An Ionotropic GABA Receptor with Novel Pharmacology at Bullfrog Cone Photoreceptor Terminals

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Key Words

Bicuculline · Cone photoreceptor · Chloride channel · Ionotropic GABA receptor · Retina · Bullfrog

Abstract

Characteristics of ionotropic γ -aminobutyric acid (GABA) receptors at bullfrog cone terminals were studied by patch clamp techniques in isolated cell and retinal slice preparations. GABA-induced inward currents from isolated cones reversed in polarity at a potential, very close to the chloride equilibrium potential, and they were completely suppressed by picrotoxin. Unexpectedly, the GABA current was dose-dependently potentiated by the well-known GABA_A receptor antagonist bicuculline (BIC), but was suppressed by gabazine, another GABA_A antagonist, and imidazole-4-acetic acid (I4AA), a GABA_C receptor antagonist. Similarly, currents induced by both GABA_A agonist muscimol and GABA_C agonist *cis*-4-aminocrotonic acid (CACA) were also potentiated by BIC. Furthermore, currents induced from cones by GABA and kainate-caused depolarization of horizontal cells in retinal slice preparations were both potentiated by BIC. All these results suggest that the ionotropic GABA receptor at the bullfrog cone terminal exhibits novel pharmacology, distinct from both traditional GABA_A and GABA_C receptors.

Introduction

As a principal inhibitory neurotransmitter in the central nervous system (CNS), including the retina, GABA acts at ionotropic GABA_A and GABA_C receptors and metabotropic GABA_B receptor [1–3]. GABA_B receptors belong to the superfamily of G-protein-coupled receptors and they activate the second-messenger pathways, phospholipase C and adenylate cyclase [4, 5]. While ionotropic GABA_A and GABA_C receptors are both directly coupled with chloride channels, they are structurally, pharmacologically and physiologically different. GABA_A receptors are hetero-oligomeric, consisting of several subunits (e.g. α , β , γ , δ , θ), and they are specifically blocked by bicuculline (BIC) and have modulatory binding sites for barbiturates, benzodiazepines, ethanol and neurosteroids [2, 6]. On the other hand, GABA_C receptors are homo-oligomeric in most cases, made up of either ρ_1 or ρ_2 subunits. The GABA_C receptor, like the GABA_A receptor, can be blocked by picrotoxin, a nonselective chloride channel blocker, and the potent antagonist I4AA [7, 8], but is insensitive to BIC. In addition, the GABA_C receptor is more sensitive to GABA than the GABA_A receptor [9–12], and currents mediated by these two types of GABA receptors are also different in kinetics [13–16].

Localization of GABA receptors in photoreceptors has been morphologically revealed in several species [17–24].

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Furthermore, ionotropic GABA receptor-mediated responses have been recorded from cones of turtle, porcine and mouse [14, 25, 26]. GABA receptors expressed in cones are thought to be involved in negative feedback from horizontal cells to these photoreceptors [26, 27], which may contribute to the receptive field surround response of bipolar cells. We recently reported that functional GABA_B receptors are expressed at the bullfrog cone terminal [28], which may provide a negative feedback mechanism for regulating signal transmission between cones and second-order neurons by modifying the amount of glutamate released from the cones. In the present work, we further studied ionotropic GABA receptors at bullfrog cone terminals in both isolated cell and retinal slice preparations using patch clamp techniques and found that these receptors possessed novel pharmacology. In particular, current responses of these receptors to GABA application were potentiated by BIC, but suppressed by picrotoxin.

Materials and Methods

Isolated Cell and Retinal Slice Preparations

Adult bullfrogs (*Rana catesbeiana*) were used in the present work. All efforts were made to minimize animal pain and discomfort in accordance with the NIH guidelines for animal experimentation and to reduce the number of animals used.

Isolated cone photoreceptors were conventionally made using a procedure described previously in detail [29] with minor modifications. The retina was isolated and cut into 8–12 pieces, and these retinal pieces were incubated in normal Ringer's with 75 U/ml papain (Washington Biochemical Corp., Freehold, N.J., USA) and 1 mg/ml cysteine (Sigma, St. Louis, Mo., USA) for 30 min at 25°C. The papain-treated pieces were stored at 4°C for up to 2 h. Retinal cells were freshly dissociated from one stored piece by mechanical trituration just before each experiment. The cell suspension was placed on a Petri dish mounted on the stage of a phase-contrast microscope (IX70, Olympus Optical, Tokyo, Japan). Cones with characteristic morphology were picked up for whole cell recording. Identification of cones as described in 'Results'. For comparison purposes, current responses to GABA application were also recorded from amacrine-like cells. These cells had a small (30–50 µm) pyriform-shaped soma and were quite similar in morphology to the monostriated amacrine cells described in tiger salamanders [30].

