).

These neurons have properties similar to those recorded from either in vivo or the in vitro hippocampal slice preparation, with one exception, input impedance, which is higher than that obtained in the other preparations. In our hands the acutely dissociated neurons had resting potentials in the range of −45 to −70 mV (n = 52) and overshooting action potentials with amplitudes of >70 mV and widths of <3 ms (n = 10). Healthy neurons responded to increasing depolarizing currents with increasing numbers of action potentials (e.g., Fig. 1*B*). In the slice preparation this assay has been used as a measure of cell health (41). The membrane time constant ranged from 20 to 36 ms (n = 8; e.g., Fig. 1*C*).

As mentioned, the major difference is the very high input impedance, ranging from 200 to 1,200 MΩ in isolated cells, compared with 30–100 MΩ for pyramidal cells recorded in the slice preparation (e.g., Ref. 31). In Fig. 1*A* responses to very small (12 pA) hyperpolarizing current pulses indicate an input impedance of ~700 MΩ. This high input impedance can probably be explained in part by the decrease in cell damage that occurs when using gigaohm-seal techniques and in part by the truncation of distal dendritic processes during dissociation, and the resultant decrease in membrane surface area of each neuron, rather than by a change in the membrane itself. As pointed out by Wong and colleagues (personal communication) this latter explanation is based on two arguments. *1)* The membrane time constant of these neurons is similar to that obtained in other preparations, indicating no difference in the specific membrane resistivity (assuming no change in specific membrane capacitance). *2)* Applying estimates of specific membrane resistivity for CA1 pyramidal cells (11) to the membrane surface area calculated from photomicrographs of the neurons result in calculated input impedances within 20% of the observed values. We calculated the membrane surface area for several cells using one of the following: *1)* "ball-and-stick" geometry for neurons with rounded somata (e.g., Fig. 1*E*, upper right panel) and one major dendrite (in this case somal diameter, dendritic diameter, and dendritic length were measured); or *2)* "truncated cone geometry" for neurons with pyramidal shaped somata (Fig. 1*E*, lower two panels) (in this case basal diameter, smallest diameter, and cell length were measured; e.g., the neuron from which the data in Fig. 7 were obtained had a calculated input resistance of 800 MΩ and a measured input resistance of 670 MΩ).

GABA (10^{-5} to 10^{-3} M in saline) was applied via a nearby pressure ejection micropipette (30). Drug solutions were applied by either bath superfusion or via a secondary pressure pipette. Drugs were obtained from Sigma Chemical (St. Louis, MO). Photomicrographs were obtained on black and white negative film, usually under Hoffman modulation contrast optics with a ×40 objective. Each of the experiments reported in this paper has been replicated on at least three neurons.

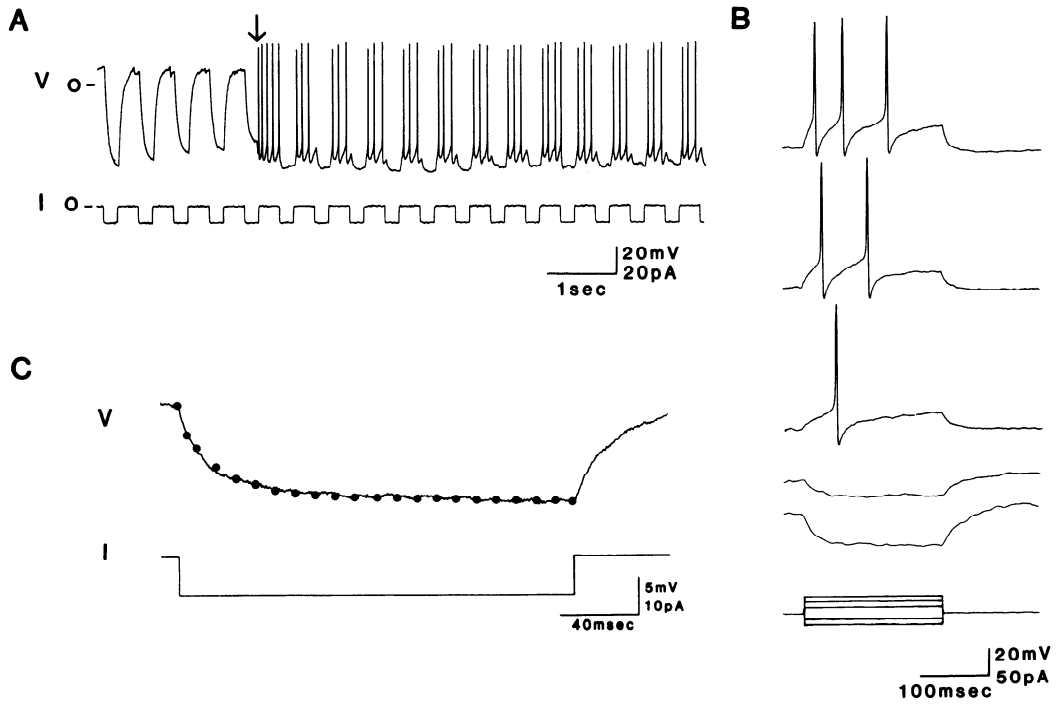

FIG. 1. Electrophysiological and morphological features of acutely dissociated neurons. *A*: rupturing the membrane to obtain a whole-cell recording under current clamp. A GΩ seal has been formed on a neuron and the amplifier is switched to current clamp mode; 200-ms constant current hyperpolarizing pulses (−12 pA) are applied at 1 Hz. Large voltage deflections are due to high (6 GΩ) seal resistance. When a pulse of suction is applied to the pipette interior (*arrow*), whole-cell recording is obtained as shown by the sudden negative shift in potential and the appearance of action potentials. Initial resting potential = −57 mV. *B*: constant current pulses are applied to different cell to elicit active and passive membrane responses. Increasing depolarizing current results in increased numbers of action potentials being elicited. *Lowest trace* is the current record. *C*: passive charging of membrane in hyperpolarized direction; response to −10 pA current. Values represented by *filled circles* were determined from the least-squares regression line for an exponential charging with a time constant of 20.0 ms. *D*: sample neurons isolated from adult guinea pig hippocampus. Photomicrographs were taken with a ×40 objective under Hoffman interference modulation microscopy. *Scale bar* in *upper-right panel* is 20 μm. Dendritic arborizations of neurons obtained from enzymatic and mechanical dissociation technique are truncated.

RESULTS

Since this preparation is relatively new, in initial experiments we extended the characterization of GABA responses obtained from the isolated neurons.

GABA responses are similar to those obtained in other preparations. Figure 2*A1* shows the currents induced by ejection of GABA-containing solutions onto the membrane of a voltage-clamped pyramidal cell. The response is voltage sensitive and monophasic with a peak conductance of 17.6 nS and a reversal potential of −64 mV (Fig. 2*A2*). Typically, GABA responses ranged from 5 to 50 nS. The falling phase of monophasic GABA-induced current (I_{GABA}) decayed with a single exponential time course at all potentials. The time constant for monophasic I_{GABA} decay was the same whether the current was inward (mean ± SE, 402.7 ± 64.8 ms, n = 10) or outward (mean ± SE, 397.7 ± 60 ms, n = 10). Biphasic responses could be obtained in some neurons with longer applications of GABA (>50 ms) to the dendritic, but not the somatic, portion of the cell. This is probably because the receptors responsible for the depolarizing phase of the biphasic response are located primarily on the dendrites (4) and the majority of the dendrites are removed during the dissociation process.

