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Bromide, in the therapeutic concentration, enhances GABA-activated currents in cultured neurons of rat cerebral cortex

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Abstract

We investigated the effect of bromide on γ-aminobutyric acid (GABA)-activated currents in cultured cerebral neurons of the rat, employing whole-cell voltage- and current-clamp techniques. Application of 100 μM GABA elicited currents whose reversal potential was 0 mV with equal concentrations of chloride in both pipette and bath solutions and more negative than −60 mV with 159 mM chloride extracellularly and 4 mM chloride inside. Bicuculline blocked the currents. These findings showed that the currents were composed of chloride flux through $GABA_A$ receptor-coupled channels. Reversal potential revealed a permeability ratio of bromide with respect to chloride (P_{Br}/P_{Cl}) of 1.51. When 100 μM GABA was applied with the extracellular solution containing 140 mM bromide and 19 mM chloride, the currents were enhanced 2.00- and 1.91-fold at the holding potentials of −20 mV and 0 mV, respectively. Extracellular solutions containing various concentrations of bromide substituted for the same amount of chloride were applied with 100 μM GABA. The therapeutic concentration of 10 mM and 20 mM bromide enhanced the currents 1.28- and 1.36-fold of the control currents at the holding potential of −20 mV, respectively. Under current-clamp recording, a larger hyperpolarization was obtained by the application of GABA with a 140 mM bromide-containing solution. These findings suggest that bromide potentiated GABA-activated currents at the therapeutic concentrations ranging from 10 mM to 20 mM, causing the larger GABA-induced hyperpolarization. It is postulated that the antiepileptic effect of bromide might occur through the potentiation of inhibitory postsynaptic potentials elicited by GABA.

Keywords: Bromide; GABA; Antiepileptic drugs; Whole-cell voltage-clamp recording; Cultured cortical neurons of rat

1. Introduction

After Locock found bromide useful in the treatment of catamenial seizures in 1857 [22], the clinical use of bromide preparations became popular within a short time. Although it was progressively superseded by other drugs, bromide continued to find some useful application in the treatment of generalized tonic–clonic seizures [20]. Later studies reappraised the usefulness of bromide to extend the spectrum of the efficacy to other types of seizures [4,8,29,31,34]. These studies suggested that the therapeutic bromide

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concentration generally ranges from 10 to 20 or 30 mM. In basic experiments, bromide was found to possess anticonvulsive activity against pentylenetetrazol-induced seizures at a concentration of approximately 30 mM [11]. When it exceeds 30 mM, side effects such as mental sluggishness, dullness, and sedation tend to appear [23].

In spite of the long history of bromide therapy against epilepsy, still undetermined are the mechanisms of action of bromide at the level of the therapeutic concentrations used against epileptic seizures. Since bromide inhibits both carbonic anhydrase and HCO_3^--ATPase as well as Cl^- transport, these actions may contribute to its anticonvulsant mechanisms [33]. In addition, because bromide has a smaller hydrated diameter than chloride [28], its passive movement across cell membranes is expected to be faster than that of chloride [16]. Consequently, the faster movement of bromide would hyperpolarize the membrane and thus prevent the spread of an epileptic discharge [7,32]. Studies on the electrophysiological mechanism of bromide actions can contribute to a better understanding of seizure disorders and can help to identify new kinds of anticonvulsants.

In many cells, the cytoplasmic Cl^- concentration is always lower than the plasma concentration, and the equilibrium potential E_{Cl} is near the resting potential [14]. Thus, like K^+ channels, Cl^- channels would be expected to oppose normal excitability and to help repolarize a depolarized cell [14]. Among many kinds of Cl^- channels proved in excitable cells, γ-aminobutyric acid (GABA)-activated Cl^- channels play important roles in generating inhibitory postsynaptic potentials (IPSPs) [2,6,17]. When anion influx through GABA-activated channels is enhanced, larger IPSPs are generated, exhibiting anticonvulsive actions. In this paper, we have tried to elucidate the mechanisms of bromide action on GABA-activated currents in cultured cortical neurons of the rat, employing whole-cell voltage- and current-clamp recording techniques.