Retinal slices were prepared following the procedures reported previously [8, 28]. In brief, the dark-adapted retina was isolated and then cut into 150-µm-thick slices in Ringer's using a manual cutter (ST-20, Narishige, Tokyo, Japan). The slices were then transferred into a recording chamber with the cut side up and they were held mechanically in place by a grid of parallel nylon strings glued onto a U-shape frame of platinum wire. All these procedures were performed under dim red illumination. The glass-bottomed recording chamber with a volume of approximately 1.0 ml was continuously perfused with the oxygen-bubbled extracellular solution,

which was fed in and out of the chamber through inlets by a peristaltic pump (Minipulse 3, Gilson Medical Electronics, Viuiers Le Bel, France) at a rate of 3–5 ml/min.

Solutions and Drug Application

For experiments with isolated cones, Ringer's with an osmolality of 250 mosm/kg H₂O consisted of (in mM) 120 NaCl, 2.0 KCl, 2.0 CaCl₂, 1.0 MgCl₂, 10 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and 10 glucose, pH adjusted to 7.4 with NaOH. All drug-containing solutions were delivered using a stepper motor-based rapid solution changer RSC-100 (Bio-Logic Science Instruments, Mundelein, France), as described in detail previously [31]. The recorded cells were completely bathed in the solution by lifting them from the dish bottom. The solution exchange could be completed in a few milliseconds.

For experiments with retinal slice preparations, Ringer's with an osmolality of 250 mosm/kg H₂O consisted of (in mM) 100 NaCl, 25 NaHCO₃, 2.5 KCl, 1.6 MgCl₂, 2.0 CaCl₂, and 10 glucose, pH adjusted to 7.4 by bubbling with 95% O₂ and 5% CO₂. GABA containing Ringer's was pressure ejected by nitrogen gas via a Picospritzer II (General Valve, Houston, Tex., USA), which was triggered by the Pulse software (PulseFit 8.65, HEKA, Lambrecht/Pfalz, Germany), and focally applied at the cone photoreceptor terminal using a pneumatic puff pipette with a motor-driven micromanipulator (MP-285, Sutter, Novato, Calif., USA). The position of the pipette with a tip size of 3–5 µm in diameter was adjusted to obtain consistent drug-induced effects. The pressure in the puff pipette was optimized for the consistency of drug delivery, and when no pressure was applied to the pipette, a small amount of the bath medium was continuously sucked into the pipette by capillary attraction, thus preventing the drug solution from leaking out. The actual concentration of GABA, muscimol and CACA at the cell membrane was surely much lower because of bulk flow, diffusion and potent uptake systems in the retina. Drugs other than GABA were applied by administering drug-containing Ringer's into the bath medium through another inlet.

To record chloride currents, the intracellular solution for whole-cell voltage clamp in both isolated cell and retinal slice preparations consisted of (in mM) 128 CsCl, 2.0 MgCl₂, 1.0 CaCl₂, 10 ethylene glycol-bis(2-aminoethylether)-N,N',N'-tetra-acetic acid (EGTA), 10 HEPES, with an osmolality of 240 mosm/kg H₂O.

All chemicals were purchased from Sigma Chemicals and freshly dissolved in Ringer's when used.

Whole-Cell Voltage Clamp Recording

The recording chamber containing retinal slices was placed on a fixed-stage microscope (Zeiss, Axioskop 2 FS Mot, Jena, Germany), which was equipped with infrared differential interference contrast (DIC) optics. A water-immersion objective with a working distance of 1.4 mm was used (Zeiss, 63W, 0.95NA). During the experiments, the cells were imaged by an infrared video camera (C2400-79, Hamamatsu, Shizuoka, Japan) and visualized on a TV monitor (Panasonic, Osaka, Japan).

Recording patch electrodes were pulled from borosilicate glass (BF150-86-10, Sutter) with a multi-stage horizontal puller (P-97, Sutter). For recording current responses from isolated cones in the voltage clamp mode, patch pipettes with a tip diameter of about 2 µm, resistance of 5–7 MΩ when filled with the intracellular solution, were used. The resistance of the electrodes used in retinal slice preparations was 8–10 MΩ. The recording pipette was connected

to a patch amplifier (EPC9/2, HEKA) and the liquid junction potential was calculated, which was 3.96 mV for experiments with isolated cells and 2.84 mV for those with slice preparations (25°C), and it was auto-compensated by the software (Pulse 8.65, HEKA). Eighty percent of serial resistance of the recording electrode was compensated and it remained unchanged during the experiments. Voltage clamp data were acquired at a sample rate of 20 kHz, filtered at 2 kHz and they were stored in computer hard disk for further analysis.

Data Processing

Data analysis was done using PulseFit 8.65 (HEKA), OriginPro 7.0 (OriginLab Corp., Northampton, Mass., USA) and Igor 4.09A (WaveMetrics, Lake Oswego, Oreg., USA). The reversal potential for the GABA receptor was determined by fitting the steady-state current response recorded when the cells were clamped at various voltage levels with a linear function:

$$I = A \times (V - E_{\text{rev}}) \quad (1)$$

where I is the steady-state current at a certain voltage level, which is determined by averaging sampled data points during 100 ms prior to the cessation of a voltage step, V is the clamping voltage, A is a constant, and E_{rev} is the reversal potential.

