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Desensitization of GABA-activated Currents and Channels in Cultured Cortical Neurons

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Application of GABA to rat neocortical neurons maintained in cell culture produced a response that declined over several seconds, even in the continued presence of agonist. The decrement could be attributed to both a redistribution of Cl^- and a true decline in GABA-induced membrane conductance, or desensitization. The extent and rate of desensitization were dose dependent in a manner similar to the dose dependence of the GABA-induced current, but were not related to the absolute magnitude of the current or to the charge transfer. Bicuculline slowed desensitization while diazepam enhanced the rate of desensitization, consistent with a localization of desensitization to the agonist-receptor binding site. When measured in the whole-cell recording mode, desensitization was voltage dependent, becoming much slower as the membrane was depolarized. Changes in extracellular or intracellular $[Ca^{2+}]$ did not appear to grossly affect the desensitization process or its voltage dependence. GABA-activated single channels, recorded in the outside-out configuration, also desensitized in the continued presence of agonist. However, desensitization differed from that seen in the same neurons in the whole-cell mode. Desensitization was considerably more rapid and did *not* show any voltage sensitivity. Moreover, single-channel responses often failed to recover after only a few exposures to agonist. Desensitization of GABA responses may play a role in the regulation of cortical inhibition, especially under conditions of intense excitatory and inhibitory synaptic activation.

Desensitization of neurotransmitter receptors represents an important mechanism for the physiological control of excitation and inhibition over time. Receptor desensitization is the change in transmitter-induced current over time that is attributable to a decrease in the induced conductance. The first, and best-studied, neurotransmitter whose desensitization was examined was ACh (Thesleff, 1955; Katz and Thesleff, 1957; Manthey, 1966; Magazanik and Vyskocil, 1970, 1975; Rang and Ritter, 1970a,b; Adams, 1975; Lester et al., 1975; Miledi, 1980; Weinstock, 1983). The rate of desensitization was found to be influenced by voltage (Magazanik and Vyskocil, 1970; Scubon-Mulieri and Parsons, 1978; Fiekers et al., 1980), calcium concentration (Manthey, 1966; Magazanik and Vyskocil, 1970; Parsons et al., 1973; Miledi, 1980; Chesnut, 1983), and direct receptor phosphorylation (Eusebi et al., 1985; Albuquerque et al., 1986; Huganir et al., 1986; Middleton et al., 1986). Desensitization of the ACh receptor has been partially characterized at the level of the single channel, where the bursts and clusters of bursts of channel openings have been interpreted as indicating the presence of two desensitized states (Sakmann et al., 1980).

The neurotransmitter GABA, acting at $GABA_A$ receptors, has been studied in some of the same ways as ACh. When GABA is applied to sensitive neurons in a variety of anatomical sites, there is often observed a decrease in efficacy over time. This can be seen in response to iontophoretic application in tissues as diverse as the rat hippocampus (Ben-Ari et al., 1979) and the Mauthner cell of the goldfish (Diamond and Roper, 1973). Application of GABA by superfusion to neurons in an intact tissue, such as spinal cord (Hackman et al., 1982), dorsal root ganglia (Desarmenien et al., 1980; Gallagher et al., 1983), or sympathetic ganglia (Brown and Galvan, 1977), leads to a response that was seen to decay over time. Similar behavior has been seen at the inhibitory junction potential in invertebrates (Sarne, 1976), as well as in chromaffin cells (Bormann and Clapham, 1985) and sympathetic ganglion cells (Adams and Brown, 1975).

It is possible that, under certain circumstances, a reduction in current without a concomitant reduction in conductance could be due to a change in the chloride gradient produced by the persistent action of transmitter. In fact, both true desensitization and a change in chloride equilibrium potential have been seen in the same neurons. Several investigators found that in mammalian hippocampal pyramidal cells or chick cerebral neurons, the decay in GABA response (with time constants of several seconds) was secondary to a change in conductance (Wong and Watkins, 1982; Numann and Wong, 1984; Thalmann and Hershkowitz, 1985; Weiss et al., 1988). However, another study found that while the current measured under voltage-clamp conditions decayed with time constants on the order of tens of seconds, the conductance was unchanged (Segal and Barker, 1984). In addition, Huguenard and Alger (1986) have shown that in acutely dissociated hippocampal pyramidal cells with low $[Cl^-]_i$, changes in the driving force underlay a decrease in current over time. As $[Cl^-]_i$ was elevated, this effect diminished and a true receptor-mediated desensitization was revealed.

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The experiments presented here examine the magnitude of desensitization of GABA-induced current in cultured rat cortical neurons at both a macroscopic and a single-channel level as well as the influence of membrane potential on this important physiological property. A preliminary report of these findings has appeared (Frosch et al., 1989).

Materials and Methods

Preparation of cultures of cerebral cortex. Cultures were prepared from embryonic day 16–17 rat embryos and maintained as previously described with some modifications (Dichter, 1978; Snodgrass et al., 1980). Cortices were collected in physiological saline (in mM: 145 Na, 3 K, 1.8 Ca, 1.0 Mg, 154 Cl, 8 glucose, buffered to pH 7.2 with 2.5 mM sodium phosphate), transferred into 2 ml of a 0.03% trypsin solution in growth medium lacking serum [Eagle's minimum essential medium without glutamine (GIBCO) supplemented with 11 mM glucose, 2 mM glutamine, and 60 U/ml penicillin and streptomycin], minced, and incubated at 35°C in a humidified 5% CO_2, 95% air environment for 120 min. The trypsin-containing solution was replaced with 2 ml of Ca/Mg-free Earle's balanced salt solution (GIBCO) and incubated for 20 additional minutes. The tissue suspension was then diluted with several volumes of growth medium with 5% rat serum, triturated, and filtered through two sheets of sterile lens paper in a Swinnex 13 mm holder to remove any clumps of cells. The yield of cells was $\sim 2 \times 10^6$ cells/fetal hemisphere with a viability of >90%. Cells were plated at a density of 4.5×10^5 viable cells/35 mm dish containing five 12 mm round cover glasses. When the non-neuronal background cells became confluent, cultures were treated with 5 μg/ml cytosine arabinoside for 48 hr. Neuronal viability was routinely maintained for 6–8 weeks and has been observed for >6 months. Coverslips were prepared by coating with calf skin collagen and polylysine or polylysine alone (Snodgrass et al., 1980).

Electrophysiology recordings. Recordings were made under direct visualization at 32–36°C. The preparation was grounded through an agar bridge, and junction potentials were minimized by using a solution between the agar bridge and the Ag/AgCl pellet identical to the chosen recording electrode solution. Electrodes were prepared from either Blue Tip hematocrit tubing or Boralex 100 μl capillary pipettes. Regardless of glass type, electrodes were coated with Sylgard Elastomer 184 (Dow Corning Co.), fire polished (Corey and Stevens, 1983), and filled immediately prior to use. Electrode resistances ranged from 4 to 8 MΩ. Recordings were made in the whole-cell and excised outside-out patch mode with an EPC-7 patch-clamp amplifier (List Electronics) using standard methods (Hamill et al., 1981). Data signals were digitized at a rate ($1/t$) of 500 Hz to 10 kHz and Bessel filtered $\leq 1/5t$ prior to presentation to the A/D interface (Ithaco 4302 dual 24 dB/octave filter). The series resistance correction, when appropriate, was made using the circuit provided within the amplifier. The match between expected and measured reversal potentials for internal anion substitutions indicated that errors due to series resistance, junction potentials, and inadequate space clamps were minimal in the measurement of GABA-induced currents.

Data acquisition and recording conditions. Experiments were controlled on line by a computer system [PDP11/23 CPU (DEC) with 256K of memory, operating under RT-11 and running BASIC 23-HP (Cheshire Data, versions 2.02 and 3.02)]. The standard bath solution used in these experiments (HEPES-buffered saline) consisted of (in mM) 140 Na, 3 K, 143 Cl, 8 glucose, and 10 HEPES, pH 7.2. For most experiments it also contained 2 mM $CaCl_2$ and 1 mM $MgCl_2$ [extracellular solution (ES)]. When desired, tetrodotoxin (TTX; Sigma) and $CoCl_2$ were added to bath solutions to block inward currents. The standard internal solution contained (in mM) 120 Cs, 25 tetraethylammonium (TEA), 1 Ca, 1 Mg, 149 Cl, 1.5 EGTA, and 10 HEPES, pH 7.2. In other experiments, 120 mM cesium aspartate was substituted for 120 mM CsCl. Both of these internal solutions are expected to have free calcium concentrations of ~0.4 μM (Caldwell, 1970).