Bicuculline methiodide inhibits GABA responses and GABA IPSPs in the vertebrate CNS (e.g., Ref. 27). Figure 2*B* illustrates that

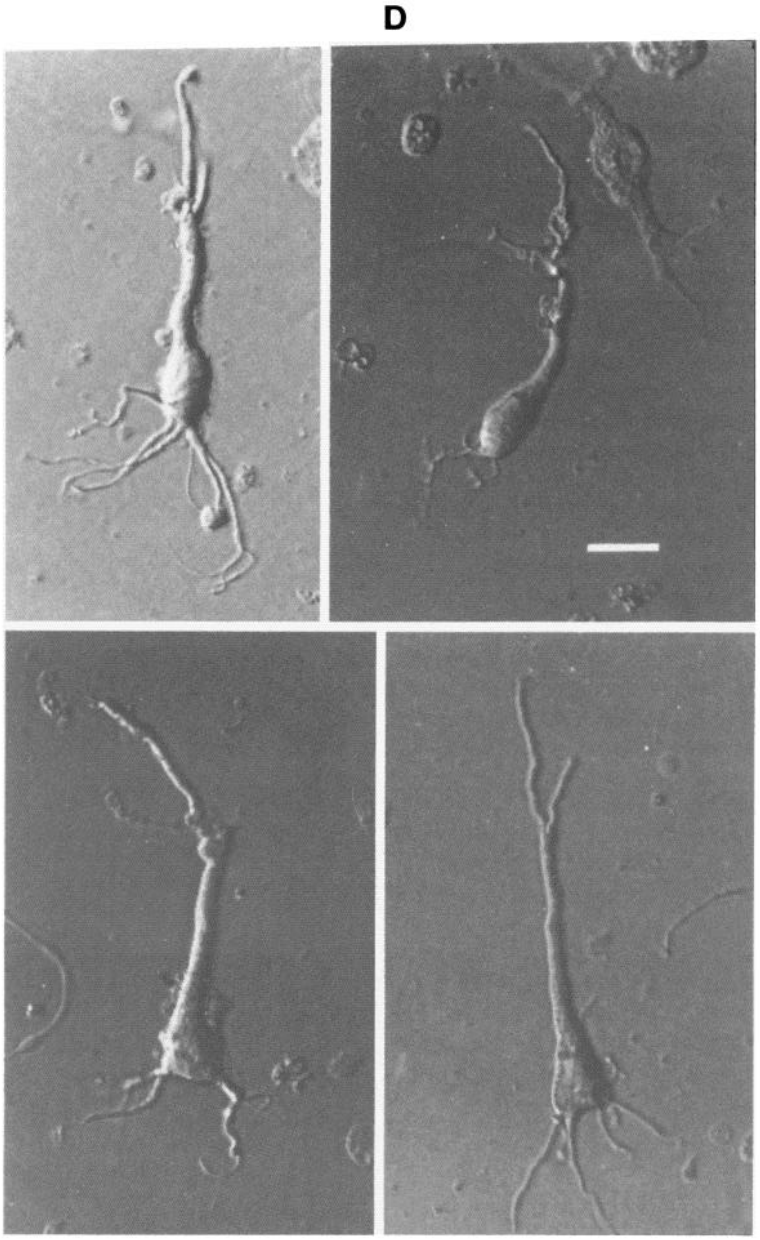

FIG. 1. (*Continued*)

this antagonist rapidly and reversibly inhibits I_{GABA} in acutely dissociated neurons. Barbiturates, specifically pentobarbital, augment GABA responses and IPSPs in the hippocampus (3, 12, 44). The addition of 100 μM pentobarbital to the superfusion medium reversibly prolongs I_{GABA} in isolated neurons (Fig. 2*C*). [Notice that, even in the presence of pentobarbital, the I_{GABA} remains monophasic, whereas a biphasic response would have resulted if depolarizing GABA receptors had been activated (3).]

The ionic dependency of the GABA responses was determined and found to be due to Cl^- current. For example, Fig. 3 shows the GABA currents obtained in two different cells

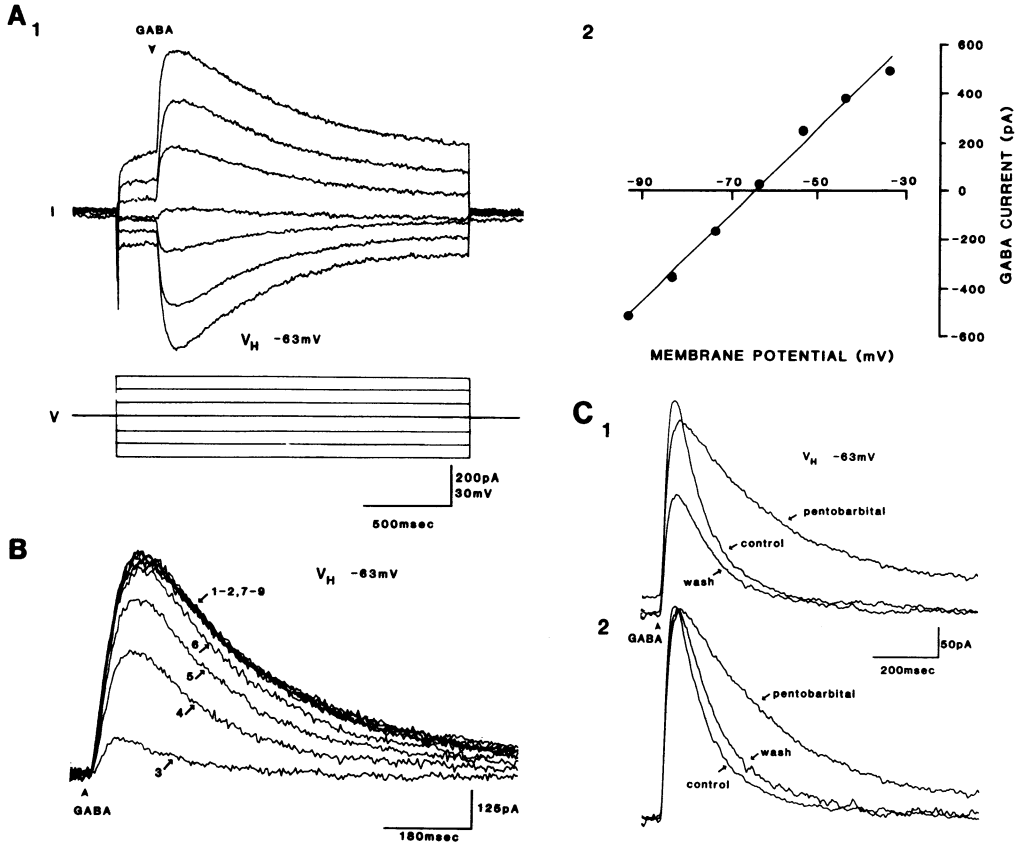

FIG. 2. GABA responses in isolated hippocampal neurons. *A1*: voltage clamp responses to GABA (1 mM, 10 ms, 80 PSI) applied via pressure ejection from a nearby micropipette. *Upper trace* is current (*I*), and *lower trace* is potential (*V*). *A2*: peak GABA response amplitudes from *A1* are plotted. Response is voltage dependent with reversal potential of −64 mV and conductance of 17.6 nS. Pipette $[Cl^-]$ = 14 mM. GABA current was measured as the difference between the peak GABA response and current 10 ms before the onset of the response. *B*: blockade of GABA response by bicuculline methiodide. GABA (1 mM, 10 ms, 50 psi) was applied to a voltage-clamped neuron at a frequency of 0.2 Hz for 9 consecutive responses. Between pulses 2 and 3, bicuculline methiodide (BMI, 100 μM, 500 ms, 5 psi) was applied via a second microejection pipette. BMI produces a nearly complete blockade of the GABA response, which is readily reversible. *C1*: prolongation of GABA response by pentobarbital. GABA (1 mM, 30 ms, 40 psi) was applied to voltage-clamped neuron at a frequency of 0.25 Hz. After recording control responses, saline containing 100 μM pentobarbital was perfused and then the control saline was washed in again. Responses in *C1* are averages of 8 traces each in control, after 4.5 min in pentobarbital, and after 4 min of wash. Because GABA current decreased gradually during the experiment, responses were normalized for peak amplitude in *C2*. Pentobarbital produces a marked, reversible slowing in the rate of decay of the GABA current. The time constants of decay (calculated from least-squares regression lines) were control, 131 ms; pentobarbital, 240 ms; and wash, 155 ms.

