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γ-Aminobutyric acid (GABA)-induced currents of skate Muller (glial) cells are mediated by neuronal-like $GABA_A$ receptors

(retina/neurotransmitters/vision/neuron–glia interaction)

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ABSTRACT **Radial glia (Muller cells) of the vertebrate retina appear to be intimately involved in regulating the actions of amino acid neurotransmitters. One of the amino acids thought to be important in mediating retinal information flow is γ-aminobutyric acid (GABA). The findings of this study indicate that enzymatically isolated skate Muller cells are depolarized by GABA and the $GABA_A$ agonist muscimol and that the actions of these agents are reduced by bicuculline and picrotoxin. Membrane currents induced by GABA under voltage clamp were dose dependent, were associated with an increase in membrane conductance, and showed marked desensitization when the concentration of GABA exceeded 2.5 μM. The responses had a reversal potential close to that calculated for chloride, indicating that the currents were generated by ions passing through channels. These data support the view that skate Muller cells possess functional $GABA_A$ receptors. The presence of such receptors on retinal glia may have important implications for the role of Muller cells in maintaining the constancy of the extracellular milieu, for neuron–glia interactions within the retina, and for theories concerning the generation of the electroretinogram.**

Glial cells can no longer be thought of as the "silent supporters" of neurons—i.e., electrically inexcitable elements whose passive properties serve to maintain the constancy of the extracellular milieu. Several voltage-sensitive currents have been ascribed to various types of glia throughout the nervous system (1–3), and there is evidence to indicate that the cellular potential of glial cells can be altered by electrogenic uptake mechanisms (4) as well as by ligand-gated ionophores (5). However, depending on the species and type of glial element, there is a striking heterogeneity in the nature of the voltage-dependent channels (6), the cellular responses to neurotransmitters (7, 8), and the underlying mechanisms mediating these reactions (9, 10).

We report here that a similar heterogeneity is evident in the electrical behavior of the radial glia (Muller cells) of the vertebrate retina, in particular with respect to their responses to the retinal neurotransmitters glutamate and γ-aminobutyric acid (GABA). Brew and Attwell (11) have shown that glutamate evokes a large inward current in isolated voltage-clamped Muller cells of axolotl and provide evidence that the current is due to an electrogenic glutamate uptake mechanism activated by intracellular potassium (12). Results of the present study show that skate Muller cells are relatively insensitive to glutamate, whereas GABA induces large current responses that are mediated primarily by a neuronal-like receptor mechanism.

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MATERIALS AND METHODS

Cell Dissociation. Skate (*Raja erinacea* and *Raja oscellata*) were obtained from the Marine Biological Laboratory (Woods Hole, MA) and maintained for periods of up to 1 month in a tank of circulating artificial seawater held at 14°C; a 12-hr light/dark cycle was used. Prior to enucleation, animals were dark adapted for 1.5 hr, anesthetized with 0.02% Tricane (A5040, Sigma), and pithed. The eyes were removed in normal room light, the anterior portion was excised, and the remaining eyecup was cut into smaller pieces and incubated for 15 min in 20 ml of an elasmobranch Ringer's solution containing 40 mg of papain (5125, Calbiochem) and 28 mg of cysteine (C7880, Sigma). The Ringer's solution consisted of 250 mM NaCl, 6 mM KCl, 20 mM $NaHCO_3$, 1 mM $MgCl_2$, 4 mM $CaCl_2$, 0.2 mM NaH_2PO_4, 360 mM urea, 10 mM glucose, and 5 mM Hepes, adjusted to pH 7.6 with NaOH. Retinae were detached from the retinal pigment epithelium and placed in 20 ml of fresh papain/cysteine Ringer's under constant agitation for an additional 45 min; they were then rinsed a minimum of five times with culture medium (L-15, GIBCO) to which 102.7 mM NaCl, 350 mM urea, 5 mM glucose, and 20 mM Hepes had been added; pH was adjusted to 7.6 with NaOH. Dissociated cells were obtained by triturating the retinae in 1 ml of the modified culture medium using a flame-polished Pasteur pipette. The cells were plated onto plastic culture dishes (Falcon 3001) containing 2 ml of the modified culture medium and stored up to 5 days at 15°C. No differences were observed in the GABA-activated currents recorded from cells within 30 min of isolation and those held in culture for 1–5 days. Prior to recording, the culture medium was replaced with the normal Ringer's solution at room temperature. Cells were viewed using an inverted microscope equipped with Hoffmann modulation contrast optics.

