Contribution of changes in the chloride driving force to the fading of $I_{\text{GABA}}$ in frog melanotrophs

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Le Foll, Frank, Olivier Soriani, Hubert Vaudry, and Lionel Cazin. Contribution of changes in the chloride driving force to the fading of $I_{\text{GABA}}$ in frog melanotrophs. Am. J. Physiol. Endocrinol. Metab. 278: E430–E443, 2000.—Chloride redistribution during typeA $\gamma$-aminobutyric acid (GABA$_\alpha$) currents ($I_{\text{GABA}}$) has been investigated in cultured frog pituitary melanotrophs with imposed intracellular chloride concentration ([Cl$^-]$) in the whole cell configuration or with unaltered [Cl$^-$], using the gramicidin-perforated patch approach. Prolonged GABA exposures elicited reproducible decaying currents. The decay of $I_{\text{GABA}}$ was associated with both a transient fall of conductance ($g_{\text{GABA}}$) and shift of current reversal potential ($E_{\text{GABA}}$). The shift of $E_{\text{GABA}}$ appeared to be time and driving force dependent. In the gramicidin-perforated patch configuration, repeated GABA exposures induced currents that gradually vanished. The fading of $I_{\text{GABA}}$ was due to persistent shifts of $E_{\text{GABA}}$ as a result of $g_{\text{GABA}}$ recovering from one GABA application to another. In cells alternatively clamped at potentials closely flanking resting potential and submitted to a train of brief GABA pulses, a reversal of $I_{\text{GABA}}$ was observed after 150 s recording. It is demonstrated that, in intact frog melanotrophs, shifts of $E_{\text{GABA}}$ combine with genuine receptor desensitization to depress $I_{\text{GABA}}$. These findings strongly suggest that shifts of $E_{\text{GABA}}$ may act as a negative feedback, reducing the bioelectrical and secretory responses induced by an intense release of GABA in vivo.

Desensitization; intracellular chloride concentration; gramicidin-perforated patch recording

In the melanotrophs of the frog pituitary pars intermedia, $\gamma$-aminobutyric acid (GABA) or GABA$_\alpha$-receptor agonists exert a transient stimulation followed by a prolonged inhibition of $\alpha$-melanocyte-stimulating hormone ($\alpha$-MSH) release in vitro (7). By using the gramicidin-perforated patch technique, which avoids artifactual alterations of the physiological intracellular chloride concentration ([Cl$^-$]; see Ref. 8), we have recently demonstrated that GABA induces an outward chloride flux from the intracellular compartment containing unusually high [Cl$^-$]. The depolarization that results from the outward current triggers the activation of voltage-operated sodium and calcium channels, leading to the initial stimulation of $\alpha$-MSH secretion. The delayed inhibitory phase of the secretory response likely originates from the strong shunting effect that accompanies the depolarization induced by high micromolar GABA concentrations and causes cessation of firing (17). Factors affecting the efficacy of the GABAergic transmission are of outstanding importance in the regulation of $\alpha$-MSH secretion because they determine which of the effects, i.e., the depolarization or the resistive shunt, predominantly controls the hormone release. In the frog melanotrophs, the GABA$_\alpha$ receptor is subject to extracellular positive or negative modulation by benzodiazepines, barbiturates, and neuroactive steroids (18–20). In addition, in this cell model, the GABA$_\alpha$ receptor function is also controlled, at the intracellular level, by the balance between phosphatase and kinase activities (4).

