Development of Responses to Excitatory and Inhibitory Amino Acids in Spiking Cells during Retinal Regeneration in the Adult Newt

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Abstract Responsiveness to amino acids in solitary spiking cells dissociated from regenerating newt retinae at different stages were studied by whole-cell patch-clamp methods in comparison with that in the normal retina. Cells from regenerating retinae of 1–2 cell thickness ("early" stage) lacked the receptors for kainate (KA), α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), N-methyl-D-aspartate (NMDA), γ -aminobutyric acid (GABA), and glycine. AMPA/KA, NMDA, GABA_A, and glycine receptors, each of which were electrophysiologically and pharmacologically similar to those in the normal retina, appeared in spiking cells before regenerating retina had segregated into two synaptic layers. During subsequent regeneration stage, the time course of appearance of amino acid receptors seems to be different between excitatory and inhibitory ones. Cells which responded to GABA and glycine gradually increased in number to normal levels, and the current amplitude induced by these amino acids also increased monotonically. The number of cells responding to the excitatory amino acids appeared to be maximal at a period of synaptic segregation. During subsequent regeneration, however, cells responded to AMPA and NMDA decreased in number and returned to the number of cells in the normal retina, while cells which responded to KA were maintained at the same number as those in the normal retina. The amplitudes of the current induced by excitatory amino acids also became maximal at a period of synaptic segregation. During subsequent regeneration, AMPA- and KA-induced currents declined to the normal level, while NMDA-induced currents were maintained.

Key words: newt retina, regeneration, spiking cell, synapse formation, amino acid receptors.

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The removal of neural retina in the adult newt eye leads to the formation of a new functional retina by neuronal transdifferentiation of the retinal pigment epithelial (RPE) cells. Such a regeneration promises to be a useful system for understanding the mechanisms of cytodifferentiation and the genesis of neural circuitry in the central nervous system.

Important components of the functional differentiation of retinal neurons during development and regeneration include the synthesis of neurotransmitters and the expression of their receptors. Recently, we have described, on the basis of immunohistochemical and electrophysiological experiments, that ganglion cells of the newt are the first retinal neurons to appear and develop their voltage-gated Na^+ and Ca^{2+} channels during retinal regeneration [1–3]. In the preceding paper, we showed that isolated spiking cells from the adult newt retina have receptors activated by AMPA, KA, and NMDA as excitatory amino acids, and by GABA and glycine as inhibitory amino acids [4].

The main object of this study was to examine the time course of the appearance of excitatory and inhibitory amino acid receptors during retinal regeneration of the adult newt. So we enzymatically dissociated spiking cells from regenerating retinæ at different stages and examined their responsiveness to amino acids with whole-cell patch-clamp techniques combined with pharmacological and ionic manipulations. Here we show that spiking cells in the regenerating retina respond to the excitatory and inhibitory amino acids before segregation of distinct synaptic layers, and that the time course of appearance of the amino acid receptors seems to be different between the excitatory and inhibitory ones.

MATERIALS AND METHODS

Preparation and dissociation procedures. Adult newts (*Cynops pyrrhogaster*) were anesthetized with 0.1% FA 100 (4-allyl-2-methoxyphenol; Tanabe), and the neural retina together with lens was surgically removed. The operated animals were maintained at about 15°C and allowed to recover. They were sacrificed on selected postoperative days (25–100 d). Eyeballs were enucleated under anesthesia and hemisected to collect the regenerating retina under a stereomicroscope. The dissociation procedures were identical to those described in a previous paper [1]. The cell suspension medium was plated onto sterile 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 the culture medium of 80% Leibovitz's L-15 (Flow Laboratories). The cells were maintained at 25°C before use. Experiments were usually performed within 12 h after plating.

Recording procedures and drug application. Details of the procedures for recording and drug application are described in our preceding paper [4]. Briefly, nystatin-perforated [5] or conventional whole-cell patch-clamp methods [6] were

Table 1. Composition of patch-pipette solutions.