Recording Procedures. Intracellular voltage recordings were obtained by using high-resistance micropipettes (60–150 MΩ when filled with 2 M potassium acetate) connected to a high-impedance amplifier (Axoprobe, Axon Instruments, Burlingame, CA). In addition, drug-induced currents were examined using the whole-cell variant of the patch-clamp technique (13). Recording pipettes, pulled from Kovar glass (7052, Garner Glass, Clairmont, CA), were used without flame polishing. The pipettes were filled with 204 mM KCl, 1 mM $CaCl_2$, 11 mM EGTA, 1 mM $MgCl_2$, 1 mM MgATP, and were connected to a low-noise current–voltage converter (L/M EPC 7, List Electronics, Darmstadt, F.R.G.). All electrical signals were stored directly on a computer disk and

Abbreviations: GABA, γ-aminobutyric acid; $[Cl^-]_o$, extracellular chloride concentration.

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also on videotape through a PCM converter (R20, Vetter, Rebersburg, PA).

Drug Application. Drugs were applied either by superfusion of the chamber or by pressure ejection from pipettes placed within 100 μm of the cell. For superfusion, a U-shaped chamber (vol, 110 μl) placed around individual cells served to minimize the volume of solution bathing the cells; flow rate was 2–5 ml·min^{-1}. Pressure ejection from either single or multibarrel pipettes was accomplished with a Picospritzer II (General Valve, Fairfield, NJ). Drugs were prepared in normal Ringer's solution, and throughout the text the concentrations refer to those in the delivery pipette. The actual concentration at the cell membrane was considerably lower than these values, but it varied with the tip diameter of the drug pipette (which ranged from $\approx$0.5 to 2 μm) and its distance from the cell. All chemicals were obtained from Sigma, with the exception of baclofen, which was a gift of Ciba–Geigy.

RESULTS

Identification of Isolated Muller Cells. Fig. 1 shows photomicrographs of two cells, which we identify as Muller cells. The cells are quite thick, possess prominent end-feet at the basal portion of the cell, and often had photoreceptors attached at the apical end. Such cells differ dramatically from presumed bipolar cells, which possess narrow axons, branching dendrites, and have much smaller terminal endings. Cells morphologically similar to those from which recordings were obtained also stained positive for antibodies directed against glutamine synthetase, a marker specifically localized to Muller cells of elasmobranch retinae (14) (results not shown: antibodies were a gift from P. Linser, C.V. Whitney Laboratory of the University of Miami).

GABA-Induced Voltage Responses. In recordings obtained with conventional intracellular microelectrodes, Muller cells had resting potentials averaging -80 ± 8.5 mV (SD; $n = 51$). On impalement, the membrane potential was typically 10–20 mV more depolarized, and the value given reflects the level obtained several minutes later after the potential had stabilized. This increase in potential, observed also by Connor *et al.* (1) in their study of turtle Muller cells, may reflect either an influx of potassium from the intracellular pipette or the formation of a better seal around the electrode. Fig. 2 shows that superfusion of 2.5 μM GABA markedly depolarized the cells and that an equivalent concentration of muscimol, an agonist specific for $GABA_A$ receptors (15), also elicited a large depolarizing response. On the other hand, 1 mM (−)-baclofen, which is known to interact with $GABA_B$ receptors (16, 17), had no effect on the membrane potential, and low concentrations of L-glutamate and L-aspartate were also without effect; 100 μM L-glutamate produced only a small depolarization.

Currents and Conductance Changes Induced by GABA. To examine the currents associated with the GABA-dependent voltage swings, we made use of the whole-cell configuration of the patch clamp recording technique (13). Fig. 3 shows changes in membrane current produced by 1.6-s pulses of GABA applied 5 s after the cell had been voltage-clamped to the potential indicated at the left of the figure; the time interval was sufficient to allow the voltage-activated currents to reach a steady state. Several important aspects of these currents are illustrated. When the cell was voltage clamped at −70 mV, GABA produced a large inward current. As the cell was clamped to successively more depolarized levels, the current became smaller, disappeared entirely at about −5 mV, and reversed to an outward current upon further depolarization. This result stands in contrast to the findings of Brew and Attwell for glutamate-induced currents observed in Muller cells of axolotl (11). Their finding that the currents could not be reversed is consistent with their suggestion that the glutamate responses were generated by an electrogenic uptake mechanism. Fig. 3 shows also that GABA increased the cell's membrane conductance. The brief 10-mV depolarizing pulses were always associated with larger responses when GABA was present; this is especially apparent when no net GABA current was evident—i.e., with the cell held at −5 mV. The reversibility of the current and the increase in conductance induced by GABA suggest that the Muller cell responses to GABA are mediated by a receptor–channel complex rather than by an electrogenic uptake mechanism.