Drugs used in these studies were applied by local microperfusion from 10–20-μm-diameter pipettes under 5–10 psi from a distance of 25–50 μm. Agents were prepared at their desired final concentrations in the bath solution and loaded into individual micro perfusion pipettes. Slow drug responses were elicited during acquisition of long epochs of membrane current. Sampling was begun prior to the micro perfusion to obtain a baseline record. Analysis of responses involved measurement of the peak induced current (I) and of the time courses of the rise and decay phases of the response. The current flowing after the peak was calculated as a fraction of the peak, and was plotted as $\ln[I(t)/I(0)]$ versus time ($t = 0$ at the time of peak current). The resulting curve was usually well described by a single regression line.

Results

Macroscopic GABA-induced currents

Change in response to GABA during a single application

The application of a moderate dose of GABA to a cultured cortical neuron results in a current that decays exponentially with a single time constant (Fig. 1). In order to measure the current and conductance changes induced by application of GABA independently, the cell was repetitively stimulated at 0.5–1 Hz with a 15–30 msec step of 10–15 mV from the holding potential in a direction away from the calculated GABA reversal potential. During stimulation, GABA was applied for 20–30 sec followed by a wash with a control solution. Results of this sort of experiment, performed at three different holding potentials to alter the size and direction of the GABA-induced currents, are presented in Figure 2*A*. The data shown in this graph, which plots the change in membrane current as a function of the change in membrane chord conductance, are well described by straight lines whose slopes match the expected driving forces. As can be seen in Figure 2*B*, the time course for changes in the GABA-induced current and GABA-induced conductance (both normalized to the maximal change observed) are identical under conditions of high (~150 mM) and symmetrical chloride concentrations. If series resistance errors were present and resulted in some of the apparent decay of current, it would be expected that the driving force would change over time; the membrane potential would shift because the voltage drop across the series resistance would increase with the increase in GABA-induced current. Under our recording conditions, this was not observed.

As previously described by Huguenard and Alger (1986), under conditions of low internal [Cl^-], GABA application results in a change in the transmembrane chloride gradient and a consequent change in the chloride equilibrium potential. Thus, the progressive decrease in the GABA-induced current that occurs under these conditions is secondary to both a change in driving force and membrane permeability to chloride. This can be seen in Figure 3*A*, which shows results from cells recorded with a pipette containing 29 mM Cl^-, and Figure 3*B*, which shows results with 10 mM internal Cl^-. As the internal chloride reservoir is decreased, the changes in the driving force for the GABA-induced current become more prominent. In order to extract the fraction of the change in current that is related to desensitization rather than the change in driving force, the data were replotted to compare directly the changes in conductance and current over time (Fig. 3*C,D*). There clearly is a contribution of true desensitization to the fading of current even under the most limiting conditions, as seen from the decrease in GABA-induced conductance change of almost 60% over 30 sec (Fig. 3*D*). Measurements made from the GABA-induced current directly, however, would overestimate the extent of desensitization.

In order to examine the relationship of desensitization to peak current, it was necessary to compare the responses (both peak current and desensitization) for a single cell, with a single dose of GABA, at a fixed holding potential at different times during the recording. Over times that are long compared to the rate of desensitization, changes occur in the response of these neurons to GABA (Stelzer et al., 1988). In Figure 4*A*, the responses of

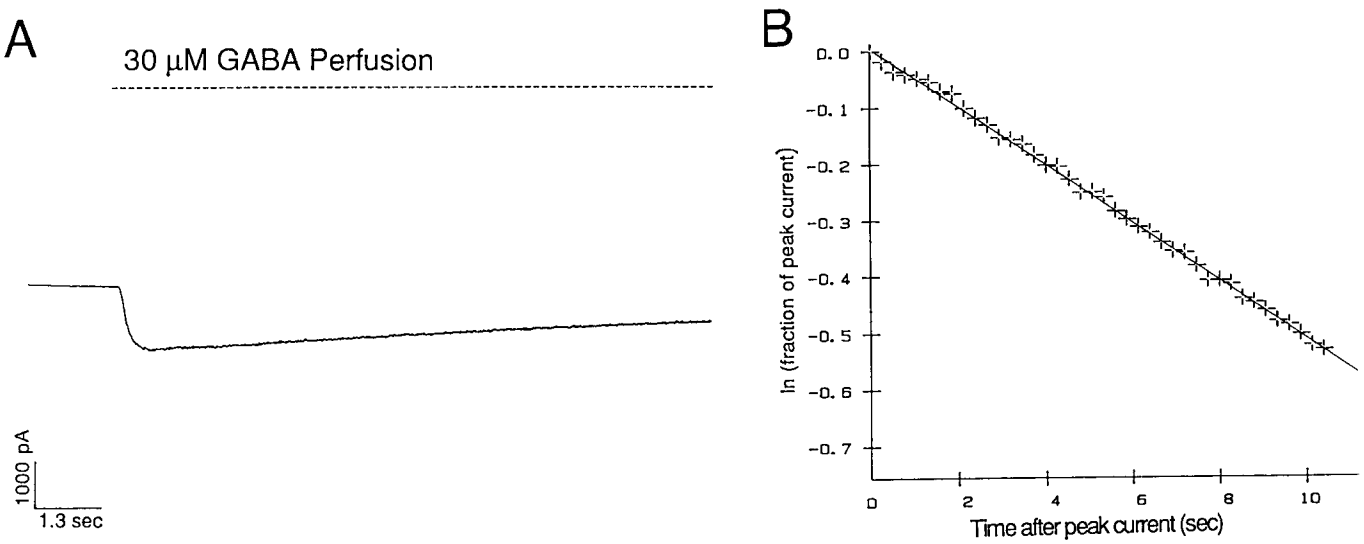

Figure 1. Response of a cultured cortical neuron to local perfusion with GABA. *A*, Application of 30 μM GABA while membrane potential was held at −60 mV produces a rapidly rising current with a slower decay phase. The bath solution contained 140 mM NaCl, 2 mM Ca-gluconate, 1 mM $MgSO_4$, 4 mM $CoSO_4$, 8 mM glucose, 10 mM HEPES-KOH, pH 7.2, 5 μM TTX; the recording electrode contained (in mM) 120 CsCl, 25 TEA-Cl, 1 $CaCl_2$, 1 $MgCl_2$, 1.5 EGTA, and 10 HEPES-NaOH, pH 7.2. *B*, A plot of the logarithm of the fraction of peak current (measured over the baseline current prior to the application of GABA) as a function of the time after the occurrence of the peak current yields a straight line with a time constant of 19.4 sec. The peak inward current, measured relative to the holding current, is 1.4 nA.

a cell to the application of GABA from the same perfusion pipette at different times during a recording can be seen to decrease over time (the measurements were taken 3, 20, and 35 min into the recording); the peak current was 59% at 20 min and 27% at 35 min relative to the level at 3 min. Despite this decrement in the absolute level of current, the rate of desensitization of GABA-induced current for this cell had *increased* (Fig. 4*B*). Although smaller GABA-induced currents decayed more rapidly here, it is possible to generate conditions where smaller currents are more stable, that is, resistant to desensitization. When recording with symmetric chloride solutions, the current at +30 mV shows slower decay than the larger currents

