Glutamate and 2-amino-4-phosphonobutyrate evoke an increase in potassium conductance in retinal bipolar cells

(2-amino-4-phosphonobutyrate receptor/ON-bipolar cell/ionic selectivity/cell culture/visual)

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ABSTRACT Although there is general agreement that L-glutamate can produce a depolarizing inward current to account for the hyperpolarizing (OFF) bipolar cell response, the conductance mechanism underlying the depolarizing (ON) response has been difficult to establish satisfactorily. To investigate the directly underlying responses, we studied the whole-cell currents controlled by L-glutamate and its analogues in solitary bipolar cells from salamander retina. We report here two groups of isolated bipolar cells: one group responded to L-glutamate with the previously described inward current [Attwell, D., Mobbs, P., Tessier-Lavigne, M. & Wilson, M. (1987) J. Physiol. (London) 387, 125–161] and a second group showed an outward current that reversed at about −70 mV. Both were associated with an increase in membrane conductance. In addition, Dl-2-amino-4-phosphonobutyrate, a compound diagnostic for ON-bipolar cell activity [Slaughter, M. M. & Miller, R. F. (1981) Science 211, 182–185], elicited outward currents that closely resembled those seen in response to L-glutamate and, furthermore, that showed a tendency to increase in conductance to potassium ions. Thus the presence of two distinct conductances controlled by L-glutamate in solitary cells would provide an alternative mechanism for generating the ON and OFF light responses at the bipolar cell level in the intact retina.

In the vertebrate retina, bipolar cells can be divided into two groups according to their response to light: depolarization in ON-center cells and hyperpolarization in OFF-center cells (1, 2). These center responses are believed to be mediated by a direct synaptic input from the photoreceptors (1, 2), which are thought to use L-glutamate or a related excitatory amino acid as a transmitter (3–8). Different postsynaptic mechanisms, therefore, appear to underlie the ON and OFF responses in bipolar cells (3, 5). Early studies reported that the ON- and OFF-center light responses were associated with a conductance increase and a conductance decrease, respectively (9, 10). Since photoreceptors hyperpolarize with illumination, resulting in a decrease in transmitter release, the transmitter appeared to cause an opening of channels in OFF-bipolar cells and an unconventional closing of channels in ON-bipolar cells. However, further studies by Saito, Toyoda, and colleagues (11–13) indicated that some of the ON-center light responses were accompanied by a conductance decrease as well as a conductance increase. Thus, it appears, in some cases, that the ON-center response may be generated by more than one conductance mechanism.

Konishi and Toyoda (14) confirmed the dual effect of glutamate on these ON-bipolar cells in isolated carp retina, where the two responses appeared to be associated with the rod and cone pathways. In other experiments, depolarizing bipolar cells in intact mudpuppy (15), dogfish (16), and goldfish (17) retinas appeared to respond to glutamate with only a decrease in conductance, associated with an extrapolated reversal potential of 0 mV. It is unclear why more than one mechanism has not been observed in other species. A more direct approach toward investigating the cellular mechanisms involved in the conductance changes may involve studying the currents evoked by putative transmitters in isolated cells. Attwell et al. (18), using acutely dissociated bipolar cells from the axolotl retina, reported finding two types of currents to glutamate using whole-cell patch-clamp techniques, with support for a conductance decrease mechanism. In addition, several groups observed slower kinetics (18–20) and washout (21, 22) of ON-bipolar cell responses, suggesting the involvement of internal messengers. Furthermore, pharmacological experiments by Slaughter and Miller (15, 23) revealed that the compound dl-2-amino-4-phosphonobutyrate (APB) selectively activated glutamate receptors on ON-bipolar cells. Seeking to confirm and extend these findings, we investigated the currents controlled by L-glutamate and its analogues in solitary bipolar cells dissociated from the salamander retina and maintained in culture.