An alternative or concomitant mechanism of regulation of the GABAergic transmission is the fading of the response induced by the agonist itself. In this regard, the time scale of the synaptic events has to be considered. For agonist exposure persisting for minutes to hours, a downregulation of receptors is responsible for a reduction of the number of $[^{3}H]$muscimol or $[^{3}H]$flunitrazepam binding sites (23, 28) due to a removal of GABA$_\alpha$ receptors from the cell surface (5). During short-term applications of GABA, electrophysiological experiments have demonstrated that the GABA$_\alpha$ current ($I_{\text{GABA}}$) declines despite the continuous presence of the agonist (3, 10, 18, 26). This early decay of responsiveness is commonly called desensitization. Accumulating evidence indicates that desensitization may involve distinct processes. A time-dependent decrease of the membrane chloride conductance in the presence of GABA has been observed in neurons and human embryonic kidney (HEK 293) cells expressing recombinant GABA$_\alpha$ receptors (9, 26, 34, 35). This phenomenon is a general feature of ligand-gated ion channels that possess nonconducting desensitized states (13, 21) and seems to be functionally equivalent to the inactivation of voltage-sensitive channels. The current decay could also result from a gradual passive redistribution of chloride between both sides of the cell membrane, leading to a shift of the $I_{\text{GABA}}$ reversal potential and a reduction of the chloride driving force ($E_{\text{GABA}}$). Such changes of $E_{\text{GABA}}$ have been described from experi-
ments using suction pipette (2), sharp microelectrode (32), or whole cell patch-clamp (12) recordings in rat or frog neurons. However, these recording configurations bring some drawbacks because of the rapid chloride exchange between the pipette and cell compartments (17), which likely introduces errors in the evaluation of the magnitude of $E_{\text{GABA}}$ displacements. The recently developed gramicidin-perforated patch technique, allowing patch-clamp recordings while preserving intact the physiological $[\text{Cl}^-]$, (1, 8, 16), represents a valuable method to investigate the ionic mechanisms responsible for the fading of $E_{\text{GABA}}$.

Herein, we have combined the standard whole cell and the gramicidin-perforated patch approaches to assess the relative contribution of genuine $\text{GABA}_A$ receptor desensitization and shifts in $E_{\text{GABA}}$ to the fading of $E_{\text{GABA}}$ in the cultured frog melanotrophs.

**MATERIALS AND METHODS**

Animals and cell cultures. Adult male frogs (Rana ridibunda; Couëtard, Saint-Hilaire de Riez, France) were used as tissue donors. Frogs were housed in a temperature-controlled room ($8^\circ C$) under an established 12:12-h light-dark photoperiod (lights on from 6:00 AM to 6:00 PM). The animals had free access to running water and were maintained in these conditions for at least 1 wk before use. Animal manipulations were performed according to the recommendations of the French Ethical Committee and under the supervision of authorized investigators. Cell cultures of frog pituitary melanotrophs were prepared as previously described (18). After anesthesia by immersion in 1% MS-222, the animals (body weight 30–40 g) were killed by cervical dislocation and anesthetization by immersion in 1% MS-222, the animals had 3–5 M $\mu g/ml$ was added. The progress of perforation was assayed by monitoring the access resistance deduced from the amplitude of the capacitive transients in response to repetitive 10-mV hyperpolarizing steps. The series resistance, typically 12 M $\Omega$ in the whole cell configuration and 14 M $\Omega$ after 10 min perforation (17), was partially (60–70%) compensated using the on-board circuitry of the amplifier. $\text{GABA}$ was dissolved in the standard extracellular solution and pressure ejected (2–4 psi) through a micropipette (2–4 $\mu m$ tip diameter). To avoid uncontrolled drug leakage, the ejection pipette was kept away and brought in close proximity (10–20 $\mu m$) to the cell soma just before microejection. Currents were recorded using an Axopatch 200A current-to-voltage converter (Axon Instruments, Foster City, CA) interfaced to a Digidata 1200 (Axon instruments) and were directly digitized with pCLAMP 6 software for further off-line analysis. Theoretical equilibrium potentials were computed with the use of the Nernst equation. Current-voltage ($I$-$V$) curves were plotted after subtracting the baseline obtained just before the agonist application from the $E_{\text{GABA}}$. $E_{\text{GABA}}$ was defined as the $x$-intercept of the $I$-$V$ curve or of the instantaneous current in a voltage step or in a voltage- ramp protocol. Slope conductance was determined by linear regression of the less-rectifying more-conducting part of the $I$-$V$ plot. Nonlinear regressions were performed using the Marquardt-Levenberg algorithm of SigmaPlot 5.0 (and Scientific, Sausalito, CA). Quantitative data are expressed as mean $\pm$ SE.

