INTRODUCTION

In mammals, rod and cone photoreceptor cells are generally found to contact two types of bipolar cells, referred to as rod bipolar cells and cone bipolar cells, respectively (Boycott and Dowling 1969; Dowling and Boycott 1966). Whereas rod bipolar cells constitute a homogeneous population, cone bipolar cells typically encompass ~10 different populations of cells (cat: Kolb et al. 1981; monkey: Boycott and Wässle 1991; rabbit: Famiglietti 1981; rat: Euler and Wässle 1995). All photoreceptors display a hyperpolarizing response to light (Tomita 1965), reducing the release of glutamate (Copenhagen and Jahr 1989). Retinal ganglion cells, however, give either a hyperpolarizing light response (OFF; sign conserving) or a depolarizing light response (ON; sign inverting) (Kuffler 1953). This sign-inverting response transformation is thought to take place at the glutamatergic connection between photoreceptors and bipolar cells in the outer plexiform layer (Werblin and Dowling 1969). All rod bipolar cells seem to be of the ON type (Dacheux and Raviola 1986). There is evidence that rod bipolar cells express a specific type of 2-amino-4-phosphonobutyrate (APB)-sensitive metabotropic (guanosine triphosphate binding protein-linked) glutamate receptor (mGluR6) (Hartveit et al. 1995; Nomura et al. 1994) and that agonist binding hyperpolarizes the membrane potential via closure of a guanosine 3′,5′-cyclic monophosphate (cGMP)-gated nonspecific cation channel, thereby generating the sign-inverting synaptic transmission (de la Villa et al. 1995; Nawy and Jahr 1990a; Shiells and Falk 1990). In rat, rod bipolar cells do not express functional (i.e., conductance-increasing) ionotropic glutamate receptors (Hartveit 1996b). Accordingly, rat rod bipolar cells should depolarize in response to an incremental light stimulus. Cone bipolar cells can be classified as either ON or OFF according to their light responses. It is generally thought that ON cone bipolar cells express an APB-sensitive metabotropic glutamate receptor (Euler et al. 1996; Müller et al. 1988), possibly mGluR6 (Masu et al. 1995), and that the intracellular signal transduction mechanism is similar to that in rod bipolar cells (de la Villa et al. 1995). OFF cone bipolar cells, however, are thought to express ionotropic glutamate receptors with integral nonspecific cation channels (de la Villa et al. 1995; Euler et al. 1996). Thus, OFF cone bipolar cells would be depolarized by glutamate, and the degree of depolarization will be maximal in darkness when release of glutamate from photoreceptors is at its maximum.

Because bipolar cells do not receive glutamatergic input at synapses other than those made by photoreceptors in the outer plexiform layer, it should be possible to distinguish OFF from ON cone bipolar cells on the basis of their response to exogenously applied ionotropic glutamate receptor agonists: OFF cone bipolar cells should be excited, whereas ON cone bipolar cells should be unresponsive. The question of the response polarity (ON vs. OFF) of specific cone bipolar cells is of considerable importance for our understanding of signal transmission in the retina. Whereas the dendrites of ON and OFF center ganglion cells stratify in different sublaminae of the inner plexiform layer (OFF cells in the distal 2/5, ON cells in the proximal 3/5) (Nelson et al. 1978), corresponding data for bipolar cells in the mammalian retina have until recently not been available (Euler et al. 1996). One possibility is that cone bipolar cells give excitatory input to ganglion cells of the same response polarity, meaning that OFF cone bipolar cells will have their
axon terminals in sublamina a and ON cone bipolar cells will have their terminals in sublamina b (Famiglietti and Kolb 1976). This has been demonstrated for the retina of carp and tiger salamander (Famiglietti et al. 1977; Hare et al. 1986). Another possibility is that axon terminals of ON and OFF cone bipolar cells are distributed throughout sublaminae a and b (Nelson and Kolb 1983) and that ON cone bipolar cells terminating in sublamina a (the “OFF region”) and OFF cone bipolar cells terminating in sublamina b (the “ON region”) mediate input to amacrine cells (Saito 1987). Alternatively, it has been suggested that some cone bipolar cells might be inhibitory and use γ-aminobutyric acid (GABA) or glycine as neurotransmitter (e.g., Cohen and Sterling 1986). This could mean that OFF center ganglion cells stratifying in sublamina a receive excitatory input from OFF cone bipolar cells and inhibitory input from ON cone bipolar cells. Correspondingly, ON center ganglion cells stratifying in sublamina b would receive input of the opposite polarity from ON and OFF cone bipolar cells (Sterling 1983). A third scheme, recently proposed by Euler et al. (1996), suggests that in the rat retina, the axon terminals of ON and OFF cone bipolar cells are indeed segregated to proximal and distal regions of the inner plexiform layer, but that the border between ON and OFF cone bipolar cells does not correspond to the expected border between ON and OFF ganglion cell dendrites.

In this investigation I recorded from morphologically identified cone bipolar cells in the rat retinal slice preparation and examined their response to ionotropic glutamate receptor agonists of both the N-methyl-D-aspartate (NMDA) and non-NMDA types. Application of NMDA did not evoke a conductance change in any bipolar cells. According to the pattern of responses to non-NMDA receptor agonists, it is predicted that the axon terminals of ON and OFF cone bipolar cells are segregated in the inner plexiform layer, with those of OFF cone bipolar cells stratifying in the distal 2/5 of the inner plexiform layer and those of ON bipolar cells stratifying in the proximal 3/5 of the inner plexiform layer. Additionally, evidence was obtained for input through gap junctions to ON cone bipolar cells. A brief account of these findings has been published in abstract form (Hartveit 1996a).

**METHODS**

The methods have previously been described in detail (Hartveit 1996b). Albino rats (25–50 days postnatal, mean age 39 days) were deeply anesthetized with halothane in oxygen and killed by cervical dislocation. After the retina was dissected free, vertical slices were cut by hand.

**Electrophysiology and infrared video microscopy**

The slices were viewed with differential interference contrast (Nomarski) and epifluorescence optics. During the experiment, imaging was performed with infrared differential interference contrast video microscopy (for details see Hartveit 1996b). The extracellular perfusing solution was continuously bubbled with 95% O₂-5% CO₂ and had the following composition (in mM): 125 NaCl, 25 NaHCO₃, 2.5 KCl, 2.5 CaCl₂, 1 MgCl₂, and 10 glucose, pH 7.4 (18–22°C).

