Responses Mediated by Excitatory and Inhibitory Amino Acid Receptors in Solitary Spiking Cells from Normal Newt Retina

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Abstract Whole-cell currents activated by L-glutamate, kainate (KA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), N-methyl-D-aspartate (NMDA), γ-aminobutyric acid (GABA), and glycine were recorded from spiking cells enzymatically dissociated from adult newt retina. Most spiking cells responded to both AMPA and KA. KA tended to be a more potent agonist for them than AMPA. The reversal potential of both AMPA and KA responses was about $-7 \text{ mV}$; this value was similar to that (about $-5 \text{ mV}$) of glutamate response. AMPA- and KA-induced currents may be carried by monovalent cations, such as Na$^+$, because their reversal potentials were sensitive to external Na$^+$. About half of the spiking cells examined responded to mixtures of NMDA and glycine. Extracellular Mg$^{2+}$ blocked completely the response to NMDA plus glycine in spiking cells held at negative membrane potentials, but not at positive membrane potentials. Almost all spiking cells responded to the inhibitory amino acids GABA and glycine. Dose-dependent desensitization was observed in both GABA and glycine responses. Currents activated by GABA and glycine were carried by Cl$^-$. Bicuculline and strychnine strongly suppressed the responses to GABA and glycine, respectively, suggesting the existence of GABA$\alpha$ receptors and conventional glycine receptors in the spiking cells.

Key words: newt retina, spiking cell, glutamate, GABA, glycine.

Certain species of adult amphibians, for instance newts and salamanders, possess the ability to regenerate a new functional retina following a removal or destruction of the original retina. The process of retinal regeneration in such species has been well studied morphologically [1–3]. However, the number of physiological studies of this regeneration system is limited.
Important components of the functional differentiation of retinal neurons during regeneration are the expression of the voltage- and transmitter-gated ionic channels specific for the activity of each of the retinal neurons. Kaneko and Saito described the appearance and maturation of the voltage-gated ionic currents of spiking cells during newt retinal regeneration using whole-cell recording techniques [4]. As an extension of our previous paper on the functional differentiation of spiking cells, it is of interest to examine the time course of the appearance of the excitatory and inhibitory amino acid receptors.

L-Glutamate appears to be a major excitatory neurotransmitter in the vertebrate central nervous system (CNS) including the retina (for reviews, see [5, 6]). It interacts with a variety of postsynaptic receptors which have been classified by their relative affinities for different glutamate analogues, such as kainate (KA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), N-methyl-D-aspartate (NMDA), and 2-amino-4-phosphonobutyrate (APB). On the one hand, γ-aminobutyric acid (GABA) and glycine appear to act as major inhibitory neurotransmitters in the CNS (for reviews, see [7, 8]).

The purpose of the present study was to examine what kinds of excitatory and inhibitory amino acid receptors exist in the spiking cells in the normal newt retina as the control to investigate the time course of the appearance of these receptors during retinal regeneration.

MATERIALS AND METHODS

Preparation. Adult newts (*Cynops pyrrhogaster*, 6–10 cm in body length) were anesthetized with 0.1% FA 100 (4-allyl-2-methoxyphenol; Tanabe) and decapitated. Eyes were enucleated and retinæ were isolated under a stereomicroscope. Solitary retinal neurons were dissociated enzymatically from the retina as described previously [4]. The cell suspension medium was plated onto plastic dishes (35 mm in diameter; Falcon, No. 3001). The dishes used in the present study were previously coated with 1% concanavalin A (Sigma, C2010) for 2 h, washed 5 times with distilled water and air-dried. After cell plating, the medium was replaced with 80% Leibovitz’s L-15 culture medium (Flow Laboratories). The cells were maintained at 25°C before use and were usually studied within 12 h after plating.

Solitary cells examined electrophysiologically had a spherical soma (15–19 μm in diameter) with a single long process (>80 μm). They showed action potentials in response to current stimulation. Cells showing such a characteristic morphology and regenerative activities were initially identified as ganglion cells using retrograde transport of horseradish peroxidase (HRP). However, in most experiment, HRP-backfilled retinæ were not used due to technical difficulties. Therefore, we referred to those cells with the characteristic morphology and regenerative activities as “spiking cells.”