**Table 1. Composition of recording solutions**

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**External NaCl KCl TEA CI CaCl$_2$ CoCl$_2$ HEPES TTX**

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Units are mM. For external solutions, pH was adjusted to 7.4 with NaOH. For pipette solutions, pH was adjusted to 7.4 using either KOH or CsOH depending on the major cation of the saline. TEA, tetraethylammonium; K$_2$-ATP, ATP potassium salt; E, external; WC, whole cell; PP, perforated patch.
Reagents. GABA, MS-222, protease type IX, collagenase type IA, Leibovitz L-15 medium, gramicidin D, HEPES, EGTA, ATP potassium salt, TTX, and tetraethylammonium were purchased from Sigma (St. Louis, MO). Kanamycin, the antibiotic-antimycotic solutions, and FCS were obtained from Boehringer Mannheim (Meylan, France). Tissue culture dishes were supplied by Costar (Cambridge, MA).

RESULTS

Whole cell $I_{\text{GABA}}$ were recorded from cultured frog melanotrophs by using either the tight-seal ruptured-patch or the gramicidin-perforated patch techniques in the voltage-clamp mode. All of the melanotrophs examined ($n = 109$) responded to GABA.

Decay of the $I_{\text{GABA}}$ in melanotrophs with imposed [Cl$-$]. In experiments carried out in the whole cell configuration, a prolonged ejection of GABA (10 µM, 15 s) elicited a current that rapidly peaked and then gradually decayed to $26.2 \pm 1.9\%$ ($n = 25$) of the maximum amplitude within 15 s (Fig. 1A). The late amplitude was defined as the residual current at the end of the GABA application. Repeated 15-s GABA ejections at 2-min intervals generated currents with reproducible peak and late amplitudes (Fig. 1B). Successive I-V relationships established at 2-min intervals from a single melanotroph displayed similar $E_{\text{GABA}}$. Moreover, the slope conductances remained unchanged.

![Fig. 1. Currents evoked by repeated administrations of $\gamma$-aminobutyric acid (GABA) in the whole cell configuration. Recordings were carried out using a low-chloride solution in the patch pipette (External 1 (E1)/Whole Cell 1 (WC1), chloride equilibrium potential ($E_{\text{Cl}}$) = $-75$ mV). A: currents evoked in a single melanotroph by 5 successive ejections of GABA (10 µM, 15 s; horizontal bars) at 2-min intervals. Holding potential was 0 mV. B: time course of the peak (●) and late (▲) currents shown in A. C: three families of currents (1, 2, and 3) evoked by GABA (10 µM, 5 s) at 2-min intervals from another cell. In each family, membrane potential was set for 2 min at values ranging from $-95$ to $+65$ mV by 20-mV steps. D: superimposed current-voltage (I-V) curves corresponding to C. Peak current amplitude was plotted against the membrane potential. Reversal potentials for families of currents 1, 2, and 3 were $-73$ (●), $-82$ (▲), and $-76$ mV (X), respectively.](http://ajpendo.physiology.org/)
were used to determine global current decay. The resulting parameters were followed throughout a prolonged (25-s) series of three successive GABA (10 µM) ejections were repeated 10 times at 2-min intervals. In each series, a brief pulse of GABA (600 ms, a) eliciting a control response was followed within 10 s by a prolonged GABA application (15 s, b), provoking a current decay. Recovery was monitored by a brief test pulse of GABA (600 ms, c) applied at incremental intervals (5-s steps) after the end of the 15-s GABA ejection. B: time course of recovery. Relative amplitude of the current evoked by pulse a was expressed as a fraction of the control current induced by pulse a and was plotted against the interpulse interval between b and c (n = 6–11). Data were fitted to a monoexponential function, yielding a time constant of 12.9 s for recovery of the control current.

(Fig. 1, C and D). To monitor the rate at which iGABA recovered from the fading provoked by a prolonged (15-s) GABA application, brief test pulses (600 ms) of GABA were delivered at incremental intervals (5-s steps, Fig. 2A). These test pulses were supposed to induce no additional current decrease. The exponential curve fitted to the data yielded a time constant (τ) = 12.9 s for recovery in the present experimental conditions (Fig. 2B).