Dose-response data of agonists or antagonists were fitted to the following Hill equations using a nonlinear least-squares method:

For activation:

$$\frac{I}{I_{\text{max}}} = \frac{1}{1 + \left(\frac{EC_{50}}{[\text{agonist}]} \right)^n} \quad (2)$$

For inhibition:

$$\frac{I}{I_{\text{max}}} = \frac{1}{1 + \left(\frac{[\text{antagonist}]}{IC_{50}} \right)^n} \quad (3)$$

where I_{max} is the steady-state response at a saturating concentration of an agonist or antagonist, which is measured by averaging sampled data points during 100 ms before the cessation of a 1-second application; EC_{50} is the concentration of the ligand producing half-maximal response; IC_{50} is the concentration of the antagonist producing half-maximal inhibition; n is the Hill coefficient. Dose-dependent potentiation of GABA (or agonist-) induced currents by BIC were also fitted to equation (2) with appropriate modification.

Results

Characterization of Ionotropic GABA Receptors at Isolated Bullfrog Cone Terminals

Isolated bullfrog cones are easily identified by characteristic morphology typical of amphibian cones. Figure 1a shows an isolated cone with the axon terminal (arrow) under DIC microscopy. Under phase-contrast microscopy, a bright oil-droplet was clearly seen in the inner segment, indicating that the cell was a single cone or the

principal member of a double cone. Cones without oil-droplets but with morphology typical of these photoreceptors may be the accessory member of double cones. These cells were also picked up for patch clamp experiments. There was no qualitative difference in regard to the characteristics of GABA-induced currents studied between these two cell types. In the present work, only data obtained in cones with oil-droplets were pooled and analyzed. Moreover, no GABA-induced current could be recorded in cones which lost axon terminals during mechanical trituration, suggesting the localization of GABA receptor on the terminals.

Figure 1b shows the current responses of an isolated cone induced by GABA of increasing concentrations when clamped at -60 mV. GABA of a concentration as low as $0.1 \mu\text{M}$ could induce a sustained inward current of 30 pA with slow activation. As GABA concentration was increased, the rise time of the current became faster, which was accompanied by a steady increase in size, but the deactivation was still rather slow. The current was saturated in size to $100 \mu\text{M}$ GABA. It was noteworthy that all these current responses were sustained and did not show desensitization. Figure 1c shows the average dose-response relationship of the GABA currents recorded from 36 cones. For constructing this relationship, the current responses of each cell to GABA of different concentrations were normalized by its response to $100 \mu\text{M}$ GABA, and the normalized data were then averaged and plotted as percentages of the maximum response. The experimental data were well fitted by the curve described with equation (2). The EC_{50} for GABA was $0.68 \pm 0.01 \mu\text{M}$ (mean $\pm$ SEM), which is clearly smaller than that of either GABA_A or GABA_C receptors reported previously [12, 32, 33], and the Hill coefficient was close to 1 (0.82 ± 0.01).

Figure 1d shows the current-voltage (I-V) relationship of the GABA currents recorded from an isolated cone at different holding potentials. According to equation (1), the E_{rev} for these currents was 2.5 mV. The average E_{rev} of 5 cones tested was 2.4 ± 0.8 mV, which is very close to the chloride equilibrium potential (E_{Cl}) (1.2 mV, 25°C) under our experimental conditions. When the chloride concentration in the intracellular solution was changed, the E_{rev} was accordingly shifted (data not shown). Moreover, the GABA currents were completely blocked by co-application of $500 \mu\text{M}$ picrotoxin (fig. 1 d, inset). All these results indicated that the GABA currents recorded from the bullfrog cones were mediated through chloride channels.

Fig. 1. GABA-induced responses from isolated bullfrog cones. **a** DIC microphotograph of an isolated cone with the axon terminal (arrow) at a high magnification ($\times 400$). An oil droplet could be clearly seen in the inner segment. The outer segment of the cell was lost during dissociation. The schematic diagram in the right shows how a drug pipette and a recording electrode were positioned. **b** Current responses of an isolated cone to GABA of different concentrations delivered by a rapid solution changer. The cell was voltage clamped at -60 mV. The currents were rather sustained with slow deactivation. GABA of $100 \mu\text{M}$ induced a maximum response. **c** Dose-response relationship of GABA currents of bullfrog cones. Steady-state currents of each cone in response to various concentrations of GABA, when voltage clamped at -60 mV, were normalized to the response of that cell to $100 \mu\text{M}$ GABA and then averaged.