Recording procedures. For electrophysiological recordings, the culture medi-
um was replaced with a control bath solution (in mM): 110 NaCl, 3.7 KCl, 1.0 MgCl₂, 3.0 CaCl₂, 1.0 Na-pyruvate, 13 glucose, 5 HEPES, and 0.001% phenol red; pH was adjusted to 7.5 with NaOH. The culture dish was mounted on the stage of an inverted microscope with phase-contrast optics (Olympus IMT-2). The bath solution was fed into the dish under gravity at a rate of 1 ml/min and withdrawn by suction. In some experiments, Na⁺-free solution was prepared by substituting choline chloride for NaCl, and Mg²⁺-free solution was made by simply removing MgCl₂ from the control solution. Modifications of Ca²⁺ and Mg²⁺ concentrations in Na⁺-free solution were made by exchanging their chloride salts for osmotically equal choline chloride.

Patch pipettes (tip diameter of 1–2 μm) were pulled from glass capillaries (0.7 mm i.d. and 1.1 mm o.d.) with a vertical electrode puller (PP-83; Narishige) and used without silicone coating or firepolishing. The patch pipette was connected with CEZ-2200 patch-clamp amplifier (Nihon Kohden), and responses were recorded and filtered at 3 kHz. Data were monitored on VC-11 memory oscilloscope (Nihon Kohden) and polygraph pen recorder (RTA-1100; Nihon Kohden), and stored on audio-visual tape with DAT data recorder (RD-120TF; Teac) for further analysis. The indifferent electrode was Ag-AgCl wire connected to the bath solution by a 3 M KCl agarose bridge. All recordings were performed at room temperature (22–24°C).

We used two kinds of whole-cell configurations by the patch-clamp method: Nystatin perforated [9] and conventional patch-clamp methods [10]. Compositions of intrapipette solutions used are listed in Table 1. In the former method, the patch pipettes were filled with one of four solutions (A–D in Table 1) containing nystatin (160 μg/ml). In the latter method, they were filled with one of three solutions (E–G in Table 1). In experiments in which cells had to be voltage-clamped, K⁺ in the intrapipette solutions was replaced with Cs⁺ to minimize voltage errors resulting from voltage-activated K⁺ currents. Concentrations of intrapipette Cl⁻ were modified by replacement of Cl⁻ with osmotically equal SO₄²⁻ and glucose. Activity coefficients were calculated from the Debye-Hückel theory at the lower limit of ionic strength.

Table 1. Composition of patch-pipette solution.

<table>
<thead>
<tr>
<th>Composition (mM)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
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<tbody>
<tr>
<td>KCl</td>
<td>138</td>
<td>0</td>
<td>10</td>
<td>0</td>
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<tr>
<td>K₂SO₄</td>
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<td>0</td>
<td>64</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CsCl</td>
<td>0</td>
<td>138</td>
<td>0</td>
<td>10</td>
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<tr>
<td>C₅SO₄</td>
<td>0</td>
<td>0</td>
<td>64</td>
<td>64</td>
<td>54</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EGTA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
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<tr>
<td>Glucose</td>
<td>0</td>
<td>0</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td>Nystatin (μg/ml)</td>
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<td>160</td>
<td>160</td>
<td>160</td>
<td>0</td>
<td>0</td>
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All solutions contained 10 mM HEPES and 0.001% phenol red. The pH was adjusted to 7.5 with 0.3 N KOH or CsOH.

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In each trial, the liquid junction potential at the electrode tip was nulled by an offset current sufficient to make the output current zero. Electrode resistance measured in the bath solution was 10 to 15 MΩ by the nystatin methods, and 2 to 5 MΩ by the conventional methods. After the electrode tip was positioned on the cell surface, a tight seal was achieved by gentle suction. Under the voltage-clamp mode, the seal resistance was measured at 10-mV steps from −80 mV. Cell-attached seal resistance of over 10 GΩ was accepted for obtaining records of higher quality. By the nystatin methods, all data were acquired from at least 15 min after tight seal formation. The series resistance was estimated from the peak amplitude of the capacitive transient currents which appeared in response to the 10 mV steps, and was 100 to 120 MΩ. By the conventional methods, the series resistance was about 10 MΩ. In the present study, holding potentials were not corrected for voltage errors resulting from the series resistances by either method. By the conventional methods, liquid junction potentials at the electrode tip which emerged in the bath solution were measured as described by Kaneko and Tachibana [11], and holding potentials were corrected for the liquid junction potentials (−4 to −12 mV). By the nystatin methods, holding potentials were not corrected for liquid junction potentials between cytosol and intrapipette solutions, because these potentials could not be measured.