2. Methods

2.1. Cell culture

Primary cultures of cortical neurons were prepared from brain hemispheres of 15-day-old Wistar rat embryos [30]. Cerebral hemispheres were dissected from the embryos and meningeal membranes were removed. After trituration with a Pasteur pipette, the resulting suspension was filtered through stainless steel meshes of progressively smaller pore sizes of 150 μm, 75 μm, and 48 μm. The filtered suspensions were centrifuged at 200 g for 5 min. The pellets obtained were then resuspended in HAM-MEM (30 mM glucose) containing 10% fetal calf serum. The resulting suspension was plated onto poly-L-ornithine-coated 35-mm dishes at a density of 5×10^4 cells/cm^2. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were maintained four to seven days before electrophysiological experiments.

2.2. Voltage-clamp recordings

Whole-cell voltage-clamp recordings, as described by Hamill et al., [13] were obtained with an Axopatch 1D (Axon Instrument, Foster City, CA). The culture medium was exchanged by bath solutions before the experiments. All solutions were filtered through a 0.22-μm membrane filter (Millipore, Bedford, MA) immediately before use. Patch electrodes were prepared from thin-walled (1.0 mm O.D.), filament-fused, borosilicate capillaries (TW100F-4, World Precision Instruments, New Haven, CT) using a programmable horizontal pipette puller (Sutter Instrument Co., San Rafael, CA). Electrode tip resistance was typically 10 to 15 MΩ. Since under whole-cell recording the pipette current declines to $I_p = -V_p/(R_s + R_c)$ in the steady state, where I_p is pipette current and R_s, R_c are series resistance and cell resistance, respectively, a low-resistance pipette should be used to keep R_s as small as possible [24]. However, we failed to make a giga-seal on the cell with a low resistance tip below 5 MΩ. GABA-activated currents were always obtained when the baseline of the current record was stabilized. No capacitative currents contaminated the baseline or affected the potential of the cell. Therefore, the potential of the pipette can be regarded as the potential of the cell. In addition, cells with long processes were never used to avoid the problems associated with spatial non-uniformities of membrane potential [12].

For voltage-clamp recording, the standard extra-

cellular solutions contained (in mM) *N*-methyl-(D)-glucamine (NMG) chloride, 140; $CaCl_2$, 7; $MgCl_2$, 1; HEPES, 10; KCl, 3; and sufficient D-glucose to bring the osmolarity to 320–325 mOsm/kg H_2O (pH 7.35–7.40). The total halide concentration in the extracellular fluid remains relatively constant, with Cl^- and Br^- tending to displace each other. Thus, various concentrations of Cl^- were replaced by the same amount of Br^- ranging from 10 mM to 140 mM, so that total amounts of anion remained constant in every experiment. In order to examine the influx of Cl^- ions at holding potentials above the Cl^- equilibrium potential, solutions used to fill the patch electrodes contained (in mM) NMG aspartate, 148; EGTA, 2; $CaCl_2$, 1; $MgCl_2$, 1; HEPES, 10; and sufficient D-glucose to adjust the osmolarity to the same value of the bath solution (pH 7.35–7.38). In some experiments, NMG aspartate was replaced by the same amount of NMG chloride to raise the concentration of Cl^- equal to the concentration in the bath solution. All recordings were conducted at room temperature (22–24°C).

2.3. Current-clamp recordings

Current-clamp recordings were obtained with an Axopatch 1D at I-mode with the patch electrode stated above. The bath solution contained (in mM) NaCl, 140; $CaCl_2$, 5; $MgCl_2$, 1; HEPES, 10; KCl, 3; tetrodotoxin (TTX), 0.002; and sufficient D-glucose to obtain an osmolarity of 320–325 mOsm/kg H_2O (pH 7.35–7.40). The recording electrodes were filled with (in mM) potassium aspartate, 155; EGTA, 2; $CaCl_2$, 1; $MgCl_2$, 1; HEPES, 10; and sufficient D-glucose to bring the osmolarity to 320–325 mOsm/kg H_2O (pH 7.35–7.40). In some experiments, 1 mM $MgCl_2$ in the pipette solution was replaced by 2 mM magnesium adenosine 5′-triphosphate (ATP) and 5 mM KCl.