The averaged data were fitted by the Hill equation (Eq. 2). Data for each point were pooled from 6 cones. The EC_{50} was $0.68 \pm 0.01 \mu\text{M}$ (mean $\pm$ SEM), and the Hill coefficient was 0.82 ± 0.01 ($n = 36$). **d** Current-voltage (I-V) curve for the GABA currents recorded in a representative cone. The averaged steady-state GABA currents (filled squares) obtained, when clamped at various voltage levels (from -60 to $+20$ mV at 20 -mV increments), were well fitted by a linear function (solid line). The reversal potential (E_{rev}) for the GABA currents obtained from this cone was 2.5 mV, very close to the chloride equilibrium potential (E_{Cl}) under our experimental condition (1.2 mV, 25°C). The inset shows that $500 \mu\text{M}$ picrotoxin (PTX) completely suppressed the current of the cone to $100 \mu\text{M}$ GABA.

Novel Pharmacology of the Ionotropic GABA Receptor at Bullfrog Cone Terminals

To determine if GABA_A and/or GABA_C receptors mediate these GABA currents, effects of BIC were first tested as a routine. Both bicuculline salt (Sigma No. B103) and free base bicuculline (Sigma No. B9130) were used in our experiments, and no difference was found. Surpris-

ingly, co-application of BIC did not suppress, but potentiated the GABA currents. Figure 2a shows how the response of an isolated cone to $100 \mu\text{M}$ GABA was dose-dependently potentiated by BIC of increasing concentrations. For this experiment, BIC continuously perfused the cell and responses to $100 \mu\text{M}$ GABA were recorded in the presence of BIC. BIC alone did not induce

Fig. 2. GABA currents of cones are potentiated by bicuculline (BIC). **a** Currents induced by 100 μM GABA from an isolated cone were potentiated by BIC in a dose-dependent manner. The BIC concentration is indicated on the right of each trace. The uppermost trace shows the current response induced by 100 μM GABA in the absence of BIC. BIC applied alone did not induce any current, and the BIC-potentiated currents were all sustained. Note that a fast deactivation component appeared, in addition to the slow component, in the responses to 100 μM GABA obtained in the presence of BIC of 10 and 100 μM . The inset shows that GABA (100 μM)-induced current from an isolated amacrine-like cell dissociated from the same retina was completely blocked by 100 μM BIC (+BIC). In contrast to the current responses of the cone, the GABA current of the amacrine-like cell showed significant desensitization. **b** Change in GABA response deactivation in the presence of BIC. Upper panel: The response of an isolated cone to 100 μM GABA

(grey trace) in the absence of BIC. The deactivation course was well fitted by a single exponential function (dark curve) with a time constant of 644.0 ms. Lower panel: The response of the same cell to 100 μM GABA in the presence of 100 μM BIC (grey trace). A double-exponential function (dark curve) was needed to fit the deactivation course of the BIC-potentiated GABA current, with two time constants of 68.9 and 825.0 ms, respectively, for the fast and slow components. **c** Dose-dependent potentiation of currents of isolated cones to 100 μM GABA caused by BIC. All responses from each cone were compared to the response of that cell to 100 μM GABA recorded in the absence of BIC (control) and represented as folds of control. Data determined from different cones in the presence of BIC of various concentrations were then averaged. The averaged data points were fitted by modified equation (2). The EC_{50} for BIC, causing a half maximum potentiation of GABA currents, was $4.0 \pm 0.2 \mu\text{M}$, and the Hill coefficient was 0.92 ± 0.04 ($n = 36$).

any discernable current. The GABA response, however, was increased from 70.9 to 89.7 pA in the presence of 1 μM BIC. When BIC concentration was increased to 10 μM , the response was further increased in size to 118.5 pA. With a further increase of BIC concentration to 100 μM , a still larger (143.1 pA) response could be obtained. The potentiation extent of the GABA response obtained at 300 μM BIC was not much different from that obtained at 100 μM BIC (data not shown). It is note-

worthy in figure 2a that a new deactivation component with faster kinetics appeared in the BIC potentiated responses, in addition to the slow deactivation component. As shown in the upper panel of figure 2b, the deactivation course of the response to 100 μM GABA recorded from the cone in normal Ringer's could be well fitted by a single exponential function with a time constant of 644.0 ms. The average time constant based on the data pooled from 18 cells was 721.6 ± 31.3 ms. Deactivation of the re-

Fig. 3. Both gabazine (SR-95531) and I4AA suppress GABA currents of isolated cones in a dose-dependent manner. **a** Dose-dependent suppression of GABA (100 μ M) currents of isolated cones by gabazine. Data from different cones were averaged after all responses from each cone were normalized to the response of that cell to 100 μ M GABA in the absence of gabazine. The averaged data points were fitted by equation (3). The IC_{50} of gabazine was $155.0 \pm 8.3 \mu$ M, and the Hill coefficient was 1.5 ± 0.1 ($n = 28$). The in-

set shows that the current induced by 100 μ M GABA from an isolated cone (open circle) was completely suppressed by 1 mM gabazine (filled circle). **b** Dose-dependent suppression of GABA currents of isolated cones by I4AA. Data were processed as in (**a**). The IC_{50} for I4AA was $718.5 \pm 34.6 \mu$ M, and the Hill coefficient was 3.0 ± 0.7 ($n = 20$). The inset shows that the current induced by 100 μ M GABA from an isolated cone (open circle) was almost completely suppressed by 1 mM I4AA (filled circle).