Drug application. We used the following drugs: l-glutamate, AMPA, KA, NMDA, APB, GABA, glycine, bicuculline, and strychnine. These drugs were purchased from Research Biochemicals Inc. (RBI) except for APB which was from Sigma.

Drugs were applied by either “pressure-application” or “bath-application.” The volume of the superfusate was kept at about 1 ml by continuously adding fresh solution under gravity at a rate of 1 ml/min through a glass pipette (about 300 μm in tip diameter) and removing the solution at the same rate by suction. In the pressure-application, a glass micropipette (1−2 μm in tip diameter) filled with a drug-containing solution was placed in close proximity (about 20 μm) to the cell soma, and the drugs were ejected by air pressure (>0.6 kgf/cm²). In the bath-application, drug solutions were gravity-fed to the dish through the same pipette as that used to supply the fresh solution. The pipette was placed about 200−400 μm away from the cell, and the stream of superfusate was directed at the cell prior to recording. The speed of fluid exchange around the cell was estimated as about 5 to 15 s with the bath-application, and about 100 to 800 ms with the pressure-application.

All values in the text with the “±” sign indicate the standard deviation (SD).

RESULTS

Responses of retinal spiking cells to excitatory amino acids

Glutamate. We examined responsiveness to l-glutamate in solitary spiking cells dissociated from the normal newt retina. Ninety (63%) of 142 cells examined
responded to L-glutamate. Figure 1a shows a typical glutamate response under the current-clamp mode. Pressure-application of 100 µM glutamate induced a depolarization accompanying spike discharges. Figure 1b shows glutamate-induced currents obtained by conventional whole-cell patch-clamp methods. The patch pipette

![Diagram]

Fig. 1. Voltage and current responses activated by L-glutamate in solitary spiking cells. a: Voltage response to L-glutamate under current-clamp mode. Recordings were made using nystatin-perforated whole-cell patch-clamp methods. The patch pipette was filled with solution A. Resting potential; −52 mV. L-Glutamate (100 µM) was applied by pressure ejection. b: Voltage dependence of glutamate-induced currents. Recordings were made under the voltage-clamp mode by conventional whole-cell patch-clamp methods. The patch pipette was filled with solution G. L-Glutamate (200 µM) was applied by pressure ejection. Holding potentials are indicated to the left of each trace. Resting potential; −42 mV. c: Current-voltage relation of glutamate-induced currents shown in b. The peak amplitude of the currents (ordinate) was plotted as a function of the holding potentials (abscissa). The reversal potential was near −5 mV in this case.
was filled with solution G in Table 1. Pressure-application of 200 μM glutamate induced an inward current in the cell at a holding potential of −80 mV. As the membrane was depolarized, the glutamate-induced inward current decreased in amplitude and reversed polarity at about 0 mV. A current-voltage relation for this cell is plotted in Fig. 1c. The average reversal potential value for 10 cells was $-5 \pm 6$ mV. The value was not significantly altered by modifications of the intracellular Cl$^-$ concentration using patch-pipette solutions E and F (data not shown). These results suggest that glutamate may act as an excitatory neurotransmitter on spiking cells in the newt retina.

**AMPA and KA.** We measured a total of 80 spiking cells for their responsiveness to glutamate analogues (AMPA and KA) in comparison with that to glutamate. These three drugs (100 μM each) were bath-applied sequentially to single cells. All cells (100%) responded to KA, 56 cells (70%) responded to AMPA, and 54 cells (about 68%) responded to glutamate. In 56 cells sensitive to both AMPA and KA, 48 cells (about 86%) the current in response to KA was larger than that

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**Fig. 2.** Whole-cell currents activated by glutamate, AMPA, and KA. Recordings were made by nystatin-perforated methods. The patch pipette was filled with solution B. Holding potential was −30 mV. a: Three drugs (100 μM each) were bath-applied to the cell sequentially. Resting potential; −58 mV. b, c: Effect of 200 μM L-glutamate or 200 μM AMPA on currents induced by 200 μM KA. Records were obtained from a single cell. Resting potential; −53 mV. Bars represent the timing and duration of the drug application.
in response to AMPA. An example of these records is shown in Fig. 2a. In the remaining 8 cells, the AMPA-induced current was somewhat larger than that induced by KA (data not shown). Two cell types could not be distinguished from each other based on their morphological features or regenerative activities. In the present study, we mainly analyzed the former type, because the later one was not always encountered.

