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L-Aspartate: Evidence for a role in cone photoreceptor synaptic transmission in the carp retina

(neurotransmitters/DL-α-aminoadipic acid/horizontal cells)

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ABSTRACT **A number of putative neurotransmitter substances and their antagonists were applied to the carp retina while intracellular recordings from L-type cone horizontal cells were made. Of all the substances tested, L-aspartate was found to be the most potent agent in depolarizing these horizontal cells in dark-adapted, partially light-adapted, and Co^{2+}-treated retinas. Furthermore, DL-α-aminoadipate, an L-aspartate antagonist, blocked the effects of both the endogenous photoreceptor transmitter and exogenously applied L-aspartate on the horizontal cells. The results suggest that L-aspartate and the natural transmitter interact with the same population of postsynaptic receptors in the horizontal cell membrane.**

In darkness, the vertebrate photoreceptor is partially depolarized by a sodium current that flows into the outer segment of the cell (1). This depolarization appears to result in a continuous release of neurotransmitter from the receptor onto second-order neurons (2). Light, by supressing the sodium current, hyperpolarizes the photoreceptor and depresses the release of transmitter (3, 4). The light response of second-order neurons (the horizontal and bipolar cells) is thus caused by a decrease of neurotransmitter secretion from the receptor terminal.

Horizontal cells and one subclass of bipolar cell [the hyperpolarizing bipolar cell (HBC)] hyperpolarize in response to light; another subclass of bipolar cell [the depolarizing bipolar cell (DBC)] depolarizes in light. It is reasonable to assume, therefore, that, if the photoreceptor transmitter acts directly on these elements, it will depolarize horizontal cells and the HBC and hyperpolarize the DBC. Blockade of transmitter release from the receptor terminals, on the other hand, should mimic the effects of light on these cell types (5–7).

A number of substances have been considered as possible candidates for the photoreceptor neurotransmitter. Attention has focused mainly on the acidic amino acids L-aspartate and L-glutamate, which have been shown to depolarize horizontal cells in several species (8–11). Furthermore, both of these amino acids have been found to depolarize the HBC and hyperpolarize the DBC (12). However, it has recently been shown that atropine, an antagonist of muscarinic acetylcholine receptors, blocks synaptic transmission between photoreceptors and horizontal cells in the turtle retina (13). In addition, γ-aminobutyric acid (GABA) has been reported to depolarize the horizontal cells in the skate (14), although in the carp and turtle retinas it appears to hyperpolarize the horizontal cells (9–11).

As yet, systematic studies on the effects of these transmitter substances and their antagonists on the cells postsynaptic to the receptors have not been described. Here we report experiments that test the relative effectiveness of a number of transmitter substances in depolarizing horizontal cells in dark- and partially light-adapted retinas and in retinas in which neurotransmission between photoreceptors and second-order cells has been interrupted with Co^{2+}. We have also studied the effects of various transmitter antagonists on the horizontal cell response.

MATERIALS AND METHODS

Preparation. Carp (*Cyprinus carpio*) were kept at room temperature in a tank filled with aerated water. Prior to an experiment, the carp were dark adapted for 10–30 min and decapitated under dim red light. The eye was enucleated and hemisected, and the posterior half of the eye cup was inverted over a piece of Ringer's solution-soaked filter paper. Fine glass rods were used to separate the retina from the pigment epithelium which remained with the eye cup. The isolated retina (receptors upward) was placed in a chamber moistened with Ringer's solution and aerated with moist oxygen.

Stimulating and Recording Systems. The dual-beam light stimulator has been described (15). One beam provided stimulus flashes and the other was used as a source of diffuse background light. Intracellular responses were obtained with micropipettes drawn out on a modified Livingston puller. The pipettes were filled with 4 M potassium acetate and had resistances, measured in Ringer's solution, of 150–800 MΩ. Standard amplification and recording techniques were used (16).

Solutions. The Ringer's solution [80 mM NaCl/22.7 mM $NaHCO_3$,/3.5 mM KCl/2.4 mM $MgSO_4$/2.3 mM $CaCl_2$/10 mM dextrose/10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)] was adjusted to pH 7.35 with 1 M NaOH. All pharmacological agents were dissolved in Ringer's solution and used within 3 hr.

Atomizing System. Custom-made fine-spray atomizers were used to deliver the drugs to the surface of the isolated retina. The volume delivered to the retina was measured by spraying a solution of [H^3]glucose onto a piece of filter paper and measuring the radioactivity by scintillation counting. The amount of spray delivered by the atomizer was reproducible within about 15% from application to application. By knowing the concentration of the test agent contained in the atomizer and making an estimate of the retinal volume, it is possible to make a rough estimate of the drug concentration applied to the horizontal cells. The value arrived at, however, depends on the nature of the assumptions made. These include the total retinal volume, the amount of surface wetting above the exposed photoreceptors, the nature of the space into which the test agent diffuses (e.g., the extracellular space or the total tissue water), and the separate rates at which the test agent diffuses into the retina and past it into the underlying layer of vitreous. Here we present values obtained by assuming the test agent to be uniformly distributed into a tissue water thickness of 100 μm. This is an overestimate in neglecting surface wetting and the diffusion gradients and is an underestimate (by about a factor of

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Abbreviations: HBC, hyperpolarizing bipolar cell; DBC, depolarizing bipolar cell; GABA, γ-aminobutyric acid.