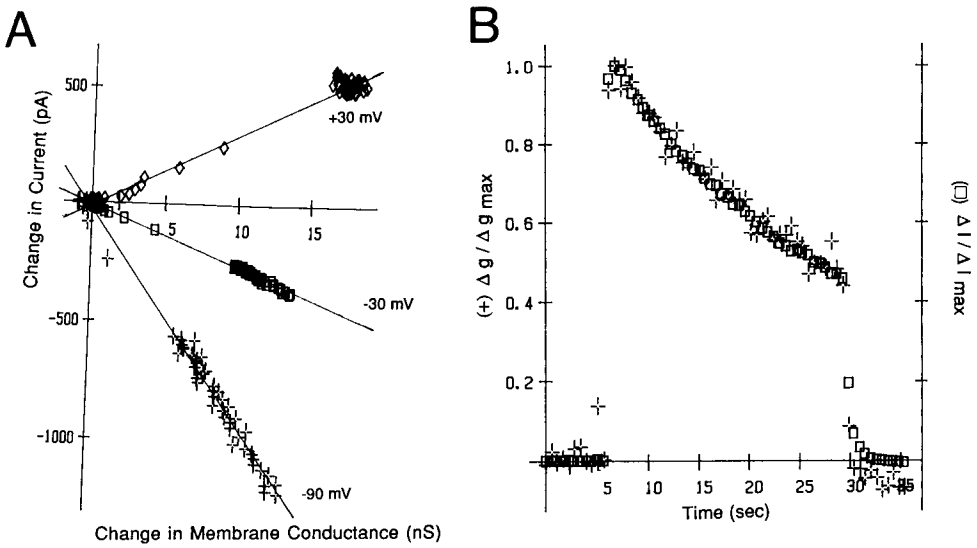

Figure 2. Stability of driving force for GABA-induced current and existence of desensitization. *A*, The change in membrane current induced by 30 μM GABA is shown as a function of the change in membrane conductance. The current was measured relative to the holding current prior to the application of GABA; the conductance was measured from the current induced by a small, short step in membrane potential. Measurements were made at three different holding potentials (*pluses*, −90 mV; *squares*, −30 mV; *diamonds*, +30 mV); the regression-fitted slopes (−88 mV, −26 mV, and +31 mV) closely match the predicted driving forces. The quality of fit for all three potentials indicated that there was no trend toward alteration in the reversal potential with application of GABA. The stimulus (15 msec voltage step of 10–15 mV) was repeated at 1 Hz. The bathing solution was ES, 5 μM TTX, and 4 mM $CoCl_2$; the recording electrode contained (in mM) 120 CsCl, 25 TEA-Cl, 1 $MgCl_2$, 1 $CaCl_2$, 1.5 EGTA, and 10 HEPES-NaOH, pH 7.2. *B*, The data taken at −90 mV from *A* are replotted as the change in current and change in conductance, normalized to the maximal change. The decay in the GABA-induced current is completely paralleled by the change in GABA-induced conductance, as expected from *A*, which indicated that the driving force is stable over time. Similar results hold at −30 and +30 mV.

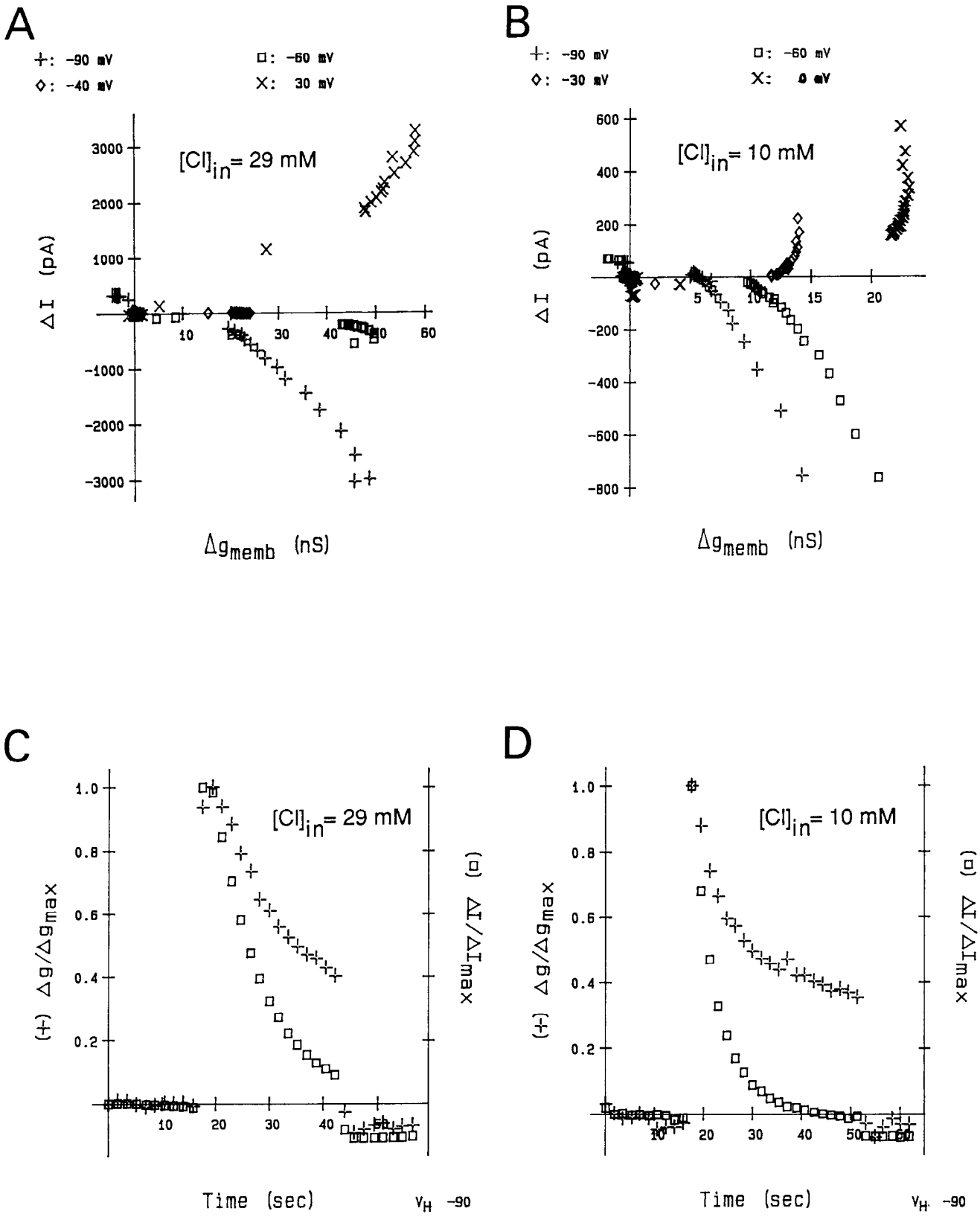

Figure 3. Conditions under which driving force can change in response to application of GABA. *A*, Data as in Figure 2*A*, with a lowered internal [Cl^-] (29 mM). It is possible to see clear nonlinearity in the relationship between the change in membrane current and the simultaneous change in membrane conductance (induced by 30 μM GABA), indicating that the driving force has changed. Recording conditions were as in Figure 2*A* except that the recording pipette contained 120 mM Cs-aspartate in place of CsCl. *B*, A further reduction in the internal [Cl^-] (10 mM) leads to an increased change in driving force. Here the internal solution had 114 mM Cs-aspartate, 6 mM CsCl, and 25 mM TEA aspartate in addition to the divalent cations, EGTA, and HEPES. *C* and *D*, Direct presentation of the difference in time course between the change in membrane current (*squares*) and the change in membrane conductance (*pluses*). As the internal [Cl^-] is reduced (*C*, 29 mM Cl^- as in *A*; *D*, 10 mM Cl^- as in *B*), the extent to which the change in current overestimates the change in conductance (desensitization) increases. *Under all ionic environments, however, there is a component of true desensitization present.*