recorded with 6 (Fig. 3*A*) or 68 mM Cl^- (Fig. 3*B*) in the intracellular pipette. The current-to-voltage (*I–V*) relations for the peak responses are shown in Fig. 3*C*. The reversal potential for I_{GABA} (E_{GABA}) is much less negative in the neuron recorded with pipette $[Cl^-]$ of 68 mM. Figure 3*D* illustrates data from 33 cells showing the relation between E_{GABA} and $[Cl^-]_i$. The line has a slope of 59 mV/decade change in $[Cl^-]_i$, which is predicted by the Nernst equation for a purely Cl^--dependent response under our recording conditions. This observation agrees with the observation of monophasic responses to somatic GABA application, and with the effects of pentobarbital, (see above) in indicating that our somatic GABA application affected hyperpolarizing GABA receptors exclusively. The actual dependence of E_{GABA} on $[Cl^-]_i$ would be identical to the predicted relation except for the deviation at 2

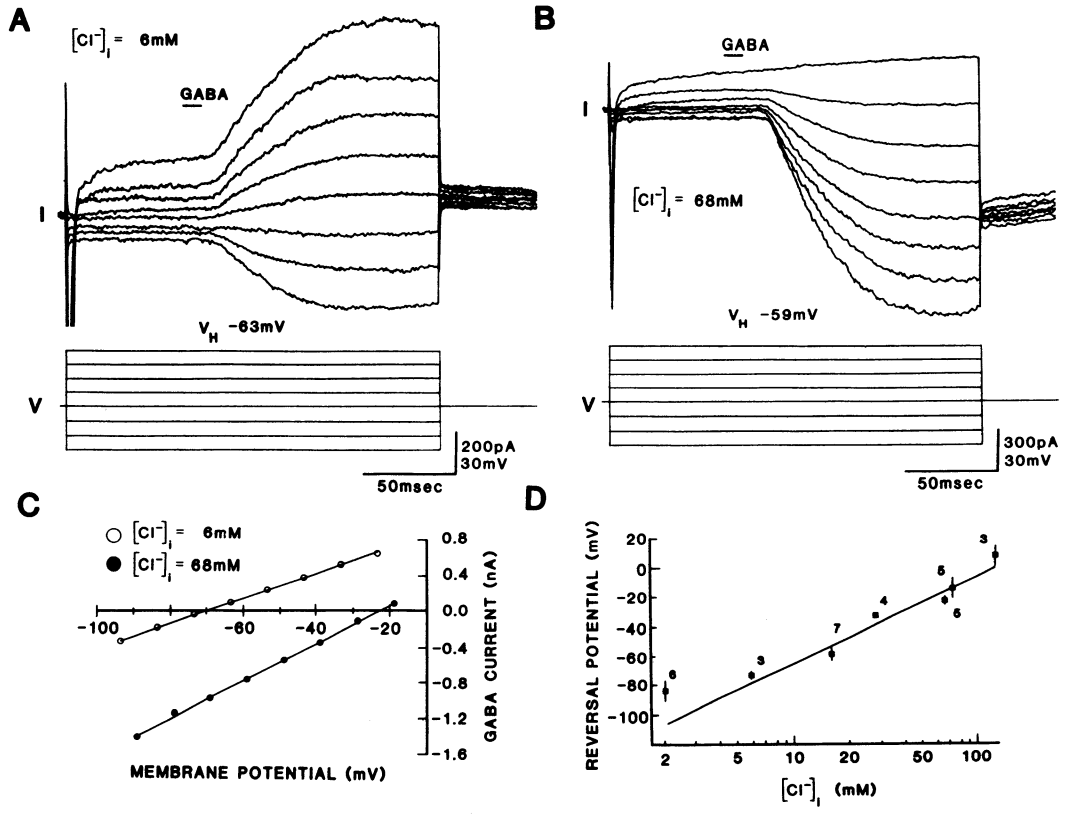

FIG. 3. Chloride dependence of GABA response. *A*: GABA currents recorded with a whole-cell clamp pipette containing 6 mM Cl^-. GABA (500 μM, 10 ms, 40 psi) was applied at 0.5 Hz. *B*: GABA currents obtained in a different neuron with pipette $[Cl^-]$ = 68 mM. GABA (500 μM, 10 ms, 50 psi) was applied at 0.5 Hz. *C*: GABA *I–V* curves for the 2 neurons in *A* (*open circles*) and *B* (*filled circles*). Reversal potential and conductance for *A* were −70.2 mV and 13.7 nS, respectively; for *B* corresponding values were −23.0 mV and 20.7 nS. *D*: E_{GABA} reversal potentials vs. pipette $[Cl^-]$ from 33 neurons. Number of cells recorded under each condition is noted on graph. Individual cells were maintained at constant holding potential (between −60 and −65 mV) and stepped for 200 ms to other potentials to determine E_{GABA}. Line has a slope of 59 mV (predicted from the Nernst equation for Cl^- at a temperature of 24°C).

mM. This deviation is probably due to inadequate buffering of $[Cl^-]_i$ in this region (see DISCUSSION).

Given these similarities between GABA responses in the dissociated neuron and in other preparations, we proceeded to examine factors affecting the lability of the responses.

Lability of GABA-activated currents during repeated application of GABA

We began by trying to determine if shifts in E_{GABA} actually occur, because relatively little is known about this phenomenon. GABA was applied at a constant frequency from an ejection pipette positioned approximately 10 μm from the cell soma. The application rate was usually 0.5 Hz. We selected this protocol because the successive GABA responses achieved a steady state at a given frequency of occurrence and holding potential and, thus, desensitization could initially be ruled out as an explanation for sudden changes in I_{GABA}. A typical experiment is shown in Fig. 4.

Figure 4 shows an isolated pyramidal cell voltage clamped at a membrane holding potential of −62 mV. As the GABA ejection pipette was positioned near the soma of the cell, transient inward currents occurred with each pulse of GABA (pipette $[Cl^-]$ in this experiment was 14 mM). When the holding potential was hyperpolarized by 10 mV to −72 mV, I_{GABA} became larger at first but faded with successive applications. When the holding potential was returned to its initial value, I_{GABA} was outward,

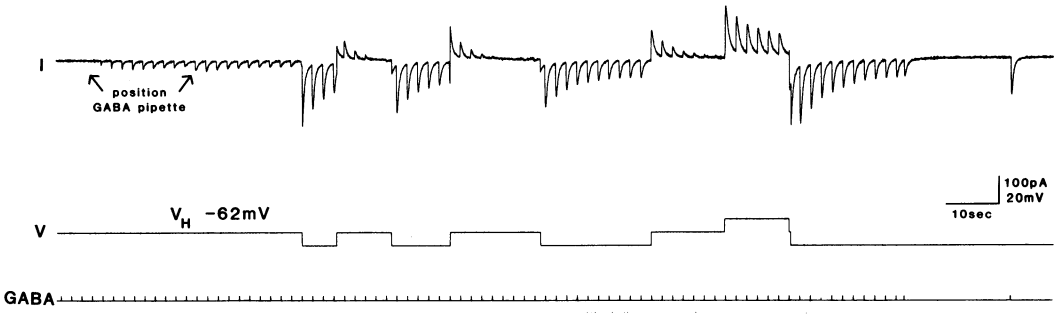

FIG. 4. Repetitive GABA application leads to rapid changes in E_{GABA}. A whole-cell clamp (pipette $[Cl^-]$ = 14 mM) was obtained and holding potential was set to −62 mV. Pressure pulses of GABA (1 mM, 20 ms, 50 psi) were applied at 0.5 Hz. As the GABA pipette was positioned near the cell, inward current responses appeared. When the holding potential was changed to −72 mV, responses became larger. After 6.3 s at −72 mV, the holding potential was returned to −62 mV, and GABA responses were then outward, indicating a change in E_{GABA}. Steps to −72 mV were repeated and then a step to −52 mV was given followed by a return to −62 mV. Note with each change in E_{HOLD} a gradual reduction in response amplitude. Finally, after several pulses were skipped, the response to a single GABA pulse was greatly enhanced.

clearly indicating that a shift in E_{GABA} had taken place. As can be seen in Fig. 4, each change in holding potential resulted in a GABA current that was initially large but faded with repeated applications at the same potential. Shifts occurred rapidly, within 2–10 s.