2.4. Drug application

GABA was dissolved to produce a 10 mM stock solution. Test solutions were prepared immediately before the experiment by diluting an appropriate amount of the stock solution in the bath solution. Drugs and ions were applied for 2 s by pressure ejection (< 2 psi; 1 psi = 6.9 kPa) from a blunt-tipped (2–3 μm diameter) micropipette placed 70 to 100 μm from the cell surface.

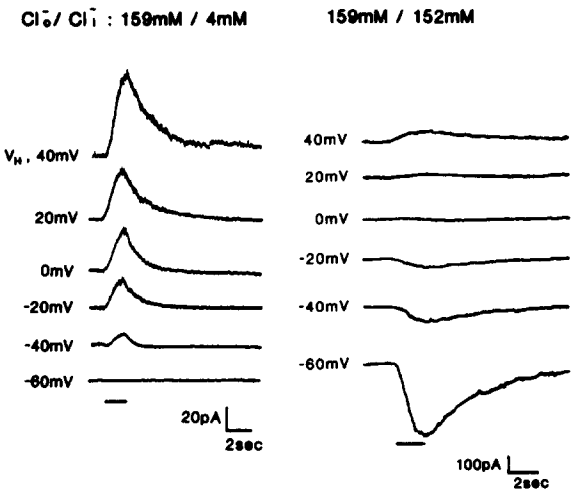

Fig. 1. 100 μM GABA was applied for 2 s with two different ionic compositions with regard to Cl^-. Left traces: With 159 mM Cl^- in the bath solution and 4 mM Cl^- in the pipette, progressively increasing outward currents were elicited as the holding potential was raised from −60 mV. Right traces: With identical Cl^- concentrations in both pipette and bath solutions, inward currents were evoked at the holding potential of −60 mV, then decreased with the elevation of the holding potential. Outward currents were observed at the potential of 20 mV and increased at the potential of 40 mV.

3. Results

3.1. GABA-activated currents

Recordings were obtained in 146 cells. With an intracellular solution containing 148 mM NMG aspartate under voltage-clamp conditions, the currents elicited during the application of 100 μM GABA reached a peak within 2 s at the holding potential above −60 mV, then decreased slightly. On cessation of the GABA application, the currents diminished gradually and returned to the baseline. The currents progressively increased as the holding potentials were raised (Fig. 1, left traces). Under the conditions of equal concentrations of Cl^- in both bath and pipette solutions, using an intracellular solution containing 152 mM Cl^-, inward currents were evoked at holding potentials more negative

than 0 mV and increased progressively toward the more hyperpolarized potentials. Conversely, outward currents were observed at holding potentials above 0 mV (Fig. 1, right traces). These currents were blocked by bicuculline (data not shown).

3.2. Effect of bromide on GABA-activated currents

With 152 mM Cl^- in the pipette, 100 μM GABA was applied with solutions containing either 159 mM Cl^- or 140 mM Br^- and 19 mM Cl^- at various holding potentials. Actual recordings from a typical cell are shown in Fig. 2. Outward currents were elicited by GABA application with 140 mM Br^--containing solution at a holding potential of 5 mV. The outward currents evoked during GABA application gradually decreased as the holding potential fell down to −8 mV. The GABA-activated currents turned inward at the holding potential of −9 mV. Inward currents were observed at the holding potential below −4 mV after the application of GABA. Since the GABA-coupled channels were still open just after the GABA application and Cl^- in the bath solution expelled Br^- from the cell surface, efflux of Cl^- overwhelmed influx of anions, resulting in inward currents. GABA application with Cl^--containing solution evoked an outward current at a holding potential of 5 mV. The outward current turned inward at a holding potential below 1 mV, then the inward current progressively increased as the holding potential fell. The reversal potential of the GABA-activated currents shifted approximately 9 mV when 140 mM Cl^- was changed to the same amount of Br^-. Additional experiments showed that the reversal potential shifted an average of 9.9 ± 3.8 mV (mean ± S.D., $n = 9$). To obtain the reversal poten-