Dose–Response Relationship. The amplitude of the GABA-induced currents was dose dependent. Fig. 4 illustrates the dose–response relationship obtained by superfusion of the

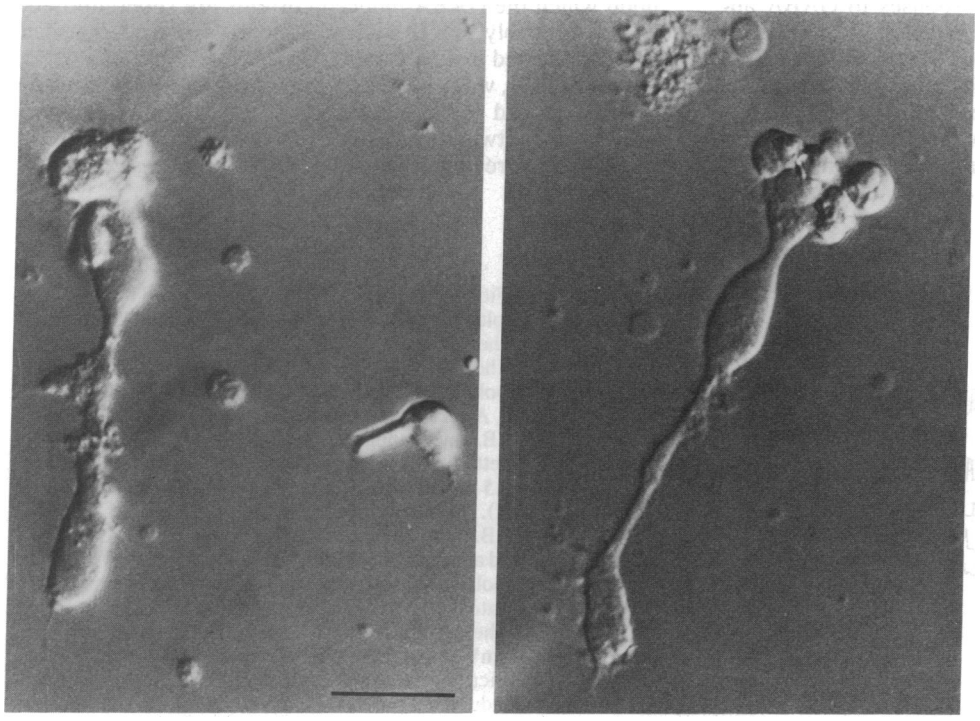

FIG. 1. Muller cells dissociated from the retina of the skate. The morphology closely resembles that of Muller cells observed in the intact retina: tufts of microvillus processes extend from the distal (upper) region to intercalate between the photoreceptors (which were still attached occasionally to some dissociated cells); the cell body is located medially, and the proximal region terminates in a conical end-foot that, *in situ*, abuts the inner limiting membrane of the retina. (Bar = 30 μm.)