To investigate whether KA and AMPA activate the same receptors as glutamate, KA was co-applied with glutamate or AMPA (Fig. 2b, c). Bath-application of KA induced an inward current at a holding potential of -30 mV. When KA was applied in the presence of glutamate, the KA-induced current was about 70% smaller than that in the absence of glutamate (Fig. 2b). Co-application of AMPA also reduced KA-induced current (to 40%) (Fig. 2c). These results suggest that glutamate receptors sensitive to both AMPA and KA exist in the newt spiking cells.

Figure 3 shows dose-response relations of currents activated by AMPA and KA. The cell was initially voltage-clamped at -30 mV by nystatin-perforated methods. Drugs were bath-applied to single cells sequentially. Fluid change around the cell required about 1 s in this experiment. Both AMPA- and KA-induced currents became detectable at a concentration of at least 10 µM (left-hand

Fig. 3. Dose-response relations for AMPA and KA in spiking cells. Recording conditions were identical to those described in Fig. 2. a: Current responses to AMPA (upper traces) and those to KA (lower traces) at 10 µM (left-hand side) and 1 mM (right-hand side). b: Normalized dose-response curves for currents activated by AMPA (▲) and KA (●). The peak amplitude of the drug-induced current at each concentration was normalized with respect to the maximal response for each cell, and plotted against the logarithm of the drug concentration. Each point represents the mean ± SD (vertical bar). Data were collected from 8 cells.

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side in Fig. 3a). Application of higher concentrations of the drugs produced larger responses. At a concentration of about 10 mM, both AMPA- and KA-induced responses became maximal (right side in Fig. 3a). The maximal current ranged from 5 to 100 pA with a mean value of 33±30 pA \((n = 7)\) for AMPA and from 12 to 185 pA with a mean value of 71±56 pA \((n = 11)\) for KA. Figure 3b shows normalized dose-response curves for AMPA (▲) and KA (●). The peak amplitude of the response was plotted as a function of the logarithm of the drug concentration. The continuous line in the figure was drawn according to the following equation:

\[
I = 100 \times \frac{A^n}{A^n + EC_{50}^n},
\]

where \(I\) is the theoretical value of the normalized response to the drugs, \(A\) is the drug concentration, \(EC_{50}\) is the drug concentration giving half-maximal response, and \(n\) is the Hill coefficient. The \(EC_{50}\) and \(n\) values were estimated beforehand from the Hill plot (not illustrated) using a least-squares fitting. The \(EC_{50}\) values for AMPA and KA were 45 and 92 \(\mu\)M, respectively. The \(n\) value was 1.1 for AMPA and 0.9 for KA.

Ionic dependency of KA- or AMPA-induced currents were examined by conventional whole-cell recordings (Fig. 4). The cell was initially voltage-clamped at 0 mV (near reversal potential of glutamate-induced currents), and were depolarized to 110 mV and then hyperpolarized to \(-110\) mV in steps of 10 mV before and during bath-application of the drug (see the bottom trace “V” in Fig. 4a as a sample record). The top trace in Fig. 4a is a sample of leakage current measured in the absence of the drug. A bath-application of 100 \(\mu\)M KA caused a small steady outward current at 0 mV (showing a baseline shift from a dotted line) and the depolarizing and hyperpolarizing voltage steps caused a large voltage-dependent current deflection (second trace). Application of 100 \(\mu\)M KA in the absence of external \(Na^+\) caused a large steady outward current at 0 mV (shift from a dotted line), and a series of voltage steps caused relatively small voltage-dependent current deflection (third trace). Figure 4b shows the current-voltage \((I-V)\) relations for the KA-induced currents measured from Fig. 4a. KA-induced currents in the control solution containing 110 mM NaCl (▲) and Na\(^+\)-free solution (□) were obtained by subtracting each leakage current from the current recorded during KA application and were plotted against step voltages. The \(I-V\) curve was near-linear in the control solution. The reversal potential value was about \(-10\) mV in this case. The mean value of the reversal potential for 7 cells was \(-7\pm5\) mV. In Na\(^+\)-free solution, KA-induced currents became outward at 0 mV, and the reversal potential was shifted to between \(-90\) and \(-100\) mV. The mean value of the reversal potential for 7 cells was \(-94\pm6\) mV. Similar reversal potential values were obtained for AMPA-induced currents (data not shown). The values were not significantly altered by modifications of the intracellular Cl\(^-\) concentration using patch-pipette solutions E and F. Changes in the external Ca\(^{2+}\) and/or Mg\(^{2+}\).
Fig. 4. Voltage dependence of KA-induced currents in solitary spiking cells. Recordings were made with conventional whole-cell patch-clamp methods. Patch pipette was filled with solution G. a: KA-induced currents in the presence and absence of external Na\(^+\). The cell was initially voltage-clamped at 0 mV, then depolarized to 110 mV in steps of 10 mV (50 ms in duration and 500 ms interval) and hyperpolarized to −110 mV in the same steps. Traces are examples of original recordings. Voltage protocol is shown in the bottom trace (V). Leakage currents in the control bath solution are shown in the upper trace. A steady bath-application of KA (100 μM) caused sustained outward current (showing a baseline shift from a dotted line). A series of voltage steps caused voltage-dependent current deflection in the presence (second trace) and absence of external Na\(^+\) (third trace). b: I-V curves for KA-induced currents measured in a. Amplitude of KA-induced current obtained from subtracting the leakage current (ordinate) was plotted as a function of the step voltages (abscissa). ■, control bath solution; □, Na\(^+\)-free solution. Reversal potential values were near −10 mV in control solution and between −90 and −100 mV in Na\(^+\)-free solution.