The electrode resistance was 4–8 MΩ and all recordings were made in the whole cell configuration of the patch-clamp technique (Hamill et al. 1981). For experiments with low intracellular chloride concentration, the electrodes were filled with a solution of the following composition (in mM): 125 cesium hydroxide, 125 gluconic acid, 4 NaCl, 1 CaCl₂, 1 MgCl₂, 15 tetraethylammonium chloride, 5 N-[2-hydroxylethy]piperazine-N’-[2-ethanesulfonic acid] (HEPES), 5 ethylendiamine-N,N,N’,N’-tetraacetic acid (EGTA), and 4 disodium ATP (Na₂ATP), pH adjusted to 7.3 with CsOH. For experiments with high intracellular chloride concentration, the solution had the following composition (in mM): 125 CsCl, 4 NaCl, 1 CaCl₂, 1 MgCl₂, 15 tetraethylammonium chloride, 5 HEPES, 5 EGTA, and 4 Na₂ATP, pH adjusted to 7.3 with CsOH. Lucifer yellow was added at a concentration of 1 mg/ml to all intracellular solutions. Liquid junction potentials were measured as described by Neher (1992) and the software (PULSE; HEKA elektronik, Lambrecht/ Pfalz, Germany) controlling the amplifier (EPC-9; HEKA elektronik) automatically corrected the holding potentials for the liquid junction potentials. Seal resistances were ≥50 MΩ for electrodes with heat polishing and 2–5 GΩ for electrodes without heat polishing. The average cell capacitance of the cone bipolar cells recorded was 3.80 ± 0.15 (SE) pF (n = 120). The software estimated and, in a few cases, compensated (up to 90%) the series resistance (30 ± 1.5 MΩ, mean ± SE). In the voltage range relevant for measurement of reversal potentials (E₀), this series resistance would produce an error of ≤1.5 mV, because the currents typically were ≤50 pA in this range. Depending on the experimental protocol, the sampling interval was varied between 250 μs and 2.5 ms.

Drugs were applied by pressure from a five-barrelled pipette complex. The concentrations of the drugs were as follows (obtained from Tocris Cookson, Bristol, UK, unless stated otherwise): 200 μM L- (+)-APB, 500 μM 3-amino-5-propyl(methyl)phosphinate (3-APMPA), 200 μM (S)-α-amino-3-hydroxy-5-methyl-4-isoxazolopropionate (AMPA), 25 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CQXN), 200 μM GABA, 200 μM glycine (May and Baker, Dagenham, UK), 250 μM kainate (Sigma, St. Louis, MO), 1 mM NMDA (Sigma), 100 or 500 μM picrotoxin (Research Biochemicals, Natick, MA, USA), 10 μM strychnine (Research Biochemicals). Solutions were either made up freshly for each experiment or were prepared from aliquots stored at −20 to −25°C.

For most cells, infrared differential interference contrast video images were digitized (ImageGrabber/24, Nectech, Hampshire, UK) and stored on computer.

**Data analysis**

For measurement of E₀, data points of current-voltage (I-V) relationships were fitted by third- to seventh-order polynomial functions by a singular value decomposition algorithm. Equilibrium potentials were calculated according to the Nernst equation. For measurement of response latency, response onset was determined by eye. Peak current was measured on fourth- to ninth-order polynomial functions fitted to relevant segments of the raw current traces. Unless otherwise stated, statistical analyses were performed with the use of Student’s t-test with a level of significance of P < 0.05. Data are presented as means ± SE (n = number of cells). Percentages are presented as percentage of control.

**Intracellular staining of cells in fixed slices**

Cells were filled by intracellular injection of Lucifer yellow in distilled water (50 mg/ml) (Hartveit 1996b). The cells were photographed at a series of focal planes on high-speed film (Kodak Tmax 400, final magnification ×100). For each cell, the negatives were scanned at 2,700 dots per inch [SprintScan 35, Polaroid (Norge) A/S, Skærø, Norway] and used to construct a montage with Adobe Photoshop (Adobe Systems, Mountain View, CA).
RESULTS

The results are based on electrophysiological recording from 120 cone bipolar cells, all of which were identified morphologically by labeling with Lucifer yellow and classified according to the criteria established by Euler and Wässle (1995). In addition, a number of axotomized cells was examined electrophysiologically, but could not be properly classified. Unless otherwise indicated, the results refer to recordings performed with low intracellular chloride concentration (see METHODS). All recordings were performed in a nominally Ca$^{2+}$-free extracellular medium (‘‘Ca$^{2+}$-free’’), but with Co$^{2+}$ added at a concentration of either 2.5 mM ($n = 5$), 3 mM ($n = 9$), or 5 mM ($n = 106$).

Morphological identification of cone bipolar cells

To establish a data base for secure identification of cells recorded in electrophysiological experiments, I injected Lucifer yellow intracellularly in 108 bipolar cells in slightly fixed retinal slices (Hartveit 1996b). Of these cells, 25 were classified as rod bipolar cells and 83 were classified as cone bipolar cells. The electrode was aimed at cell bodies around the middle of the inner nuclear layer, toward the typical location of cone bipolar cells (Euler and Wässle 1995) and away from the location of rod bipolar cells (Greferath et al. 1990). In this way, cone bipolar cells with axon terminals stratifying at various depths of the inner plexiform layer were filled. Cone bipolar cells have fine terminal axonal branches, with a relatively wide lateral extension. They have small vesicular expansions along the terminal branches instead of the large knob-shaped swellings characteristic of rod bipolar cells. In their study, Euler and Wässle (1995) described nine different types of cone bipolar cells altogether. All these types were observed in the present study (Fig. 1) and will be referred to as CB1–CB9.