If the fading of I_{GABA} in Fig. 4 were due to the contribution of another conductance in addition to Cl^-, voltage steps of different polarities should produce different effects. For example, if an incrementing inward current contributed to the gradual decrease in outward current during depolarizing steps, then during hyperpolarizing steps, when I_{GABA} itself was initially inward, the additional incrementing inward current should result in an increase, or at least a slower rate of change, in successive GABA currents. Although changes in I_{GABA} during trains of GABA pulses appeared to be the same regardless of the polarity of the voltage step, subtle differences might still exist. Therefore, we also compared the time courses of change in I_{GABA} when stepping to hyperpolarizing or depolarizing potentials. Figure 5 shows that no systematic difference occurred in the rate of change of I_{GABA} whether the steps were depolarizing or hyperpolarizing. The upper panels depict the responses at two different application rates (0.5 Hz in *A* and 0.05 Hz in *B*), and the lower panels show the rate of decay of I_{GABA} on semilogarithmic scales. The rate of decay of I_{GABA} was independent of the direction of voltage step but highly dependent on the rate of GABA application (cf. Fig. 5, *C* and *D*). The rate of decrease in I_{GABA} was reduced with slower application rates [10.0 ± 0.89 s at 0.5 Hz (Fig. 5*C*) vs. 43.5 ± 4.1 s at 0.05 Hz (Fig. 5*D*)].

Figure 5*B* also illustrates that overlap of I_{GABA} during trains of GABA pulses (e.g., Figs. 4*A* and 5*A*) was not necessary to produce the fading of I_{GABA}. At a rate of 0.05 Hz, I_{GABA} disappeared completely between pulses. However, even in this case, the peak I_{GABA} faded after each change in holding potential.

At this point the extent to which desensitization might contribute to the fading of I_{GABA} was not clear. For example, toward the end of the experiment in Fig. 4, nine GABA pulses were skipped. This resulted in an increase in the size of the last GABA response that could have been due to either a recovery of the transmembrane chloride distribution (a change in E_{GABA}) or recovery from desensitization (a change in g_{GABA}).

Contribution of driving force and conductance to changes in I_{GABA}

The protocol illustrated in Fig. 6 was designed to address this issue. Twin hyperpolarizing voltage pulses (20 mV, 40 ms) were applied to the membrane immediately before the onset of I_{GABA} and at its peak to measure membrane conductance. The difference in the currents elicited by the two conductance pulses provides an estimate of g_{GABA}. The holding potential was altered periodically to elicit

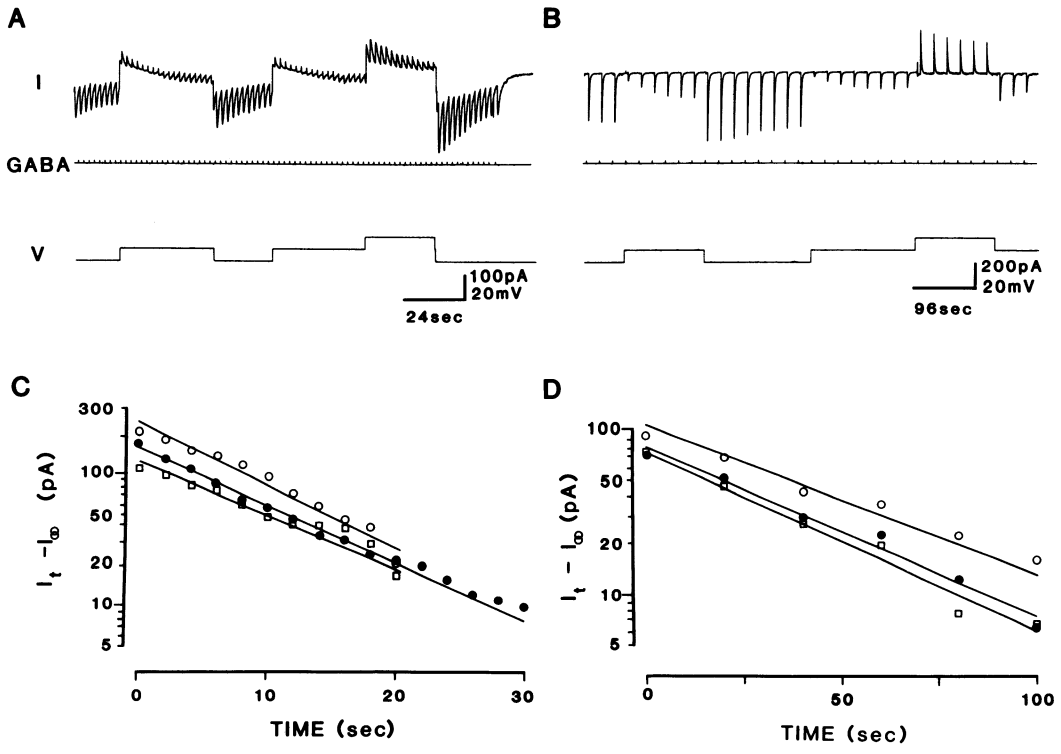

FIG. 5. The time course of the change in I_{GABA} is dependent on rate of application but not change in potential. *A*: GABA was applied repetitively (1 mM, 10 ms, 80 psi) at 0.5 Hz while potential was varied through the following sequence of voltages: −72, −62, −72, −62, −52, and −72 mV. GABA responses diminish after each change in holding potential. Pipette $[Cl^-]$ = 14 mM. *B*: in the same neuron GABA was applied repetitively at 0.05 Hz while potential was varied through same sequence of voltages as in part A. GABA responses slowly diminish after each change in holding potential. *C*: semilog plots of the rate of decay of peak GABA currents from *A* at different potentials (*open square*, currents on transition from −62 to −72 mV, time constant = 10.6 s; *filled circle*, from −62 to −52 mV, time constant = 10.4 ms; and *open circle*, from −52 to −72 mV, time constant = 8.96 s). *D*: semilog plots of the rate of decay of peak GABA currents from *B* at different potentials (*open square*, currents on transition from −72 to −62 mV, time constant = 39.9 s; *open circle*, from −62 to −72 mV, time constant = 48 s; and *closed circle*, from −62 to −52 mV, time constant = 42.7 s).

changes in I_{GABA}. Traces in the lowest row in Fig. 6 are expanded portions of the upper, continuous trace taken at the numbered points. Trace 1 and the initial portion of trace 2 show the responses to the first and last pulses of GABA applied at the holding potential of −72 mV. The peak currents elicited at the holding potential declined throughout this period, however, g_{GABA}, as measured by the current step during the second conductance pulse, was apparently not affected. The same general pattern was observed throughout the course of the experiment. Trace 2 shows the responses before and immediately after the voltage step from −72 to −42 mV. Responses just before and after the return step from −42 to −72 mV are indicated in trace 3. Trace 4 shows a response immediately on returning to −72 mV and trace 5 shows the response 40 s later. In each case I_{GABA} showed a time-dependent reduction (or even reversal, cf. 4 and 5) with little change in g_{GABA}.

Experiments like that shown in Fig. 6 made possible a computation of an estimate of the reversal potential of I_{GABA} during each pulse from

$$I_{GABA} = g_{GABA}(E_{HOLD} - E_{GABA}) \quad (1)$$

where E_{HOLD} is the membrane holding potential and I_{GABA} is the peak GABA current. GABA conductance was calculated according to the following formula

