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γ-Aminobutyric acid receptor channels in adrenal chromaffin cells: A patch-clamp study

(adrenal medulla/single chloride ion channels/diazepam)

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ABSTRACT **We have studied membrane channels activated by γ-aminobutyric acid (GABA) in adrenal medullary chromaffin cells by using patch-clamp techniques. These channels share many properties with GABA-receptor channels in the central nervous system. They are chloride-selective, blocked by the GABA antagonist bicuculline, and reversibly desensitized at high GABA concentrations. The dose–response curve has a slope of 2 in the Hill plot, indicating a bimolecular binding reaction of GABA to the receptor. Single-channel currents display multiple conductance states as do glycine-activated chloride channels in mouse spinal neurons. Gating properties of GABA-activated channels, as described by a sequential model for agonist-activated channels, are similar to gating properties in central neurons. GABA-induced currents are potentiated by diazepam, indicating that anxiolytic drugs like the benzodiazepines might be involved in the regulation of anxiety states in the peripheral nervous system.**

In the mammalian central nervous system γ-aminobutyric acid (GABA) is an inhibitory transmitter (see ref. 1) that causes an increase of the postsynaptic membrane conductance to chloride ions (2–4). The peripheral nervous system, in contrast, uses mainly adrenergic receptors to regulate inhibitory activity. Adrenergic regulation is unlikely to occur in adrenal medulla, because cholinergic stimulation, via splanchnic nerve terminals, releases large amounts of epinephrine and norepinephrine from medullary chromaffin cells. One mechanism underlying control of catecholamine release could be modulation by substance P, which enhances desensitization of the acetylcholine receptor (5). Recently, Kataoka *et al.* (6) reported that chromaffin cells are capable of synthesizing, storing, and releasing GABA. These cells also possess high-affinity binding sites for [^{3}H]muscimol, a potent GABA receptor agonist, and for [^{3}H]flunitrazepam, a benzodiazepine receptor ligand. We have asked whether these binding sites represent GABA receptors coupled to Cl^- channels, as in the central nervous system. Using patch-clamp techniques (7), we found GABA-dependent Cl^- channels in isolated bovine chromaffin cells. These channels, which might be involved in the control of catecholamine release, can be modulated by the benzodiazepine compound diazepam.

MATERIALS AND METHODS

Cell Culture. Single bovine chromaffin cells were enzymatically isolated from bovine adrenal medulla as described (8, 9). The cells were plated at densities of 5000–50,000 cells per dish on poly(lysine)-coated glass plates or on 35-mm Falcon tissue culture dishes. Spherical cells (10–20 μm diameter) were selected for electrophysiologic recording, but more differentiated cells gave similar results. Cells were used 1–8 days after culturing.

Electrophysiology. All cells were bathed in standard saline solution at room temperature (21–23°C). The extracellular solution was 140 mM NaCl/2.8 mM KCl/2 mM $MgCl_2$/1 mM $CaCl_2$/10 mM Hepes/NaOH, pH 7.4. The intracellular solution was either 143 mM KCl/2 mM $MgCl_2$/1 mM $CaCl_2$/11 mM EGTA/10 mM Hepes/KOH, pH 7.2, or 120 mM CsCl/20 mM tetraethylammonium chloride/2 mM $MgCl_2$/1 mM $CaCl_2$/11 mM EGTA/10 mM Hepes/NaOH, pH 7.2. To determine Cl^- selectivity, chloride was partially substituted by isethionate (2-hydroxyethanesulfonate). Liquid junction potentials were corrected as described (8). GABA was purchased from Sigma and bicuculline methiodide from Pierce. Diazepam was a gift from Hoffmann-La Roche (Grenzach-Wyhlen, F.R.G.).

We used whole-cell, outside-out, and cell-attached variations of the patch-clamp technique (7). Whole-cell recordings in current clamp gave resting potentials of −60 to −70 mV. We used low-resistance (1–2 MΩ) soft-glass pipettes in most whole-cell recordings (low series resistance) and hard-glass pipettes (Pyrex) with resistances of 10–20 MΩ for outside-out patches (lower noise). We recorded the current and voltage outputs from the patch-clamp amplifier (EPC-7, List Electronic, Darmstadt, F.R.G.) on magnetic tape.