Composition (mM)	A	B	C	D	E
KCl	138	0	0	0	0
CsCl	0	138	10	10	138
Cs ₂ SO ₄	0	0	64	64	0
EGTA	0	0	0	5	5
Glucose	0	0	64	64	0
Nystatin (μ g/ml)	160	160	160	0	0

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.

applied for electrophysiological recordings. The patch pipette solutions used are listed in Table 1. The control bath solution contained (in mM) 110 NaCl, 3.7 KCl, 1.0 MgCl₂, 3.0 CaCl₂, 1.0 Na-pyruvate, 13.0 glucose, 5.0 HEPES, and 0.001% phenol red (pH 7.5 adjusted with 0.3 N NaOH). In some experiments, Na⁺-free and Mg²⁺-free solutions were made, and the Ca²⁺ concentration in Na⁺-free solution was modified as described previously [4]. Solutions containing the following drugs, L-glutamate, AMPA, kainate (KA), NMDA, APB, GABA, glycine, bicuculline, and strychnine, were prepared immediately before the experiments. The solutions were applied either by pressure through glass pipettes (pressure-application) or by adding directly to the perfusing solution (bath-application). All data obtained in the present study are expressed as the mean $\pm$ SD.

Definition of stages of regenerating retinae. To specify the stage of regeneration, regenerating retinae on selected postoperative days were fixed with Bouin's fixative and cryosectioned. Regenerating retinae were divided into 3 groups ("early," "intermediate," and "late"). An early regenerating retina is 1–2 cells thick, comprising pigmented cells and cells with nonpigmented cytoplasm. An intermediate retina is characterized by an increase in cell thickness with no segregation into distinct synaptic layers. A late retina characteristically has two synaptic layers.

Retinal regeneration was temperature-dependent. For operated animals kept at 22°C, the early, intermediate, and late stages roughly corresponded to 18–20, 24–26, and more than 30 d after surgery [1]. In the present study, operated animals were kept in an incubator at a temperature of about 15°C. This allowed us to separate the stages of regeneration more easily. At this temperature, early, intermediate, and late stages corresponded to 25–30, 40–50, and more than 55 d after surgery. The late stage was further divided into two groups, I and II. Late-I and -II stages corresponded to 55–65 and 90–100 d after surgery. Spiking cells at both stages were not morphologically distinguishable, but could be distinguished in their different responsiveness to drugs (see text).

To avoid overlap of morphological stages caused by inherent variabilities of regeneration, each stage of regeneration was separated by several days.

RESULTS

Morphological and electrical properties of spiking cells in regenerating retinæ

Cells dissociated from regenerating retinæ were tested for their ability to generate action potentials by injecting a depolarizing current. Sensitivity to excitatory and inhibitory amino acids were studied only in the spike-generating cells. Figure 1 shows representative voltage and current responses of the spiking cells dissociated from different stages of regenerating retinæ. At the early stage, cells with non-pigmented cytoplasm have a relatively large cell body ($18\text{--}28\text{ }\mu\text{m}$ in diameter) and lack processes. About half of them (23/44 cells) responded to a depolarizing current with slow-rising action potential of long duration (upper trace in Fig. 1a). At later stages, almost all cells which were characterized by round somata of about $15\text{--}19\text{ }\mu\text{m}$ in diameter, with or without single processes, showed action potentials. Their action potentials were shorter in duration (upper traces in Fig. 1b and c). Current records (lower traces in Fig. 1) were obtained from the corresponding cells by a depolarization to -30 mV from a holding potential of -80 mV . Voltage-gated inward currents increased in amplitude during regeneration. A transient outward current was also developed in the older cells. Mean values of the resting potential and maximum inward current of spiking cells at four different stages are listed in Table 2 in comparison with those of the normal spiking cells.

Fig. 1. Representative voltage responses (upper traces) and their corresponding membrane currents (lower traces) of spiking cells dissociated from regenerating retinæ at three different stages. Recordings were made by nystatin-perforated methods. Patch pipette was filled with solution A. Under current-clamp mode, the cell was initially hyperpolarized by constant current injection at about -80 mV (dotted line) and depolarized above threshold. Under voltage-clamp mode, the cells were depolarized from a holding potential of -80 to -30 mV . a: Thirty days after surgery (early stage). Resting potential: -35 mV . b: Forty-seven days after surgery (intermediate stage). Resting potential: -43 mV . c: Fifty-six days after surgery (late-I stage). Resting potential: -48 mV .