$$g_{GABA} = (\Delta I_{peak} - \Delta I_{base\ line})/\Delta E \quad (2)$$

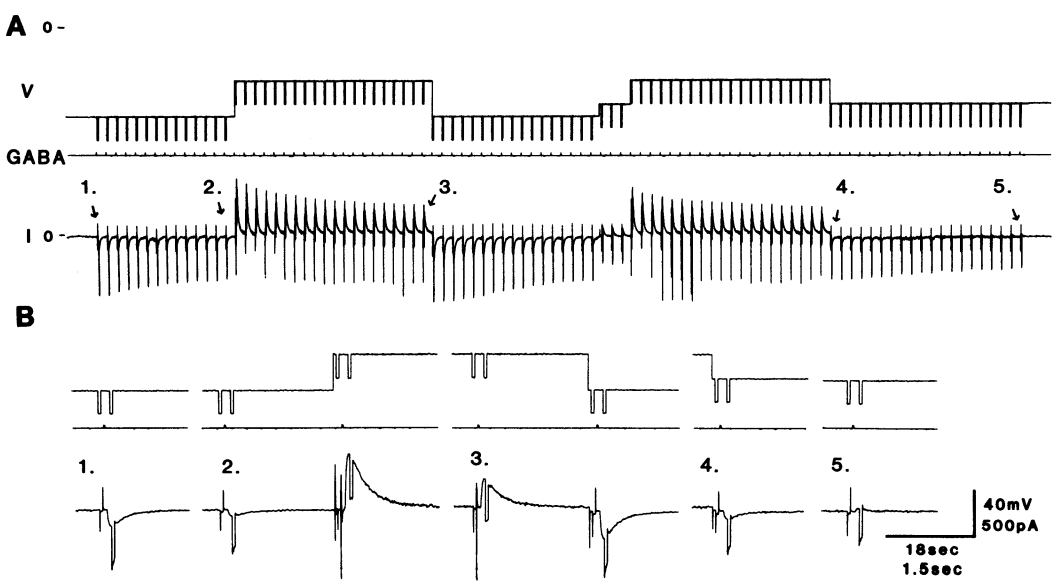

FIG. 6. Conductance changes during change in GABA currents. *A*: in this experiment, GABA (1 mM, 20 ms, 60 psi) was applied at 0.5 Hz, and a 40-ms 20-mV hyperpolarizing pulse was applied just before and at peak of each GABA response (see lowest row for details). Pipette [Cl^-] = 14 mM. In this way any change in conductance that occurred during repetitive GABA application could be monitored. *B*: selected records from experiment shown in *A*. *1*: initial GABA response at onset of train of repetitive pulses; *2*: 2 responses, just before and just after, first transition in holding potential (−72 to −42 mV); *3*: 2 responses, just before and just after, second voltage transition (−42 to −72); *4*: initial response on returning holding potential to −62 mV (inward); *5*: final response at holding potential to −62 (outward). In each case conductance activated during peak of GABA current is approximately the same (see Fig. 7). Therefore changes in I_{GABA} current are due to changes in E_{GABA}.

where ΔI_{peak} is the current response to a conductance pulse delivered at the peak of the GABA response, $\Delta I_{base\ line}$ is the current response to a conductance pulse just before the GABA ejection, and ΔE is the magnitude of the conductance pulse, −20 mV. Figure 7 plots the result of such computations for the experiment shown in Fig. 6. During the experiment g_{GABA} remained at approximately 15 nS, but I_{GABA} changed rapidly with each pulse. The change in current can be explained mainly by the change in driving force, seen in the lowest panel as a change in E_{GABA}, toward the holding potential in each case.

In Fig. 7 estimates of E_{GABA} were based on a two-point linear extrapolation. Although g_{GABA} has been reported to be a nonlinear function of membrane potential (12, 21, 37, 42), this was not prominent within the voltage range studied in our experiments. When nonlinearity was apparent, it was in the same direction as found by others, but very minor (e.g., Fig. 8*C*). Nevertheless, in some experiments we also measured E_{GABA} directly following step changes in E_{HOLD}.

A representative experiment of this type is shown in Fig. 8. GABA ejection was repeated at 0.5 Hz during 200-ms voltage steps at E_{HOLD} = −63 mV (Fig. 8*A*). After E_{HOLD} was changed to −43 mV, four GABA pulses were given and then a series of GABA responses was obtained with the same step voltage commands (Fig. 8*B*). The shift in holding potential resulted in a 9-mV change in E_{GABA} from −73 to −64 mV (Fig. 8*C*). This change was reversible within 1 min after returning to the original holding potential (not shown). The slight decrease in the maximum conductance activated by GABA was due to the gradual long-term fading in the GABA response that was observed in several experiments (e.g., Fig. 7, middle panel). Whether this is due to desensitization is not known. In any case, this gradual effect cannot explain the rapid alterations in I_{GABA}. Direct measurements of E_{GABA} gave the same result as our previous indirect

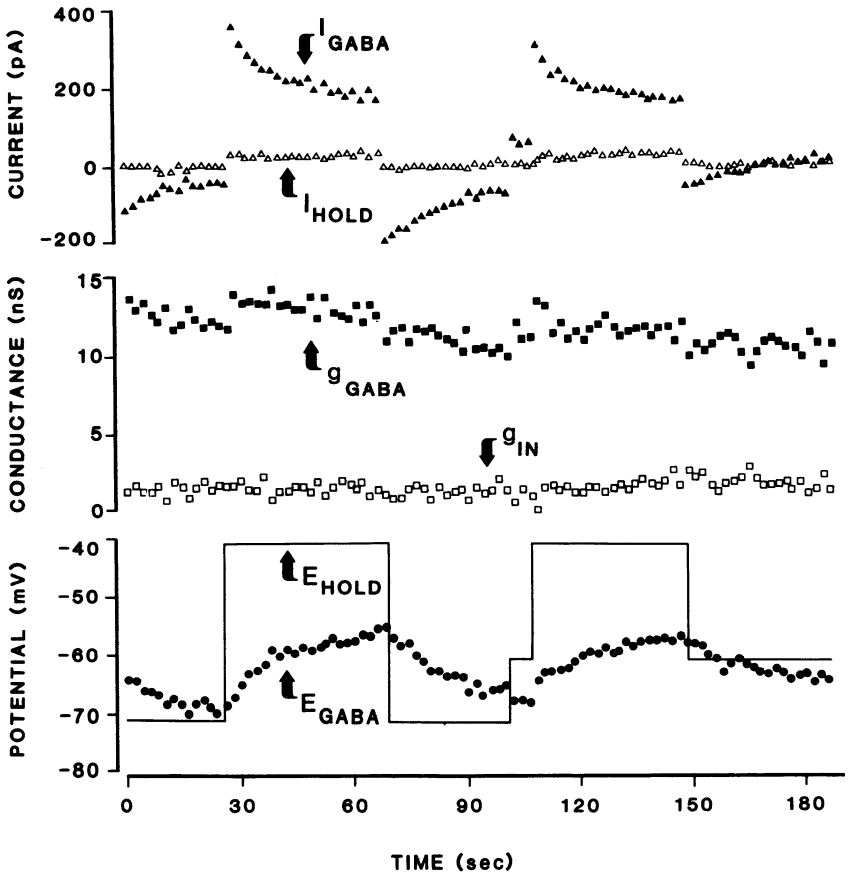

FIG. 7. GABA currents, conductances, and equilibrium potentials from the experiment shown in Fig. 6. *Upper panel* represents holding (I_{HOLD}) and GABA currents (I_{GABA}) at each GABA response. *Middle panel* graphs input conductance (g_{IN}) and g_{GABA}. *Lower panel* shows holding potential (E_{HOLD}) and GABA equilibrium potential (E_{GABA}). All values calculated as described in text, *Eqs. 1* and *2*. As can be seen, changes in I_{GABA} associated with changes in E_{HOLD} (*top panel*) are accompanied by changes in E_{GABA} (*lower panel*) and not by changes in g_{GABA} (*middle panel*).

measurements; therefore, any error due to nonlinearity of g_{GABA} was negligible within the voltage range we were investigating.

If the fading of I_{GABA} is due solely to the redistribution of Cl^-, then activation of g_{GABA} at E_{GABA} should be without effect on the driving force. The experiment in Fig. 9 is an example of an experiment that tested this hypothesis. GABA was applied repeatedly at 0.1 Hz and the holding potential was adjusted until I_{GABA} disappeared (i.e., $E_{HOLD} = E_{GABA}$). Thereafter, although g_{GABA} was reasonably large, I_{GABA} remained undetectable (i.e., E_{GABA} did not change). When the holding potential was eventually depolarized by 20 mV to −36 mV, I_{GABA} became evident and then decreased with time. On returning the holding potential to E_{GABA}, I_{GABA} slowly disappeared. Values for g_{GABA} and E_{GABA} (derived as in Fig. 7) are presented. Again these data indicate that changes in I_{GABA} are related to changes in E_{GABA} and also argue against the contribution of more than one ionic species to I_{GABA} (see DISCUSSION).