We perfused single cells under voltage clamp two ways: in one method we perfused the bath (volume <1 ml) at a rate of 1 ml/min; the bath could be completely exchanged in 2–3 min. For fast application, we used a U-shaped glass capillary tube with an outlet hole (≈100 μm) placed next to the cell. A perfusion pump controlled the rate of flow through the U-tube (0.1 ml/min). Rapid switching by an electronic valve controlling the outflow resulted in concentration changes of the solution bathing the cell within 100 msec (5, 8). We applied the same perfusion methods to outside-out patches.

Analysis. We digitized all single-channel data at 0.4 msec intervals after filtering at 1000 Hz. For details of analysis see ref. 10. We filtered whole-cell recordings of cell current at 50 or 100 Hz, and we plotted these on an X-Y recorder from a digital oscilloscope.

RESULTS

Whole-Cell Recordings. Fig. 1*A* shows the current evoked by external perfusion of a chromaffin cell in standard solution containing 10 μM GABA. The extracellular solution was initially standard saline and was then gradually changed to standard saline containing 10 μM GABA, a concentration which desensitized the cells only slightly. The cell was held at −70 mV in the whole-cell recording mode. The internal

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Abbreviation: GABA, γ-aminobutyric acid.

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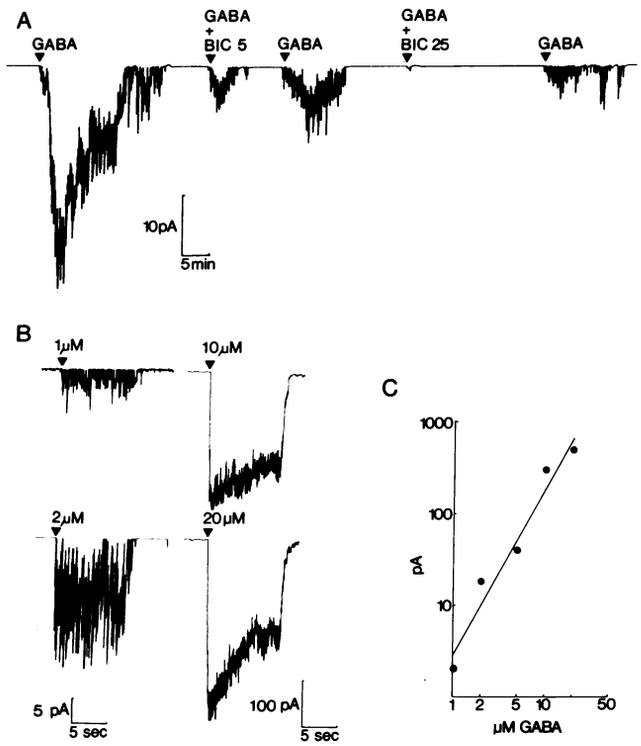

FIG. 1. Whole-cell recording from chromaffin cell. (*A*) GABA-induced inward currents from single chromaffin cell. Capacitance was 6.8 pF. The cell was voltage-clamped at −70 mV. Chloride concentration on both membrane faces was 145 mM. Bath perfusion with 10 μM GABA or 10 μM GABA plus bicuculline was begun at times indicated by the arrowheads. Bicuculline at 5 μM (BIC 5) inhibited the response to 22% of the peak response to GABA alone, and 25 μM bicuculline (BIC 25) almost completely blocked the response. Recovery was very slow and was incomplete on this time scale. (*B*) Responses of a single chromaffin cell to different GABA concentrations at −70 mV membrane potential. Capacitance was 5.1 pF. GABA was applied in standard extracellular solution (see *Materials and Methods*) via the U-tube for 9.5-sec pulses, and GABA-induced current was recorded. All records are from one cell. (*C*) Dose–response curve for the data in *B*. The peak response is plotted as a function of GABA concentration. The point at 20 μM GABA was corrected for desensitization. The points were best fit by a straight line of slope 1.9 (r = 0.998).

perfusion of chromaffin cells is complete within 15 sec (8). With our intracellular solution, chloride concentrations of 145 mM were therefore rapidly obtained on both sides of the membrane. At negative membrane potentials, chloride ions leave the cell, giving rise to an inward current. In 10 μM GABA, the peak response was 37 pA. In 10 μM GABA plus 5 μM bicuculline, the current was reduced to 8.2 pA (22% of peak). Bicuculline blocks the GABA response (11) by competing with GABA for the receptor (12). After washing the bath with standard saline solution and applying GABA again, recovery from bicuculline was incomplete. In 10 μM GABA plus 25 μM bicuculline, almost all channels were blocked. Again, recovery was incomplete, even after >15 min of washing the bath with standard saline.