In another set of experiments, a protocol was developed to assess the relative contribution of the decrease of the GABA-dependent membrane conductance (gGABA) and the shift of EGABA to the decay of iGABA. Both parameters were followed throughout a prolonged (25-s) GABA exposure, by applying repeated voltage ramps at 3.5-s intervals. A very fast sweep rate (800 mV/s) was employed to minimize the influence of the ramp on the global current decay. The resulting I-V relationships were used to determine EGABA and the slope gGABA (Fig. 3, A and B). In these experiments, the intrapipette chloride concentration (33 mM) was close to the physiological [Cl−]i (27 mM) previously measured in the frog melanotrophs (17). To verify that the voltage ramps did not influence the values of EGABA and gGABA, control experiments were performed on single cells. In all cells studied (n = 8), the values of EGABA and gGABA obtained using voltage ramps did differ from that measured with voltage steps (data not shown). In the presence of GABA, a time-dependent decrease of gGABA occurred. Concomitantly, a gradual shift of EGABA toward 0 mV was observed (Fig. 3, B and C). Although iGABA fell down to 33 ± 6% of the peak current, gGABA dropped to 44 ± 6% of the control, and EGABA markedly drifted from −31.5 ± 0.9 to −20.0 ± 3.2 mV (n = 8, Fig. 3, D-F).

The role of the chloride flux in the decay of iGABA was further studied for various chloride driving forces. Therefore, the above-described repeated-ramp protocol was used at three different holding potentials (Fig. 4, A-C). At −80 mV, the decay phase of the GABA-evoked inward current was accompanied by a decrease of gGABA, which was associated with a slight shift of EGABA toward more negative potentials. At −35 mV, a value close to the chloride equilibrium potential (ECl) in the present recording conditions, GABA failed to elicit any current or noticeable change in EGABA. Nevertheless, a time-dependent decrease of gGABA was still detected. At +30 mV, GABA generated a robust outward current characterized by a high initial conductance compared with that measured at negative potentials. Thereafter, gGABA markedly decayed during GABA exposure. In addition, EGABA sharply decayed toward 0 mV (Fig. 4, D and E).

Fading of the iGABA in melanotrophs with unaltered physiological [Cl−]i. In cells studied in the whole cell configuration, chloride exchange between pipette and cell compartments could distort the time course of the current. In this respect, the gramicidin-perforated patch technique provides an alternative approach that allows recordings of iGABA without imposing [Cl−]i (1, 16, 17). In recordings performed in the gramicidin-perforated patch configuration, a prolonged GABA ejection (10 µM)
Fig. 3. Redistribution of chloride ions during the response to GABA in the whole cell configuration. Melanotrophs were held at 0 mV with a physiological internal chloride concentration (E2/WC3, E_Cl = −31 mV). A: current evoked by a prolonged GABA application (10 µM, 25 s; horizontal bar). High-speed depolarizing triangular voltage ramps (800 mV/s, from 0 to +75 mV and from +75 to 0 mV) followed by mirror-image hyperpolarizing ramps were applied before (a) and during (b-h) the GABA application. B: superimposed instantaneous I-V relationships corresponding to A. The leak current (a) has been subtracted from the currents recorded during GABA exposure (b-h). C: enlarged detail of the I-V curves shown in B. Arrows indicate the zero-current potential for each I-V curve. D-F: changes in I_GABA (D), GABA reversal potential (E_GABA; E), and GABA conductance (g_GABA; F) occurring during the prolonged GABA administration. Relative current amplitude and slope conductance in c-h were expressed as a fraction of their respective values in b. Each point corresponds to the mean ± SE of data obtained as in A from 8 cells.
Fig. 4. Dependence of the changes in $E_{\text{GABA}}$ and $g_{\text{GABA}}$ on the holding potential ($V_h$) in a single melanotroph. A-C: recordings were performed in the whole cell configuration with a physiological internal chloride concentration ($E_{\text{Cl}} = -31$ mV). Currents were evoked by prolonged GABA applications (10 µM, 25 s; horizontal bars) at holding potentials of $-80$ (A), $-35$ (B), or $+30$ (C) mV. High-speed triangular voltage ramps were applied before and during GABA exposure, as described in Fig. 3. Voltage ramp pulses ran from $-5$ to $-135$ mV (A), $+35$ to $-115$ mV (B), and $+105$ to $-45$ mV (C). Resulting instantaneous I-V curves are presented below the current traces. D-E: changes in $g_{\text{GABA}}$ (D) and $E_{\text{GABA}}$ (E) accompanying the $I_{\text{GABA}}$, at holding potentials of $-80$ ($\bullet$), $-35$ ($\blacksquare$), or $+30$ ($\triangle$) mV. Relative slope conductance measured at the different times was expressed as a fraction of the initial conductance at $-80$ mV.
µM, 15 s) elicited a decaying current. Repeated 15-s GABA ejections at 2-min intervals generated currents that gradually vanished (Fig. 5A). Peak and late amplitudes of $I_{\text{GABA}}$ declined with similar time courses (Fig. 5B). To establish successive I-V relationships from a single melanotroph, families of $I_{\text{GABA}}$ at increasing membrane potentials were recorded at 2-min intervals (Fig 5C). Interestingly, from one family of currents to another, the I-V curves exhibited an irreversible drift of $E_{\text{GABA}}$ from −25 to 0 mV, without any marked modification in the slope conductances (Fig. 5D).