MATERIALS AND METHODS

Preparation. Solitary bipolar cells were dissociated from aquatic-phase tiger salamander Ambystoma tigrinum retina, either with papain (24) or with dispase/collagenase. For the dispase/collagenase (0.5 mg/ml and 2 mg/ml, respectively), the tissue was incubated for 1.5–2 hr at 37°C; otherwise, the procedures remained the same. The cells were maintained on a monolocular antibody substrate, Sal-1 (25), and in serum-free medium containing bovine serum albumin (1 mg/ml), 1× minimal essential medium (MEM) vitamin mixture, 0.1× MEM essential amino acids, 0.1× MEM nonessential amino acids, 1% medium 199, insulin (20 μg/ml), 5 mM taurine, thyroxine (2 μg/ml), transferrin (10 μg/ml), and gentamicin (10 μg/ml) in amphibian salt solution (see below) for up to several weeks. Cells were studied typically within the first few hours to days of plating: no obvious changes in response properties were observed over this time. Bipolar cells were identified by their characteristic morphology (26). Although there was variability in bipolar cell shape, the cells from which recordings were made generally possessed an ovoid body with a prominent Landolt club.

Electrophysiology. Responses to glutamate and its analogues were recorded in the whole-cell mode of the patch-clamp technique (27). Patch pipettes were pulled on a BB-CH-PC (Mechanex) puller in a three-step fashion, used directly from the puller, and typically contained 100 mM D-aspartate, 7.5 mM NaCl, 2.5 mM NaH2PO4, 1 mM MgCl2, 2 mM Hepes, 1 mM Na2ATP, 0.05 mM EGTA, and 18 mM sucrose, adjusted to pH 7.0 with KOH. The pipette resistances before forming a seal ranged from 5 to 20 MΩ, with a

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Abbreviation: APB, Dl-2-amino-4-phosphonobutyrate.
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median of 10 MΩ; corrections for tip potentials were made
(28). The currents were amplified using a Yale Mark V
patch-clamp amplifier and were low pass filtered at 1 kHz.
Data were stored on videotape following digitization, on
chart paper, and in a Zenith Z-386 personal computer used to
run the pCLAMP acquisition and analysis software from Axon
Instruments (Foster City, CA).
Although series resistance and cell input resistance and
capacitance were not routinely monitored, these measure-
ments were made on separate bipolar cells under identical
recording conditions. The series resistance after rupture of
the membrane was measured from the amplitude of the
capacitative transients in response to a series of voltage steps
from the holding potential in voltage clamp and calculated
according to $R_p = V/I (t = 0)$ (where $V$ = voltage, $I$ = current,
and $R_p$ = the pipette resistance) as described in Tessier-
Lavigne et al. (29). The series resistance ranged from 25 to 35
MΩ, averaging 29.4 ± 3.2 MΩ (mean ± SD; $n = 10$). The
voltage error due to the series resistance was estimated to be
<4 mV, for which no correction was made. The cell input
resistance, $R_{in}$, and capacitance, $C$, were derived from
voltage responses to current steps in current clamp. Calculated
from the equation describing the steady-state current as $I = $V/$R_{in}$, the $R_{in}$ ranged from 4 to 10 GΩ, with an average of 7.8
± 1.7 GΩ (mean ± SD; $n = 7$). The capacitance was derived
from $\tau$, the time constant of the capacitative current, obtained
using the CLAMPFIT curve-fitting program, as $C = \tau/R_{in}$,
assuming $R_{in} \gg R_p$, and ranged from 2.0 to 4.0 pF, with an
average of 2.4 ± 0.7 pF (mean ± SD; $n = 7$).
Amphibian salt solution contained 108 mM NaCl, 3 mM
KCl, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 0.5 mM MgSO₄, 1 mM
NaHCO₃, 0.5 mM NaH₂PO₄, 0.1 mM choline chloride, 16
mM glucose, 1 mM sodium pyruvate, 2 mM Hepes, and
0.001% phenol red, adjusted to pH 7.3 with NaOH. All drugs
were dissolved in external medium and were applied after the
total membrane current reached steady state by pressure
ejection from a second pipette (2- to 3-μm tip diameter)
positioned near the cell. Responses of both polarities were
observed in cultures from either dissociation procedure, but
there was a tendency to see more cells with outward currents
in the dispase/collagenase preparation. Sodium L-glutamate
was obtained from Sigma; APB, Cambridge Research Bio-
chemicals (Valley Stream, NY); papain and collagenase,
Worthington; and dispase, Boehringer Mannheim.
The cells were generally superfused with amphibian salt
solution on a cooled stage maintained at 12°C. For ion-
substitution experiments, the increase in potassium chloride
concentration was accompanied by an equal decrease in
sodium chloride concentration to keep the osmolarity constant.
The patch pipette contained 100 mM KCl, 4 mM MgCl₂, 10 mM NaH₂PO₄, 1.1 mM Na₂ATP, 0.05 mM EGTA, 0.1–0.2 mM cyclic GMP, adjusted to pH 7.0 with KOH.