Direct and indirect responses evoked by kainate

Kainate activates both high-affinity AMPA/low-affinity kainate receptors (‘‘AMPA receptors’’) and low-affinity AMPA/high-affinity kainate receptors (‘‘high-affinity kainate receptors’’) (reviewed by Hollmann and Heinemann 1994). Little desensitization is observed when kainate, as opposed to AMPA, is applied to a cell expressing AMPA receptors. Thus it is convenient to use kainate as a probe of both AMPA receptors and high-affinity kainate receptors. External Ca$^{2+}$-dependent transmitter release was blocked by recording in Ca$^{2+}$-free extracellular medium with addition of Co$^{2+}$, known to block voltage-gated Ca$^{2+}$ channels (Nowycky 1991). It has been observed, however, that application of non-NMDA receptor agonists under these conditions can evoke the release of GABA in the inner plexiform layer, possibly from amacrine cells (Hartveit 1996b). This indirectly mediated kainate response was characterized by slow kinetics (slow onset, long rise time, slow decay) and it was blocked by a combination of GABA$_A$/GABA$_C$ receptor antagonists. In contrast, directly mediated responses were characterized by relatively rapid kinetics (fast onset, short rise time, fast decay) and they could only be blocked by non-NMDA receptor antagonists, not by GABA$_A$/GABA$_C$ receptor antagonists. The same distinction between directly and indirectly mediated responses was observed for cone bipolar cells in the present study. For some cells, kainate evoked only an indirectly mediated response, indicating that the cells do not express functional ionotropic glutamate receptors. For other cells, kainate was able to evoke both a directly mediated and an indirectly mediated response. This indicates that these cells express functional ionotropic glutamate receptors but that the mechanism underlying the indirectly mediated response is also operative.

.cells with direct responses. An example of a cone bipolar cell (CB4) with a directly mediated response to kainate is shown in Fig. 2. Kainate was applied for 3 s and evoked
a short-latency (latency to start 0.07 s) inward current (peak amplitude −64 pA) when the cell was voltage clamped at −70 mV (Fig. 2A). When kainate was applied together with the non-NMDA receptor antagonist CNQX (Honore et al. 1988), the response was strongly suppressed (Fig. 2B; n = 21 of 21). To investigate the I-V relationship of the current, the membrane potential was ramped from −100 mV to +40 mV (100 mV/s), first in the absence of kainate, then during the application of kainate, and finally after kainate had been washed away (Fig. 3A; CB3). For each condition, an I-V relationship was constructed by plotting the evoked current against the corresponding potential (Fig. 3B). The net current evoked by kainate was constructed by subtracting the current evoked in the absence of kainate from the total current evoked during drug application (Fig. 3C). The current reversed direction at a membrane potential of approximately −5.5 mV. This is very close to the expected value of ~0 mV for a nonselective cation current through ionotropic glutamate receptor channels (Ascher and Nowak 1988) and far from the equilibrium potential for chloride (ECl = −45.5 mV; see below).

For several cells with direct kainate responses, longer-lasting application of kainate, used during sampling of I-V curves, caused a drift of the Erev in a negative direction (Fig. 4). Each of the I-V curves in Fig. 4A is constructed from a single sweep. For the first sweep the Erev was −5.5 mV. With continued application of kainate, the Erev became more negative (−18.3 mV), and in the last sweep compiled, Erev was −26.5 mV (Fig. 4A). This is closer to ECl (−45.5 mV) than to 0 mV. On the basis of results obtained for rod bipolar cells (Hartveit 1996b), a likely interpretation is that a second response with slower onset, mediated indirectly by release of GABA, contributed to the total response. For comparison, four cells were tested with voltage ramps during application of GABA. The Erev was −47.5 ± 1.0 mV, very close to ECl (Fig. 4B; CB2). When kainate was coapplied with picrotoxin (100 μM) and 3-APMPA (500 μM), the negative drift of the Erev was blocked (Fig. 4C; CB2). Picrotoxin is a noncompetitive GABA_A receptor antagonist and 3-APMPA is a competitive GABA_C receptor antagonist (Woodward et al. 1993). GABA_C receptors on rat bipolar cells are largely resistant to picrotoxin (Feigenspan and Bornmann 1994). This suggested that kainate was evoking the release of GABA. Coapplication of kainate with 100 μM picrotoxin alone was not sufficient to block the drift in Erev. When kainate was coapplied with CNQX, both the direct and the indirect response components were blocked (not shown; n = 8 of 8). However, when kainate was applied in combination with a series of consecutive voltage ramps, the first response in the series was often relatively uncontaminated, with Erev close to 0 mV (Fig. 4A). This made it possible to isolate the indirect from the direct response. Thus, when the first ramp response was subtracted from a ramp response obtained later in the same series, the difference corresponded roughly to the contribution from the indirect GABAergic response (Fig. 4A). Separate tests with and without GABA antagonists (Fig. 4C) were less successful in revealing this relationship, probably because the conductance in the control condition varied too much over time. For some of the cells in which kainate evoked a current with Erev drifting from 0 mV toward ECl (Erev typically approximately −20 mV), the conductance remained at an increased level in the first control responses obtained after offset of kainate application, but Erev moved closer to ECl, suggesting that the indirect response decayed more slowly than the direct response.

**Cells without direct responses.** A representative example of a cone bipolar cell (CB8) with only an indirect response to application of kainate is shown in Fig. 5, A and B. No response was observed during a 3-s application at a holding potential of −70 mV (Fig. 5A). When the duration of drug application was prolonged, during compilation of I-V curves, a clear response was observed (Fig. 5B). The Erev was −46.3 mV, close to ECl (−45.5 mV), suggesting that the response to kainate was indirectly mediated by release of GABA. Confirming this, coapplication of kainate with picrotoxin and 3-APMPA blocked the response (Fig. 5B). The response was also blocked by CNQX (n = 3 of 3; not shown), indicating that it ultimately depended on non-NMDA receptors. Application of 100 μM picrotoxin alone was insufficient to block the kainate-evoked response (n = 4 of 4), suggesting that blockade of both GABA_A and GABA_C receptors is needed to block the response. When ECl was changed to +1.5 mV by increasing the intracellular chloride concentration, the Erev of the indirect kainate response followed the change in ECl, being −7.0 mV for the cell illustrated in Fig. 5C (CB8). Similar results were obtained for two other cells. During long-lasting application of kainate at a holding potential of −70 mV, the slow kinetics of the response could be directly observed (Fig. 5D).