Table 2. Membrane properties of spiking cells in normal and regenerating retinae.

Membrane properties	Retinae				
	Normal	Early	Intermediate	Late I	Late II
Resting potential (mV)	-52 ± 11 (66)	-31 ± 7 (23)	-41 ± 9 (156)	-45 ± 9 (72)	-52 ± 12 (31)
Max. voltage-gated inward current (pA)	532 ± 261 (65)	115 ± 111 (17)	251 ± 122 (97)	462 ± 220 (68)	529 ± 265 (29)

Values are mean $\pm$ SE. The number of cells tested is shown in parentheses. Max. means maximum.

Time course of appearance of responses to excitatory amino acids

AMPA and KA responses. We examined the effects of potent glutamate agonists, AMPA and KA, on spiking cells at different stages of regeneration. Whole-cell recordings were performed by nystatin-perforated patch-clamp methods. A spiking cell was initially voltage-clamped at -30 mV and then the drugs ($100 \mu\text{M}$ each) were bath-applied to the cell sequentially. Figure 2 shows the

Fig. 2. The time course of appearance of AMPA and KA responses in spiking cells during regeneration. Appearance rate (%) of the drug-sensitive cells are plotted against different stages of regeneration in comparison with those in the normal retina (Normal). See text for the number of cells examined for each stage. Recordings were made by nystatin-perforated methods. Patch pipettes were filled with solution B. Holding potential was -30 mV. AMPA and KA ($100 \mu\text{M}$ each) were bath-applied to single cells in turn. Percentage of cells which responded to KA is shown as the total bar height, in which diagonal area shows the percentage of cells which responded to AMPA as well. E: early; I: intermediate; L.I.: late I; L.II.: late II. Data at Normal are quoted from the preceding paper [4].

percentage of spiking cells responded to the drugs at different stages of regeneration in comparison with those in the normal retina. At the early stage (E), none of the cells tested ($n=23$) responded to either AMPA or KA with detectable currents. At the intermediate stage (I), cells sensitive to the drugs appeared. Twenty-seven out of 44 cells tested (61%) responded to KA at this stage. Of these, 21 responded to AMPA as well, and the remaining 6 cells responded to KA only. At the late-I stage (L.I), all of the spiking cells tested ($n=18$) responded to both AMPA and KA. At the late-II stage (L.II), all of the spiking cells tested ($n=19$) responded to KA (100%), 13 of which responded to AMPA as well, and the remaining 6 cells responded to KA only. These appearance rates were identical to those in the normal retina (Normal).

Figure 3 shows example records of AMPA- and KA-induced currents (a and b) and mean values of these currents (c) at different stages of regeneration. Since the cell size remained almost the same at the intermediate and later stages (see above), the response amplitude to the drugs was compared without correction for the cell surface area.

In regenerating retinæ at different stages as well as the normal retina, KA-induced currents tended to be larger in amplitude than AMPA-induced currents. At the intermediate stage, the mean value of the AMPA-induced currents for 17 cells was 6 ± 4 pA and that of the KA-induced currents was 12 ± 10 pA. At the late-I stage, the mean value of the AMPA-induced currents was 36 ± 24 pA and that of the KA-induced current was 87 ± 61 pA. These values were significantly larger than those obtained from the major spiking cells in the normal retina. At the late-II stage, most cells ($n=11$) sensitive to both drugs showed a similar response pattern to those observed in spiking cells at previous stages (data not shown). The mean values of AMPA- and KA-induced currents were 16 ± 14 and 45 ± 32 pA, respectively. These values were very close to those obtained from the major spiking cells in the normal retina (15 ± 14 pA for AMPA, 43 ± 34 pA for KA, $n=48$).

A few spiking cells from regenerating retinæ had AMPA responses which were somewhat larger in amplitude than the KA responses. In the present study, however, we did not analyze these responses, because such cells were rarely encountered.