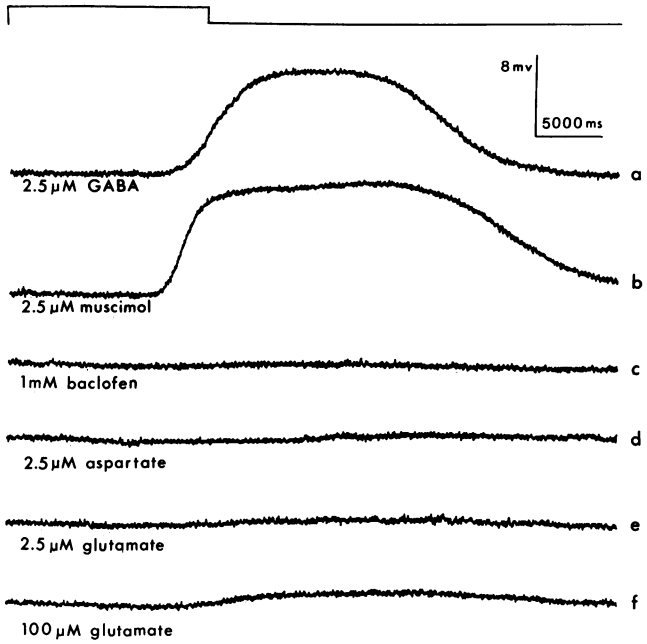

FIG. 2. Voltage responses of a Muller cell to superfusion of 2.5 μM GABA, 2.5 μM muscimol, 1 mM (−)-baclofen, 2.5 μM L-aspartate, 2.5 μM L-glutamate, and 100 μM L-glutamate. The resting potential of this cell was −84 mV. Top trace, timing of solution switch; delay in response onset reflects time for solution to enter chamber.

cells with GABA; in each case, the cells were voltage clamped to −70 mV. The threshold for the generation of these currents was reached at a concentration of ≈0.1 μM, and responses grew thereafter monotonically for concentrations up to ≈5 μM. However, the response obtained at 500 μM GABA was less than that obtained at some lower concentrations due to the rapid desensitization of the response at high drug concentrations (see below).

Effects of $GABA_A$ Antagonists. The effects of GABA were significantly reduced by application of bicuculline, a known antagonist of responses at $GABA_A$ receptor sites (18, 19). The superimposed series of traces in Fig. 5*A* illustrates the consistency of successive current responses to GABA applied to one cell using 400-ms pressure pulses from a nearby micropipette. However, after "puffing" 500 μM bicuculline onto the cell for 10 s from one of the adjacent barrels, the responses to GABA were greatly reduced (Fig. 5*B*); after a short period of time, the responses recovered to near control values. Picrotoxin also reduced the responses induced by

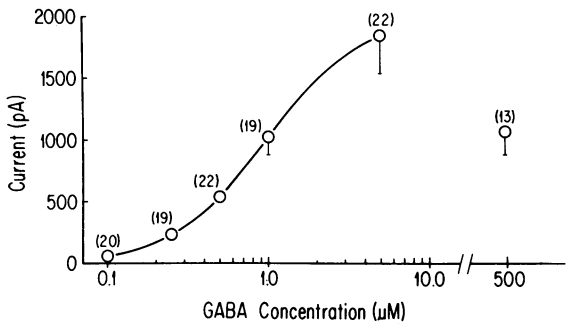

FIG. 4. Dose–response curve for the drug-induced currents obtained during superfusion with GABA. The minimum effective concentration of GABA appears to be ≈0.1 μM, with a half-maximal response occurring at concentrations near 1 μM. The smaller response to 500 μM GABA appears to be the result of rapid desensitization of the response. Curve was drawn by eye; numbers above points reflect the number of cells examined at each concentration. Error bars indicate SD; at the lower drug concentrations, the magnitudes of the SDs were smaller than the size of the symbols.

GABA (data not shown), but the recovery time was greatly prolonged. The ability of bicuculline and picrotoxin to suppress the GABA-induced currents and the stimulatory action of muscimol noted earlier provide pharmacological evidence that these currents are mediated via a $GABA_A$ receptor.

Ionic Mechanism of the GABA Response. To explore the ionic basis of the GABA-induced currents, the extracellular chloride concentration ($[Cl^-]_o$) was varied, while the internal concentration of chloride was held constant at 204 mM—i.e., the chloride concentration within the recording pipette. Fig. 6*A* shows GABA-induced currents obtained under standard conditions—i.e., with $[Cl^-]_o$ at 266 mM. To minimize problems associated with desensitization (see below) and variations in the driving force on chloride accompanying prolonged changes in chloride conductance, brief (30 ms) pulses of relatively low drug concentrations (1 μM) were used. As shown previously (Fig. 3), inward currents predominate at negative holding potentials and reverse polarity at positive holding potentials; in this particular instance, the reversal potential was approximately −15 mV. With positive holding potentials there is initially a small sag in the current responses upon which the GABA-induced currents are superimposed. This is probably due to voltage-activated currents that had not yet reached a steady state.