concentrations (3 to 30 mM) also did not modify the currents induced by AMPA and KA in Na\(^+\)-free solution (data not shown). These results suggest that Na\(^+\) is a main component of the AMPA- and KA-induced inward currents.

**NMDA.** We tested a total of 40 spiking cells for their responsiveness to NMDA at a holding potential of −80 mV. Nineteen cells (about 48%) responded to the mixture of NMDA and glycine in the absence of external Mg\(^2+\). Six of these cells also responded to NMDA alone. Figure 5 shows a typical NMDA response. Bath-application of NMDA alone did not induce a detectable response. However, when co-applied with glycine, NMDA elicited a small inward current accompanied
Fig. 5. Whole-cell currents activated by NMDA in the spiking cells. 
a: NMDA-induced current and the potentiating effect of glycine. The recording was made by nystatin-perforated methods in the absence of extracellular Mg$^{2+}$. Bath-application of NMDA (250 $\mu$M) alone did not cause any significant response but caused an inward current in the presence of glycine (1 $\mu$M). Bars represent the timing and duration of the drug application. Holding potential was $-80$ mV. 
b: Effect of Mg$^{2+}$ on the NMDA-induced current. Bath-application of NMDA (250 $\mu$M) plus glycine (1 $\mu$M) caused an inward current in Mg$^{2+}$-free solution. Application of Mg$^{2+}$ (1 mM) suppressed the inward current. c: I-V relations for the NMDA response. Recordings were made by conventional patch-clamp methods. Patch pipettes were filled with solution G. Recording procedures were the same as those in Fig. 4. The voltage steps were applied to the same cell before and during application of NMDA (250 $\mu$M) plus glycine (1 $\mu$M). Points in the graph represent the data obtained in the control bath solution containing 1 $\mu$M Mg$^{2+}$ (■) and in the Mg$^{2+}$-free solution (□) from the same cell.

by a relatively large noise increase (Fig. 5a). The mean NMDA response potentiated by glycine was 30±22 pA ($n=19$).

The response to NMDA plus glycine was blocked, when 1 mM Mg$^{2+}$ was added to an Mg$^{2+}$-free perfusing solution (Fig. 5b). Since the block of NMDA responses by Mg$^{2+}$ has been shown to be voltage-dependent in other preparations [12–15], we also examined whether this is the case in newt spiking cells. Figure 5c shows I-V curves for currents activated by a mixture of NMDA and glycine in the

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absence and presence of Mg$^{2+}$ in the external solution. The recording procedures for NMDA-induced currents were similar to those in Fig. 4. In the absence of Mg$^{2+}$ (□), the $I-V$ curve was almost linear and the reversal potential was about 0 mV. In the presence of Mg$^{2+}$ (■), currents at negative membrane potentials were completely blocked without significant effect on the currents at positive membrane potentials.

**APB.** We tested a total of 5 cells for their responsiveness to 50–500 μM APB. None of them responded to this drug.

**Responses of retinal spiking cells to inhibitory amino acids**

We tested a total of 50 cells for their responsiveness to GABA and glycine using whole-cell patch-clamp methods. All cells (100%) examined responded to GABA and 48 cells (96%) to glycine.