The peak GABA-induced current per chromaffin cell in response to 10 μM GABA ranged from ≈5 pA to 400 pA. We cannot explain the large variability from cell to cell. The mean of the current from 12 cells was 10.5 ± 9.3 μA/cm^2 (assuming a cell capacitance of 1 μF/cm^2). There was no significant correlation of the size of GABA-induced current with the number of days in culture after day 1.

We obtained the dose–response curve to GABA by fast application of GABA to a whole-cell in voltage clamp via the U-tube (Fig. 1*B*). At 1 μM GABA, only one or two channels were open at any one time, but at 20 μM the GABA response was large and rapidly desensitized. Fig. 1*C* shows a double-logarithmic plot of peak response vs. GABA concentration. We corrected the point at 20 μM for desensitization by extrapolating to time zero (see below). The straight line, obtained by a least-squares fit, had a slope of 1.9. This indicates that two molecules of GABA must bind to the receptor before the channel can open (13). Experiments on spinal neurons have shown that the concentration range used here is indeed low compared with the microscopic dissociation constant K_d (unpublished data).

GABA-induced currents were desensitized with GABA concentrations >5 μM (see Fig. 1*B*). Fig. 2*A* shows a response of a chromaffin cell to a long U-tube application of 20 μM GABA. The current rapidly decreased following the initial peak of 400 pA to near base line after 80 sec of exposure to GABA. The current I as a function of time t was given by I = [210 pA · exp(−t/2.8 sec)] + [315 pA · exp(−t/20.8 sec)] + 5 pA. The initial peak was obtained by extrapolation to t = 0 and amounted to 530 pA. The two time constants, 2.8 sec and 20.8 sec, agree with observations obtained under identical conditions from GABA-activated channels in mouse spinal neurons (unpublished data). Cholinergic receptors at the frog neuromuscular junction also show biphasic desensitization (14).

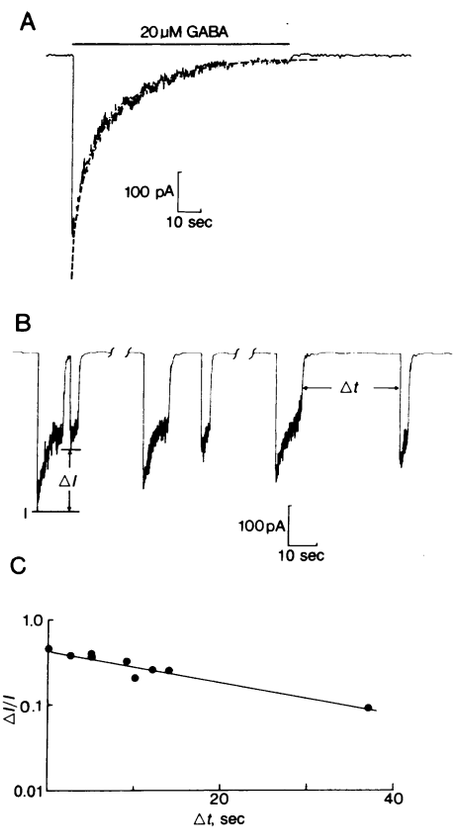

FIG. 2. Time course of desensitization and resensitization. (*A*) Application of 20 μM GABA via the U-tube resulted in a rapid decrease of whole-cell current following the initial peak. Cell was clamped at −70 mV at symmetrical 145 mM Cl$^-$. The time course of desensitization was fit by two exponentials plus base line, as shown by the dashed line (see text). (*B*) Recovery from desensitization after application of 20 μM GABA for 9.5 sec. At various time intervals (Δt) after desensitizing pulses, recovery was measured by applying test pulses of GABA. The difference, ΔI, between peak current induced by the desensitizing pulse (I) and that in response to the test pulse was calculated. (*C*) Plot of log(ΔI/I) as a function of Δt. The time constant for recovery was 23 sec.