sponse of the same cell recorded in the presence of 100 μ M BIC, on the other hand, should be fitted by a double exponential function with two time constants: one was 68.9 ms for the fast component and other was 825.0 ms for the slow component (lower panel). The average time constants ($n = 18$) were 90.7 ± 7.4 ms and 937.8 ± 56.0 ms, respectively. For comparison purpose, we also tested the effects of BIC on GABA currents recorded from isolated bullfrog amacrine-like cells. As shown in the inset of figure 2a, the response of this amacrine-like cell to 100 μ M GABA showed desensitization and was completely suppressed by co-application of 100 μ M BIC. Similar observations were obtained in all 6 amacrine-like cells tested. Figure 2c shows the potentiation of the response to 100 μ M GABA as a function of BIC concentration. The current responses of each cell to 100 μ M GABA in the presence of BIC of different concentrations were normalized by that obtained in normal Ringer's (control response). The normalized data were averaged and plotted as folds of the control response. The curve described with modified equation (2) fitted the data points well, yielding a value of $4.0 \pm 0.2 \mu$ M ($n = 36$) for BIC causing half-maximum potentiation. The Hill coefficient was close to 1 (0.92 ± 0.04). It should be noted, however, that responses to GABA of lower concentrations, say 1 μ M, could be blocked by BIC of much higher concentrations, say 1 mM (data not shown).

The BIC-potentiated responses were insensitive to the GABA_B receptor agonist baclofen (100 μ M, $n = 4$) or antagonist saclofen (200 μ M, $n = 5$) (data not shown). The E_{rev} for these currents (2.2 ± 0.6 mV, $n = 6$) was almost unchanged, indicating that they were also mediated through chloride channels.

It was of interest that gabazine (SR-95531), another selective GABA_A receptor antagonist, did not potentiate the GABA currents of bullfrog cones, but suppressed them in a dose-dependent manner. Figure 3a shows the effects of gabazine of increasing concentrations on current responses of cones to 100 μ M GABA. The data were processed in a way similar to that for the BIC-induced potentiation. Curve fitting using equation (3) yielded an IC_{50} of $155.0 \pm 8.3 \mu$ M and a Hill coefficient of 1.5 ± 0.1 ($n = 28$). The GABA current was suppressed by gabazine of a concentration as low as 30 μ M and almost abolished by 1 mM gabazine (fig. 3a, inset). Similarly, the GABA_C receptor antagonist I4AA suppressed GABA currents, and the dose-suppression relationship obtained on the basis of the data pooled from 20 cones is shown in figure 3b. The curve described with equation (3) did not fit the data points quite well, but it was obvious that 1 mM I4AA almost completely suppressed the current response to 100 μ M GABA (fig. 3b, inset). The IC_{50} for I4AA was $718.5 \pm 34.6 \mu$ M and the Hill coefficient was 3.0 ± 0.7 ($n = 20$).

Fig. 4. Dose-response relationship of muscimol-induced currents and BIC-potentiated muscimol currents of isolated cones. **a** Dose-dependent relationship of muscimol-induced currents of isolated cones. The data were processed as in figure 1c. All responses from each cone were normalized to the response of that cell to 500 μM muscimol. The EC_{50} of muscimol was $19.8 \pm 4.0 \mu\text{M}$, and the Hill coefficient was 0.92 ± 0.08 ($n = 30$). The inset shows the current induced by 500 μM muscimol (MUS) from an isolated cone, which was sustained with rather slow deactivation. **b** Dose-response relationship of BIC potentiated muscimol (500 μM) currents of iso-

lated cones. The data were processed in a similar way to that performed for the data shown in figure 2c. All responses from each cone were plotted as folds of the response induced by 500 μM muscimol from that cell in the absence of BIC. The EC_{50} for BIC, producing a half maximum potentiation of muscimol currents, was $9.7 \pm 1.2 \mu\text{M}$ and the Hill coefficient was 0.98 ± 0.01 ($n = 36$). The inset shows that the current response of the cell shown in the inset of (**a**) (grey trace) was significantly potentiated by 3 μM BIC (dark trace).

We further examined current responses of cones to the GABA_A receptor agonist muscimol. Muscimol application also induced sustained inward currents, which were rather similar in waveforms to the GABA currents (cf. response in fig. 4a, inset, with those in fig. 1b). The average dose-response relationship obtained from 30 cones yielded an EC_{50} of $19.8 \pm 4.0 \mu\text{M}$ and a Hill coefficient of 0.92 ± 0.08 . In the inset of figure 4b, the response of an isolated cone to 500 μM muscimol, inducing a maximum response, was clearly potentiated in the presence of 3 μM BIC. The potentiation was dose-dependently increased and the EC_{50} of BIC for 500 μM muscimol-induced currents was $9.7 \pm 1.2 \mu\text{M}$ ($n = 36$), as yielded by the relationship shown in figure 4b, and the Hill coefficient (0.98 ± 0.01) was almost unchanged from that obtained in the absence of BIC. Again, BIC of much higher concentrations, say 1 mM, could suppress the response to muscimol of a rather low concentration, say 10 μM (data not shown).