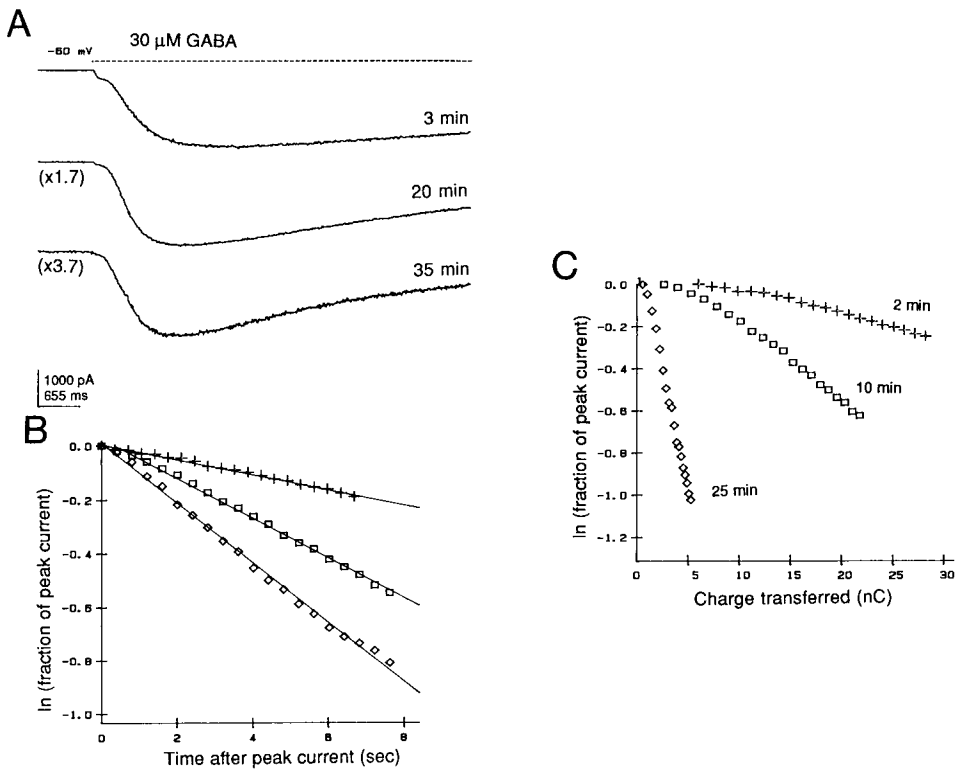

Figure 4. Rates of desensitization are not dependent on current flow or charge transfer. *A*, Responses of a neuron to application of 30 μM GABA from a single microperfusion pipette at various times into a recording (3, 20, and 35 min). As the recording progressed, the peak GABA-induced current decreased, while the rate of desensitization increased. The bath solution was ES, 5 μM TTX, and 4 mM $CoCl_2$; the recording pipette contained (in mM) 120 CsCl, 25 TEA-Cl, 1 $MgCl_2$, 1 $CaCl_2$, 1.5 EGTA, and 10 HEPES-NaOH, pH 7.2. Agonist was applied for the time indicated by the *broken line*; the small, early irregularities of the response reflect flow artifacts during the initiation of superfusion. (Traces at 20 and 35 min were scaled up to the approximate size of the 3 min trace for illustrative purposes.) *B*, Desensitization rates for the data shown in *A*. The rate of desensitization increased from a time constant of 35.6 sec (3 min, *pluses*) to 13.5 sec (20 min, *squres*) to 9 sec (35 min, *diamonds*) over the time that the peak current decreased to less than one-third of its original value. *C*, Data (at different times into the recording) were analyzed by plotting the natural logarithm of the fractional current remaining (relative to the peak induced current) as a function of the integral of the induced current record up to that point. If the decrease in current were related to the amount of charge transferred, the points from different applications of GABA should lie along a single curve. However, the rate of desensitization was not related to the amount of charge transferred as can be seen by the failure of these different data sets to superimpose when plotted in this manner.

at −60 to −90 mV (see below). The noncorrespondence between the rate of desensitization and the magnitude of the GABA-induced current is important because it suggests that series resistance errors are not adequate to explain the current decay. It is possible that the amount of charge transferred across the membrane during the application of GABA could lead to a decrease in apparent current flow over time because of electrode polarization leading to a decreased capacity of the electrode to pass current. The data can be plotted to show the GABA-induced current as a function of the charge transferred by calculation of the integral of the current record (Fig. 4*C*). If the amount of charge transferred (or the net flux of chloride) explained the decay in the current observed, then the various data sets would be expected to fall along a single curve, with each measurement series extending a different distance along the path. Since the data for the different responses fall along different curves, there must be a contribution to desensitization from a factor(s) other than charge movement.

Dose dependence

The extent and rate of desensitization were dose dependent in a manner similar to the dose–response relationship for GABA-induced current (Fig. 5). Higher concentrations of GABA applied to the same cell resulted in faster rates of desensitization. The shape of the dose–response relationship for desensitization was more difficult to determine than for GABA-induced current, since application of maximally desensitizing doses of GABA often resulted in significantly incomplete recovery. Additionally, it was not possible to measure accurately the very slow rates of desensitization found typically with doses less than the K_d for peak induced current (about 20 μM). However, it was clear that for any given cell, the desensitization curve corresponded relatively well with the curve for GABA-induced current (Fig. 5*C*).

Pharmacological manipulation of desensitization

When coapplied with GABA, bicuculline methiodide (BMI) leads to a reduction in current and the desensitization rate of the residual current is considerably reduced (Fig. 6*A*), just as if a lower concentration of GABA had been applied. The results shown here demonstrate the reversible increase in the decay time constant (from 20–25 sec to >150 sec).

In contrast, the rate of desensitization of the residual GABA-activated current at an incomplete blocking dose of picrotoxinin (PTX) was not different from the normal range of decay rates observed for GABA alone (Fig. 6*B*). For this experiment, mea-

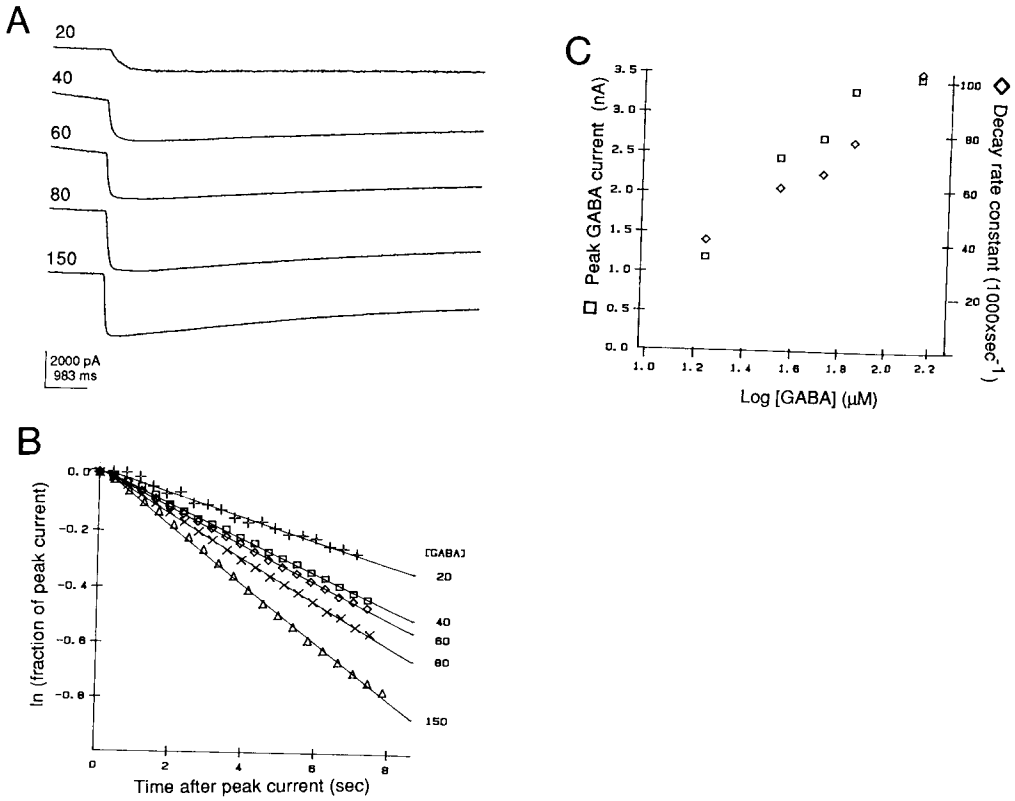

Figure 5. Dose–response relationship for desensitization of GABA-induced current. *A*, Records of current responses to application of increasing concentrations of GABA. As the [GABA] is increased, the peak current grows, as does the rate of desensitization. Recordings were made in a ES, 5 μM TTX, 4 mM $CoCl_2$ bath with (in mM) 120 CsCl, 25 TEA-Cl, 1 $MgCl_2$, 1 $CaCl_2$, 1.5 EGTA, and 10 HEPES-NaOH, pH 7.2, in the pipette (37°C). Membrane potential was held at −60 mV. Applications were not made in order of increasing dose to avoid any contribution of changes in desensitization over time. The interval between applications was greater than 45 sec, and recovery was complete. *B*, Responses shown in *A* plotted as decay curves to show the single exponential nature of the desensitization of GABA-induced current for all doses tested. The time constants measured, along with the agonist dose, are 24.2 sec (20 μM), 16.5 sec (40 μM), 15.2 sec (60 μM), 12.9 sec (80 μM), and 9.7 sec (150 μM). *C*, Data plotted as a dose–response relationship for both the GABA-induced current (*squares*) and the desensitization rate (*diamonds*). For this purpose, the inverse of the time constant (the rate constant) has been used. The dose–response relationships for the two processes (peak current and desensitization) correspond well.

surements were made in a bath solution that included PTX (20 μM), and the perfusion pipette contained GABA (30 μM) in addition to PTX. (Coapplication of GABA and PTX, in the absence of pretreatment with PTX, was complicated by the interaction of the on- and off-rates of the two drugs and could not be used for these purposes.) The currents found when recording in this paradigm were extremely small (<150 pA) compared to the normal response to such a dose of GABA, yet the desensitization was within the normal range of that seen in the absence of PTX (even to the faster extreme).