**Voltage and current responses to GABA.** Figure 6a shows typical voltage responses to GABA. Cells were initially depolarized at suprathreshold level (about −30 mV) by constant current injection to maintain spike discharges. When GABA was applied by pressure ejection during excitation, it hyperpolarized the cell membrane and suppressed ongoing spikes. To investigate the ionic mechanisms underlying the hyperpolarizing responses evoked by GABA, we measured the reversal potential of GABA-induced current by conventional whole-cell recordings. Figure 6b shows the voltage dependence of GABA-induced currents obtained in the control bath solution containing 122 mM Cl$^-$ . The recording was made using a CsCl-containing patch pipette (solution E). Pressure-application of GABA induced an inward current in the cell at a holding potential of −95 mV. As the membrane was depolarized, the GABA-induced current decreased in amplitude and reversed polarity from inward to outward between −75 and −55 mV. The reversal potential value was about −65 mV in this cell.

The dependence of the reversal potential of GABA-induced current on intracellular Cl$^-$ concentration was tested by replacement of intrapipette Cl$^-$ with SO$_4^{2-}$. The average reversal potential values measured with pipette solutions E (10 mM Cl$^-$), F (30 mM Cl$^-$), and G (138 mM Cl$^-$) were −65 ± 5 mV ($n = 8$) at 10 mM Cl$^-$, −39 ± 6 mV ($n = 11$) at 30 mM Cl$^-$, and 1 ± 5 mV ($n = 12$) at 138 mM Cl$^-$ (Fig. 6c). These values agreed with the theoretical values of Cl$^-$ equilibrium potential calculated from the Nernst equation with Cl$^-$ activities, suggesting that Cl$^-$ was a main ion involved in the GABA-induced current.

**Voltage and current responses to glycine.** Figure 6d shows typical voltage responses to glycine. Like GABA, glycine hyperpolarized the cell membrane and inhibited spike discharges. Figure 6e shows the voltage-dependence of a glycine-induced current obtained in the control bath solution (122 mM Cl$^-$). Pressure-application of glycine induced an inward current at the holding potential of −95 mV. As the cell was depolarized, the glycine-induced current decreased in amplitude and reversed its polarity between −75 and −55 mV. The reversal potential value was about −65 mV in this cell.
The reversal potential of the glycine response, like GABA, was found to depend on the intracellular Cl⁻ concentration. Figure 6f shows the quantitative relationship between the average reversal potential and intrapipette Cl⁻ concentration. The average reversal potential values were $-64 \pm 7$ mV ($n=6$) at 10 mM Cl⁻, $-42 \pm 8$ mV ($n=7$) at 30 mM Cl⁻, and $1 \pm 5$ mV ($n=6$) at 138 mM Cl⁻. These values resembled those of GABA-activated currents.

**Dose-response for GABA and glycine.** Figure 7 shows dose-response relations of currents activated by GABA and glycine. The cell was initially voltage-clamped at 0 mV, and the drugs were bath-applied. Both GABA- and glycine-induced outward currents became detectable at a concentration of at least 3 μM. The magnitude of the current was steady during continuous application of the drug (on the left in Fig. 7a and b). Application of higher concentrations produced larger responses which produced desensitization in a concentration-dependent manner. At a concentration of about 300 μM, both GABA and glycine responses became
maximal (on the right in Fig. 7a and b). The maximal current ranged from 30 to 480 pA with a mean value of 153±140 pA (n=9) for GABA and from 80 to 470 pA with a mean value of 226±137 pA (n=9) for glycine. The response to glycine recovered completely from desensitization within 5 min, while the response to GABA took more than 10 min to recover. Figure 7c shows normalized dose-response curves for GABA (●) and glycine (▲) in which the peak amplitude of the response is plotted as a function of the logarithm of the drug concentration. The EC₅₀ values for GABA and glycine were 21.6 and 22.4 μM, respectively. The n value for GABA and glycine were 1.6 and 1.4. These values may be underestimated because of a partial desensitization due to slow application of drugs.

Effects of GABA and glycine receptor antagonists. We tested effects of bicuculline, an antagonist of GABAₐ receptors, and of strychnine, an antagonist of glycine receptors, in spiking cells. A typical result is shown in Fig. 8. The cell was initially voltage-clamped at 0 mV. After recording the response to GABA or glycine, the effect of their antagonists were tested. Application of bicuculline suppressed the GABA-induced current strongly (upper traces in Fig. 8a), but the glycine-induced current only partially (lower traces in Fig. 8a). On the other hand, strychnine suppressed the glycine response strongly (lower traces in Fig. 8b), but the GABA response only partially (upper traces in Fig. 8b). These results suggest that GABA receptors in newt spiking cells are mainly comprised of GABAₐ receptors and that glycine receptors are the conventional type.