**Responses presumably mediated by APB receptors.** Kainate acts as an agonist at the APB receptor(s) expressed by bipolar cells in the mudpuppy retina (Slaughter and Miller 1983), and similar observations were made for rod bipolar cells in the rat retina (Hartveit 1996b). Narrow
and Jahr (1990b) reported that the agonist-evoked response of APB receptors on bipolar cells runs down very quickly, most likely because it is mediated by a metabotropic glutamate receptor. Accordingly, to avoid interference from responses mediated by APB receptors, neither guanosine triphosphate nor cGMP was added to the pipette solution. To check for APB responses, a total of nine cone bipolar cells (3 CB3, 2 CB5, 2 CB6, 1 CB7, 1 CB8) were tested with application (3 s) of APB at −70 mV. None of the cells responded. However, of a total of 119 cone bipolar cells tested with application of kainate at a holding potential of −70 mV, 5 cells (1 CB5, 2 CB7, 2 CB8) responded with an outward current (9.3 ± 3.0 pA). An example of the effect of kainate on one of these cells (CB5) is shown in Fig. 6. Two of the cells were also tested with voltage ramps. One cell (CB8) responded with a conductance increase and an $E_{\text{rev}}$ at −45.1 mV, most likely due to an indirect effect mediated by chloride ions (see above). The other cell (CB5) responded with a conductance decrease and an $E_{\text{rev}}$ of −2.3 mV, but the response did not recover to the predrug baseline. A conductance decrease was not observed for any other cells. Accordingly, it seems clear that an interaction of kainate with APB receptors is not a problem in these experiments.

**Responses to kainate among the various types of cone bipolar cells**

This section presents the results for direct and indirect responses to kainate as observed for the different types of cone bipolar cells. On the basis of these results, I propose that CB1–CB4 are off cone bipolar cells because they display short-latency, direct kainate responses. CB5–CB9 are proposed to be on cone bipolar cells because they do not display direct kainate responses. The short-latency kainate responses observed in some of these cells (CB5–CB8) are most likely conveyed through gap junctions from AII amacrine cells. Indirect kainate responses, mediated by GABA, are observed in both on and off cone bipolar cells.
FIG. 4. *I-V* relationships for direct and indirect \([\gamma\text{-aminobutyric acid (GABA)}\text{ mediated}]\) kainate responses in presumed OFF cone bipolar cells (voltage ramp \(-100\, mV\) to \(+40\, mV\); 100 mV/s). A: application of kainate alone (200 \(\mu M\)). Traces marked 1, 2, and 3 were recorded in succession at intervals of 3.5 s. Note slow increase of current and drift of \(E_{\text{rev}}\) from \(-5.5\, mV\) in trace 1 to \(-26.5\, mV\) in trace 3. Stippled trace marked (3-1) is the difference between trace 3 and trace 1 (heavily smoothed for clarity) and is an approximation of the *I-V* relationship for the indirect, long-latency response component in isolation (CB3, same cell as in Fig. 3). B: *I-V* relationship of GABA response (CB2; voltage ramps \(-100\, mV\) to \(+40\, mV\); 100 mV/s). C: *I-V* relationships for sum of direct and indirect response (Kainate) and pharmacologically isolated direct response [Kainate + picrotoxin + 3-aminopropyl(methyl)phosphinic acid (3-APMPA)]. CB2: chloride equilibrium potential \((E_{\text{Cl}})\) is marked by vertical arrows in A–C. Low intracellular chloride concentration.

FIG. 5. Kainate-evoked whole cell current responses in presumed ON cone bipolar cells. A: at a holding potential of \(-70\, mV\), 3-s application of kainate (200 \(\mu M\); horizontal bar) did not evoke a short-latency response (CB8). Low intracellular chloride concentration. B: *I-V* relationship of the kainate-evoked response during voltage ramps \((-100\, mV\) to \(+40\, mV\); 100 mV/s) with longer-lasting kainate application. Note that \(E_{\text{rev}}\) is close to \(E_{\text{Cl}}\) (1). The conductance increase was blocked when kainate was coapplied with picrotoxin and 3-APMPA. Same cell as in A. C: *I-V* relationship for the kainate response during recording with high intracellular chloride concentration (CB8). Notice how \(E_{\text{rev}}\) followed the new \(E_{\text{Cl}}\) (+1.5 mV; D). D: at a holding potential of \(-70\, mV\), 15-s application of kainate (horizontal bar) evoked a long-latency, slowly rising and slowly decaying inward current (same cell as in C).
CB1 (n = 2). For one cell, application of AMPA at −70 mV evoked a short-latency inward current (peak amplitude −25 pA). The latency to onset of the response was 0.43 s. The cell was not examined with voltage ramps during drug application. For the other cell, application of kainate at −70 mV evoked a short-latency (0.31 s) inward current (peak amplitude −116 pA). When tested with longer-lasting kainate application (in the presence of picrotoxin and 3-APMPA), the $E_{\text{rev}}$ was −8.9 mV.

CB2 (n = 11). All cells gave a short-latency response to kainate at a holding potential of −70 mV (−56 ± 7.1 pA). The mean latency to onset was 0.24 ± 0.05 s (range 0.10–0.57 s). The response to kainate was strongly reduced by CNQX (n = 4 of 4). The $E_{\text{rev}}$ was −10.7 ± 2.1 mV (n = 7), relatively close to the expected $E_{\text{rev}}$ (−0 mV), but three of the cells showed a drift of the $E_{\text{rev}}$ in the negative direction ($E_{\text{rev}}$ approximately −20 mV). When one of these cells was tested with coapplication of kainate and picrotoxin and 3-APMPA, the $E_{\text{rev}}$ changed to −2 mV.

CB3 (n = 19). All cells displayed a short-latency response to kainate at −70 mV (−39 ± 5.8 pA). The mean latency to onset was 0.23 ± 0.03 s (range 0.10–0.63 s). The response was blocked by CNQX (n = 3 of 3). $E_{\text{rev}}$ was −8.5 ± 1.8 mV (n = 11). A drift of $E_{\text{rev}}$ in a negative direction was seen for several of the cells, but was blocked by picrotoxin and 3-APMPA (n = 3 of 3).

CB4 (n = 8). Kainate evoked a short-latency response at −70 mV (7 of 7; −77 ± 15 pA). The latency to start was 0.19 ± 0.04 s. In the last cell, AMPA evoked a short-latency response with a peak amplitude of −8 pA (latency to start 0.42 s). CNQX strongly suppressed the response to kainate (n = 3 of 3). $E_{\text{rev}}$ of the kainate-evoked response was examined in two cells (in the presence of picrotoxin for 1 cell) and was −8.2 and +8.9 mV.