This observation of a normal rate of desensitization in the presence of reduced current supports the conclusion that desensitization is receptor mediated and is independent of the current flow through the channel or the redistribution of ions across the membrane. Additionally, the continued desensitization in the presence of a pharmacological blocker, PTX, which is thought to be localized to a site distinct from the agonist binding site (White et al., 1985), together with the bicuculline experiments, helps to localize the desensitization process to the agonist-receptor binding site. While the extent of block induced by the doses used of these two compounds differed from cell to cell, there was no correspondence between the extent of block and the degree of desensitization present for either of these compounds.

As reported in other systems (chromaffin cells, Bormann and Clapham, 1985; chick central neurons, Mierlak and Farb, 1988), the coapplication of GABA with diazepam (DZP) led to an enhanced rate of desensitization when compared to the response to GABA alone (Fig. 6*C*). This may be equivalent to the generation of an effectively higher dose of GABA, since the increased affinity for GABA induced by benzodiazepines will yield a higher concentration of ligand-bound receptor complexes. Increasing the concentration of the agonist would produce a similar effect, as long as the dose range involved is near the middle of the dose–response curve. However, an additional factor may be involved, as the rate of desensitization increased more than that expected from the increase in the absolute magnitude of the induced current (Mierlak and Farb, 1988).

Responses to muscimol were compared with those to GABA in a series of cells. At concentrations producing comparable peak currents, the two agonists produced indistinguishable desensitization (Fig. 6*D*).

Dependence of desensitization on the membrane potential

The desensitization process was influenced by the holding potential; as the membrane was more depolarized, the time constant of desensitization increased (Fig. 7). By making direct measurements of the GABA-induced conductance change over time, it can be shown that the voltage dependence of desensitization is independent of the internal $[Cl^-]$. As seen in Figure 8, the extent of decay of the conductance change is dictated by the holding potential under all three ionic conditions studied (*A*, $[Cl^-]_i$ = 149 mM; *B*, $[Cl^-]_i$ = 29 mM; *C*, $[Cl^-]_i$ = 10 mM). These results also provide further substantiation for a lack of a connection between the magnitude of the current and the degree of desensitization since, when the internal chloride is mostly replaced with the impermeant species aspartate (as in Fig. 8*C*) and the reversal potential for GABA-induced current is close to −50 mV, more current flows at a potential of 0 mV than at −60 mV or −30 mV, and yet the degree of desensitization still correlates well with the holding potential.

The voltage dependence of desensitization was seen most eas-

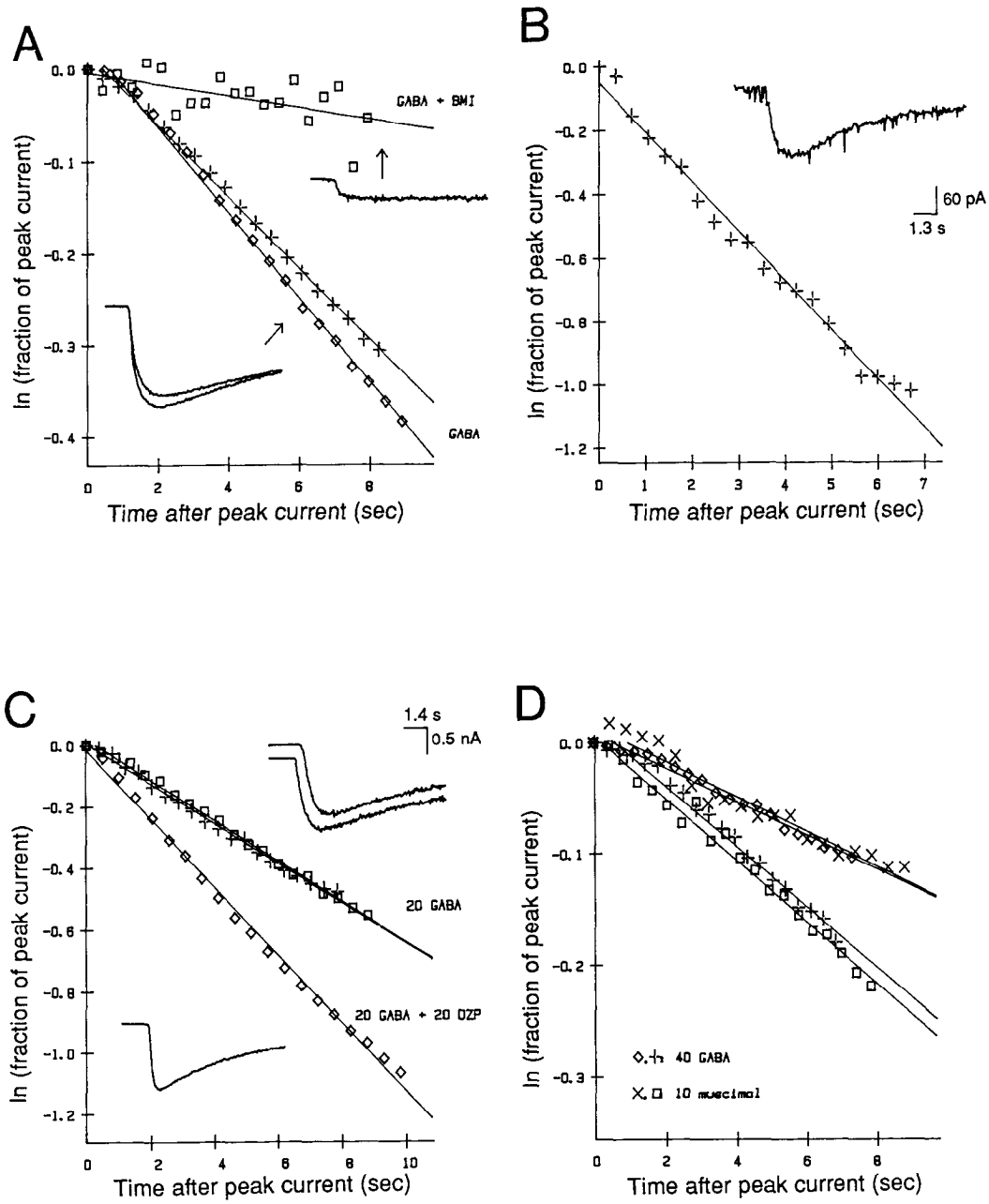

Figure 6. Pharmacological manipulation of desensitization of GABA-induced current. *A*, Desensitization measurements of GABA current (30 μM) in the presence and absence of BMI (10 μM) shows that the presence of BMI leads to a reduction in the rate of current decay, which is reversible [time constants are 25.6 sec for GABA (*diamonds*), ~150 sec for GABA with BMI (*squares*), and 21.6 sec for recovery (*pluses*)]. Membrane potential was maintained at −60 mV. The *inset* traces show the actual current records (calibration as in *C*). *B*, When GABA (30 μM) was applied to a neuron in a bath containing PTX, the current induced was markedly smaller than normal (<150 pA) yet decayed with a time constant in the normal range (6.8 sec). The *inset* in the graph shows the actual current record. Membrane potential was maintained at −60 mV. *C*, Desensitization rates for a neuron in response to GABA (20 μM) or the combination of GABA with DZP (20 μM). The cell was tested twice with GABA alone showing a peak current of 1.3 nA of inward current at −60 mV and a decay time constant of 15.3 sec (*squares* and *pluses*). When tested next with the combination of GABA and DZP (*diamonds*), the peak current was enhanced nearly 100% (to 2.5 nA of inward current). At the same time, the rate of desensitization was increased; the time constant dropped to 9 sec. These changes were only slowly reversible, presumably secondary to the lipophilic nature of this benzodiazepine. The *insets* show the actual current records with the appropriate calibration. The membrane potential was maintained at −60 mV. *D*, Fitted desensitization time courses of responses to GABA (40 μM) and muscimol (10 μM), at both −60 mV and +30 mV, show comparable desensitization for the two agonists. The doses of agonist were selected to match roughly the peak GABA-induced current [−1.7 nA and 896 pA for GABA at −60 mV (*pluses* and *squares*) and +30 mV (*diamonds* and *crosses*) vs. −1.6 nA and 625 pA for muscimol]. The desensitization time courses are essentially indistinguishable.

ily with midrange doses of GABA. Clearly, when doses were examined that did not desensitize at −60 mV, no desensitization was seen at more positive membrane potentials either. Similarly, when maximal doses were applied (as high as 150 μM), the difference between the rate of desensitization at near physiological values of membrane potential and at strongly depolarized levels was reduced. When working with the dose range of 20–50 μM GABA, the general range of time constants for the