The ionic dependence of the KA-induced current was examined by ion substitution experiments and reversal potential measurements (Fig. 4). Cells examined here were obtained from regenerating retina at the intermediate stage. In Fig. 4a, bath-application of KA ($100\mu\text{M}$) induced inward current at a holding potential of -80mV . When Na^+ was removed from the perfusing solution, the KA-induced current disappeared. The reversal potential of KA response was measured by conventional whole-cell patch-clamp techniques in the presence and absence of external Na^+ (Fig. 4b). Reversal potentials were measured as described in the preceding paper (Fig. 4 in [4]). In the presence of Na^+ (■), the relationship between currents activated by KA ($100\mu\text{M}$) and membrane potentials was almost linear and the reversal potential was close to 0mV . The mean value of the reversal

Fig. 3. Whole-cell currents activated by AMPA and KA in spiking cells dissociated from regenerating retinas at different stages. Whole-cell recordings were made with nystatin-perforated methods. Patch pipettes were filled with solution B. Holding potential was -30 mV. AMPA and KA ($100 \mu\text{M}$ each) were bath-applied to single cells sequentially. a: At intermediate stage. The cell was isolated from retina 40 d after surgery. Resting potential: -38 mV. b: At late-I stage. The cell was isolated from a retina 58 d after surgery. Resting potential: -50 mV. c: Change in amplitude of AMPA ($\blacktriangle$) and KA responses ($\bullet$) in spiking cells during regeneration. Mean value ($\pm$ SD) of the drug-induced currents are plotted against different stages of regeneration in comparison with those in the normal retina. *Indicates that the mean value at each regeneration stage is significantly larger than that in the normal retina ($p < 0.05$ using one-tailed t -test).

potential was -2 ± 2 mV ($n=5$). In Na^+ -free solution ($\square$), an outward current appeared at 0 mV and the reversal potential shifted to around -80 mV. The mean value of the reversal potential was -91 ± 6 mV ($n=3$). To eliminate potassium currents which make it difficult to maintain voltage above -20 mV, cesium chloride solution was used as the patch pipette solution (solution E). Therefore, the

Fig. 4. Ionic dependency of KA-induced current in the spiking cells dissociated from regenerating retinæ at the intermediate stage. a: Na^+ dependency of KA-induced current. A cell was dissociated from retina 40 d after surgery. The recording was made by nystatin-perforated methods. The patch pipette was filled with solution B. Holding potential was -80 mV . Inward current induced by KA ($100 \mu\text{M}$) disappeared in Na^+ -free KA solution. b: I - V relation for KA response in the presence and absence of Na^+ in the bath solution. A cell was obtained from retina 48 d after surgery. Recordings were made by conventional patch-clamp methods. The patch pipette was filled with solution E. The cell was initially voltage-clamped at 0 mV , then depolarized to $+80 \text{ mV}$ in steps of 10 mV (duration, 50 ms ; interval, 500 ms) and hyperpolarized to -110 mV at the same steps. Amount of currents activated by KA ($100 \mu\text{M}$) in the presence and absence of Na^+ were plotted as a function of the voltage steps (see Fig. 4 in our preceding paper [4] for details). In the control bath solution (111 mM Na^+) (■), reversal potential was very close to 0 mV . In Na^+ -free solution (□), reversal potential was around -80 mV .

outward current observed in Na^+ -free solution appeared to be carried by mainly Cs^+ .

At the late-I stage, the reversal potential of the KA response was $-4 \pm 6 \text{ mV}$ ($n=17$) in the control bath solution, and was $-92 \pm 3 \text{ mV}$ ($n=6$) in the Na^+ -free solution. At the late-II stage, the reversal potential of KA response was $-4 \pm 3 \text{ mV}$ ($n=5$) in the control bath solution, and was $-90 \pm 6 \text{ mV}$ ($n=4$) in the Na^+ -free solution. These values were almost identical to those in the normal retina.

Modifications of the Cl^- concentration in the patch-pipette solution (10–138 mM) and of the Ca^{2+} concentration in the Na^+ -free solution (3–30 mM) appeared not to affect the KA response significantly in any spiking cells tested (data not shown), suggesting that the KA response was mediated by monovalent cation channels.

The reversal potentials of the AMPA response at the late-I and late-II stages were -4 ± 1 mV ($n=3$) and -5 ± 1 mV ($n=3$) in the control solution. These values were almost identical to those of the KA response.