Current responses of cones to the GABA_C receptor agonist CACA were also studied. CACA induced a sustained current as well, but with fast deactivation (fig. 5a,

inset), which was clearly different from either GABA- or muscimol-induced current. The average dose-response relationship of the CACA current yielded an EC_{50} of $43.7 \pm 5.4 \mu\text{M}$, and a Hill coefficient of 1.9 ± 0.5 ($n = 42$). The CACA current was potentiated by BIC of concentrations lower than 20 μM , but it was suppressed at higher concentrations of BIC. The effects of BIC of low (3 μM) and high concentrations (200 μM) on the current response of a cone to 100 μM CACA are shown in the insets of figure 5b and figure 5c, respectively. While the CACA current was significantly potentiated by 3 μM BIC, it was completely suppressed by 200 μM BIC. Figure 5b shows that the CACA current steadily increased in size with increasing concentrations of BIC in a range of 0–20 μM . These data points appeared to follow a linear relation, but could not be fitted well by equation (1). A possible explanation may be that the BIC-induced suppression might have emerged, along with the potentiation, when the concentration of BIC was higher than 1 μM so that the potentiated currents obtained in a range of 1–10 μM of BIC concentration might have been suppressed to some extent. In other words, the GABA currents obtained in

Fig. 5. Effects of BIC on CACA-induced currents of isolated cones. **a** Dose-dependent relationship of CACA-induced currents of isolated cones. All responses from each cone were normalized to the response of that cell to 100 μM CACA. The data points were fitted with equation (2), yielding an EC_{50} of $43.7 \pm 5.4 \mu\text{M}$ and a Hill coefficient of 1.9 ± 0.5 ($n = 42$). The inset shows that 100 μM CACA induced a sustained inward current from an isolated cone with fast activation and deactivation. **b** Dose-dependent potentiation of CACA currents by BIC of concentrations less than 10 μM . The potentiation was almost linearly related to the logarithm of BIC concentration. The inset shows that the current of the cell to 100 μM CACA shown in (a) (grey trace) was potentiated by 3 μM BIC (dark

trace). **c** Dose-dependent suppression of CACA currents by BIC of concentrations, higher than 30 μM . The data points were fitted with equation (3). The IC_{50} of BIC was $82.0 \pm 3.0 \mu\text{M}$ and the Hill coefficient was 3.4 ± 0.4 ($n = 30$). The inset shows that the current of the cone to 100 μM CACA shown in the inset of (a) (grey trace) was completely suppressed by 200 μM BIC (dark trace). **d** Muscimol (MUS, 500 μM) and CACA (100 μM) induced inward currents from the same isolated cone, with deactivation of different kinetics. When MUS (500 μM) and CACA (100 μM) were co-applied to the same cone terminal, the current induced was quite similar to the muscimol current. Similar results were obtained in 7 cones.

this range of BIC concentrations might have been larger if no such suppression existed. The amplitude of the CACA currents recorded in the presence of BIC of concentrations higher than 20 μM was plotted as a function of BIC concentration in figure 5c. The current was dose-dependently suppressed by BIC. Even though the curve described with equation (3) fitted the data points reasonably well, it might have been distorted due to the BIC-induced potentiation occurring at the lower concentration range.

There was a possibility that muscimol- and CACA-induced currents may be mediated by two subtypes of ionotropic GABA receptors. To test this possibility, we compared the current response to a mixture of muscimol and CACA with those induced by muscimol or CACA alone. The upper and middle traces in figure 5d are the responses of an isolated cone to 500 μM muscimol and 100 μM CACA, respectively, both of which produced maximum responses. The response induced by co-application of muscimol of 500 μM and CACA of 100 μM

Fig. 6. Potentiation of GABA currents of cones in retinal slice preparations. **a** DIC microphotograph of a bullfrog retinal slice at a high-magnification ($\times 630$), showing the distal part of the retina. Cones are easily identified by characteristic morphology and oil droplets. The terminal of a cone is indicated by arrow. The schematic diagram in the right shows how a puff pipette and a recording electrode were positioned. **b** GABA of 1 mM focally puffed to the terminal of the cone shown in (**a**), which was voltage clamped at

-60 mV, induced an inward current (control). Note that activation and deactivation of the current were both much slower than those recorded from isolated cones. The GABA current was greatly potentiated in the presence of $200 \mu\text{M}$ BIC added in the bath medium, but completely blocked when $500 \mu\text{M}$ picrotoxin (PTX) was added in the bath medium along with $200 \mu\text{M}$ BIC (+BIC+PTX). The GABA current returned to the control level following washout with Ringer's.

(bottom trace) was obviously not the algebraic sum of the muscimol response and the CACA response, but almost identical to the muscimol response. It is reasonable to speculate that the effect caused by muscimol of the saturating concentration ($500 \mu\text{M}$) might have occluded the action of CACA on this ionotropic GABA receptor or vice versa. Similar results were obtained in 6 other cones. These results suggest that muscimol and CACA may act at a single receptor type.