Fig. 6. Voltage and current responses activated by GABA (a–c) and glycine (d–f) in solitary spiking cells. a and d were recorded by nystatin-perforated whole-cell patch-clamp methods. c, d and e, f were recorded by conventional whole-cell patch-clamp methods. a: Voltage response to GABA under current-clamp mode. The patch pipette was filled with solution C. Pressure ejection of GABA (30 μM) suppressed ongoing spikes. Resting potential; −56 mV. b: Voltage dependence of GABA-induced currents. The patch pipette was filled with solution E. Holding potentials are indicated to the left of each trace. Currents induced by 30 μM GABA reversed the polarity between −55 and −75 mV. c: Cl⁻ dependence of GABA-induced current. Reversal potentials of the GABA-induced current (ordinate) were plotted as a function of the logarithm of intrapipette Cl⁻ concentration (abscissa). Solution E (10 mM Cl⁻), F (30 mM Cl⁻), and G (138 mM Cl⁻) were used for the patch pipette solutions. Data represent the mean±SD (vertical bars) at each intrapipette Cl⁻ concentration. The number of cells tested is shown in parentheses. The straight line represents the equilibrium potential for Cl⁻ calculated by the Nernst equation. d: Voltage response to glycine under current-clamp mode. Pressure ejection of glycine (30 μM) suppressed ongoing spikes. Resting potential; −48 mV. e: Voltage-dependence of glycine-induced currents. Currents induced by 30 μM glycine reversed the polarity between −55 and −75 mV. f: Cl⁻ dependence of glycine-induced current. The patch-pipette solutions and method of plotting data were identical to those described in c.
Fig. 7. Dose-response relations for GABA and glycine in spiking cells. Recordings were made by nystatin-perforated methods. Cells were voltage-clamped at 0 mV, and the drugs were bath-applied. Patch pipettes were filled with solution D. a: Current responses to GABA at 3 µM (left) and 300 µM (right). b: Current responses to glycine at 3 µM (left) and 300 µM (right). c: Normalized dose-response curves for currents activated by GABA (○) and glycine (▲). The peak amplitude of the drug-induced current at each concentration was normalized with respect to the maximal response for each cell, and plotted against the logarithm of the drug concentration. Each point represents the mean ± SD (vertical bar). Data were collected from 8 cells.

DISCUSSION

In the present paper, we describe receptors activated by AMPA, KA, and NMDA as excitatory amino acids and by GABA and glycine as inhibitory amino acids in solitary spiking cells, presumably ganglion cells, dissociated from the adult newt retina. These measurements will serve as a baseline for our study of the appearance of these receptors during retinal regeneration, presented in the following paper [16].

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Fig. 8. Effects of antagonists on GABA- and glycine-induced currents. Recordings were made with nystatin-perforated methods. Cells were voltage-clamped at 0 mV, and drugs were bath-applied. Patch pipettes were filled with solution D. 

**a:** Effects of bicuculline (Bi) on GABA and glycine responses. Application of GABA (30 μM) or glycine (30 μM) caused an outward current. Bicuculline (10 μM) suppressed the response to GABA strongly (upper trace), but the glycine response only partially (lower trace).

**b:** Effects of strychnine (Str) on GABA and glycine responses. Strychnine (1 μM) suppressed the response to GABA (10 μM) only partially (upper trace), but suppressed the response to glycine (30 μM) strongly (lower trace).

Excitatory amino acid receptors

L-Glutamate is thought to mediate excitatory synaptic transmission from bipolar cells to amacrine and ganglion cells in vertebrate retinae [17–20]. Synaptically released glutamate can act via a variety of postsynaptic receptors which have been classified by their affinities for different glutamate analogues. In the present study, we observed that there are at least two classes of glutamate receptors (AMPA/KA and NMDA varieties), but we were not able to detect responses to APB. In our previous paper [21], we reported that APB activated glycine receptors in ganglion cells isolated from newt retina. This may not be correct, because the APB which we used (RBI) was contaminated by glycine.

**AMPA/KA receptors.** In most spiking cells of newt retina, KA induced larger currents than those induced by AMPA. A few reports on the retinal ganglion cells of other species suggested that AMPA and KA activate separate glutamate receptor populations [13, 15, 22]. However, in the newt retina, currents activated by KA in spiking cells were suppressed by application of glutamate or AMPA, suggesting that KA and AMPA activate the common glutamate receptors.