NMDA response. The time course of appearance of NMDA responses in spiking cells is shown in Fig. 5 (bar graph). We examined effects of NMDA on 10 cells at the early stage of regeneration, and none of them responded to NMDA (250 μM) plus glycine (1 μM) in the absence of external Mg^{2+} . On the other hand, 15 of 40 cells (38%) responded to the drugs at the intermediate stage. At the late-I stage, all of the cells tested ($n=12$) responded to NMDA plus glycine. However, at the late-II stage, the rate of cells sensitive to the drug decreased to 9 of 20 cells (45%). This appearance rate was identical to that in the normal retina.

Figure 6 shows whole-cell currents activated by NMDA (250 μM) plus glycine (1 μM). A cell dissociated from the intermediate regenerating retina responded to NMDA in the absence of external Mg^{2+} with a noise increase at a holding potential of -80 mV (Fig. 6a). Application of Mg^{2+} (1 mM) in the bath solution suppressed this current noise. The mean amplitude of NMDA-induced currents at the intermediate stage was 13 ± 8 pA ($n=15$). Figure 6b shows a representative NMDA-induced current recorded from a cell dissociated at the late-I stage. Bath

Fig. 5. Appearance rate (%) of NMDA responses in spiking cells (bar graph) and mean values ($\pm$ SD) of the drug-induced current (line graph) during regeneration. Recordings were made by nystatin-perforated methods. Patch pipettes were filled with solution B. Holding potential was -80 mV. NMDA (250 μM) plus glycine (1 μM) was bath-applied to the cell in the absence of external Mg^{2+} . Otherwise presentation of the data is identical to that in Fig. 2.

Fig. 6. Whole-cell currents activated by NMDA in spiking cells and block of their responses by Mg^{2+} . Recordings were made by nystatin-perforated methods. Patch pipettes were filled with solution B. Holding potential was -80 mV. NMDA ($250 \mu\text{M}$) plus glycine ($1 \mu\text{M}$) was bath-applied to the cell in the absence of external Mg^{2+} . a: NMDA-induced current fluctuations recorded from a cell dissociated from retina 48 d after surgery. Application of Mg^{2+} (1 mM) suppressed the noise. Resting potential: -43 mV. b: NMDA-induced current recorded from a cell dissociated from retina 60 d after surgery. Application of Mg^{2+} (1 mM) suppressed the current. Resting potential: -47 mV. The timing of the drug application is indicated by the bars.

application of NMDA plus glycine in the absence of Mg^{2+} induced an inward current with noise at a holding potential of -80 mV. Application of Mg^{2+} in the bath solution caused complete suppression of the inward current. The mean amplitude of NMDA-induced currents was 30 ± 11 pA ($n=12$). This value was almost identical to those of the late-II regenerating and normal retinæ. The mean values of NMDA-induced currents at different regeneration stages are shown in a line graph in Fig. 5 in comparison with those in normal retina.

APB response. We examined the effects of APB, which is known to selectively activate metabotropic glutamate receptors in the vertebrate retina, on spiking cells dissociated from regenerating retinæ at different stages. However, we could not detect any responses to the drug in the cells at every stage. This result coincides with the fact that spiking cells in the normal retina lack APB receptors [4].

Time course of appearance of responses to inhibitory amino acids

GABA response. We examined the effects of GABA ($30 \mu\text{M}$) on 23 cells at the early stage of regeneration, and none of them responded to GABA. Spiking cells sensitive to the drug appeared at the intermediate stage. Twenty-seven of 45 cells (60%) responded to the drug with an outward current at a holding potential of -30 mV. At the late-I and late-II stages, all of the cells tested ($n=21$ and 18)

responded to the drug. The appearance rate of the GABA response at different stages is shown as a bar graph in Fig. 7 in comparison with that of normal retinae.

The mean value of GABA-induced currents at a holding potential of -30 mV was 25 ± 17 pA ($n=27$) at the intermediate stage. The mean value of the GABA-induced current increased to 46 ± 30 pA ($n=21$) at the late-I stage and 56 ± 28 pA ($n=18$) at the late-II stage. These values are shown in a line graph in Fig. 7 in comparison with that of normal retinae.