We measured the recovery from desensitization by applying test pulses at various time intervals after exposing the cell to standard pulses of 20 μM GABA. Fig. 2*B* shows the experimental protocol with 9.5-sec pulses (20 μM GABA) followed at various time intervals Δt by test pulses (20 μM GABA). The difference between peak current induced by the standard pulse (I) and the test pulse is ΔI. In Fig. 2*C*, $\log(\Delta I/I)$ is plotted as a function of Δt. Recovery was approximated by a single exponential with a time constant of 23 sec. This value compares to 7 sec reported for nicotinic acetylcholine receptor channels in bovine chromaffin cells (5).

As in other preparations (13, 15), the chromaffin cell channel gated by GABA is chloride-selective. In symmetrical Cl^- concentrations of 145 mM, GABA-activated currents recorded in the whole-cell mode had a reversal potential close to 0 mV. When we replaced Cl^- in the pipette solution with equimolar concentrations of isethionate, a large impermeant anion for Cl^- channels, the reversal potential shifted according to the Nernst equation. For example, for 45 mM internal Cl^- the reversal potential was −28 mV.

Single-Channel Measurements. To study single GABA-gated channels we used outside-out patches (13, 16), and GABA was applied from the bath or by the U-tube. Fig. 3*A* shows currents through single GABA-activated channels from an outside-out patch in 10 μM GABA at −70 mV. The chloride concentration was 145 mM on both sides of the membrane. Apart from the main current level of 3.2 pA, there were at least two sublevels of 2.1 pA and 1.2 pA. Steps of 0.8 pA occurred rarely in this patch (arrow). Plotting current as a function of membrane potential, we obtained three conductance levels (Fig. 3*B*) of the channel: 45 pS, 31 pS, and 18 pS. In addition, an 11-pS conductance was present. The number of substates and the dominant conductance levels varied from patch to patch. As in Fig. 3 and in most other patches, the dominant conductance level was 45 pS. In some patches, however, this 45-pS level was not present. These conductance levels correspond closely to conductance levels of GABA- and glycine-activated Cl^- channels in spinal cord neurons, which are 46 pS, 31 pS, and 20 pS at 22°C (16). However, the 46-pS conductance level was shown to be the glycine main state in spinal cord neurons (16), whereas glycine, up to concentrations of 50 μM, did not open channels in chromaffin cells. Substates were identified as substates rather than overlaps of more than one channel by their appearance in bursts (see ref. 16).

Gating Properties of GABA-Activated Channels. We investigated the gating mechanism of GABA-activated channels on outside-out patches, using 5–10 μM GABA. Single-channel events occurred at low frequency (0.5–3 sec^{-1}). Fig. 4 shows the results from a patch exposed to 10 μM GABA at −70 mV. The amplitude histogram (Fig. 4*A*) has a single peak at 2.9 pA; in other words, only one conductance state (42 pS) was present. The distribution of closed times (Fig. 4*B*) includes 610 out of a total of 905 intervals between openings. This means that 295 closing events were longer than 50 msec. The distribution was fit by the sum of two exponentials. The slow component had a time constant of 20.3 msec, representing the average time between elementary current pulses. The fast time constant of 1.4 msec refers to the short closing gaps within elementary current pulses, since the channel presumably reopens several times during a single occupancy of the receptor (Nachschlag phenomenon) (17). Any group of openings separated by time intervals shorter