Both AMPA and KA elicited non-desensitizing responses in most spiking cells. However, in a few cells, AMPA induced responses with rapidly desensitizing components (8/40 cells). Such a response has been commonly observed in tigray horizontal cells and goldfish ganglion cells [15, 23]. The appearance of a low AMPA response with rapid desensitization in this experiment may not be simply explained by technical problems of drug application, because we could obtain
substantially the same results using "U-tube" concentration clamp methods (fluid exchange time around the cell was 90–150 ms) [24]. There are observations that concanavalin A (ConA) reduces glutamate receptor desensitization [23, 25]. Since we used ConA as an attachment factor of dissociated cells to culture dishes, responses to AMPA in spiking cells attached on ConA tightly may have been influenced.

AMPA/KA receptors have been thought to selectively conduct monovalent cations (for reviews, see [26, 27]). However, molecular biological studies have shown that various combinations of different subunits alter both the $I$-$V$ relation and $\text{Ca}^{2+}$ permeability (for reviews, see [27, 28]). The $\text{Ca}^{2+}$ permeable AMPA/KA receptors were electrophysiologically identified in bipolar cells in the adult salamander retina [29], horizontal cells in the adult catfish retina [30], and ganglion cells in the developing rat retina [31]. In the newt spiking cells, $I$-$V$ curves for AMPA and KA responses in the control solution were nearly linear. The reversal potentials of the AMPA and KA responses were dependent on external $\text{Na}^+$ concentrations but independent of external $\text{Ca}^{2+}$ or $\text{Mg}^{2+}$ concentrations. Outward currents recorded in the $\text{Na}^+$-free solution appear to be carried by intracellular $\text{Cs}^+$, because the patch-pipette solution used contained only CsCl. These results suggest that AMPA/KA receptors in the newt spiking cells contain monovalent cation channels.

**NM2DA receptors.** NM2DA sensitive receptor is one of the most widely studied glutamate receptors in the vertebrate CNS (for reviews, see [5, 26]) including retina [13–15]. This receptor is coupled with channels which are highly permeable to $\text{Ca}^{2+}$ as well as $\text{Na}^+$ and $\text{K}^+$ [32]. The NM2DA-induced current is blocked by extracellular $\text{Mg}^{2+}$ in a voltage-dependent manner and potentiated by glycine [12–15, 33, 34]. In the present study, we also detected such NM2DA responses in the newt spiking cells.

Roughly half of the spiking cells examined responded to NM2DA. This incidence is lower than those of ganglion cells in other species [13–15]. The low response rate might be explained by a species difference. However, we must also mention that enzymatic dissociation caused the lack of the NM2DA sensitivity of hippocampal neurons from rat [35].

**Inhibitory amino acid receptors**

Retinal spiking cells are known to receive inhibitory inputs from amacrine cells which release GABA and/or glycine as a neurotransmitter [36–38]. Indeed, we could observe that GABA- and glycine-activated currents in the newt spiking cells. They were dependent upon intracellular $\text{Cl}^-$ concentration and their reversal potentials followed the Nernst equation for $\text{Cl}^-$. This result agreed well with the general knowledge that GABA and glycine directly inhibit spiking cells by activating $\text{Cl}^-$ conductances [36, 39, 40] (for review, see [8]).

**GABA receptors.** In the vertebrate retina, GABA is known to activate at least three classes of receptors: $\text{GABA}_A$, $\text{GABA}_B$, and $\text{GABA}_C$ [24, 36, 39–43] (for review, see [8]). The $\text{GABA}_A$ and $\text{GABA}_C$ receptors contain $\text{Cl}^-$ channels.

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Bicuculline selectively blocks responses mediated by GABA_A receptors, but not those mediated by GABA_C receptors [42, 43]. A finding that the GABA responses in the newt spiking cells were strongly suppressed by bicuculline suggests the existence of GABA_A receptors. In a few spiking cells, however, a part of the GABA-induced current was resistant to the application of mM order bicuculline (data not shown). Therefore, possibility of the existence of GABA_C receptors in newt spiking cells must be examined in detail.

**Glycine receptors.** Most spiking cells examined responded to glycine as well as GABA in an inhibitory manner. Because glycine responses involved a Cl^{-} conductance increase, and since they were predominantly suppressed by strychnine, we concluded that the newt retinal spiking cells possess conventional glycine receptors, as described in the isolated ganglion cells from goldfish [40] and rodent retinæ [41].

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