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![Figure 1](image-url)

**Fig. 1.** Membrane currents induced by L-glutamate (Glu) in isolated bipolar cells. (a) Voltage dependence of L-glutamate-induced outward currents. The whole-cell currents of a voltage-clamped bipolar cell in response to 300-ms applications of 500 μM L-glutamate were determined at the holding potential, indicated to the left of each trace, after the voltage-dependent currents had reached steady state. The magnitude of the steady-state current just before the drug application is shown to the right in a and c. The resting membrane potential was −39 mV. For the purpose of clarity, an offset of 5 pA (a) or 30 pA (c) has been introduced between each pair of current traces, which have been resistance–capacitance filtered with a time constant of 100 ms. (b) Current–voltage relation of the L-glutamate-induced outward current shown in a. The peak amplitude of the current (ordinate) is plotted as a function of membrane potential (abscissa). The response reversed polarity around −75 mV. (c) Voltage dependence of the L-glutamate-induced inward currents. The whole-cell currents of a voltage-clamped bipolar cell in response to 300-ms applications of 250 μM L-glutamate are shown. The resting membrane potential was −32 mV. (d) Current–voltage relation of the L-glutamate-induced inward current shown in c. The peak amplitude of the inward current (ordinate) is plotted as a function of the holding membrane potential (abscissa). The response reversed polarity around +12 mV.
RESULTS

1-L-Glutamate-Induced Responses. To investigate the basis of the ON and OFF responses, we studied the currents controlled by L-glutamate in isolated salamander bipolar cells by using the standard whole-cell patch-clamp technique (27). From a population of 101 cells, 72 showed either an inward or an outward current, both of which were stable and could be observed for at least an hour after achieving the whole-cell configuration. While bipolar cells were identified by morphology, it was not possible to predict response polarity based on any distinguishing characteristic, consistent with the findings of Hare et al. (26).

Fifty-one of the bipolar cells responded with outward currents to the local application of L-glutamate (100–500 μM) at the resting membrane potential (V_m = −43.4 ± 10.3 mV) (Fig. 1a). The current reversed polarity from outward to inward around −70 mV (−69.2 ± 4.0 mV; n = 25) and was accompanied by an increase in membrane conductance. The maximal amplitudes were typically 15–20 pA, under our recording conditions. The time to peak of the response was notably slow, on the order of 1.0–1.5 sec from the onset of L-glutamate application. The relationship between the peak amplitude of the response and voltage (Fig. 1b) could not be fitted with a straight line, and there was a tendency toward inward rectification at less negative potentials, which is seen more clearly in other examples.

The remaining 21 bipolar cells responded with a large inward current to L-glutamate (100–500 μM) at the resting membrane potential (V_m = −37.7 ± 8.2 mV), which reversed to outward at membrane potentials above +3 mV (3.0 ± 8.6 mV, n = 11) (Fig. 1c). The inward current showed a short rise time (100–300 msec), and the amplitudes reached ≈100 pA, or severalfold larger on average than those of the outward current seen in the first group of bipolar cells. The peak amplitude of the inward current showed little rectification over the range of voltage from −100 to 50 mV (Fig. 1d). In the few bipolar cells tested, kainate (25–50 μM, n = 4 out of 12) and quisqualate (100 μM, n = 2 out of 5) elicited inward currents similar to those evoked by L-glutamate.