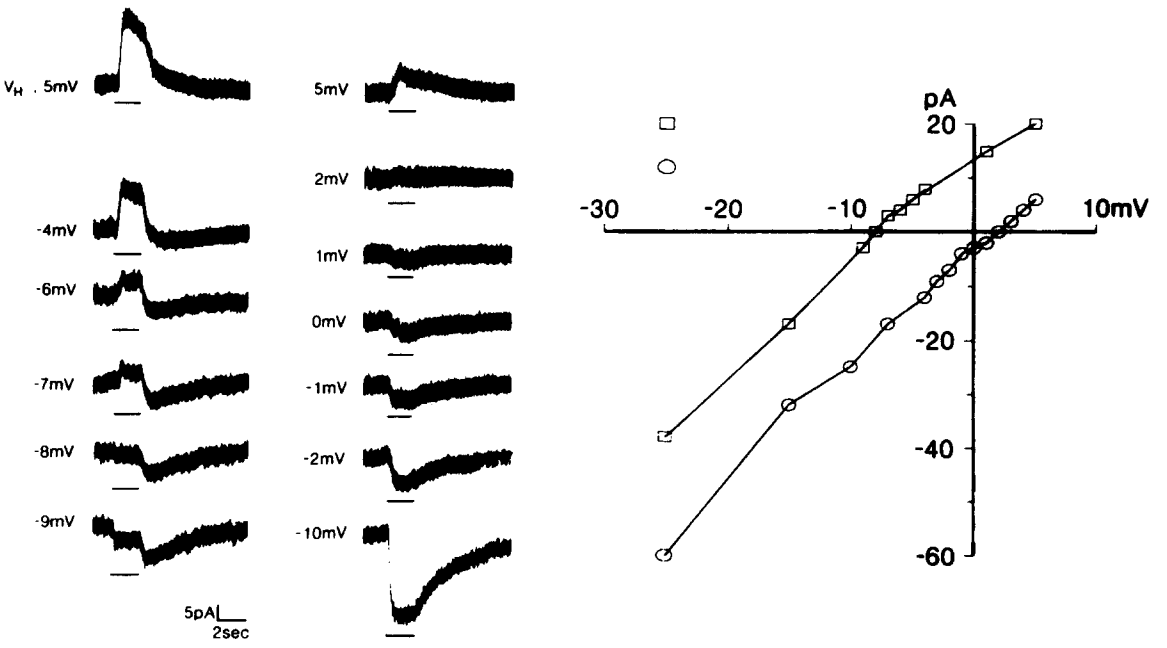

Fig. 2. With 152 mM Cl^- in the pipette, 100 μM GABA was applied with solutions containing either 140 mM Br^- and 19 mM Cl^- or 159 mM Cl^- in the same cell. Left: During the application of GABA with 140 mM Br^- solution, outward currents were elicited at the holding potential of 5 mV, then decreased as the holding potential was decreased. No currents were evoked at the potential of −8 mV. Note that inward currents were elicited after the application of GABA at the holding potentials below −4 mV. Since GABA-activated channels were still open just after the application of GABA and Br^- ions were expelled by Cl^- around the cell, the dominant Cl^- efflux over Cl^- influx caused inward currents at the potentials. It suggested that either an increase of Br^- influx or a decrease of Cl^- efflux was produced by the application of Br^-. The reversal potential of GABA-activated currents with the identical concentration of Cl^- was approximately 1 mV. Right: *I–V* curve shows that inward currents were suppressed by the external Br^- at the negative potentials, while outward currents were augmented. □: 140 mM Br^- + 19 mM Cl^-; ○: 159 mM Cl^-.

tial, V_o, of agonist-activated currents, two or three current values on either side of the reversal potential were used for interpolation. In experiments with a mixture of Cl^- and other monovalent anions, A^-, the Goldman–Hodgkin–Katz potential equation [10,15] was used to calculate the relative permeability (P_A/P_{Cl}) of anion A^- with respect to Cl^-:

$$V_o = -\frac{RT}{F}\ln\frac{[Cl^-]_o + P_A/P_{Cl}[A^-]_o}{[Cl^-]_i + P_A/P_{Cl}[A^-]_i}.$$

The subscripts 'o' and 'i' denote external and internal ion species, respectively, and R, T and F have their usual meanings. The reversal potential reveals a relative permeability ratio of the channels to Br^- with respect to Cl^- (P_{Br}/P_{Cl}) of 1.51.