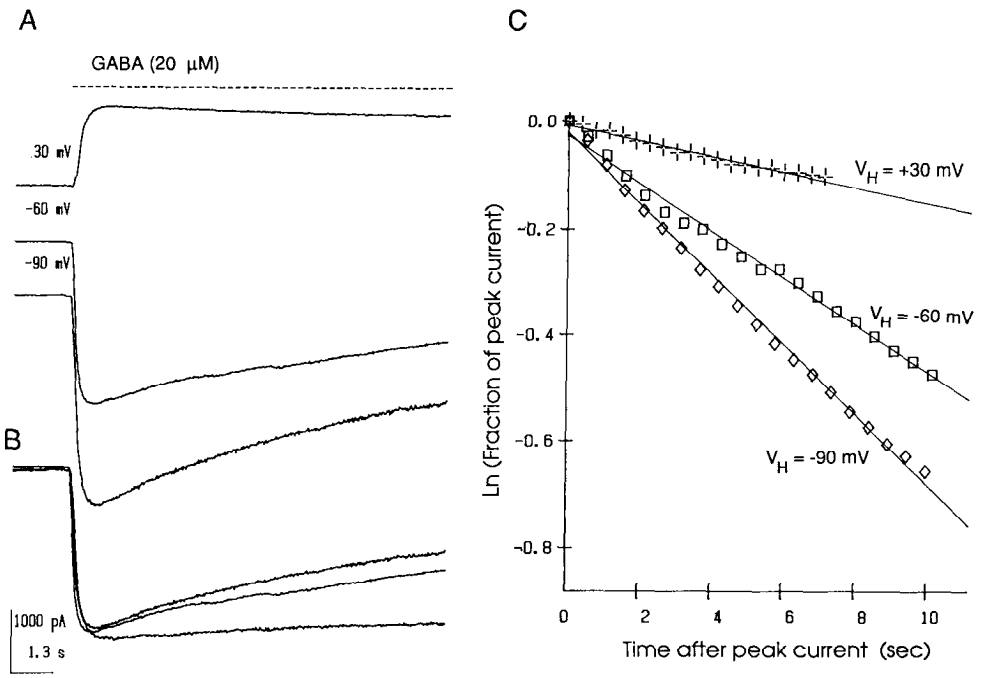

Figure 7. The rate of desensitization depends on membrane potential. *A* and *B*, Records of responses of a neuron to 20 μM GABA at three holding potentials. At the *top* of the figure, the unscaled records are shown. As the membrane potential is depolarized from −90 mV to −60 mV and finally to +30 mV, the rate of desensitization is slowed. This can be seen in *B*, where the data are replotted in a scaled manner to match peak currents, and the current trace at +30 mV is inverted. The voltages were tested in the order −60 mV, then +30 mV, and finally −90 mV to avoid errors from changes in response characteristics over time. Agonist was applied for the time indicated by the *broken line*. *C*, Decay time constants from the data shown in *A*. The calculated time constants from regression analysis of a log plot of the decay time course are 69.4 sec at +30 mV, 22.5 sec at −60 mV, and 15.1 sec at −90 mV. For all three values of holding potential, the data are well described by a single exponential fit.

decay phase was from 4.3 to 67 sec (27.4 ± 17.3, mean ± SD; $n = 20$) at −60 mV and from 11.3 sec to unmeasurable (treated as a value of 200 sec for calculations) (mean, 144 ± 75 sec) at +30 mV. The ratio of time constants of +30 mV to −60 mV for this same set was 2.6 to 10 (5.9 ± 3).

The difficulties involved in obtaining ideal spatial control when recording in voltage-clamp mode from cultured cortical neurons can be substantial. In order to demonstrate that the voltage dependence of the desensitization was not an artifact of inadequate space clamping, cultures were treated with colchicine at doses sufficient to produce loss of dendritic trees and to generate a rounded soma (10–50 ng/ml for 24 hr). Compared to control cells with elaborate geometries, these neurons demonstrated similar rates of desensitization to GABA and voltage dependence of this desensitization (data not shown).

Temporal stability of desensitization of GABA-induced current

It was possible to make measurements over a long period of time in some cells in order to examine the effects on the GABA response. For these experiments, in which recordings lasted up

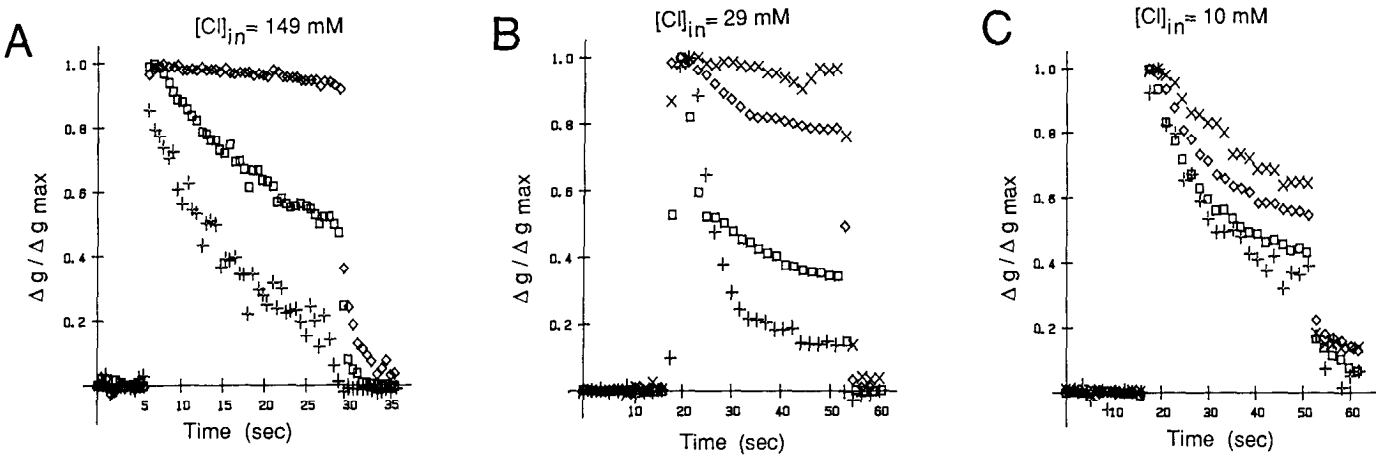

Figure 8. Voltage-dependent desensitization of GABA-induced current is present under conditions of lowered internal [Cl^-]. *A*, The directly measured changes in GABA-induced conductance decrease as a function of time at three membrane potentials with high internal [Cl^-] (*pluses*, −90 mV; *squares*, −60 mV; *diamonds*, +30 mV). As expected from previously shown data, the desensitization process is more rapid at more hyperpolarized levels of membrane potentials. Bath solution contained ES, 5 μM TTX, and 4 mM $CoCl_2$; recording electrode contained (in mM) 120 CsCl, 25 TEA-Cl, 1 $MgCl_2$, 1 $CaCl_2$, 1.5 EGTA, and 10 HEPES-NaOH, pH 7.2. GABA was applied at 30 μM. *B*, Data similar to *A* except that the internal [Cl^-] was reduced to 29 mM by replacement of 120 mM CsCl with 120 mM Cs-aspartate. All other solutions remained the same. The results show a voltage dependence to the rate of desensitization here as well (*pluses*, −90 mV; *squares*, −60 mV; *diamonds*, −30 mV; *crosses*, +30 mV). *C*, Another cell recorded with internal [Cl^-] of 10 mM also shows the same phenomenon (*pluses*, −90 mV; *squares*, −60 mV; *diamonds*, −30 mV; *crosses*, 0 mV). Internal solution was (in mM) 114 Cs-aspartate, 6 CsCl, 25 TEA aspartate, 1 $MgCl_2$, 1 $CaCl_2$, 1.5 EGTA, and 10 HEPES-NaOH, pH 7.2.