To evaluate the relative contribution of changes in $g_{\text{GABA}}$ and $E_{\text{GABA}}$ to the decay of $I_{\text{GABA}}$ recorded in the gramicidin-perforated patch configuration, the repeated-ramp protocol described in Fig. 3 was used. Under these conditions, the late current amplitude decreased to $28 \pm 6\%$ of the peak during a 25-s GABA (10 µM) ejection. Concomitantly, $g_{\text{GABA}}$ diminished to $45 \pm 2\%$ of its control value, and $E_{\text{GABA}}$ shifted from $−34.8 \pm 2.2$ to $−20.9 \pm 2.3$ mV ($n = 8$, Fig. 6, A-C). In addition, in all melanotrophs ($n = 5$) submitted to three consecutive repeated-ramp protocols at 2-min intervals, long-
Fig. 6. Redistribution of chloride ions during the response to GABA in the gramicidin-perforated patch configuration. Currents were recorded using a CsCl and gramicidin D solution in the patch pipette (E2/PP2, ECl = -0.6 mV). Holding potential was 0 mV. High-speed triangular voltage ramps were applied before and during 25-s GABA (10 µM) exposures, as described in Fig. 3. A-C, with changes in IgABA (A), EGABA (B), and gGABA (C) occurring during the prolonged GABA administrations. Relative current amplitude and slope conductance were expressed as a fraction of their initial values. Each point indicates the mean ± SE of data obtained from 8 cells. Only one GABA pulse was delivered per cell under study. D: three currents (1, 2, and 3) were evoked by prolonged GABA applications at 2-min intervals in a single melanotroph. The corresponding instantaneous I-V curves focus on the current reversal potentials. E: changes in EGABA observed during successive IgABA 1 (●), 2 (■), and 3 (▲) shown in D.
lasting and additive shifts of $E_{\text{GABA}}$ were observed. As shown in the representative example of Fig. 6, D and E, the $E_{\text{GABA}}$ drifted from $-39.6$ to $-29.3$ mV, $-31.9$ to $-22.7$ mV, and $-24.4$ to $-17.2$ mV throughout the first, the second, and the third GABA ejection, respectively.