Extracellular solutions containing various concentrations of Br^- substituted for the same amount of Cl^- were applied to the cell with 148 mM NMG aspartate-containing solution in the pipette. The solution containing 140 mM Br^- applied without GABA caused no current at potentials below 40 mV (data not shown). Next, solutions containing Cl^- and Br^- were applied with 100 μM GABA to examine the enhancement ratio of the GABA-activated currents. Fig. 3 shows the actual recordings of GABA-activated outward currents elicited by solutions with 152 mM Cl^-/0 mM Br^- (control solution) and 12 mM Cl^-/140 mM Br^- (upper traces: right). The currents were enhanced 2.00 ± 0.39 and 1.91 ± 0.44 (mean ± S.D., $n = 9$) at holding potentials of −20 mV and 0 mV, respectively. (The values were acquired from the cells which were voltage-clamped at both −20 mV and 0 mV.) The enhancement ratios at the holding potential of −20 mV obtained from four-day and seven-day old cells were 2.04 ± 0.37 (mean ± S.D., $n = 8$) and 1.95 ± 0.57 (mean ± S.D.,

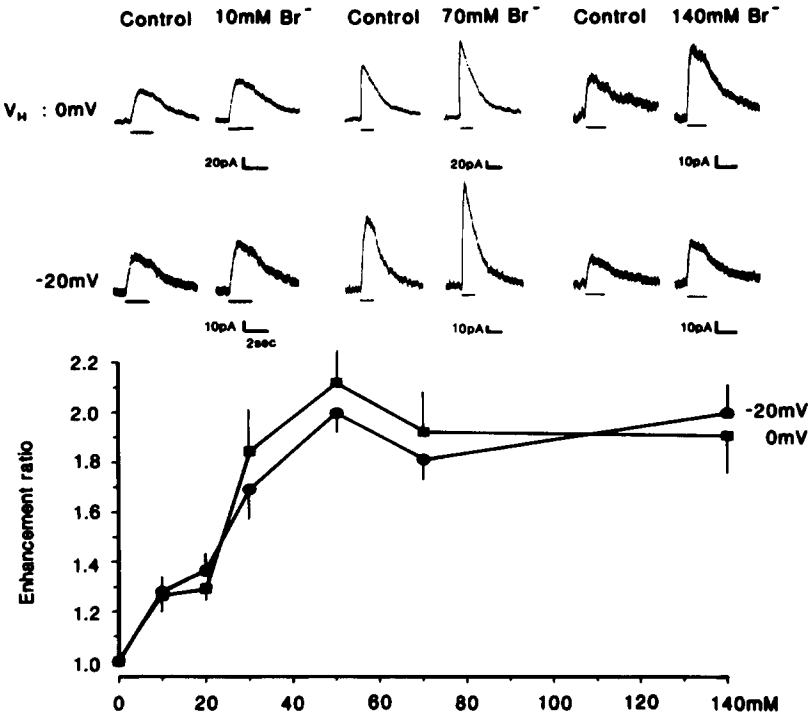

Fig. 3. Upper traces: Actual voltage-clamp recording at holding potentials of −20 mV and 0 mV. Solutions with various concentrations of Br^- substituting for the same amount of Cl^- were applied with 100 μM GABA. The GABA-activated outward currents were enhanced by the Br^--containing solutions. The enhancement ratio was estimated from peak of the currents obtained by the control solution and the Br^--containing solution at the same potential, and then plotted against the Br^- concentrations (lower plot; error bar shows mean ± S.D. of 6–22 cells per point). The enhancement ratio increased as the Br^- concentration was raised to 50 mM, and then became saturated thereafter.

$n = 4$), respectively. Since both values were identical, experiments were done using four- to seven-day old cells. As the typical recordings show (upper traces: middle), the enhancement ratios of the currents elicited by 70 mM Br^- were 1.81 ± 0.37 and 1.92 ± 0.60 (mean $\pm$ S.D., $n = 12$) at holding potentials of -20 mV and 0 mV, respectively. The 10 mM Br^--containing solution enhanced the currents 1.28 ± 0.16 and 1.27 ± 0.20 (mean $\pm$ S.D., $n = 7$) at holding potentials of -20 mV and 0 mV, respectively, as compared with the solution containing 152 mM Cl^-/0 mM Br^- (upper traces: left). Similarly, solutions containing 20 mM, 30 mM, and 50 mM Br^- were tested at holding potentials of -20 mV and 0 mV. The plots show the enhancement ratio of the GABA-activated outward currents by the various concentrations of Br^--containing solutions with respect to 152 mM Cl^- solution (lower plots). The enhancement ratio increased linearly up to approximately two-fold at 50 mM Br^-, then remained saturated until 140 mM.