Desensitization occurs with a slower time course than changes in E_{GABA}

The previous experiments demonstrated that repeated application of GABA leads to rapid changes in E_{GABA} in isolated hippocampal neurons. This does not rule out a contribution of desensitization (i.e., an actual de-

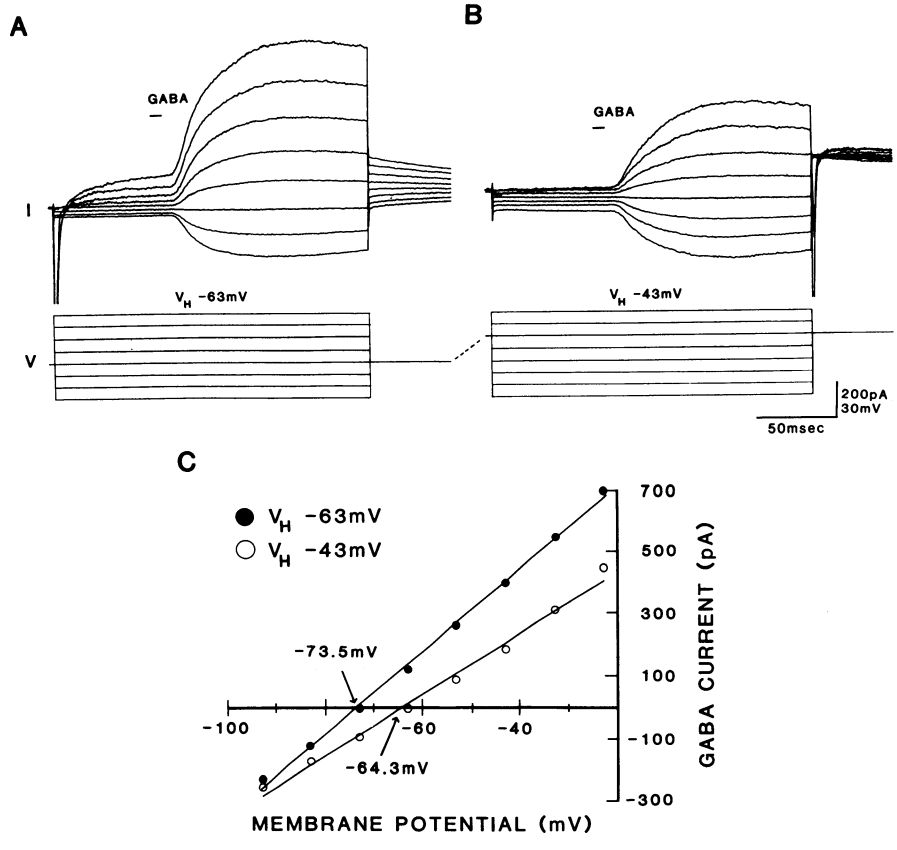

FIG. 8. Direct measurement of changes in E_{GABA} caused by changes in E_{HOLD}. *A*: GABA *I–V* relation was obtained at holding potential of −63 mV (pipette $[Cl^-]$ = 6 mM, 100 μM GABA, 10 ms, 40 psi). GABA responses were obtained every 2 s (0.5 Hz). *B*: in the same neuron holding potential was changed to −43 mV, 4 GABA pulses were applied at 0.5 Hz, and then *I–V* relation was determined immediately. *C*: *I–V* relation for *A* and *B* are plotted. At holding potential of −63 mV, E_{GABA} = 73 mV; at E_{HOLD} of −43 mV E_{GABA} = −64 mV. There was a gradual decline in g_{GABA} throughout this experiment.

crease in g_{GABA}) to the fading of I_{GABA} under these circumstances (cf. Ref. 36). Experiments such as those depicted in Fig. 10 were performed to elucidate the relative contributions of the two processes during prolonged application of GABA. In these experiments a 10-s application of GABA leads to an I_{GABA} that faded with time. By application of repetitive conductance pulses both before and during the GABA application, g_{GABA} and E_{GABA} could be estimated from *Eqs. 1* and *2*. Figure 10, *C* and *D*, represents the normalized I_{GABA} and g_{GABA} levels during the application of GABA. When pipette $[Cl^-]$ was 16 mM, both I_{GABA} and g_{GABA} diminished with time, but I_{GABA} decreased much faster than g_{GABA} (Fig. 10*C*). For example, at 2 s I_{GABA} decreased to 45% of peak, whereas the conductance was still maintained at 80% of peak (Fig. 10*C*). The change in I_{GABA} not accounted for by the change in g_{GABA} was caused by a change in the driving force. Since the membrane potential was constant at E_{HOLD}, this implies a change in E_{GABA}. As a final test of the hypothesis that changes in $[Cl^-]_i$ account for shifts in E_{GABA}, pipette $[Cl^-]$ was increased to 68 mM to buffer $[Cl^-]_i$ more effectively, i.e., to reduce the effects of small Cl^- flux. In this case (Fig. 10, *B* and *D*) the decrease in I_{GABA} paralleled exactly the decrease in g_{GABA}. The experiment in Fig. 10*B* represents the most complete contribution of desensitization to decreased I_{GABA} that we have

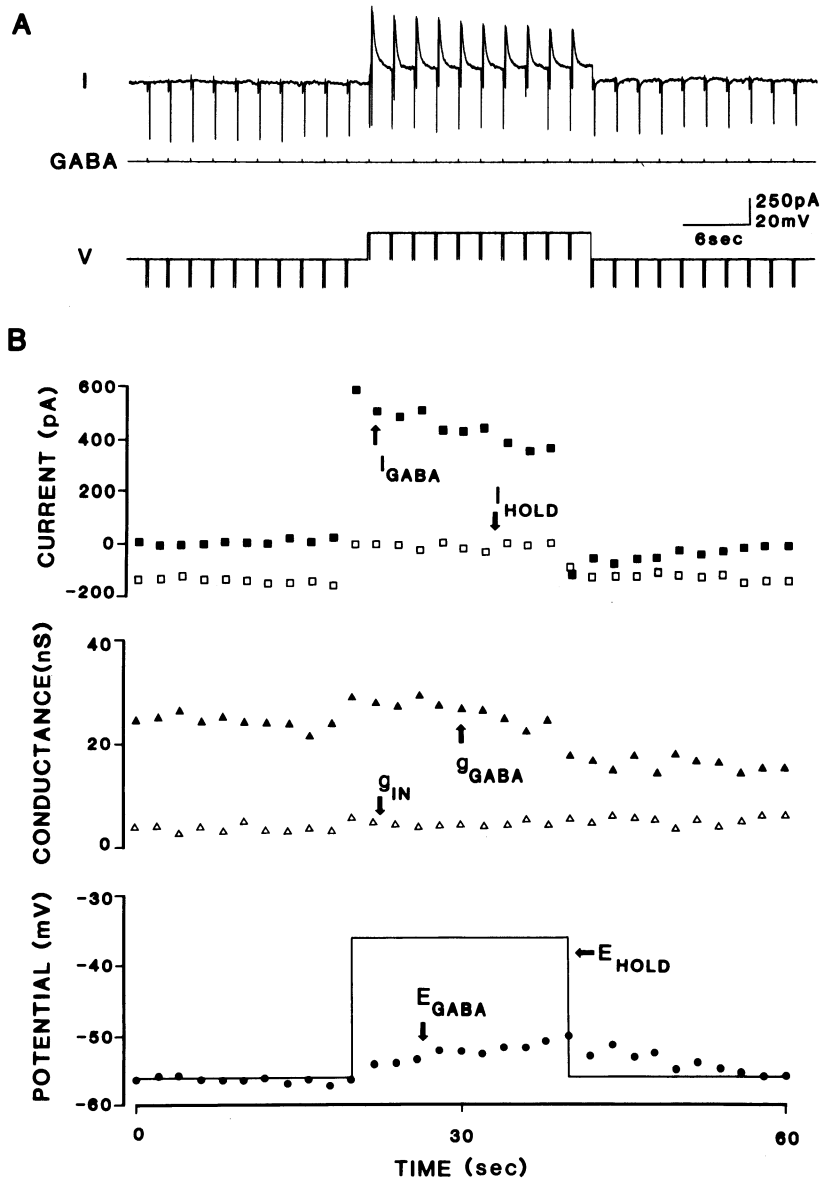

FIG. 9. GABA responses do not change in the absence of I_{GABA}. *A*: an experiment similar to that shown in Fig. 6 was performed. GABA (100 μM, 4 ms, 50 psi) was applied at 0.5 Hz, with holding potential adjusted to E_{GABA}. No change occurred in I_{GABA} during this time. However, as soon as the membrane potential was depolarized by 20 mV, a gradual change in the driving force, with a concurrent decrease in I_{GABA}, occurred. Pipette $[Cl^-]$ = 16 mM. *B*: calculations of GABA current, conductance, and equilibrium derived from *Eqs. 1* and *2* in text. Here it can be seen that even when g_{GABA} was moderate (25 nS), there is no change in the E_{GABA} until there is GABA-activated current flowing across the membrane.

seen. However, the general conclusion holds for all cases: shifts in E_{GABA} play a smaller role in decreases in I_{GABA} when $[Cl^-]_i$ is high, than when it is low. We have also noted that with 68 mM Cl^- in the pipette, maximum desensitization to a 10-s pulse of 100-μM GABA was 86 ± 2% (n = 7) compared with 43 ± 3% with 16-mM Cl^- in the pipette (n = 4).