The persistent drift of $E_{\text{GABA}}$ toward more depolarized potentials observed in intact melanotrophs could be accounted for by a continuous accumulation of chloride in the intracellular compartment, through specific transport systems and/or chloride channels in addition to the GABA$_A$ receptor. To address this issue, a protocol was designed and applied to single cells ($n = 5$, Fig. 7). First, three successive families of $I_{\text{GABA}}$ were recorded at 2-min intervals. $E_{\text{GABA}}$, deduced from the corresponding I-V relationships, shifted from its native value ($-38$ mV) to a more depolarized and stable level ($-11$ mV). Thereafter, to lower $[\text{Cl}^-]$, the cell was submitted to a series of five GABA ejections at $-80$ mV. Subsequently, $E_{\text{GABA}}$ measured from a first family of $I_{\text{GABA}}$ was $-35$ mV, a value close to that obtained initially. In a second determination, $E_{\text{GABA}}$ shifted to $-10$ mV, as already observed at the beginning of the recording session. Finally, after another series of five GABA ejections at $-80$ mV, again $E_{\text{GABA}}$ ($-36$ mV) recovered (Fig. 7, A and C).

The possible influence of $E_{\text{GABA}}$ changes on the postsynaptic response of melanotrophs to GABA was investigated by using an experimental procedure closer to physiological conditions. The cell was alternatively clamped at potentials ($-50$ and $-40$ mV) close to the resting potential of the melanotrophs ($-46.3 \pm 1.5$ mV, $n = 29$). A train of brief GABA pulses (10 μM, 100 ms, 1.1 Hz) was delivered. At $-50$ mV, the GABA-evoked inward current was initially large and gradually diminished over the course of the experiment. In contrast, at $-40$ mV, the inward current was markedly weaker and reversed after 150 s recording (Fig. 8).

**DISCUSSION**

In the frog melanotrophs bathed in HEPES-buffered media, chloride is the exclusive charge carrier of the $I_{\text{GABA}}$ (17, 20). By using the gramicidin-perforated patch technique, we have recently found that, in this cell model, $E_{\text{GABA}}$ is $-10$ mV positive to the resting potential. As a consequence, GABA provokes a depolarization, triggering action potentials. The voltage change due to GABA is underlaid by a dramatic increase of the membrane conductance, which shunts the voltage-activated currents. Thus the initial burst of activity is followed by a shunt-induced abolition of firing, with GABA damping membrane potential at $E_{\text{GABA}}$ (17). We now demonstrate that, in frog melanotrophs with unaltered $[\text{Cl}^-]$, $E_{\text{GABA}}$ is unexpectedly a labile parameter. It appears that long-lasting shifts of $E_{\text{GABA}}$ can account for a GABA$_A$ receptor use-dependent fading of the response to GABA.

The main purpose of our study was to decipher, in the frog melanotrophs, the mechanisms involved in the fading of $I_{\text{GABA}}$ occurring in the presence of the agonist. In the whole cell configuration, GABA generated a decaying current that was reproducible over repeated agonist applications at 2-min intervals. This indicates that the parameters ($E_{\text{GABA}}$ and $g_{\text{GABA}}$) controlling the current flow were modified throughout the agonist exposure and recovered within the delay between the GABA pulses. The kinetics of recovery ($t = 12.9$ s) were found to be similar to that described in neurons (3, 13, 15). Recovery may involve both true reactivation, i.e., removal of desensitized states (13), and restoration of the initial transmembrane chloride gradient by ion diffusion between the patch pipette and the cell interior (17). The observation that successive I-V curves were superimposed confirmed that neither the chloride driving force nor the conductance varied from one GABA application to another. In contrast, by using the repeated-ramp protocol to follow $E_{\text{GABA}}$ throughout a prolonged GABA exposure, it appeared that $E_{\text{GABA}}$ gradually and transiently drifted during the chloride flow. The shift of $E_{\text{GABA}}$ toward potentials corresponding to lower driving forces reflects a passive redistribution of chloride, which leads, together with the decrease of $g_{\text{GABA}}$, to the fading of $I_{\text{GABA}}$ in the continued presence of the agonist. This observation is in good agreement with previous findings in rat and frog neurons (2, 12, 32). To verify the role of the chloride flux in the current decay, the variations of $E_{\text{GABA}}$ and $g_{\text{GABA}}$ have been monitored at various holding potentials. The data reveal that the displacement of $E_{\text{GABA}}$ was more pronounced at increasing chloride driving forces and was inverted when $I_{\text{GABA}}$ reversed. In these experiments, the initial increase or decrease of $g_{\text{GABA}}$ observed at holding potentials of $+30$ and $-80$ mV, respectively, was attributed to the voltage sensitivity of the GABA$_A$ receptor channel. Altogether, the present results demonstrate that, in frog melanotrophs, the GABA-evoked chloride flux induces transient time- and driving force-dependent shifts of $E_{\text{GABA}}$.