To investigate the charge carrier underlying the GABA response, we measured the reversal potential of GABA response in different intrapipette Cl^- concentrations by conventional whole-cell recordings (Fig. 8a, b). The voltage-dependence of the GABA-induced current was obtained from cells at the intermediate stage of regeneration. Figure 8a shows a reversal potential measurement using a patch pipette solution containing 10 mM Cl^- (solution D) in the control bath solution containing 122 mM Cl^- . At a holding potential of -45 mV, GABA induced a small outward current the amplitude of which increased with depolarization and decreased with hyperpolarization. The polarity of the current reversed at about -65 mV in this case. Figure 8b shows a reversal potential measurement using a patch pipette containing 138 mM Cl^- (solution E). The polarity of the current reversed at about 2 mV.

The mean values of the reversal potentials were -65 ± 9 mV ($n=12$) at an internal Cl^- concentration of 10 mM and 2 ± 3 mV ($n=6$) at 138 mM. These values agreed with the theoretical values of Cl^- equilibrium potential calculated from the

Fig. 7. Appearance rate (%) of GABA responses in spiking cells (bar graph) and mean values ($\pm$ SD) of the drug-induced current (line graph) during regeneration. Recordings were made with nystatin-perforated methods. Patch pipettes were filled with solution C. Holding potential was -30 mV. GABA ($30 \mu\text{M}$) was bath-applied to the cell. Otherwise presentation of the data is identical to that in Fig. 2.

Fig. 8. Reversal potential measurements (a and b) of GABA responses in the spiking cells and blocking effect of bicuculline on GABA responses (c). Cells were dissociated from regenerating retinae 40d after surgery. In a and b, recordings were made by conventional patch-clamp methods in the control bath solution (122 mM Cl^-). The cell was initially voltage-clamped at a given holding potential (left of each trace), and GABA (30 μM) was applied by pressure ejection. In a, the patch pipette was filled with solution D (10 mM Cl^-). GABA-induced current reversed its polarity at about -65 mV. In b, the patch pipette was filled with solution E (138 mM Cl^-). Reversal potential of the current was about 2 mV. c: Effect of bicuculline (10 μM) on the current activated by GABA (10 μM). Recordings were made by nystatin-perforated methods. The patch pipette was filled with solution C. Holding potential was 0 mV. The GABA response was completely suppressed by bicuculline. After washout of the drug, the GABA response recovered partially (right).

Nernst equation with Cl^- activities: -65 mV (10 mM Cl^-) and 3 mV (138 mM Cl^-). Similar reversal potential values of GABA-induced current were obtained at later stages. These results suggest that Cl^- is a main charge carrier of the GABA-induced current.

We tested the effects of bicuculline, an antagonist of GABA_A receptors, on spiking cells dissociated from the retina at the intermediate stage. An example is shown in Fig. 8c. A cell was initially voltage-clamped at 0 mV by nystatin-perforat-

ed methods. Application of $10\ \mu\text{M}$ GABA induced an outward current. This current was completely suppressed by the application of $10\ \mu\text{M}$ bicuculline. The effect of bicuculline was reversible. Similar blocking effect of bicuculline was observed in all cells examined at later stages (data not shown). These results imply that GABA receptors expressed in spiking cells can be categorized into GABA_A receptors identical to those in normal spiking cells [4].

Glycine response. We examined the effects of glycine ($30\ \mu\text{M}$) on 23 cells at the early stage of regeneration. None of them responded to glycine. Spiking cells sensitive to the drug appeared at the intermediate stage. Twenty-nine of 45 cells (about 64%) responded to the drug with an outward current at a holding potential of $-30\ \text{mV}$. At the late-I stage, 20 out of 21 spiking cells (about 95%) responded to the drug and at the late-II stage, all of the spiking cells examined ($n=18$) responded to it as spiking cells in normal retina did. The appearance rate of the glycine response at different regeneration stages is shown in a bar graph in Fig. 9.