CB5 (n = 9). Both CB5 and CB6 are narrowly stratifying cells with similar overall morphology, and there is only a minor difference between them concerning the level of stratification in the inner plexiform layer (Fig. 1). Accordingly, it might be argued that classification of a cell as either CB5 or CB6 is unreliable. However, it can be consistently observed that CB5 and CB6 stratify immediately distal and proximal, respectively, to a narrow band of higher optical density in the inner plexiform layer (Euler and Wässle 1995). This band is located slightly proximal to the middle of the inner plexiform layer, approximately at the border between stratum (S) 3 and S4 (Fig. 7). Therefore, by comparing the stratification level in relation to the optically dense band, it is usually not difficult to distinguish between CB5 and CB6 cone bipolar cells.

Of the nine cells classified as CB5, four cells displayed a short-latency inward current in response to a 3-s application of kainate at −70 mV (−16 ± 3.4 pA; latency to onset 0.53 ± 0.06 s). When one of the cells was tested with voltage ramps, the $E_{\text{rev}}$ was +21.7 mV. The significance of this result did not become clear until a larger number of cells classified as CB6, CB7, and CB8 was examined (see below). Two of the cells also responded to AMPA with short-latency inward currents (2 of 2; −15 and −11 pA; latency to start 0.18 and 0.19 s). One cell responded to kainate with an APB-receptor-like response (Fig. 6; see above). The last four cells did not respond to kainate at all. The morphology of the cells was normal, including the presence of dendrites in the outer plexiform layer. Two of these cells were tested with longer-lasting application of kainate. $E_{\text{rev}}$ was −45.4 and −34.6 mV, suggesting the presence of an indirect response ($E_{\text{Cl}} = −45.5$ mV).

CB6 (n = 16). Eight cells displayed a short-latency response to kainate at −70 mV (in the presence of picrotoxin and 3-APMPA; Fig. 8A; −23 ± 10 pA). The mean latency to start was 0.27 ± 0.09 s. Coapplication of kainate with CNQX reduced the response (Fig. 8B; n = 3 of 3). Two of the cells were further tested with kainate during voltage ramps. For both cells, $E_{\text{rev}}$ started out at more positive potentials and drifted in a negative direction from one sweep to the next during the application of kainate (Fig. 8C). The final (steady-state) $E_{\text{rev}}$ was −5.7 and −18.8 mV. This suggested a mixture of short-latency direct and long-latency indirect responses with different $E_{\text{rev}}$. Five of the eight cells with short-latency responses were tested with voltage ramps and application of kainate in the presence of picrotoxin and 3-APMPA. This considerably reduced the drift of $E_{\text{rev}}$. Surprisingly, for three cells the current could not be reversed at all (up to +40 mV) and for the other two cells the current reversed at positive potentials (+7.4 and +33.0 mV, respectively; Fig. 8D).

Eight other cells did not display a short-latency response to kainate. Five of these cells were tested with longer-lasting application of kainate in combination with voltage ramps. Four cells displayed a conductance increase. The $E_{\text{rev}}$ was −46.3 ± 3.4 mV ($E_{\text{Cl}} = −45.5$ mV; n = 3) and +9.7 mV ($E_{\text{Cl}} = +1.5$ mV; high intracellular chloride concentration; n = 1).

CB7 (n = 27). The results for CB7 were quite similar to those for CB5 and CB6 cone bipolar cells described above. Of 27 cells tested, 13 responded with short-latency inward currents at −70 mV (−19 ± 3.1 pA). The mean latency to start was 0.49 ± 0.18 s. Seven of these cells were tested with kainate during voltage ramps. The $E_{\text{rev}}$ drifted in a negative direction from more positive potentials (Fig. 9, A and C) and the steady-state $E_{\text{rev}}$ varied considerably, from −44.7 mV to +31.0 mV (mean −9.3 ± 9.6 mV). When kainate was coapplied with picrotoxin and/or 3-APMPA to four of the cells, the current reversed at +22.4 mV for one cell and could not be reversed for the other three cells (up to +40 mV; Fig. 9, B and D). For three CB7 (and 2 CB6) cells tested with kainate alone and kainate coapplied with picrotoxin and/or 3-APMPA, there was a significant differ-
FIG. 7. Stratification level of axon terminals for presumed ON cone bipolar cells of type 5 and 6 in video-enhanced, infrared differential interference contrast image of a vertical slice of rat retina. Imaging with ×40 objective and ×1.6 lens. The inner plexiform layer has been divided into 5 strata of equal thickness, labeled 1–5 (left). Notice narrow longitudinal band of higher optical density at the border between stratum 3 and stratum 4. Arrows labeled 5 and 6: level of stratification of axon terminals of cone bipolar cells type 5 and 6, respectively. Arrowhead: cell body and primary dendrite of a presumed AII amacrine cell. A proportionally scaled image of an AII amacrine cell (modified from Perry and Walker 1980; their Fig. 16B) has been overlaid to illustrate level of stratification of the cell’s lobular appendages and arboreal dendrites. The retinal layers are indicated by abbreviations (ONL, outer nuclear layer; other abbreviations as in Fig. 1). Scale bar: 30 μm.
FIG. 8. Characteristics of kainate-evoked whole cell current responses of a presumed ON cone bipolar cell (CB6). A: 3-s kainate application (200 μM; horizontal bar) at −70 mV evoked a short-latency inward current. B: suppression of response of the same cell during coapplication of kainate and CNQX (25 μM; horizontal bar). C: I-V relations for kainate response. Traces marked 1 and 2 were recorded in succession at an interval of 3.5 s. Note slow increase of current and drift of \( E_{\text{rev}} \) from approximately −40 mV in trace 1 to −0 mV in trace 2. Vertical arrow: \( E_{\text{Cl}} \). D: I-V relationship for response to kainate coapplied with picrotoxin and 3-APMPA to block indirectly mediated GABA response; \( E_{\text{rev}} \) approximately −40 mV.