3.3. Current-clamp recordings

Current-clamp recordings were performed to observe the actual change of membrane potentials elicited by 100 μM GABA with Br^--containing solutions. Typical recordings are shown in Fig. 4. The membrane potential was hyperpolarized from -20 mV to -27 mV by the application of GABA with a 157 mM Cl^--containing solution, while in the same cell, GABA with solutions containing 140 mM Br^- and 17 mM Cl^- hyperpolarized the membrane potential from -20 mV to -35 mV. In a total of ten cells, hyperpolarization obtained by the application of solutions containing 0 mM Br^-/157 mM Cl^- and 140 mM Br^-/17 mM Cl^- was 6.9 ± 0.7 mV (mean $\pm$ S.D.) and 14.1 ± 1.6 mV (mean $\pm$ S.D.), respectively. These differences are statistically significant by Wilcoxon's test ($P < 0.01$). Furthermore, the GABA application with solution containing 20 mM Br^- hyperpolarized the membrane potential 3.5 ± 0.8 mV (mean $\pm$ S.D., $n = 6$) more than the application with the control solution.

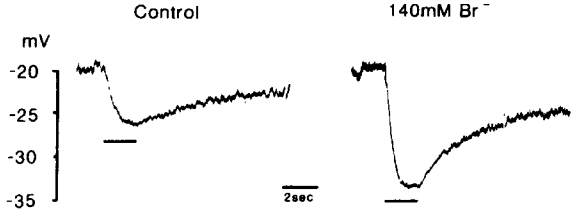

Fig. 4. Current-clamp recordings. Typical recording shows that GABA with 155 mM Cl^- (control solution) hyperpolarized the cell membrane from -20 mV to -27 mV (left), while in the same cell, the membrane potential was hyperpolarized to -34 mV by the application of GABA with a 140 mM Br^--containing solution (right).

4. Discussion

Bromide is distinguished from other antiepileptic agents in that it is a simple inorganic anion, which is also recognized to exhibit sedative actions [23]. In addition, bromide is reported to be a successful remedy for generalized tonic–clonic seizures (GTCs) and other types of seizures (photosensitive epilepsy, symptomatic localization-related epilepsy, and alternating hemi-grand mal seizure) [4,8,31,34]. The therapeutic serum bromide concentration ranges from 10 mM to approximately 30 mM in humans [4,8,31,33,34] and approximately 30 mM in pentylenetetrazol-induced seizures in mice [11]. The main disadvantage of bromide is the low ratio between the therapeutically effective dose and its toxicity, manifested mostly as sedation and psychic disturbances [19]. In humans, the toxic symptoms frequently develop at serum concentrations of 15 to 30 mM [33]. In mice, the TD_{50} is approximately 50 mM [11]. Following oral ingestion, bromide rapidly distributes, replacing the same amount of chloride, and enters the brain from the plasma. Since the CSF-to-plasma ratio (0.79 to 1.0) of bromide is higher than the brain-to-plasma ratio (0.19 to 0.3) [26], a large amount of bromide should exist in the extracellular space.

As to the mechanisms of actions of bromide, both the inhibition of carbonic anhydrase and HCO_3^--ATPase are thought to contribute to its anticonvulsant actions [33]. In addition, bromide is thought to compete with chloride for GABA-gated Cl^- channels [7,32] because of the smaller hydrated diameter of bromide [28]. However, general agreement on bromide's antiepileptic mechanism of action are lacking. The major inhibitory neurotransmitter in the brain is GABA, and any means of severely diminish-

ing GABA synaptic activity (e.g., inhibition of synthesis, direct block of the receptor) or blocking GABA receptor-mediated events immediately induces seizures [21]. This does not constitute proof that a similar mechanism exists in human epilepsy, but it provides a basis for the investigation of seizure phenomena in both animal models and humans. Thus, it is postulated that enhancement of GABAergic synaptic transmission in the brain contributes anticonvulsive actions.