APB-Induced Outward Currents. From previous pharmacological studies on APB, characterizing it as an agonist for ON-bipolar cells in the intact retina (15, 23), one would expect APB to mimic the action of the endogenous photo-receptor transmitter. In a separate study of 40 bipolar cells, APB (50–100 μM) induced outward currents in 27 cells at the resting membrane potential (V_m = —40.4 ± 8.0 mV), and its application was associated with an increase in membrane conductance. The remaining 13 cells gave no response; we never observed inward currents to APB. The APB-induced outward current resembled the glutamate-evoked outward current in amplitude and time course. A feature of the outward current that was more prominent in this example was the inward rectification seen at depolarized membrane potentials (Fig. 2b).

Ionic Selectivity of Outward Currents. Like the glutamate-induced outward current, the APB-induced current reversed at about −70 mV (−71.1 ± 16.5 mV; n = 3), suggesting the involvement of chloride or potassium ions as possible charge carriers. The reversal potential of the APB-induced current, however, was not significantly shifted (−78.3 ± 5.1 mV; n = 9) when determined with a high chloride concentration in the patch pipette (109 mM), which changed E_Cl from −63 mV to −2 mV. In contrast, the reversal potential was found to depend on the potassium concentration. When the bath contained 12 mM or 48 mM potassium, the reversal potential of the outward current shifted to −42 mV (−42.3 ± 9.6 mV; n = 7) or −18 mV (−18.2 ± 2.3 mV; n = 5), respectively, from the −78 mV observed in 3 mM potassium (Fig. 3a–c). The relationship of the reversal potential as a function of external potassium concentration remained fairly linear over the range of 3–48 mM potassium and produced a slope of 50 mV per decade (Fig. 3d), in reasonable agreement with the 57 mV per decade predicted from the Nernst equation for a single species of monovalent ion. Deviation from the Nernst prediction suggests that, while potassium may be the predominant charge carrier, we cannot exclude a small role for sodium or calcium ions. In the present study, we did not test L-glutamate and APB on the same cells; however, based on the overall similarities of the outward currents, it seems reasonable to assume that the outward currents seen with L-glutamate and APB both arose from an increase in potassium conductance.

From recent experiments done using retinal slices, Nawy and Jahr (22) describe the suppression by glutamate and APB of a cGMP-activated conductance in depolarizing bipolar cells. The inclusion of cGMP (100–200 μM) in our recording
pipette seemed to increase the size of the APB-induced outward current but did not affect its reversal potential, response polarity, or stability. We did not observe the development of an obvious, large inward current in those bipolar cells upon rupture of the membrane patch. It is unclear why we have not observed the cGMP-activated current in isolated cells; it may require the addition of other factors (e.g., GTP) in the patch pipette to be activated.

**DISCUSSION**

In a study of acutely dissociated bipolar cells, Attwell et al. (18) observed an inward current in response to L-glutamate that reversed polarity around \(-12\) mV. This inward current is similar to the one described in this report, with a reversal potential of \(+3\) mV, and suggests a relatively nonspecific conductance increase to monovalent cations, typical of vertebrate glutamate channels (30). This cell type would correspond to the OFF-bipolar cell, which is depolarized in the dark and hyperpolarized with light when the release of glutamate is decreased or stopped. Such a mechanism for the OFF response is consistent with the L-glutamate-induced depolarizations, accompanied by a conductance increase, seen in solitary bipolar cells from the skate retina (31).