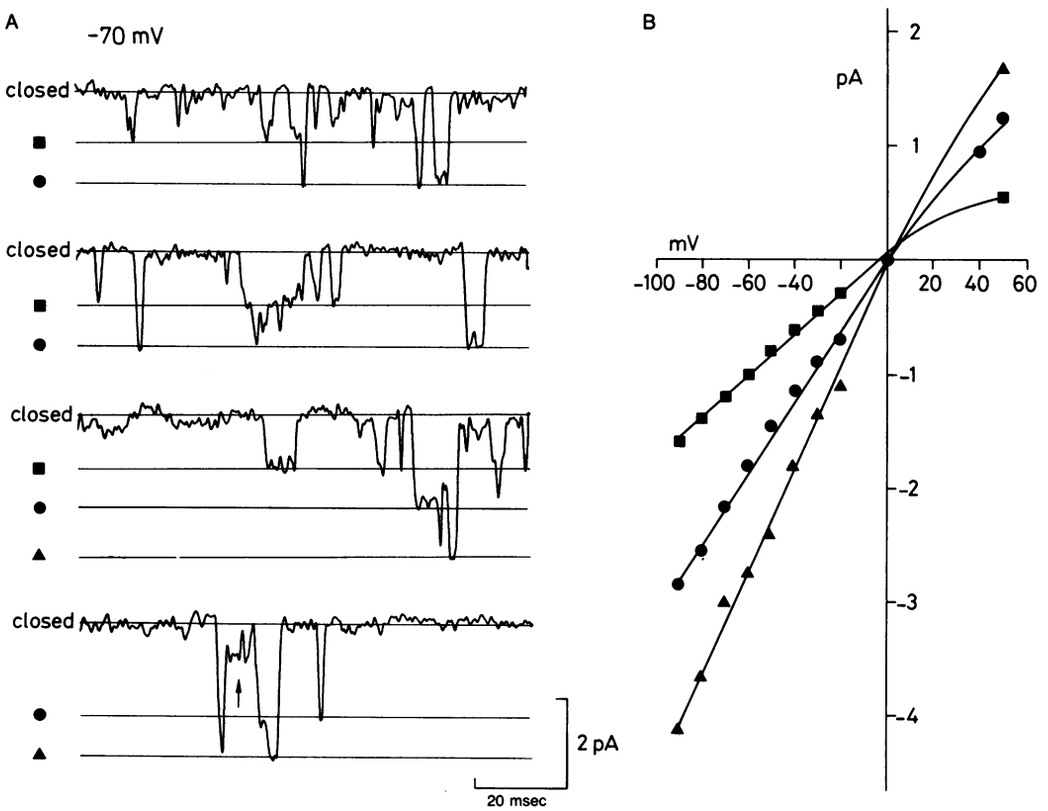

FIG. 3. Single-channel currents activated by GABA, recorded from an outside-out patch. Chloride concentration was 145 mM on both sides of the membrane. K^+ was replaced by Cs^+ in the pipette solution. After addition of 10 μM GABA in standard saline to the bath, single-channel currents started to appear. (*A*) Traces of recordings at −70 mV membrane potential display several conductance states. The current levels were 3.2 pA (▲), 2.1 pA (●), and 1.2 pA (■). Occasionally, small steps of 0.8 pA were observed (arrow). (*B*) Current-voltage relationship from the same experiment for the three conductance states. The points were fit by straight lines in the voltage range −100 to 0 mV. The slope conductances were 45 pS (▲), 31 pS (●), and 18 pS (■). At positive membrane potentials, single-channel current showed rectification.