In our experiments, the reversal potentials of GABA-activated currents obtained with two different ionic conditions of Cl^- concentrations in both extracellular and intracellular solutions showed that the currents were composed of Cl^- flux. In addition, the blockade of the currents by bicuculline proves the channels coupled to the $GABA_A$ receptor [1]. The shifts of reversal potentials of GABA-activated currents observed by the application of GABA in a 140 mM Br^--containing extracellular solution revealed that the relative permeability ratio of the $GABA_A$-coupled channels to Br^- with respect to Cl^- is 1.51, which is favorably comparable to the values obtained in other experiments [5,9,27]. The higher relative permeability might be explained by the smaller hydrated diameter of Br^- than Cl^-. In our experiments with a low concentration of internal Cl^-, greater GABA-activated outward currents were elicited by the application of a Br^--containing solution than a solution containing solely Cl^-, suggesting that bromide enters the cells through the $GABA_A$-activated channels more easily than Cl^-. However, in other studies, the actual single-channel conductances of GABA-activated channels for Br^- was smaller than for Cl^- [5,9,27]. Bormann et al. [5] assumed that binding sites within the channel might hinder the bromide molecule from penetration. Using I^- instead of Br^- in the bath solution to examine the membrane conductance, Robertson [27] observed a marked increase in inward current at negative potentials with I^- externally and inferred that changing the external anion from Cl^- to I^- facilitated Cl^- exit from the cell. Since a very low concentration of Cl^- was used in the pipette in our experiments, Cl^- may not form enough efflux to be facilitated, then is overwhelmed by the influx of Br^-. Therefore, the outward currents might possibly be enhanced by the existence of external Br^-. Furthermore, Robertson [27] showed that greater outward currents were obtained by the application of I^- with GABA externally at positive potentials in symmetrical Cl^- solutions. Considering the outward direction formed by the influx of I^- at the potentials, these data are consistent with our results. In addition, Fetima-Shad and Barry [9] surmised that the anion influx at positive potentials was greater than the anion efflux at negative potentials on the basis of the conductance ratios determined from single-channel conductance with symmetric substitution of bromide and other halide ions. Since we observed Br^- influx rather than Cl^- efflux, the outward currents consisting of Br^- influx might be enhanced in our experiments.

Applications of a 20 mM Br^--containing solution revealed a 1.14- and 1.30-fold enhancement of the currents at holding potentials of -20 mM and 0 mV, respectively. A solution containing 30 mM Br^-, where toxicity frequently develops in humans, enhanced the currents by 1.82-fold, which is close to the value obtained by 140 mM Br^-. The low value of TD_{50} may be explained by the steep slope of the ratio up to 30 mM. Since influx of the anion induces a more negative membrane potential, which forms an inhibitory postsynaptic potential (IPSP) [2,6,17], the enhancement of anion influx implies that more hyperpolarization can be attained. Although other kinds of Cl^- channels have been found in neurons [25], $GABA_A$-coupled Cl^- channels are known to be the main system for producing IPSP [18]. Therefore, the enhancement ratio of approximately 1.30 obtained by a therapeutic concentration of Br^- (20 mM) seems favorable enough to hyperpolarize the membrane potential. Current-clamp recordings clearly showed that more hyperpolarization was obtained by a 140 mM Br^--containing solution with GABA. A therapeutic concentration of 20 mM Br^- hyperpolarized the membrane potentials more than the control solution. The ionic conditions used in the current-clamp recordings were consistent with physiological ionic compositions. These findings suggest that the application of Br^- with GABA enhanced the GABA-activated currents and elicited a larger amount of anion influx, resulting in the generation of a larger IPSP. Balcar et al. [3] reported that Br^- selectively antagonized picrotoxin- and pentylenetetrazol-induced seizures without effects on any of the GABAergic factors (metabolism, transport character-

istic of the receptor-associated GABA-binding site) and speculated that it might act on Cl^- channels associated with GABA. Our study is favorably consistent with their speculations. In conclusion, our study suggests that the antiepileptic action of bromide might occur through the enhancement of IPSPs elicited by GABA.

Acknowledgments

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