The mean value of glycine-induced currents at a holding potential of $-30\ \text{mV}$ was $20 \pm 8\ \text{pA}$ ($n=29$) at the intermediate stage. Glycine-induced currents increased to $51 \pm 38\ \text{pA}$ ($n=20$) at the late-I stage and $60 \pm 34\ \text{pA}$ ($n=18$) at the late-II stage and reached values near normal levels. These values are shown as a line graph in Fig. 9.

To investigate the charge carrier underlying the glycine response, we measured the reversal potential of the glycine response using different intrapipette Cl^- concentrations with conventional whole-cell recordings (Fig. 10a, b). Figure 10a

Fig. 9. Appearance rate (%) of glycine responses in spiking cells (bar graph) and mean values ($\pm\text{SD}$) of the drug-induced current (line graph) during regeneration. Recordings were made with nystatin-perforated methods. Patch pipettes were filled with solution C. Holding potential was $-30\ \text{mV}$. Glycine ($30\ \mu\text{M}$) was bath-applied to the cell. Otherwise presentation of the data is identical to that in Fig. 2.

Fig. 10. Reversal potential measurements (a and b) of glycine responses in the spiking cells and blocking effect of strychnine on glycine responses (c). Cells were dissociated from regenerating retinæ 40 d after surgery. Recordings were made by conventional patch-clamp methods in a and b, and by nystatin-perforated methods in c. Recordings were made with in the control bath solution (122 mM Cl^-). In a and b, the cell was initially voltage-clamped at a given holding potential (left of each trace), and glycine (30 μM) was applied by pressure ejection. In a, the patch pipette was filled with solution D (10 mM Cl^-). The glycine-induced current reversed its polarity at about -65 mV. In b, the patch pipette was filled with solution E (138 mM Cl^-). Reversal potential of the current was about 2 mV. c: Effect of strychnine (1 μM) on the current activated by glycine (30 μM). The patch pipette was filled with solution C. Holding potential was 0 mV. The glycine response was completely suppressed by strychnine. After washout of the drug, the glycine response recovered (right).

shows the voltage-dependence of glycine-induced currents which were obtained from a cell dissociated from the retina at the intermediate stage. The control bath solution contained 122 mM Cl^- and the pipette solution contained 10 mM Cl^- (solution D). At a holding potential of -45 mV, glycine induced a small outward current, the amount of which increased with depolarization and decreased with hyperpolarization. The reversal potential of the current was about -65 mV in this

case. Figure 10b shows a reversal potential measurement using a pipette solution containing 138 mM Cl^- (solution E). The polarity of the current reversed at about 2 mV.

The mean values of the reversal potential were -62 ± 1 mV ($n=5$) at 10 mM Cl^- and 2 ± 3 mV ($n=7$) at 138 mM Cl^- . These values agreed with the theoretical values of Cl^- equilibrium potential. Similar reversal potential values of the glycine-induced current were obtained at the later stages. These results suggest that glycine-induced currents observed in the regenerating retinae are mediated by an increase in Cl^- conductance.

We tested the effects of strychnine, an antagonist of glycine receptors, on spiking cells dissociated from the intermediate retina. The cell was initially voltage-clamped at 0 mV with nystatin-perforated methods. The application of 30 μM glycine induced an outward current. This current was completely suppressed in the presence of 1 μM strychnine (Fig. 10c). The effect of strychnine was reversible. A similar blocking effect of strychnine was observed in all cells examined at later stages (data not shown). These results imply that the glycine receptors expressed in spiking cells during regeneration are identical to those in normal spiking cells [4].

DISCUSSION

Immature spiking cells. Cells with non-pigmented cytoplasm dissociated from the early regenerating retinae (1–2 cells thick) responded to a depolarizing current with a slow-rising action potential of long duration mediated by an increase in Na^+ conductance. They were probably immature spiking cells [1]. In our previous paper, we reported that cells with such immature activities developed spikes in culture which are identical to those in normal ganglion cells [2]. It has been reported that ganglion cells are the first retinal neurons to differentiate during development and regeneration [3, 7] (for review, see [8]). None of the immature spiking cells responded to either AMPA, KA, NMDA, and APB, or GABA and glycine. This could be due to the lack of receptors for the amino acids at the early stage.