Responses evoked by NMDA

A total of 17 cone bipolar cells was tested with NMDA. Application of NMDA at a holding potential of −70 mV did not evoke a change in conductance in any of the cells (Fig. 11A). Cells tested were classified as CB2, CB3, CB5, CB6, CB7, and CB8. Because glycine is a coagonist at the NMDA receptor (Johnson and Ascher 1987), NMDA was coapplied with 1 μM glycine (and 10 μM strychnine to block strychnine-sensitive glycine receptors). The channels linked to NMDA receptors are blocked by Mg\(^{2+}\) and a series of other divalent cations, including Co\(^{2+}\), in a voltage-dependent manner (Nowak et al. 1984). However, even when application of NMDA was combined with voltage ramps (−100 mV to +40 mV; 100 mV/s), no conductance increase was observed in any of the cells tested (\( n = 8 \); Fig. 11B). Cells tested were classified as CB2, CB3, CB6, CB7, and CB8. For some cells, the duration of application was increased up to 60 s. As a control, NMDA responses with the characteristic J-shaped I-V relationship (negative slope conductance) in the same range as for intact cells with short-latency responses. With longer-lasting application of kainate during voltage ramps, the \( E_{\text{rev}} \) was −0 mV for all cells (−3.0 ± 1.1 mV; \( n = 9 \); Fig. 10C). Lack of reversal or a drift of the \( E_{\text{rev}} \) in a negative direction was never observed. This result supports the idea that the drift of \( E_{\text{rev}} \) in a negative direction observed for intact cells is caused by recruitment of longer-latency GABAergic input in the inner plexiform layer.

Response characteristics of kainate on amacrine cells

There is no reason to believe that any amacrine cells, with short-latency inward currents evoked by kainate (mean latency to start 0.17 ± 0.02 s; \( n = 27 \)), have mistakenly been classified as OFF cone bipolar cells in the present study. First, the morphology is clearly different, with amacrine cells having longer processes, typically extending further in the lateral direction and/or stratifying more diffusely in the inner plexiform layer. Corresponding to this, the membrane capacitance is higher in amacrine cells than in cone bipolar cells (10.5 ± 2.0 pF vs. 3.80 ± 0.15 pF; \( n = 120 \); \( P < 0.01 \)).
FIG. 9. Characteristics of kainate-evoked whole cell current responses of 2 different presumed ON cone bipolar cells (A and B; CB7; C and D, CB7). A and C: I-V relations for kainate response. Traces marked 1 and 2 were recorded in succession at an interval of 3.5 s. Note slow increase of current and drift of \( E_{\text{rev}} \) from \(-40 \text{ mV} \) or greater in trace 1 to approximately \( 0 \text{ mV} \) in trace 2. Vertical arrow: \( E_{\text{Cl}} \).

B and D: I-V relationship for response to kainate coapplied with 3-APMPA (B) or picrotoxin and 3-APMPA (D) to block indirectly mediated GABA response; \( E_{\text{rev}} \) greater than \(-40 \text{ mV} \). Low intracellular chloride concentration.

Furthermore, the peak amplitudes of kainate-evoked currents in amacrine cells (\(-369 \pm 73 \text{ pA} \); range \(-75 \text{ to } -1,680 \text{ pA} \)) were considerably larger than and almost nonoverlapping with the peak amplitudes for cone bipolar cells with short-latency responses (\(-37 \pm 3.7 \text{ pA} \); range \(-4.3 \text{ to } -127 \text{ pA} \); \( n = 71; P = 0.0001 \)). Accordingly, when kainate evoked a short-latency inward current in a cell, the peak amplitude often served as a reliable prediction of the identity of the cell recorded from.

All (narrow-field bistratified) amacrine cells are of particular interest here because they contact axon terminals of cone bipolar cells via gap junctions in the proximal part of the inner plexiform layer (cat: Kolb and Famiglietti 1974; rabbit: Dacheux and Raviola 1986; rat: Chun et al. 1993). In recordings of four AII amacrine cells (Fig. 12A), application of kainate at a holding potential of \(-70 \text{ mV} \) evoked short-latency (0.19 \pm 0.02 s), large inward currents (peak amplitude \(-1,062 \pm 308 \text{ pA} \)) that could be strongly suppressed by coapplication of CNQX, thus confirming the results of Boos et al. (1993) (Fig. 12, B and C). When application of kainate was combined with voltage ramps (\(-100 \text{ mV} \text{ to } +40 \text{ mV}; 100 \text{ mV/s} \)), the \( E_{\text{rev}} \) was \(-0.3 \pm 1.7 \text{ mV} \) (Fig. 12D; \( n = 3 \), low intracellular chloride), the expected value for a current through nonselective cation channels.

**DISCUSSION**

The main finding in this study is that the different morphological types of cone bipolar cells in the rat retina can be classified into ON- or OFF-type cells depending on which type(s) of kainate (and/or AMPA) responses they display. First, all cells of types CB1–CB4 displayed direct responses, observed as short-latency inward currents at \(-70 \text{ mV} \) and a corresponding \( E_{\text{rev}} \) close to 0 mV. I propose that these cells are OFF cone bipolar cells. Second, some cells (among CB5–CB9) did not display short-latency responses to kainate. Other cells of the same types (CB5–CB8), however, displayed short-latency kainate responses, but the evoked current either could not be reversed or reversed at positive potentials. I propose that these cells (CB5–CB9) are ON cone bipolar cells and that the short-latency kainate responses are mediated via gap junctions with AII amacrine cells. Third, indirect responses were often observed during longer-lasting application of kainate, in both ON and OFF...
polar cells), no drift of $E_{\text{rev}}$ toward $E_{Cl}$, suggestive of an indirect response, could be observed. The release of GABA in Ca$^{2+}$-free extracellular medium with 5 mM Co$^{2+}$ was discussed in a previous study of rod bipolar cells (Hartveit 1996b) and thus will not be repeated here.

**OFF cone bipolar cells (CB1–CB4)**

For the types CB1, CB2, CB3, and CB4, kainate (or AMPA) evoked a short-latency inward current, characteristic of a direct response, in every cell tested. In addition, for a number of cells there was evidence that kainate evoked an indirect response mediated by GABAergic input onto the axon terminals of these cells. This was observed as a gradual drift of the $E_{\text{rev}}$ from ~0 mV toward $E_{Cl}$ during longer-lasting application of kainate. Indeed, with perfect voltage clamp, the observed $I-V$ curve would simply be the linear sum of the $I-V$ curves for the two response components (direct and indirect). This drift of $E_{\text{rev}}$ was blocked when kainate was coapplied with the GABA$\alpha$ receptor antagonist picrotoxin and the GABA$\gamma$ receptor antagonist 3-APMPA, and it was not observed in axotomized bipolar cells. In both of these conditions the evoked current reversed close to 0 mV, as

cone bipolar cells. It is surprising that Euler et al. (1996) only observed indirect kainate responses in ON cone bipolar cells and not in OFF cone bipolar cells. Two lines of evidence suggest that these indirect responses are mediated by GABA acting on the axon terminals of cone bipolar cells in the inner plexiform layer. First, for some axotomized bipolar cells without short-latency kainate responses (presumably ON cone bipolar cells), no indirect response to kainate could be observed. Second, for other axotomized bipolar cells with short-latency responses to kainate (presumably OFF cone bi-