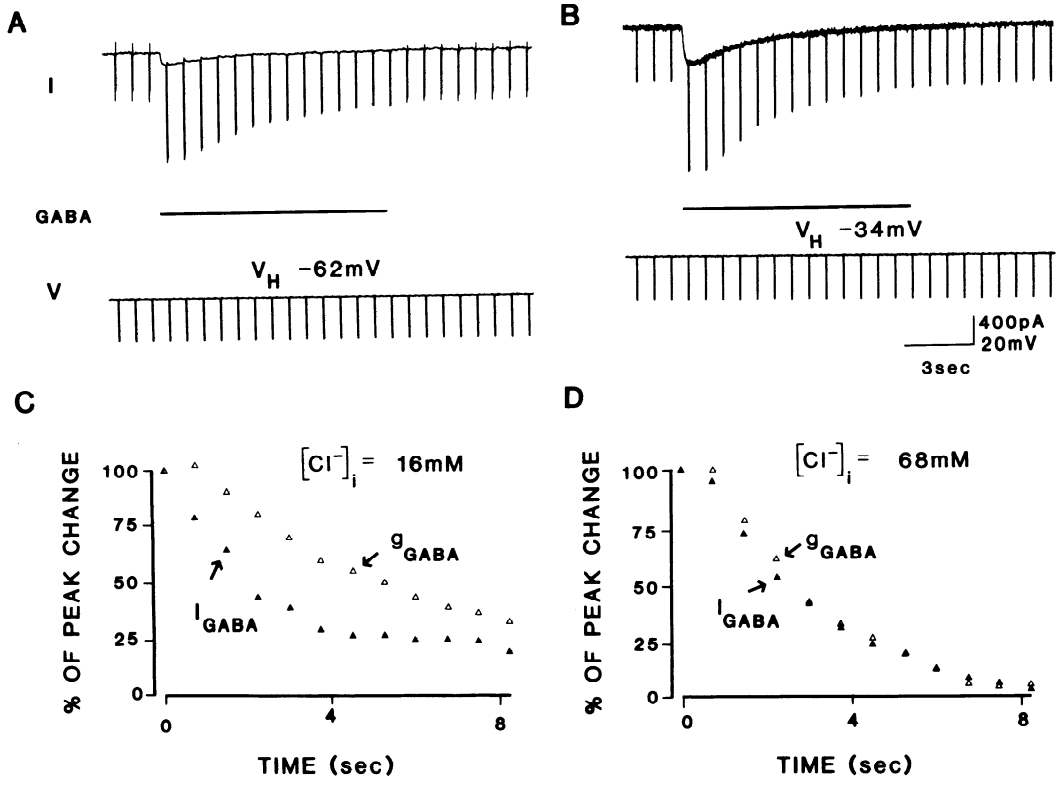

FIG. 10. Strongly buffering $[Cl^-]_i$ prevents shifts in E_{GABA}. *A*: a prolonged pulse of GABA (100 μM, 10 s, 10 psi) was applied to a neuron and 30-mV hyperpolarizing conductance pulses were given every 0.75 s. The pipette $[Cl^-]$ was 16 mM and GABA response faded during prolonged application. *B*: 10-s application of GABA (100 μM, 10 psi) to a different neuron recorded with pipette containing 68 mM Cl^-. *C*: percentage changes in g_{GABA} and I_{GABA} of response shown in *A*. Although g_{GABA} decreased markedly, I_{GABA} faded even more rapidly. *D*: comparison of changes in g_{GABA} and I_{GABA} from the response in *B*. In this case decrease in current was paralleled by change in conductance, indicating little or no change in GABA equilibrium potential.

GABA responses in tissue-cultured mouse spinal cord neurons

As a control for the reliability of the acutely dissociated neuron preparation we replicated several experiments on tissue cultured spinal cord neurons (generously supplied by G. K. Bergey; cf. Ref. 40). A series of GABA pulses produced a fairly constant I_{GABA} until a step in E_{HOLD} was applied. I_{GABA} at first increased and then decreased. Analysis of the response revealed the same rapid shifts in E_{GABA} as seen in acutely dissociated hippocampal neurons. In 16 determinations in four cells E_{GABA} shifted 6.5 ± 2 mV per 10-mV change in E_{HOLD} (cf. Figs. 7 and 9). In these cells there was also a more complete desensitization when pipette $[Cl^-]$ was elevated (76 ± 5% with pipette $[Cl^-]$ = 68 mM, n = 4; vs. 46 ± 5% with pipette $[Cl^-]$ = 6 mM, n = 4).

DISCUSSION

We have used the acutely dissociated hippocampal neuron preparation to investigate experimental questions that could not be addressed adequately in other mammalian brain preparations. The advantages of this model system include visualization of the neuron under study, which facilitates voltage clamping of cells, and precise localization of transmitter application; freedom from complicating factors due to a restricted extracellular space; and lack of interference from synaptic transmission or transmitter uptake mechanisms. The main

disadvantage is that, as a relatively new preparation, it represents an as yet incompletely understood departure from other preparations. Our observations confirm that there is reasonable agreement between many of the properties of these neurons and those of neurons in the in vitro slice preparation. We have extended the characterization of isolated neurons to include various features of GABA responses. Enzymatic treatment, although obviously a matter of concern, has not yet revealed major deleterious effects. As one type of control we, and others (21, 36), have now used a variety of enzymes, and qualitative differences in cell viability have not yet surfaced. We (McCarren and Alger, unpublished observations) have also maintained intracellular recordings from CA1 pyramidal neurons for up to 2 h after beginning perfusion with saline containing papain, 1 mg/ml. Synaptic transmission in the slice begins to fail after ~1 h, but there are no obvious differences in electrophysiological properties of individual neurons. In particular, input resistance does not change and eventually the impalements are lost spontaneously, probably because of mechanical instability created by digestion of the extracellular matrix. Finally, we have also studied tissue-cultured spinal cord neurons using whole-cell clamp techniques. Neurons in this preparation clearly differ in many ways from acutely dissociated neurons. The fact that our major findings can be duplicated exactly in the tissue-cultured cells gives confidence in our results.

We have demonstrated that E_{GABA} is a surprisingly labile parameter. Shifts in E_{GABA} occur quite readily. Both direct and indirect measurements indicated that alterations of I_{GABA} produced on stepping to new holding potentials were due to this factor. At a constant holding potential near the normal resting potential of hippocampal neurons (−60 to −65 mV) the reversal potentials obtained for I_{GABA}, determined by brief voltage steps, were close to the predicted E_{Cl} for the given $[Cl^-]_i$, i.e., to pipette $[Cl^-]_i$. However, prolonged changes in the holding potential caused clear shifts (e.g., Figs. 4, and 7–9) in the reversal potential of the GABA response. We thus confirm previous inferences that shifts in transmitter equilibrium potential can influence transmitter-activated responses (10, 16, 18, 29, 42). We have also shown that shifts in E_{GABA} are not necessarily due to contamination by excitatory postsynaptic potential (EPSPs) or to changes in extracellular ion concentrations. Moreover, our data indicate that shifts in E_{GABA} occur rapidly enough and in response to small enough GABA applications to be physiologically relevant. A typical GABA response had a peak conductance of ~15 nS, i.e., equivalent to the activation of ~750 GABA-activated channels (assuming a single channel conductance of 20 pS, see Refs. 21, 42). The close correspondence between build-up in $[K^+]_o$ and shift in E_{IPSP} found by McCarren and Alger (31) may have been due in part to a depolarizing action of $[K^+]_o$, rather than to Donnan equilibrium-like effects of $[K^+]_o$ changes on $[Cl^-]_i$. We also found that, in experiments in which direct comparisons were made, shifts in E_{GABA} may occur more rapidly than decreases in g_{GABA}. We observed that desensitization with very high levels of pipette $[Cl^-]$ was more complete than with low levels of pipette $[Cl^-]$. This factor may have to be taken into account in studies of desensitization.