When $[Cl^-]_o$ was lowered to 16 mM (Fig. 6*B*), the GABA-induced inward currents reversed at a much more depolarized level (between +50 and +70 mV). The relationship between the reversal potential of the response and the

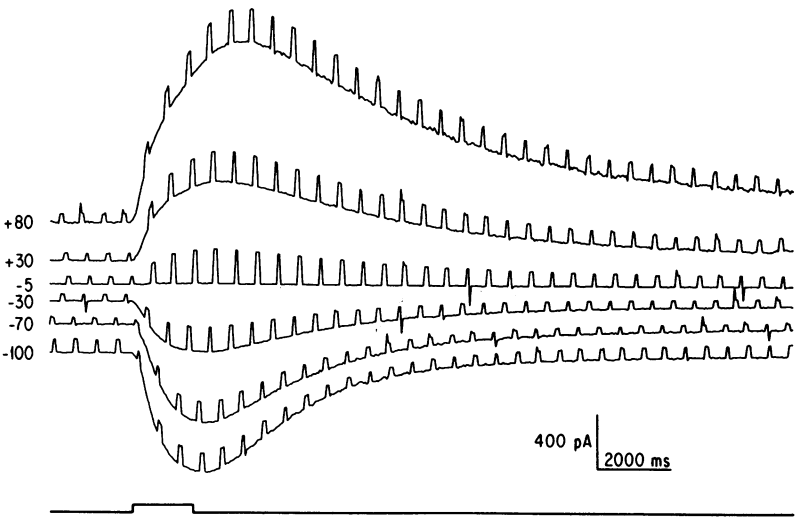

FIG. 3. Whole-cell currents generated by GABA in a voltage-clamped (−70 mV) Muller cell. The potential of the cell was jumped to a new steady level (indicated to the left of each trace), after which 10-mV 100-ms depolarizing pulses were applied at a frequency of 1.8 Hz. After the currents had reached a new steady state, GABA was applied from one barrel of a four-barrel pipette by a 1.6-s pressure pulse (pipette tip diameter, ≈0.5 μm; GABA concentration in the pipette was 100 μM). Bottom trace: duration of pressure ejection. GABA induced an inward current when the cell was held at negative potentials greater than −5 mV; at more depolarized levels, the responses became increasingly greater outward currents. In addition, note the increase in the current responses (upward deflections) to brief 10-mV depolarizations after the addition of GABA, indicating a drug-induced increase in membrane conductance.

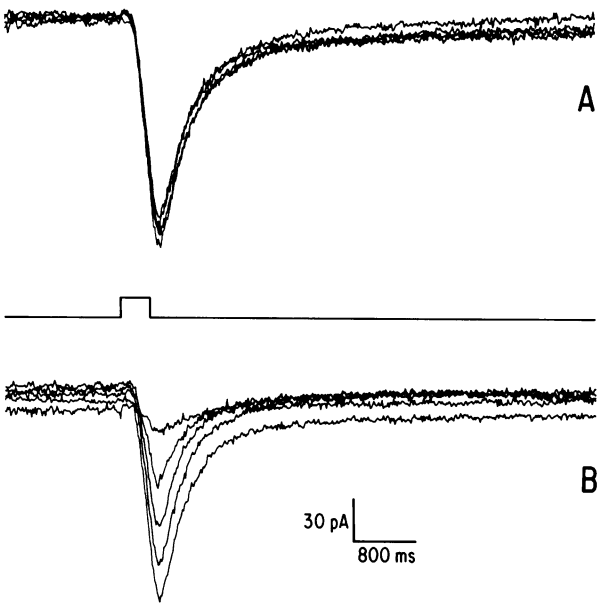

FIG. 5. Bicuculline block of the responses to GABA. (*A*) Four superimposed current responses from a single cell voltage clamped at −70 mV to brief applications of GABA pressure ejected from one barrel of a nearby four-barrel pipette; the concentration within the pipette was 100 μM. Middle trace, pressure ejection timing; 10 s separate each episode. (*B*) Responses after the application of 500 μM bicuculline. Bicuculline was applied from another barrel of the four-barrel pipette for 10 s; GABA was then puffed onto the cell as in *A*. The initial response is much smaller, with subsequent applications giving larger and larger responses as the bicuculline diffuses away.

concentration of extracellular chloride is depicted in Fig. 6*C*. The close agreement between the experimentally determined values for the reversal potential and the values calculated from the Nernst equation (indicated by the straight line in Fig. 6*C*) is a good indication that the currents induced by GABA result from the activation of a chloride conductance.