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**FIG. 10.** Characteristics of response to kainate in a putative OFF cone bipolar cell with axon cut in the inner plexiform layer. A: 3-s application of kainate (200 µM; horizontal bar) evoked a short-latency inward current. B: coapplication of CNQX (25 µM) blocked the response to kainate. C: $I-V$ relationship of kainate response during voltage ramps (-100 mV to +40 mV; 100 mV/s), $E_{\text{rev}}$ ~ 0 mV. There was no drift of the $I-V$ curve or any indication of an indirect kainate response. Low intracellular chloride concentration.

**FIG. 11.** Lack of response to N-methyl-D-aspartate (NMDA) in cone cells without short-latency kainate responses (presumably ON cone bipolar cells), no indirect response to kainate could be observed. Second, for other axotomized bipolar cells with short-latency responses to kainate (presumably OFF cone bi-
expected for nonselective cation channels integral to ionotropic glutamate receptors. Furthermore, no cells classified as CB1–CB4 ever responded to kainate with an outward current at −70 mV, the response expected for an APB type of glutamate receptor. Taken together, these results suggest that the cone bipolar cells morphologically classified as CB1–CB4 correspond physiologically to OFF cone bipolar cells, expressing conventional ionotropic non-NMDA glutamate receptors.

The axon terminals of CB1–CB4 stratify in S1–S2 of the inner plexiform layer (Fig. 1). This is exactly the location where the lobular appendages of All amacrine cells provide glycineric (Pourcho and Goebel 1985) synapses onto the axon terminals of cone bipolar cells (rat: Chun et al. 1993; rabbit: Strettoi et al. 1992; cat: McGuire et al. 1984), thereby conveying a scotopic signal to the OFF pathway. This point has been illustrated in the right half of Fig. 7, where an image of a Golgi-stained rat All amacrine cell (Perry and Walker 1980, their Fig. 16B) has been overlaid on a video image of a retinal slice, as seen with infrared differential interference contrast optics. This pattern of connectivity is consistent with the assignment of CB1–CB4 as putative OFF cone bipolar cells. Conversely, if a cone bipolar cell with an axon terminal stratifying vitreal to S1–S2 is an OFF cone bipolar cell (as suggested by Euler et al. 1996), it would not be in a position to receive a scotopic signal of the adequate polarity. For CB2, which expresses the calcium-binding protein recoverin (Euler and Wässle 1995), there is ultrastructural evidence for input from All amacrine cells (Han et al. 1996).

**ON cone bipolar cells (CB5–CB9)**

The classification of CB1–CB4 as OFF cone bipolar cells on the basis of the observation that kainate (or AMPA) evoked a short-latency direct response in every cell is relatively straightforward. For many cells of types CB5–CB9, kainate did not evoke a short-latency response, but indirectly mediated responses were sometimes observed during longer-lasting application of kainate. These indirect responses were slow in onset, reversed close to and followed changes in $E_{\text{rev}}$, and could be blocked by a combination of picrotoxin and 3-APMPA. These characteristics were identical to those described above for OFF cone bipolar cells and to those observed previously for rod bipolar cells (Hartveit 1996b). In addition, a few cells responded to kainate with an outward current at −70 mV, typical for APB-like responses. On the basis of these results, one would expect that CB5–CB9 do not express functional (conductance increasing) ionotropic glutamate receptors and therefore would correspond physiologically to ON cone bipolar cells.

However, for every type of bipolar cell in this group (except CB9, where only 2 cells were recorded), some of the cells tested displayed a short-latency response to kainate. In this respect, the cells responded very similarly to types CB1–CB4 and it might be argued that they are OFF cone bipolar cells and that CB5–CB8 contain a mixture of ON and OFF type cells. However, when the $I-V$ relationship was examined with voltage ramps, the kainate-evoked current could not be reversed (up to +40 mV) or reversed at potentials markedly above 0 mV, suggesting that the short-latency responses were not direct responses mediated by the cone bipolar cell under test. For some cells with evidence of an additional indirect GABAergic response, a similar lack of reversal was observed when the indirect response was blocked by picrotoxin and 3-APMPA. Importantly, the short-latency kainate response was suppressed by CNQX, indicating that it was mediated by conventional non-NMDA receptors. The series resistance measured for these cells (from the recording electrode to the cell interior) was similar to that for the rest of the cells (~30 MΩ). Given the small size of the evoked currents (10–30 pA), it is not likely that the voltage drop across this series resistance is sufficient to explain the deviation of the $E_{\text{rev}}$ from the expected value (~0 mV). Indeed, for a presumed OFF cone bipolar cell that was recorded initially with a much higher series resistance (160 MΩ), the voltage drop was sufficiently high that the current evoked by kainate could not be reversed. However, when the series resistance dropped to 42 MΩ, $E_{\text{rev}}$ was ~0 mV.

The lack of reversal for presumed ON cone bipolar cells with short-latency kainate responses does suggest, however, that a space clamp problem might be important, in the sense that a high resistance was present between the pipette and the site where the current was generated (Armstrong and Gilly 1992). A simple explanation for the lack of adequate space clamp is that the current is generated at ionotropic glutamate receptors on All amacrine cells and conducted through gap junctions between the arboreal dendrites of All amacrine cells and the axon terminals of cone bipolar cells. In the rat retina, ultrastructural studies have demonstrated that All amacrine cells contact the axon terminals of cone bipolar cells via gap junctions in S3 and S4 in the inner plexiform layer (Chun et al. 1993). This is exactly the location needed to contact the axon terminals of cone bipolar cells CB5, CB6, CB7, and CB8 (Figs. 1 and 8). Indeed, ultrastructural evidence for gap junctions between the axon terminals of (recoverin-positive) CB8 cone bipolar cells and All amacrine cells has recently been presented (Han et al. 1996). None of the results obtained in the present study demonstrate directly that the responses are mediated through gap junctions located at the axon terminals of the cone bipolar cells, but it should be noted that for axotomized bipolar cells with short-latency kainate responses, lack of reversal was never observed ($E_{\text{rev}}$ ~ 0 mV).