Because of the relatively rapid ion equilibration between the pipette and cell compartments, the whole cell recordings may lead to an underestimation of the magnitude of $E_{\text{GABA}}$ changes during the response to GABA. To avoid such a limitation, the fading of $I_{\text{GABA}}$ has been examined using the gramicidin-perforated patch approach (16, 17). In the gramicidin-perforated patch recordings, the decay of $I_{\text{GABA}}$ observed during a single 25-s GABA exposure was underlaid by a fall of $g_{\text{GABA}}$ and a shift of $E_{\text{GABA}}$, which were characterized by magnitudes and time courses very similar to those obtained from whole cell recordings. Thus we conclude that, in the whole cell configuration, the chemical access to the intracellular compartment did not alter the transient changes of $E_{\text{GABA}}$ caused by the chloride flux itself. This probably originates from the fact that chloride exchanges between pipette and cell compartments are much less intense than chloride fluxes evoked by GABA through membrane channels. However, when GABA was repeatedly ejected while the physiological $[\text{Cl}^-]$ remained unaltered, $I_{\text{GABA}}$ gradually vanished from one GABA application to the next. This response pattern markedly differed from that obtained in the whole cell recordings in which repeated $I_{\text{GABA}}$ were reproducible. Interestingly, in the gramicidin-
Fig. 7. Reversibility of the shifts of $E_{\text{GABA}}$ in the gramicidin-perforated patch configuration. A: recordings were carried out using a KCl and gramicidin D solution in the patch pipette (E1/PP1, $E_{\text{Cl}} = -0.6$ mV). A single melanotroph was submitted to the voltage-clamp protocol indicated above the current traces. In this representation of the voltage protocol, the time scale is not constant. Repeated GABA (10 µM, 5 s) ejections were performed at 2-min intervals, as indicated by the vertical arrows. The stimulation procedure included 6 $I$-$V$ protocols (noted 1 to 6) and two series of 5 successive GABA ejections at $-80$ mV. B: superimposed $I$-$V$ curves obtained from the $I$-$V$ protocols shown in A. □, △, ○, ■, ▲, and • correspond to the families of currents 1, 2, 3, 4, 5, and 6, respectively. C: changes in $E_{\text{GABA}}$ during the stimulation procedure followed in A. The values of $E_{\text{GABA}}$ were deduced from the $I$-$V$ curves in B.
perforated patch configuration, successive GABA ejections resulted in increased shifts of $E_{\text{GABA}}$ that persisted during washout while $E_{\text{GABA}}$ recovered. Moreover, the present work demonstrates that the long-lasting redistributions of chloride ions were highly reproducible and mostly depended on the direction of the evoked chloride flux. Hence the possible contribution of a resting chloride permeability to the regulation of $[\text{Cl}^-]_i$ is likely negligible. Taken together, the above-described observations indicate that, in intact frog melanotrophs, $[\text{Cl}^-]_i$ is severely altered by the passive chloride flux through the GABA$_A$ receptor channels and seems not to be subject to any other short-term regulation.