In addition, Attwell et al. (18) reported an outward current in response to 1 mM glutamate that reversed polarity above \(-13\) mV and was associated with a conductance decrease, which we did not observe. The outward current we found was clearly different because it reversed at \(-70\) mV, was associated with a conductance increase, and could be elicited by 100 \(\mu\)M glutamate. This bipolar cell would correspond to an ON-bipolar cell type as it would depolarize with light from the shutting off of a hyperpolarizing current, present in the dark when transmitter release is high. Our finding that the ON-bipolar cell response can be explained on the basis of a selective increase in conductance to potassium has support from intracellular work in the carp retina by Saito, Toyoda, and colleagues (11–13). They proposed two mechanisms that appeared to mediate the ON-bipolar cell response: one in which light caused a conductance increase to sodium and another in which light caused a conductance decrease to potassium and/or chloride. While the conductance decrease response may correspond to the former, our data are consistent with the latter mechanism of a conductance decrease...
to potassium ions in response to light. It is unclear whether
this response correlates with a cone-dominant ON-center
response in the salamander as in fish. In addition, Karschin
and Wässe (32) have found conditions where APB induced
a conductance increase among the majority of responding
bipolar cells isolated from rat retina. A glutamate-induced
potassium-dependent conductance increase has also been
reported at the lobster neuromuscular junction by Miwa and
Kawai (33) and appears to involve a guanine nucleotide-
binding regulatory protein mechanism (34), suggesting a need
for GTP in the pipette solution in future experiments.

There are, however, reports that seem to be in contradic-
tion to our findings. In particular, the effect of glutamate
and APB on depolarizing bipolar cells has been associated
only with a conductance decrease from intracellular recordings
in the intact mudpuppy (15), dogfish (16), and goldfish (17)
retinas, suggesting that the neurotransmitter closes channels
with a reversal potential above the resting potential. We did
not observe a conductance decrease response to glutamate,
perhaps due to the high concentration threshold for glutamate
(1 mM) with isolated cells as reported by Attwell et al. (18).
In the original work on APB in the intact retina (15, 16),
concentrations of 50 μM–1 mM APB were used to block the
light response and produced a conductance decrease in
ON-bipolar cells. More recently, Nawy and Copenhagen (17)
reported similar effects at 2–10 μM APB. Using relatively
high concentrations of APB (50–100 μM) on isolated bipolar
cells, we observed a potassium-dependent outward current
that mimicked the outward current seen in response to L-glutamate. It could be that the conductance mechanism for
the outward current is localized to a greater extent on more
distal dendrites in the intact retina and that these are more
frequently removed during the dissociation procedure, re-
sulting in a reduction of the maximum amplitude (and, hence,
detectability) of APB and glutamate responses.

In addition, most of these intact preparations were studied in
the rod-dominated, dark-adapted state. From the work of
Saito and colleagues (12–13), it seems probable that cone
bipolar cells utilize different ionic mechanisms from the rod
bipolar cells in mediating the ON response. It is conceivable
that the same receptor may be coupled to different ino-
onephores or that subtypes of the APB receptor may exist.
Indeed, Kondo and Toyoda (14) and Nawy and Copenhagen
(17) have provided evidence for different glutamate receptor
subtypes subserving the rod and the cone pathways on the
same cell. Dopamine has been shown to modulate the glu-
tamate responses of horizontal cells (35), and it appears that
humoral agents associated with adaptation may also modu-
late the glutamate responses of bipolar cells (36). Finally, it
may be that glutamate and APB evoke an increase in potas-
sium conductance in only a subset of ON-bipolar cells and
that the others operate by means of a conductance decrease
response or that both mechanisms are used by the same cell
and one is somehow lost upon dissociation or is masked in a
combined response to glutamate (14).

In conclusion, the prime finding of this study is the
identification of two classes of solitary bipolar cells from
salamander retina possessing glutamate-induced currents
with different ionic selectivities, providing an additional
mechanism for generating membrane potential changes of
opposite polarity to the previously described conductance
decrease response. In particular, we report the identification
of a potassium-dependent current in response to L-glutamate
and to APB in a subset of bipolar cells, which may correspond
to one type of ON-bipolar cell response.

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