Behaviors of GABA Currents at Cone Axon Terminals in Retinal Slice Preparations

To exclude the possibility that potentiation by BIC of GABA currents might be an artifact produced when cones were dissociated, we tested effects of BIC on current responses of cones recorded in retinal slice preparations while GABA was applied to their terminals. In these preparations, cones were easily identified by their localization and characteristic morphology (fig. 6a). When 1 mM GABA was focally puffed to the terminal of the cone, as

indicated by the arrow in figure 6a, a sustained inward current was elicited (fig. 6b, upper trace). Due to bulk flow and diffusion barrier in the retina, activation and deactivation of the current were both much slower than those obtained from isolated cones (cf. responses in fig. 1b). When the preparation was perfused with $200 \mu\text{M}$ BIC, the current response of the cone to 1 mM GABA was significantly potentiated (fig. 6b). In the presence of $500 \mu\text{M}$ picrotoxin, along with $200 \mu\text{M}$ BIC, 1 mM GABA no longer induced any discernable current.

Whole-cell recordings were further made from cones when the preparation was perfused with $40 \mu\text{M}$ kainate, which is supposed to induce the release of GABA from horizontal cells by causing large depolarization of these cells [34, 35]. The result of such an experiment is shown in figure 7a. As expected, perfusion with $40 \mu\text{M}$ kainate induced a sustained inward current from the cone with a 5-second delay. Again, the current was potentiated by the addition of $200 \mu\text{M}$ BIC to the perfusate but was completely suppressed by $500 \mu\text{M}$ picrotoxin. We further de-

Fig. 7. Inward currents are induced from cones by kainate application in retinal slice preparations. **a** An inward current with a 5-second delay was recorded in a cone when 40 μ M kainate (KA) was added into the bath medium. The experiment was performed in the presence of a background light (10.1 μ W/mm²). The GABA current induced by kainate perfusion (grey trace) was potentiated by BIC (200 μ M) added into the bath medium, but completely suppressed by picrotoxin (PTX). The cone was held at -60 mV. **b** I-V relationships of the kainate (40 μ M)-induced current responses of the cone

shown in **(a)** recorded in the absence and presence of 200 μ M BIC. Both curves yielded an E_{rev} of about +5 mV, close to the E_{Cl} under our experimental conditions (5.1 mV, 25°C). These curves were achieved by subtracting the control currents to the voltage ramp ranging from -80 to +40 mV in normal Ringer's from the data obtained in the presence of 40 μ M kainate (grey curve) and 40 μ M KA + 200 μ M BIC (dark curve). Similar results were obtained in 5 other cones.

terminated the reversal potentials for the kainate-induced current and the BIC-potentiated kainate current (fig. 7b). The I-V relationships determined by voltage ramps for these currents indicated that the reversal potentials were about +5 mV in both cases, very close to the E_{Cl} (5.1 mV, 25°C) under our experimental conditions. Similar results were obtained in 5 other cones.

Discussion

Ionotropic GABA Receptor of Novel Pharmacology at Bullfrog Cone Terminals

Immunocytochemical studies have demonstrated labeling of GABA_A and GABA_C receptor subunit isoforms at the cone axon terminal in several species [17, 20–24]. Electrophysiologically, it was first reported that GABA-induced chloride currents from isolated turtle cones were completely blocked by BIC, suggesting involvement of GABA_A receptors [26]. In salamander retinal slice preparations, Wu [36] showed that a light step elicited a depolarization in cones, while the outer segments of these cells were truncated off. The depolarization was only partially suppressed by BIC, suggesting the possible existence of GABA_C receptors at the cone terminal. Using a photoreceptor-glia co-culture model, it was further found that

GABA responses of adult porcine cones consisted of two distinct components, a BIC-sensitive one and an I4AA-sensitive one [25]. This is also the case for mouse cones in situ [14].

In the present work, we studied ionotropic GABA receptors at the bullfrog cone terminal. Responses of bullfrog cones to focal application of GABA to their terminals were indeed mediated by chloride channels. EC_{50} for GABA of the receptor is much lower than that for GABA_A receptors (10–100 μ M), but close to that for GABA_C receptors (1–5 μ M). The responses mediated by this receptor were sustained without desensitization and they were activated by CACA and suppressed by I4AA, but not modulated by pentobarbital (200 μ M, $n = 12$, data not shown). These pharmacological properties are reminiscent of those, typical of traditional GABA_C receptors. But the responses mediated by this receptor to GABA, muscimol and CACA are all potentiated by BIC, which is widely used to distinguish GABA_C receptors from GABA_A receptors. To our best knowledge, this peculiar phenomenon is without precedent. It was of interest that muscimol-induced currents were all potentiated by BIC in a range of 1–1,000 μ M (fig. 4b), whereas CACA-induced currents were potentiated by BIC at lower concentrations (<10 μ M), but suppressed at higher concentrations (>30 μ M) (fig. 5b, c). These observations suggest a

possibility that there might be two populations of receptors. While this possibility could not be ruled out, we tend to think, according to the occlusion experiments shown in figure 5d, that there is only a single type of GABA receptors with novel pharmacology at the cone terminal.