The presence of a short-latency kainate response in some, and not other, cone bipolar cells in each of groups CB5–CB8 is most likely caused by a dynamic regulation of the open probability of the gap junctions between the All amacrine cells and the cone bipolar cells. Gap junction permeability can be regulated by intracellular second messengers, and Mills and Massey (1995) recently reported that tracer coupling between All amacrine cells and cone bipolar cells can be blocked by cGMP. This suggests that at a given time an ON cone bipolar cell is not able to respond to both kainate and APB. Under conditions with low intracellular cGMP concentration, the cell will be coupled to All amacrine cells, display a short-latency kainate response, and function as a component in the rod pathway. It will not, however, be able to respond to application of APB with an outward current. Conversely, under conditions with high intracellular cGMP concentration, the cell will not be coupled to All amacrine...
FIG. 12. Characteristics of kainate-evoked responses in AII amacrine cells. A: cell filled with Lucifer yellow. A series of negatives taken with epifluorescence optics at different focal planes was scanned and assembled to generate a single image. Top horizontal bar: indicates border between the inner nuclear layer and the inner plexiform layer. Bottom horizontal bar: border between the inner plexiform layer and the ganglion cell layer. Each bar is 15 \( \mu \text{m} \). B: response to application of kainate (200 \( \mu \text{M} \); horizontal bar) at a holding potential of −70 mV. C: strong suppression of response to kainate (200 \( \mu \text{M} \); horizontal bar) by coapplication of CNQX (25 \( \mu \text{M} \)). D: I-V relationship of kainate response during voltage ramps (−100 mV to +40 mV; 100 mV/s). Notice \( E_{\text{rev}} \) close to 0 mV. The small spikelike deflections from the I-V curve between −60 and −40 mV correspond to voltage-activated sodium currents and were caused by escape from the voltage clamp. They appear as outward currents because they were present only in the control trace, which was subtracted from the trace sampled during application of kainate. Low intracellular chloride concentration.

Direct evidence for expression of non-NMDA ionotropic glutamate receptors was obtained for four AII cells, confirming the results of Boos et al. (1993). Glutamatergic input to AII amacrine cells most likely comes from rod bipolar cells (Chun et al. 1993; Strettoi et al. 1990, 1992). In the direct recordings from AII amacrine cells, with potentially less of a space clamp problem and better voltage control compared with the indirect recordings through the cone bipolar cells, kainate-evoked currents reversed close to 0 mV. Accordingly, if the short-latency kainate-evoked currents recorded in the presumed ON cone bipolar cells are indeed generated in AII amacrine cells, the lack of reversal is due to lack of voltage control of the AII amacrine cell(s) when the recording pipette is located at the cone bipolar cell. Even in cases in which the gap junction resistance between the cone bipolar cell and the AII amacrine cell would be relatively low, the large amplitude of the kainate-evoked current in the AII amacrine cells would cause a substantial voltage drop between the two cells, causing the measured \( E_{\text{rev}} \) to become more positive than 0 mV. Further evidence that the observed kainate responses are indeed evoked in AII amacrine cells will depend on the ability to suppress the responses by blocking these gap junctions.
Which ionotropic glutamate receptors are expressed by cone bipolar cells?

Functional (conductance increasing) ionotropic receptors of the NMDA type were not detected for any of the cells (CB1, CB4, and CB9 were not tested). This is generally consistent with results for the NR2 subunit mRNAs (A–D) from in situ hybridization experiments (Brandstätter et al. 1994), but raises a problem for the NR1 subunit mRNA, which seemed to be expressed by cells located throughout the inner nuclear layer. It remains to be seen whether NR1 mRNA is also expressed at the protein level and whether it is incorporated into a functional glutamate receptor. In mammalian cells, NR1 is incapable of forming a functional homomeric receptor (Boeckman and Aizenman 1994). The possibility should be considered, however, that (some) bipolar cells might express various non-NMDA and/or NMDA receptor subunits unrelated to the glutamatergic input from photoreceptor cells in the outer plexiform layer. One function could be that of autoreceptors at the axon terminals in the inner plexiform layer. If that is the case, these and previous results (Hartveit 1996b) suggest that ligand binding to the receptor subunit(s) is not accompanied by a detectable increase in membrane conductance. Interestingly, evidence for such receptors has recently been found in crayfish (Parnas et al. 1996).

As to the molecular identity of the ionotropic non-NMDA type receptor subunits expressed by the presumed OFF cone bipolar cells, the responses of some cells to AMPA suggest that at least some cells express high-affinity AMPA receptors (Hollmann and Heinemann 1994), consistent with previous immunocytochemical evidence for expression of glutamate receptor B in the outer plexiform layer (Peng et al. 1995). Further work, however, is required to determine the molecular specificity of glutamate receptors expressed by different ON and OFF cone bipolar cells.

Stratification of ON versus OFF cone bipolar cells in the inner plexiform layer

On the basis of the results presented in this investigation, I suggest that cone bipolar cells CB1–CB4 express functional (conductance increasing) ionotropic non-NMDA glutamate receptors and therefore correspond physiologically to OFF cone bipolar cells. Cone bipolar cells CB5–CB9 do not express such receptors and therefore correspond physiologically to ON cone bipolar cells. Within this scheme, the OFF sublamina of the inner plexiform layer will encompass S1 and S2, whereas the ON sublamina will encompass S3–S5. Accordingly, the OFF/ON border will not be located in the middle of the inner plexiform layer (as proposed by Euler et al. 1996), but rather will be shifted toward the outer retina. Rat retinal ganglion cells stratify in two distinct levels, with the border shifted toward the outer retina (Bunt 1976; Famiglietti and Vaughn 1981; Peichl 1989), but it has not yet been demonstrated that they correspond physiologically to ON and OFF center cells. The same shift of the ON/OFF border toward the outer retina can be observed in cat retina (Nelson et al. 1978; Peichl and Wässle 1981). The results presented in this study suggest that the functional stratification of cone bipolar cells in the rat retina will parallel that for ganglion cell dendrites, with OFF and ON cone bipolar cells providing excitatory input to OFF and ON center ganglion cells, respectively. Definitive evidence for this scheme will require recording of light-evoked responses from various types of cone bipolar cells and ganglion cells.

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