Desensitization of the Response to GABA. A phenomenon characteristic of other cell types displaying currents mediated by $GABA_A$ receptors is a rapid decrease in the response to high concentrations of GABA when applied for a fairly long period of time (20, 21). This alteration in response, referred to as desensitization, was also observed in the responses of Muller cells to GABA. Desensitization became apparent usually when the concentration of GABA exceeded ≈2.5 μM and developed much more rapidly as the concentration of GABA was increased. For example, current responses to 0.5 μM GABA reached a steady level, remained constant during perfusion, and then declined rapidly as GABA was removed from the chamber. With 5 μM GABA, on the other hand, responses peaked and then declined during drug application. The reduction with time appeared not to be due to a reduction in the driving force, as the reversal potential of the steady-state portion of the response was almost identical to the reversal potential of the response peak.

DISCUSSION

The data presented here are consistent with the conclusion that Muller cells of the skate possess functional $GABA_A$ receptors. The responses to GABA (*i*) are mimicked by muscimol, (*ii*) are reduced by bicuculline and picrotoxin, (*iii*) are associated with an increase in cellular conductance, (*iv*) display rapid desensitization, and (*v*) are highly dependent on the chloride concentration gradient across the cell membrane. For the most part, these observations are similar to recent findings by Kettenmann and co-workers (22, 23)

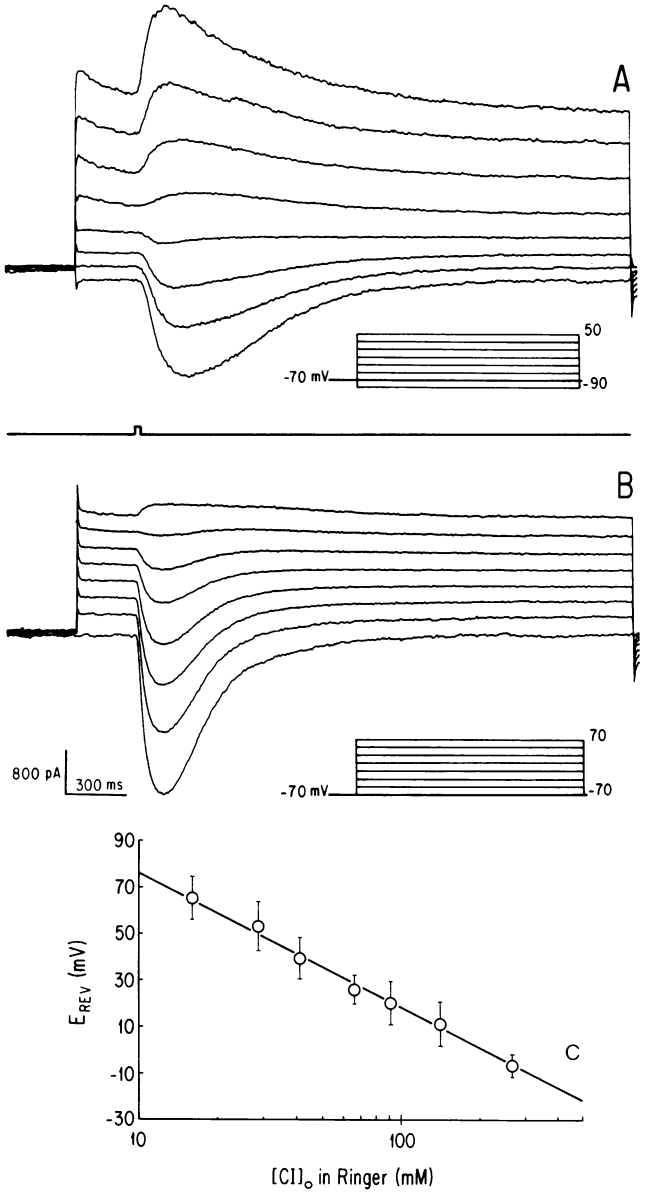

FIG. 6. The dependence of the GABA-induced responses on chloride. Cells were whole-cell clamped as described (voltage protocol is shown in the bottom portion of the figure) and solutions containing different concentrations of chloride perfused over the cell; 1 μM GABA was pressure ejected from a nearby single-barrel pipette. (*A*) Responses in normal Ringer's solution. (*B*) Responses in a Ringer's solution in which all of the NaCl had been replaced by Na-isethionate. The GABA currents now reverse at about +60 mV. (*C*) Relationship between the reversal potential (E_{REV}) and $[Cl^-]_o$. Data were obtained from eight cells; error bars are SDs. Responses were first recorded in normal Ringer's solution, followed by solutions containing increasingly lower concentrations of Cl^-; the cells were finally returned to normal Ringer's solution to ensure that the alteration in reversal potential was reversible. The line drawn through the data represents the calculated values of $[Cl^-]_o$ at the various concentrations of extracellular chloride; its slope (58 mV per decade) is that predicted by the Nernst equation.

indicating that cultured astrocytes from rat cortex are depolarized by GABA and that the ionic mechanism underlying the potential change results from a transient increase in chloride conductance similar to that of the neuronal $GABA_A$ receptor.