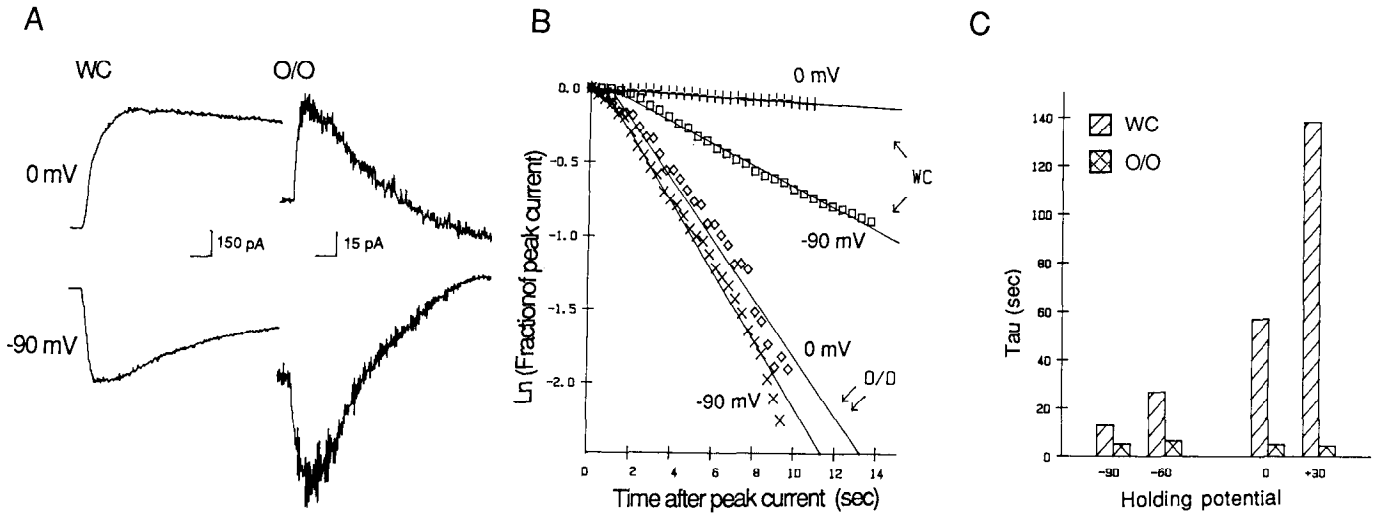

Figure 9. Comparison of desensitization of GABA responses in whole-cell (*WC*) and O/O recordings. *A*, Current records in response to 30 μM GABA from a whole-cell recording (*left*) and an O/O patch formed from the same cell (*right*) show some striking differences. The whole-cell records, as demonstrated previously, exhibit the voltage dependence normally observed for GABA desensitization. In contrast, the decay of the patch responses appears to be unaffected by alterations in membrane potential (time calibration, 1.3 sec for both sets of tracings). The desensitization of patch current to a level lower than resting may reflect an alteration of the leakage current in response to superfusion. *B*, Plot of the data shown in *A*. For whole-cell records, the fitted time constants are 13.8 sec at −90 mV and 118 sec at 0 mV; for the O/O patch, 4.3 sec at −90 mV and 5 sec at 0 mV. *C*, Data from 13 cells, at a variety of voltages, showing the influence of membrane potential on desensitization rates in the whole-cell mode (*hatched bars*) but not the O/O patch mode (*crosshatched bars*). Data were used from cells recorded with a variety of ionic compositions. Data were included for cells from which measurements were made at the same potential in both the O/O and whole-cell modes. (Data were not available at all potentials for all cells.)

to an hour, responses were tested with pairs of GABA applications with a short wash between them. Between pairs of measurements, the cell was allowed to rest briefly for about 1 min. At a given holding potential, the peak current in response to GABA decreased over time—although at a slower rate than reported for acutely dissociated hippocampal neurons (Stelzer et al., 1988)—and the rate of desensitization *increased* (data not shown). The gradual rundown of the GABA-induced current could not be explained by a loss of the standard resensitization mechanism, since near the end of the recording the paired GABA responses remained comparable. The rate of desensitization increased at both +30 mV and −60 mV, but remained strongly influenced by membrane potential.

Lack of influence of modulator candidates on properties of GABA-induced current

GABA applications in normal (2 mM) and high (10 mM) Ca^{2+}-containing bath solutions show effectively no differences in their voltage-dependent desensitization (data not shown). All of the expected desensitization characteristics of the GABA response were also present when the internal calcium was as high as 10 μM (1 mM Ca^{2+}, 1 mM EGTA) or as low as ~1 nM (0 added Ca^{2+}, 5.5 mM EGTA). Recordings were also obtained from a series of neurons in which the pipette solution contained ATP (1.5 mM), GTP (0.1 mM), cAMP (0.1 mM), and cGMP (0.1 mM) in addition to the standard electrolyte. The responses to GABA, the desensitization of GABA-induced current, and its voltage dependence were all unaffected. For a series of six cells, there was a range of τ values at −60 mV of 19.1–63 sec (mean, 33 ± 16 sec), and a range of 57 to >200 sec (mean, 120 ± 64) at +30 mV; the ratio of τ values at the two voltages ranged from 2.6 to 5.4 (mean, 3.7 ± 1.3). These results were indistinguishable from the control measurements presented earlier. Forskolin and phorbol 12-myristate 13-acetate (PMA) were used in attempts to alter desensitization kinetics of GABA-induced currents through phosphorylation by cAMP-dependent protein kinase A (PKA) or protein kinase C (PKC). Cells were exposed to these agents in growth media for 1 hr prior to recording. For forskolin, similar treatments have been shown to increase the cAMP content in these cultures (P. A. Rosenberg, personal communication). The inability to use each cell as its own control necessitated looking only for clearly unusual responses. No consistent changes were seen for cells treated with forskolin (60 μM) or PMA (100 nM).

Microscopic GABA-induced currents

Characterization of the time course of GABA-activated channels

Outside-out (O/O) patches obtained from cortical neurons that contained GABA-activated channels almost always contained numerous channels rather than a single channel (M. Frosch and M. Dichter, unpublished observations). The responses of these patches to GABA were characterized by a rapid rise to a peak current followed by a decay, often complete within several seconds (Fig. 9*A*). The decay phase could often be described by a single exponential component for most of its time course (Fig. 9*B*). The general characteristics of this process were similar to the observed desensitization of GABA-induced current as measured in the whole-cell recording mode as considered above. The most prominent differences between desensitization in these patches and in whole-cell recordings were in the absolute rates of desensitization and the effect of membrane potential on the rate of desensitization. The rate of decay of current in patch recordings was faster than the rate when measured in the whole-cell recording mode at the same value of membrane potential. This can be seen in Figure 9*A*, which shows the decay of GABA-induced current in a whole-cell recording and in an O/O patch formed from the same cell. The rate of desensitization in the

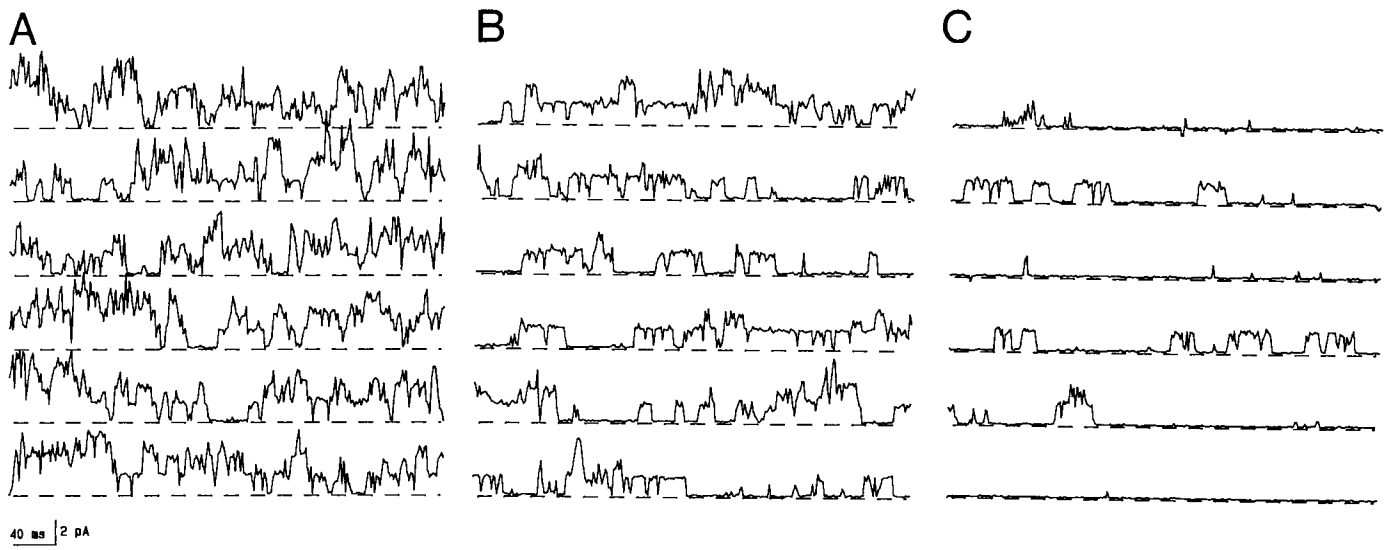

Figure 10. Single GABA-activated channel amplitudes are stable during desensitization. *A,* Sequential records, each representing 400 msec of channel activity from an O/O patch desensitized with GABA ($V_H = -60$ mV; equimolar Cl^- on both sides of the membrane). The first block of data occurred 7.2 sec into the superfusion. There are clearly several channel levels present here, although complete closings are seen. *Broken lines* represent zero current flow. Data were sampled at 2.5 kHz and filtered at 2 kHz. *B,* Data as in *A,* but starting 12 sec into the superfusion. The constancy of the single-channel amplitude can be seen by comparison with the adjacent records from *A.* There are many more single openings here, with rare transitions to levels with more than two channels open. *C,* A final set beginning after 22 sec. The channels are mostly closed, although well-defined openings are present. This patch predominantly displayed the 27 pS conducting state.