An important issue is whether GABA activated a conductance for one or more ionic species. As mentioned previously, GABA can activate both hyperpolarizing and depolarizing responses, and conductances for different ionic species may be involved. Hyperpolarizing GABA responses can result from activation of a bicuculline-sensitive Cl^- conductance or a bicuculline-insensitive, baclofen-activated K^+ conductance (20, 34). However, the receptors responsible for both the depolarizing GABA response and the bicuculline-insensitive K^+ response are located primarily on the dendrites. The loss of dendritic membrane in the dissociation procedure, coupled with precise localization of GABA application to the cell soma, contributed to our ability to obtain Cl^--dependent responses. Pharmacological and physiological evidence indicates that our responses must have been almost exclusively Cl^- dependent: *1)* E_{GABA} was strongly dependent on the $[Cl^-]$ gradient; *2)* in two of three cells bicuculline blocked I_{GABA} entirely, and baclofen (1 mM) produced no conductance change in the three cells (data not shown); *3)* GABA induced a monophasic, single exponential response; *4)* I_{GABA} remained monophasic in the presence of pentobarbital (a powerful enhancer of depolarizing GABA responses); and *5)* re-

petitive GABA application at E_{GABA} did not lead to shifts in E_{GABA}. This last point is important because the reversal potential for a transmitter that activates more than one ionic conductance simultaneously is not an actual equilibrium potential, but a state in which the lack of measured current is caused by equal and opposite currents, rather than by the true absence of current. If I_{GABA} did involve multiple ionic fluxes, activating g_{GABA} at E_{GABA} could cause changes in E_{GABA}, because individual ionic fluxes would result in unequal changes in the equilibrium potentials for the ionic species involved.

As noted earlier, the plot of E_{GABA} versus pipette Cl^- (Fig. 3) indicated a minor deviation from a Nernst prediction in the region of low $[Cl^-]_i$. Similar findings have been reported previously by others regarding purely Cl^--dependent responses (e.g., Refs. 33, 38). The simplest explanation is that in the region of low Cl^- we were unable to control $[Cl^-]_i$ adequately and, rather than the assumed value of 2 mM, $[Cl^-]_i$ never declined below ~5 mM. Alternatively, the data in Fig. 3 can be fit reasonably by a Goldman-Hodgkin-Katz equation that assumes that $P_{Na}/P_{Cl} = 0.02$. However, beyond the deviation of the line in Fig. 3, we are unable to account for any of our results simply by assuming a slight Na contribution. In particular, the finding that quantitatively identical changes in E_{GABA} occur during both depolarizing and hyperpolarizing voltage steps effectively rules out any simple mechanism by which a fixed 2% Na contribution might explain the recorded shifts in E_{GABA}. To account for our results by Na influence we would have to postulate not only that GABA activates Na conductance but that P_{Na}/P_{Cl} changes in a fashion which is dependent on both membrane voltage and on rate of GABA application. No independent data support these postulates. The fit to the data could not be improved by adjusting P_K/P_{Cl}, suggesting that a K^+ conductance was not involved. Thus, the parsimonious explanation is that the responses we recorded are due to Cl^- and that $[Cl^-]_i$ buffering is not complete.

Note that $[Cl^-]_i$ control by the recording pipette is not instantaneous, i.e., the pipette does not constitute a $[Cl^-]$ clamp; if it did, then shifts in E_{GABA} could not have occurred. Fenwick et al. (19) found that intracellular ion concentrations equilibrated with pipette concentrations with a time constant of ~6 s. Our data are compatible with a similar delay.

Eccles and colleagues (6, 18) used a technique (13) of altering intracellular ionic concentrations by passing current through the recording microelectrode and electrophoresing ions into hippocampal pyramidal neurons in vivo to study the anionic permeability of the postsynaptic membrane. Some of our results resemble theirs. We confirm their observation that recovery from either loading or depleting $[Cl^-]_i$ occurred with the same time constant. Perhaps coincidentally, the actual value of their time constant was about 20 s (at an IPSP stimulation rate of 1–2 Hz), and this compares favorably with our measurements of 10 s (GABA application rate of 0.5 Hz) to 40 s (at a rate of 0.05 Hz). We believe our Cl^- loading effect is different from theirs, however. The current injection levels in the present experiments were much smaller than in the earlier reports (10–100 pA vs. 10 nA). In addition, if our shifts in E_{GABA} were due to an electrophoretically induced increase or decrease in $[Cl^-]_i$, we would expect this effect to be very dependent on the magnitude and duration of the current used to voltage clamp the membrane at new levels of E_{HOLD} and less so on the GABA-activated currents. Nevertheless, we found E_{GABA} to be heavily influenced by the rate of GABA application, when different rates were examined at the same voltage levels in the same cell (e.g., Fig. 5). Thus it appears that shifts in E_{GABA} were due mainly to Cl^- flux through GABA-activated Cl^- channels, with perhaps a smaller contribution due to Cl^- flux through a resting permeability. Actually, flux through a transmitter-activated channel would probably also be a significant factor in systems such as the in vivo hippocampus in which, due to the occurrence of spontaneous IPSPs and a potentiating effect of the anesthetic used (pentobarbital) on IPSPs (35), a substantial contribution to resting Cl^- permeability may be due to a transmitter-activated conductance.

To what extent can our conclusions be applied to neurons in other preparations? In view of the differences discussed earlier it would be unwise to extrapolate without exercising caution. However, we anticipate that our findings will have some generality. For example, actual $[Cl^-]_i$ control in isolated neurons recorded with large whole-cell clamp electrodes should

be better than in neurons recorded with high resistance intracellular pipettes, and so shifts in $[Cl^-]_i$ might be expected to occur even more readily when high resistance pipettes are used. In addition, shifts in E_{GABA} occur with GABA responses that are quite comparable in magnitude to inhibitory synaptic responses recorded in hippocampal neurons (12, 32). Shifts in E_{GABA} of the kind produced by our experimental protocol would be especially important physiologically when IPSPs are activated concurrently with membrane depolarizations, as is frequently the case (31). On the other hand, dissociated neurons are maintained at a cooler temperature than is typical for the slice preparation (~24 vs. 30–37°C). In general, studies in slices have revealed that low temperatures result in responses which differ quantitatively rather than qualitatively (45) from those seen at higher temperatures. We might thus expect the duration, or magnitude, of shifts in E_{GABA} to be different in neurons maintained at higher temperatures. E_{IPSP} in hippocampal neurons in the slice preparation is unaltered when these cells are maintained for up to 30 min at 22°C (3). Thus, either the mechanism that maintains the Cl^- gradient is not very sensitive to temperature changes over this range or the activity of such a mechanism does not readily respond to maintain the Cl^- gradient in the face of acute changes in the gradient. We have recorded from several dissociated neurons at 30°C and have obtained results virtually identical to those at 24°C. For instance, at 30°C I_{GABA} disappeared in about 4 s during a long GABA pulse, due to changes in g_{GABA} and E_{GABA}, just as in Fig. 10 at 24°C. Nevertheless, we cannot exclude the possibility that low temperature, or some other aspect of the dissociation procedure, results in decreased efficiency of a metabolically dependent Cl^- transport mechanism. Such an effect could alter the degree to which the ion accumulation and depletion phenomena, which we have demonstrated, actually influence IPSPs in vivo. At the moment it is difficult to evaluate this hypothesis, because almost no information is available regarding Cl^- transport in hippocampal neurons in any preparation (5, 6).

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