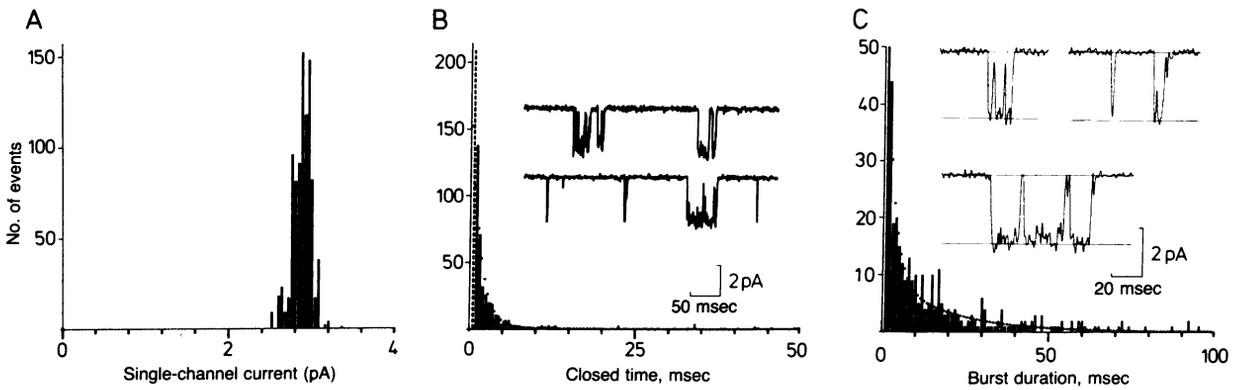

FIG. 4. Gating of GABA-activated channels. Outside-out patch recording with standard intracellular CsCl solution at −70 mV. GABA was applied in bath solution at a concentration of 10 μM. (*A*) Amplitude histogram of events has a single peak at 2.9 pA. (*B*) Distribution of time intervals between channel openings. From a total number of 905 closed times, 610 events shorter than 50 msec are shown in the histogram. The distribution is fit by the sum of two exponentials (dotted curve) with decay times of 1.4 msec and 20.3 msec. From the area under the fastest component, 348 short closings were obtained. (*Inset*) Two current traces at low time gain. (*C*) Distribution of burst durations. Bursts were selected as any series of openings separated by time intervals not longer than 5 msec (see text). (Examples of bursts at higher time resolution are shown in the *Inset*.) The distribution is fit by the sum of two exponentials (dotted curve) with decay constants of 2.5 msec and 20 msec. The number of bursts is 188. The area under the fastest component represents 41% of the total area.

than a certain value is defined as a burst (18, 19). We have chosen a threshold value of intervals that minimizes misassignments in the classification of intervals into intra- and interburst intervals (5, 20). The value was 5 msec in this case. We then constructed a histogram of burst duration (Fig. 4*C*). This distribution is fit by the sum of two exponentials with decay constants of 2.5 msec and 20 msec. They correspond to short openings and to bursts of reopenings. From the ratio of bursts to short closing gaps (188:348), the elementary current pulse of 20 msec was interrupted, on average, 1.9 times by gaps of 1.4 msec and therefore consists of 2.9 successive openings. Although the short events comprise 41% of the total number of bursts, they contribute only 9% of the total current. The mechanism is unclear but they may represent openings of the channel from the monoliganded state of the receptor (13, 17). In central neurons, the burst duration is around 50 msec at 22°C (13, 21).

We described the time course of channel gating with a reaction scheme similar to the one proposed by del Castillo and Katz (22) but modified to be consistent with the dose–response behavior of GABA-induced whole-cell currents:

$$A + R \underset{k_{-1}}{\overset{2k_1}{\rightleftharpoons}} AR + A \underset{2k_{-1}}{\overset{k_1}{\rightleftharpoons}} A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^*. \quad [1]$$

Two GABA molecules A bind to the receptor R in a stepwise manner to form the GABA–receptor complex A_2R, which isomerizes to A_2R^*, the open channel. For simplicity, we assumed the rate constants k_1 and k_{-1} to be identical in both binding reactions. The rate constants α, β, and k_{-1} (for channel closing, channel opening, and dissociation of agonist, respectively) can be inferred from the burst duration t_b, the closing-gap duration t_g and the number of closing gaps per burst n_g (18, 19):

$$\beta = 2k_{-1}n_g, \quad [2]$$

$$\alpha = \frac{n_g + 1}{t_b - n_g t_g}, \quad [3]$$

and

$$k_{-1} = \frac{1}{2\,t_g(n_g + 1)}. \quad [4]$$

The calculated rate constants were $\alpha = 167\ sec^{-1}$, $\beta = 467\ sec^{-1}$ and $k_{-1} = 123\ sec^{-1}$. We obtained similar results from two other patches. These values are close to those reported for hippocampal (21) and spinal neurons (unpublished data).