The mechanisms underlying the BIC-induced potentiation remain to be further explored. A working model is proposed here for the explanation of the phenomenon. The Hill coefficient is known to be related to the number of co-operative site or binding site of a receptor [37, 38]. The Hill coefficients of this GABA receptor were all close to 1 for GABA-, muscimol-induced and BIC-potentiated currents, but they were larger than 1 for CACA-induced and gabazine-, I4AA-suppressed currents. While the traditional GABA_A receptor is thought to have two binding sites for GABA [12, 32, 33], our results raised a possibility that GABA and muscimol may share an identical binding site on the receptor whereas either antagonists (e.g. gabazine and I4AA) or agonist CACA may each possess two binding sites. While BIC binds to the same site for GABA on traditional GABA_A receptors [39], BIC may be able to bind to the antagonist site, in addition to the GABA site in this unique GABA receptor. When BIC binds to the GABA site, it may synergetically act on this receptor with GABA, thus potentiating the GABA current. The fast component of GABA currents appearing in the presence of BIC (fig. 2b) may be due to an allosteric activation mechanism, as occurs in the recombinant GABA_A receptors containing a mutated β_2 subunit [40]. On the other hand, BICs binding to the antagonist site would suppress the GABA current. When the concentration of BIC is raised to a rather high level, so that the suppression effect of BIC overwhelms the potentiation effect, the GABA current would then be suppressed by BIC. It is a real puzzle that BIC and gabazine differentially act on this GABA receptor, while these two GABA_A receptor antagonists are thought to bind to identical sites [41]. A possible explanation for the different actions may be that they differentially modulate the site(s) that is(are) involved in direct gating of the channel, since they are shown to work as allosteric inhibitors of channel opening of the GABA_A receptor and induce conformational changes of the receptor [40]. In this regard, it is noted that gabazine was reported to block GABA_C responses in perch horizontal cells [42].

It should be also noted that the CACA-induced currents always possess rather fast activation and deactivation, implying that CACA may bind to and dissociate from this receptor with kinetics faster, as compared to other agonists (e.g. GABA, muscimol), and have a differ-

ent gating mechanism. Actually, the fast deactivation of the CACA currents somewhat bears a resemblance to the characteristic kinetics of perch-p2A homo-oligomeric GABA receptors expressed in *Xenopus* oocytes [43].

Novel Pharmacology of the Ionotropic GABA Receptor May Be Related to a Unique Subunit Composition and/or an Assembly with an Unrevealed New Subunit

Recent evidence suggests that there is a great diversity of ionotropic GABA receptors, which are different in kinetics, affinity for agonists and antagonists [2], and the existence of ionotropic GABA receptors with unique pharmacological properties has been demonstrated. In the chick embryo retina GABA receptors expressed on amacrine and ganglion cells can be blocked by BIC and positively modulated by barbiturates, but they are insensitive to picrotoxin [44]. In cat retina heterogeneous types of GABA_A receptors are segregated into different zones: those in the distal retina are sensitive only to BIC whereas those in the proximal retina are sensitive to both BIC and picrotoxin [45]. In lobster central neurons (olfactory projection neurons and thoracic neurons) ionotropic GABA receptors appear to differ in pharmacology from vertebrate GABA_A and GABA_C receptors in that they are insensitive to BIC and gabazine, and exhibit other unique agonist potency sequences [46]. This diversity may be ascribed to the composition of ionotropic GABA receptors. These receptors are pentameric complexes of subunits, known as α , β , γ , δ , ϵ , π , and ρ subunits, and a new θ subunit was recently added to this list [47]. Most of the subunits have several members. Different subunit assemblies provide the basis for the functional diversity of ionotropic GABA receptors. It is known, for instance, that different α , β subunits underlie distinct affinities of GABA binding and γ subunits confer the sensitivity to benzodiazepines on the receptor [2]. It was recently found that GABA_A receptor subunits γ_2 and δ confer unique kinetic properties on recombinant GABA receptor currents in mouse fibroblasts [48]. Therefore, the novel pharmacology of the ionotropic GABA receptor expressed at the bullfrog cone terminals should be resulted from a unique subunit composition, different from that of traditional GABA_A or GABA_C receptors. Indeed, co-assembly of GABA_A and ρ subunit has been shown to contribute probably to the molecular organization of GABA_C receptors in the retina [49–51]. It is also highly possible that an unrevealed new subunit may be assembled. Even though we are not sure whether ionotropic GABA receptors of similar pharmacology may be found in other central neu-

rons or other species, it is definitely of interest to determine what kind of subunit composition may be responsible for the BIC-induced potentiation and to uncover the site(s) BIC and gabazine may differentially act on using recombinant GABA receptors containing different subunits.

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