O/O patch was more rapid (with time constants for the decay process of 5–6 sec) than in the whole-cell recording mode. Moreover, the rate of desensitization was the same for the patches at a membrane potential of −90 mV as it was at 0 mV (Fig. 9*B*). In contrast, in whole-cell recordings, the rate of desensitization was strongly influenced by the value of the membrane potential (a decay time constant of 13.8 sec at −90 mV compared to insignificant decay at 0 mV).

This pair of differences between the decay rates observed in excised patch and whole-cell recordings from the same cell was a consistent finding. The use of the lowered internal concentration of chloride for the whole-cell measurements leads, if anything, to an overestimate of the decay in GABA-induced conductance seen at 0 mV, as shown above. The advantage of these ionic solutions was the ability to make observations of patch responses while holding at 0 mV—the reversal potential for nonselective cation channels, which otherwise would have contaminated the records, making direct measurement difficult. Data from a series of cells have been pooled in Figure 9*C,* showing that the patch responses decayed in a more rapid manner than whole-cell responses. The influence of membrane potential on the rate of decay of GABA response was lost when the transition from whole-cell to O/O patch was made. This appeared to happen as soon as the excised patch recording was obtained, and contrasts strikingly with the overall stability of the voltage dependence of desensitization when examined for long periods of time during whole-cell recordings.

The amplitude of the response of a patch to the application of GABA is given as the product of three terms: the probability of a channel being open (p), the total number of channels in the patch (N), and the single-channel current (i). Of these three, the first and third variables are candidates to be time dependent, while it is unlikely that the structural variable (number of channels in the patch) would change over time. From the examination of a rare patch that had a relatively small number of channels (about 12–15, based on the peak current with initial application of GABA), it can be seen that the number of channels open at any given time changes from $\gg 1$ at early times to < 1 later into the application of GABA (Fig. 10). These records also make clear the relative stability of the single-channel amplitude over time—while it is possible that there are fluctuations, there is no decay in unitary conductance that matches the observed decay in current. Thus, the effect of desensitization is to decrease the single-channel open probability.

Discussion

When GABA is applied to cultured rat cortical neurons, the response (either measured as the change in induced current or membrane conductance) decays over time, as the cell continues to be superfused. This process has been identified as receptor-mediated desensitization, signifying that the actual response to the agonist has decreased, rather than a change in the expression of the response. This desensitization is dose dependent in a manner that correlates well with the activation of the current and is reliably described by a single exponential process. During whole-cell recording, the desensitization is not irreversible; rather, after intervals on the order of tens of seconds, repeated application of GABA induces an identical current, which decays over time. Thus, there is a process of resensitization that removes channels from the desensitized state and returns them to a condition from which they can again be activated by application of GABA.

Studies of desensitization at the single-channel level address the mechanism of desensitization. The measurement of desensitization at the channel level was expedited by the tendency of O/O patches that are sensitive to the application of GABA to contain numerous channels. Application of GABA to these patches induces a current that often reaches tens of picoamperes at maximum and decays with an exponential time course. The data shown here indicate that the decay in current over time

reflected a change in the open channel probability rather than a change in single-channel amplitude. Desensitization, therefore, represents a single-channel event with a decrease in the open probability over time. A similar mechanism was described for GABA-activated channels in chick cerebral neurons (Weiss, 1988), although the distinction between reversible desensitization of channels and persistent "rundown" of channels in these O/O patches was not made.

Desensitization of GABA-induced current can be modulated with pharmacological agents. When the decay of GABA-induced current is examined in the presence of a competitive ligand for the GABA site (bicuculline), the decay of the residual current is slowed. This is consistent with a model in which the GABA receptor complex (GRC) must have its two GABA sites occupied with agonist ligands prior to shifting into the desensitized state (but see Macdonald et al., 1989a,b). When bicuculline is present, the dwell time in the doubly bound $GABA_2$-GRC state might be reduced, thereby reducing desensitization. In contrast, in the presence of PTX, the kinetics of GABA action reveal only minimal changes in decay rate. Since PTX interacts with a site distinct from the GABA binding site, this is consistent with a model that proposes that the desensitization transition is related to the interaction of GABA with its receptor sites and unrelated to the passage of current. Finally, inclusion of a benzodiazepine increased rates of desensitization as would be expected from an agent that enhanced GABA binding. An alternative model could be proposed with a separate binding site for GABA that modulates desensitization independent from the agonist-gated sites; however, our experiments do not address this possibility.

Enhancement of phosphorylation by PKA and PKC stimulation, the inclusion of nucleotides, and alterations in $[Ca^{2+}]_i$ or $[Ca^{2+}]_o$ were all without apparent effect on the desensitization rate or the voltage dependence of desensitization. In freshly dissociated hippocampal neurons, it has been shown that Mg-ATP appears to be required to slow the rate of rundown of GABA current (Stelzer et al., 1988). This group did not find any influence of this phosphorylation-enhancing factor on desensitization, although they did not examine the effects of voltage.

The most striking characteristic of desensitization during whole-cell recording, in the present study, is the influence of membrane potential: as the membrane potential is depolarized, the rate decreases. This modulation depends on the absolute level of the membrane potential rather than the amount or direction of flow of GABA-induced current. A similar phenomenon has been observed in rat hippocampal neurons in culture (Oh and Dichter, 1992). Interestingly, however, in another preparation, postnatal rat retinal ganglion cells, we have found *no* influence of membrane potential on the rate of desensitization of GABA-induced current (Tauck et al., 1988); the rate remained rapid at various voltages and was similar in both the whole-cell and O/O patch configuration (Lipton, 1989). This difference between central neurons of the same species raises the possibility of variation in subunit composition of the complex (Betz, 1990). The desensitization of channels in O/O patches in cortical neurons is quite different, however, from that observed in whole-cell recordings. The current induced by GABA in patches desensitizes more rapidly than the whole-cell current; additionally, there appears to be *no* influence of membrane potential on the single-channel response. In fact, desensitization rates measured in patches at 0 mV are typically as fast as or faster than those seen in whole-cell recordings at extremely hyperpolarized levels. The absence of voltage sensitivity in the rate of desensitization, as well as the overall relative speed of desensitization when GABA-activated channel responses are examined in excised O/O patches, indicates that the influence of membrane potential observed in the whole-cell configuration is *not* an intrinsic channel property. That is, the effect of membrane potential of slowing the rate of desensitization as the cell is depolarized is mediated through some other membrane component that does not accompany the GABA-activated channels into the excised patch of membrane.

Desensitization has the important characteristic of decreasing the effect of the transmitter. In cell culture, at least, neurons may demonstrate "tonic" partial GABA receptor desensitization due to ongoing spontaneous inhibitory synaptic action (Dichter and Frey, 1989). At the potentially high concentration of GABA present in the synaptic cleft, the importance of desensitization would be large. The shifts in ions that have been found by other investigators and are present in this system under certain recording conditions (e.g., when intracellular $[Cl^-]$ is low) also decrease the apparent effect of GABA (the shift in membrane potential or the induced current); however, the shunting action of GABA would not be decreased by ionic shifts.

The process of desensitization and its regulation by membrane potential suggests the possibility for intrinsic modulatory influences on GABA-mediated inhibition. Alteration of the voltage dependence of desensitization could represent a "fine-tuning" mechanism for cortical GABAergic inhibition. Furthermore, the voltage dependence of GABA desensitization might also be important in abnormal states such as epilepsy. For example, as a cell becomes depolarized by excitatory neurotransmitters, the inhibitory action of GABA would become more prominent; GABA desensitization is less pronounced at more positive membrane potentials. Future studies of synaptic currents under normal and abnormal conditions will be necessary to define further the role of voltage-dependent GABA desensitization in the functioning of the nervous system.

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