Onset of chemosensitivity. Responses to the excitatory and inhibitory amino acids appeared in spiking cells before the retinal cells differentiated to form the distinct synaptic layers. According to the present macroscopic analysis, both of the excitatory and inhibitory amino acid receptors seem to appear at the intermediate stage. Our previous immunohistochemical studies on the newt regenerating retina suggest that ganglion cells differentiate earlier than bipolar and amacrine cells [3, 7]. This, in turn, suggests that neurotransmitter receptors may develop earlier than the neurotransmitters themselves. The appearance of neurotransmitter receptors before synaptic formation was first described in neuromuscular systems (for reviews, see [9, 10]) and reported also in many studies of developing nervous systems [11, 12] including the retina [13, 14]. The physiological meaning of receptor expression before synaptogenesis remains to be explored.

It has been reported that neurotransmitter receptors of certain embryonic neurons have different ionic permeabilities from those of mature cells. For example, in the developing rat retina, ganglion cells express Ca^{2+} permeable AMPA/KA receptors [15]. In the rat hippocampus during development, NMDA-evoked inward currents are less voltage-dependent in immature neurons than in adult neurons [16]. In the present study, however, many of the characteristics of excitatory and inhibitory amino acid responses, i.e. the reversal potential, ionic dependency, and pharmacological property, did not change substantially between the regenerating and normal retinæ.

Development of chemosensitivity. In the Rohon-Beard neurons of the *Xenopus* spinal cord [11] and hippocampal neurons of rat brain [17], the sensitivity to glycine increases at early stages of development and then disappears later, although GABA receptors persist. While, in regenerating newt retina, both GABA- and glycine-sensitive cells gradually increased in number to normal levels, and the current amplitude induced by these amino acids also increased monotonically. A gradual increase in the receptor density has been reported for GABA_A receptors in the developing chick brain [18] and glycine receptors in the chick embryo spinal cord [19]. A monotonous increase in GABA synthesis has also been reported during newt retinal regeneration [20]. It remains to be determined whether the increase in GABA- and glycine-induced currents of spiking cells during regeneration is due to an increase in the number of receptors or in conductances of single channels, or both.

In contrast, the time course of appearance of responsiveness to the excitatory amino acids are complicated. The number of cells which responded to AMPA, KA, and NMDA appeared to be maximal at the late-I stage. At the late-II stage, however, the number of cells which responded to AMPA and NMDA decreased to near those in the normal retina, while cells responded to KA were maintained at the same number as those in the normal retina. The amplitudes of currents induced by these amino acids also became maximal at the late-I stage. At the late-II stage, the amplitude of AMPA- and KA-induced currents declined to the normal level, while that of NMDA-induced currents was maintained. Somewhat different developmental changes in the excitatory amino acid receptors have been reported in other preparations. For example, receptor binding assay of radioactive amino acids during development of chick embryo retina demonstrated that the density of AMPA-sensitive receptors transiently increased at the early stages of development and declined at subsequent stages, although those of KA- and NMDA-sensitive receptors persisted [21].

So far we have no systematic explanation for the complicated response pattern of spiking cells to individual excitatory amino acids. To explain it, we must at least take into account an unavoidable experimental limitation, that is, the dissociation procedures sheared off processes normally present in intact retinal cells. Excitatory amino acid receptors might be expressed to a large extent over somata at an earlier stage of synaptogenesis, and later become localized at post-synaptic sites on

dendrites which are lost in the dissociation procedures. Neurotransmitter receptors which concentrate at sites of synaptogenesis and decrease elsewhere during development have been described in neuromuscular synapses (for review, see [9]), developing cerebellar Purkinje cells [12, 22], and cultured retinal cells [13].

The functional significance of a transient increase in the number of amino acid receptors is not been fully understood. However, recent studies suggest that some amino acids may promote neurite outgrowth and synapse formation via activation of their receptors [23–27] (for reviews see [28, 29]). The transient increase in NMDA receptors during development is of particular interest, because antagonists block activity-dependent plasticity [30] or establishment of topographical maps in the brain [31, 32]. The possibility that the transient increase in the amino acid receptors at the late-I stage play a role in synaptogenesis during subsequent retinal regeneration remains to be elucidated.

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