To influence the response to GABA in vivo, the shifts of $E_{\text{GABA}}$ must be driven by deviations of membrane potential from the native $E_{\text{Cl}}$ to maintain a significant chloride flux throughout the presence of GABA in the synaptic cleft. This implies that GABA released in synapses formed between hypothalamic nerve endings and melanotrophs activates only a few channels so that the resistive shunt still remains negligible and does not result in a voltage clamp of the membrane at $E_{\text{Cl}}$. Accumulating evidence now supports the occurrence of such a situation in vivo (6, 17, 24, 27, 29). Moreover, it has recently been proposed that, during an inhibitory postsynaptic current, a substantial number of postsynaptic receptors must be exposed to subsaturating concentrations of GABA because of the time course of GABA release, the action of transmitter clearance mechanisms, the cleft configuration, and the “spillover” of GABA, which leads to activation of extrasynaptic GABA$_A$ receptors (11). As a result, GABA must actually open only a part of the postsynaptic GABA$_A$ receptor channels for relatively long periods, contrary to that often obtained by rapid jumps of saturating GABA concentrations (1 mM, 0.5–3 ms) in patch experiments. In addition, modification of the membrane potential is a very common mechanism shared by a panoply of neurotransmitters and neuropeptides in frog melanotrophs (for review, see Ref. 33). It has also been shown that inhibitory postsynaptic potentials are often associated with depolarizations in neurons (22, 30, 31). These voltage fluctuations likely contribute to move the membrane potential beyond $E_{\text{Cl}}$. Herein, we demonstrate...

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**Fig. 8.** Inversion of $I_{\text{GABA}}$ elicited by a train of GABA pulses in a cell alternatively clamped at 2 membrane potentials in the gramicidin-perforated patch configuration. Current recordings were performed using a KCl and gramicidin D solution in the patch pipette ($E_{\text{PP1}}$, $E_{\text{Cl}} = -0.6$ mV). A single melanotroph was alternatively held at $-50$ or $-40$ mV at 15-s intervals (top). $I_{\text{GABA}}$ (bottom) were evoked by a train of brief GABA pulses (10 µM, 100 ms) applied at the frequency of 1.1 Hz (middle). Extended current traces shown at bottom display inversion of $I_{\text{GABA}}$ at $-40$ mV in the second part of the recording. Horizontal dashed lines superimposed to the current traces indicate the zero-current levels at $-40$ and $-50$ mV.
that variations of membrane potential in a restricted range (10 mV) spanning the resting potential, imposed during a train of 100-ms GABA pulses, were sufficient to induce the reversal of $I_{\text{GABA}}$. This indicates that crucial changes of $E_{\text{GABA}}$ may occur after small variations of membrane potential and in response to GABA pulses, which are both relevant to physiological conditions.

The findings of the present work are schematically illustrated in Fig. 9. In the whole cell configuration, the transient shift of $E_{\text{GABA}}$ induced by the chloride flow combines with the gradual decline of $g_{\text{GABA}}$ to entail the decay of $I_{\text{GABA}}$. The patch pipette solution does not instantaneously damp but rather slowly buffers the $[\text{Cl}^-]$. In the gramicidin-perforated patch configuration, the physiological $[\text{Cl}^-]$, remains unaltered, and the redistribution of chloride persists over the successive GABA ejections. As a result, the shift of $E_{\text{GABA}}$ leads to a tachyphylaxis phenomenon. This latter observation suggests that, in melanotrophs, $[\text{Cl}^-]$ is not subject to a short-term regulation.

Among these lines, it must be mentioned that, so far, no information is available concerning the regulation of $[\text{Cl}^-]$ by pumps, channels, or any metabolically dependent active transport systems in melanotrophs. Nevertheless, because $[\text{Cl}^-]$ is less than extracellular chloride concentration, mechanisms responsible for a net chloride extrusion may exist in these cells. In addition, in the present work, we have intentionally employed HEPES-buffered saline without CO$_2$ equilibration precisely to eliminate any involvement of HCO$_3^-$ in the
information concerning the role of chloride, HCO\textsubscript{3}^- in the above-reported results would provide interesting buffered gramicidin-perforated patch recordings and studies of the response to GABA in melanotrophs.

The regulations of I\textsubscript{GABA} reported herein have some functional significance. It is speculated that, in physiological conditions, the melanotrophs can be switched between excitatory and inhibitory modes of GABA signaling. For instance, the shift of E\textsubscript{GABA} could be considered as a negative feedback of the GABAergic inputs, regulating the amplitude of the response as a function of the intensity and duration of previous chloride fluxes.

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