Modulation of Cl^- Channels by Diazepam. We tested the effect of diazepam, a clinically important benzodiazepine-receptor ligand, on GABA-activated Cl^- channels. Benzodiazepines enhance GABA-mediated synaptic transmission by increasing the total current flowing through the channels (23, 24). Fig. 5 shows whole-cell recordings from a chromaffin cell voltage-clamped at −70 mV. For the top trace, 10 μM GABA was applied in standard saline solution via the U-tube and the evoked inward current was recorded. The peak response was 65 pA. For the middle trace, the same GABA concentration plus 10 μM diazepam was added. The response was 145 pA at peak, and the current was desensitized. For the bottom trace, we again applied 10 μM GABA after 15 min of washout with saline solution. On average,

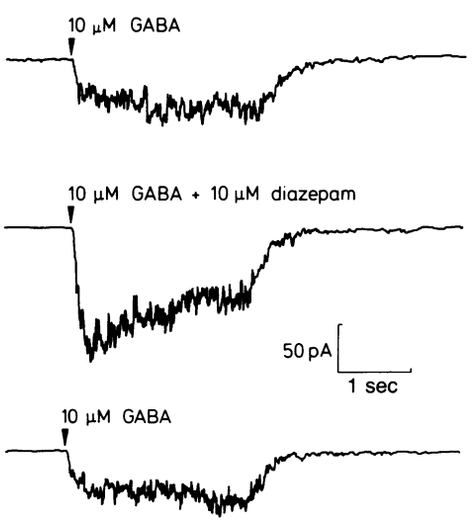

FIG. 5. Effect of diazepam on GABA-activated inward currents. The cell was clamped at −70 mV. Intracellular Cl^- concentration was 145 mM. Top trace shows a response of 65 pA evoked by 10 μM GABA applied in standard saline solution via the U-tube. After addition of 10 μM GABA plus 10 μM diazepam (middle trace), peak current was 145 pA and desensitization was more rapid. Bottom trace was obtained with 10 μM GABA after 15 min of washing the bath with saline.

GABA-activated currents in the presence of diazepam were potentiated by a factor of 2.1 ± 0.2 (mean ± SEM, for 4 cells). Consistently, the time course of desensitization appeared to be faster compared to control. The same behavior was found in cultured spinal neurons (unpublished data).

DISCUSSION

We have investigated the conductance and gating properties of GABA-activated membrane channels in bovine medullary chromaffin cells. Whole-cell recordings showed that these channels were selective for Cl^- ions and could be blocked by bicuculline. This indicates that they are coupled to $GABA_A$ receptors (see ref. 25). Cells responded to GABA in a dose-dependent manner with an increase in current that was proportional to the square of the GABA concentration. At ≥ 10 μM GABA, the currents decreased following the initial peak response (desensitization). The time course of desensitization was described by the sum of two exponentials. The recovery from the desensitized state of the receptor channels back to the activatable state was approximated by a single exponential.

GABA-activated single-channel currents from excised membrane patches usually displayed at least three different conductance levels, similar to glycine-activated channels in mouse spinal neurons. Glycine, on the other hand, did not open channels in chromaffin cells. It has been proposed that there is only one class of multistate Cl^- channel, the main conductance adopted by the channel being determined by the receptor (i.e., for GABA or glycine) (16). That the higher glycine conductance is activated by GABA in chromaffin cells might indicate a difference in the coupling mechanisms of brain and peripheral GABA receptor-channel complexes.

Elementary current pulses appeared as bursts of openings separated by short closing gaps. The burst duration was shorter than that reported for GABA receptor channels in central neurons, but there was also an excess of short events which may represent openings from the monoliganded state of the receptor. From the time course of single-channel gating, we were able to calculate some of the rate constants in terms of the modified del Castillo–Katz (22) reaction scheme for agonist-activated channels. The results compare quite well to those reported for GABA receptor channels in central neurons.

Chromaffin cells are stimulated by acetylcholine released from nerve terminals. This results in membrane depolarization and subsequent release of catecholamines and of GABA. Under physiological conditions, GABA-activated Cl^- channels conduct current in the opposite direction to acetylcholine receptor channels and are therefore inhibitory. The magnitude of the inhibitory current increases with depolarization (see ref. 21).

GABA receptor Cl^- channels in chromaffin cells can be modulated by the benzodiazepine compound diazepam. In whole-cell recording, GABA-activated currents were strongly potentiated by diazepam, indicating an interaction with the GABA receptor-channel complex. Since anxiolytic benzodiazepines modulate GABA receptor function in chromaffin cells, the regulation of anxiety states by these drugs may not be restricted to central GABAergic systems.

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