Because skate Muller cells exhibit avid uptake of exogenous GABA (24, 25), there is the possibility that the responses observed here are linked somehow to an electrogenic uptake mechanism. Our findings suggest otherwise. If the

uptake of GABA is electrogenic, then under our experimental conditions it does not contribute significantly to the observed voltage and current responses. It is possible that the carrier mechanism may have been damaged by enzymatic dissociation or that the currents generated by the uptake mechanism may be very small in comparison with those produced by activation of the $GABA_A$ receptors. Alternatively, $GABA_A$ receptor activation may be elicited at much lower concentrations of GABA than comparable currents induced by the process of electrogenic uptake.

The presence of $GABA_A$ receptors on Muller cells has important implications for theories concerning the generation of the *b*-wave of the electroretinogram, a transretinal potential of considerable diagnostic value in ophthalmology (26, 27). It is generally believed that the *b*-wave is the extracellular expression of radial current flow generated by a potassium-mediated depolarization of the Muller cells (28–30). This hypothesis was challenged recently by findings showing that micromolar concentrations of glutamate evoke large currents in Muller cells of the axolotl, due presumably to an electrogenic uptake mechanism for this neurotransmitter (11). Our work suggests that the GABA-induced currents in skate Muller cells can be very large and may also be a factor in generating transretinal potentials. In this regard, it is interesting to note that GABA and its antagonists have been shown to exert a wide range of effects on electroretinographic responses (31–34). Furthermore, electrophysiological and immunohistochemical studies suggest that GABA may serve as a neurotransmitter in horizontal cells, interplexiform cells, and amacrine cells of elasmobranch retinae (35–37). However, it should be kept in mind that the response of the Muller cell to GABA in the intact skate retina is not known and that it is clearly dependent on the chloride equilibrium potential (E_{Cl^-}), a value that has yet to be determined for cells *in situ*.

What role $GABA_A$ receptors might play in governing Muller cell function is not yet clear. Like glial elements elsewhere in the nervous system, Muller cells have been implicated in the clearance of potassium released into the extracellular space by neuronal activity (38, 39). Both the permeability of the cell membrane to potassium and energy-dependent processes are thought to participate in maintaining the stability of the extracellular potassium concentration (40). The glial cell reaction to the neuronal release of GABA may represent another component in this homeostatic system. During an elevation of extracellular potassium a GABA-mediated increase in chloride conductance might prevent the membrane potential from reaching the new E_{K^+}, thereby increasing the driving force on potassium, and enabling potassium (as well as chloride) to continue to flow into the cell. This is consistent with a mechanism suggested by Boyle and Conway (41) and Hodgkin and Horowicz (42) to explain the passive uptake of potassium into muscle fibers. In addition, the GABA-sensitive chloride channels of some neurons appear to be permeable also to bicarbonate ions (43). If this proves to be true also for Muller cells, then the glial reaction to GABA may influence intracellular pH with potentially important consequences for cellular metabolism.

The question of whether Muller cells of other species will respond similarly to those of skate is also of interest. Elasmobranch Muller cells appear to be unique among the fish in their ability to take up exogenous GABA (44); amphibian Muller cells also do not show significant uptake of GABA (44). It may be that Muller cells in these species do not play a prominent role in the regulation of extracellular GABA and therefore would not require receptors to sense its presence. Indeed, in preliminary experiments, we were unable to record receptor-mediated responses to GABA from goldfish Muller cells. However, mammalian Muller cells do possess uptake mechanisms for GABA (45–47); it is not known whether these cells possess $GABA_A$ receptors similar to the Muller cells of skate.

Finally, it is important to recall that glial cells not only take up neuroactive substances, they are capable of releasing them under appropriate circumstances (48, 49). Indeed, depolarization by veratridine or by raising extracellular potassium concentration promotes a calcium-dependent release from Muller cells of preloaded radiolabeled GABA (50). It is possible, therefore, that the voltage changes due to GABA affect the glial release of neuroactive agents, which modulate, in turn, the responses of neighboring neurons and glia.

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