5 if extracellular space is 20% of tissue volume) in assuming that the test agents are not confined to the extracellular space. These are thus nominal values that give the order of magnitude of the test agents' concentration and are primarily useful for comparisons among the different test agents. The relative concentrations of the agents are not dependent on assumptions about the distribution of the agents within the tissue and are thus accurate to within 15%.

Identification of Cell Type. Horizontal cell responses are encountered in the carp retina just below the receptor layer, approximately 100–150 μm from the outer retinal surface. Penetration of a horizontal cell was signaled by a potential drop of 20–30 mV. Horizontal cells that receive input exclusively from cones and that hyperpolarize to all wavelengths of light were most commonly recorded. These are termed luminosity (L-type) cells, and all results reported in this paper are from such cells. All drugs were tested on at least 10 L-type cone horizontal cells; most drugs that caused significant effects were tested on 25–50 cells. Horizontal cells that receive input from rods also hyperpolarize in response to light; however, these cells were encountered only infrequently and no conclusions regarding their responses to the applied substances could be made.

RESULTS

Fig. 1 shows the effects of five drugs on the responses of dark-adapted L-type cone horizontal cells. Each trace represents a record from a different horizontal cell. L-Aspartate, L-glutamate, and carbachol, a powerful acetylcholine agonist that is poorly inactivated by acetylcholinesterase, were all effective in transiently depolarizing carp horizontal cells. However, in the dark-adapted carp retina, GABA consistently hyperpolarized horizontal cells whereas glycine in a wide range of concentrations (0.1–5 mM) did not significantly affect the horizontal cell membrane potential or its light-evoked responses. It is unlikely, therefore, that either GABA or glycine can be the photoreceptor transmitter.

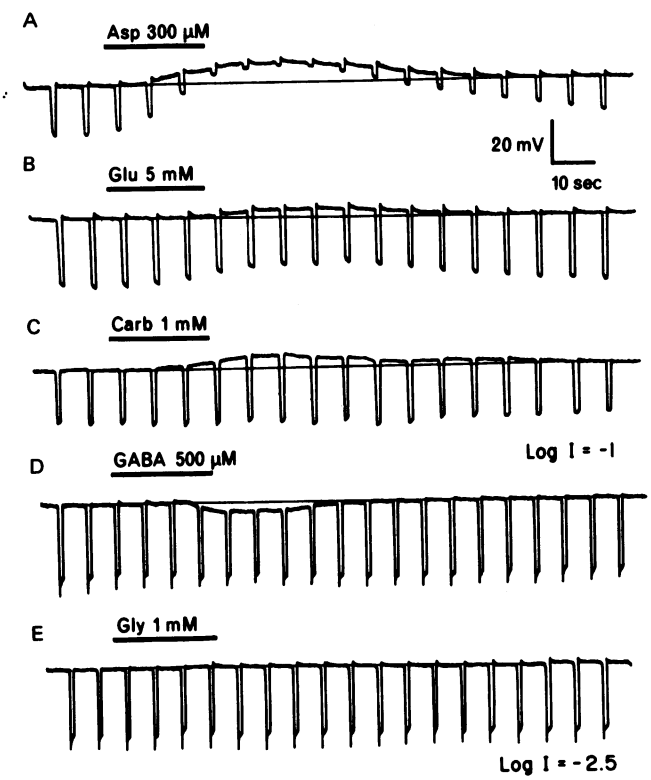

FIG. 1. Effects of L-aspartate, L-glutamate, carbachol, GABA, and glycine on five different L-type cone horizontal cells in the carp retina. The line above each trace indicates the duration of drug application (~20 sec) and the numbers above the lines are the calculated drug concentrations at the synaptic regions. One-second full-field flashes were delivered to the retina at 7-sec intervals. Aspartate, glutamate, and carbachol transiently depolarized the horizontal cell, GABA hyperpolarized the cell, and glycine did not affect the membrane potential. Note that aspartate not only depolarized the horizontal cell at a low concentration but also substantially decreased the light-evoked responses. Concentrations of test agents are nominal estimates (see *Materials and Methods*).

Of the three substances that depolarized the horizontal cells, aspartate was the most effective. At a concentration of 300 μM, aspartate produced the same or a greater degree of depolarization of a horizontal cell than did 5 mM glutamate or 1 mM carbachol. Furthermore, this concentration of aspartate almost completely eliminated the light-evoked responses of the horizontal cells. With 5 mM glutamate and 1 mM carbachol, on the other hand, the light-evoked responses were decreased minimally (glutamate) or often slightly enhanced (carbachol). Only with substantially higher concentrations of glutamate (~10 mM) or carbachol (5–10 mM) were the light-evoked responses decreased significantly in size.

Co^{2+}-Treated Retinas. In the experiments shown in Fig. 1, drugs were applied onto a dark-adapted retina in which the receptors were continuously releasing neurotransmitter. Thus, the effects of any exogenously applied drugs presumably were superimposed on a substantial background of natural transmitter. Furthermore, if the drugs affected the receptor itself, this could significantly alter transmitter release which would result in a change of horizontal cell potential. To eliminate these difficulties, retinas were treated with 1 mM Co^{2+} to isolate the horizontal cells from receptor influence. After supression of receptor transmission, various concentrations of drugs were again applied to the retina.

Fig. 2 shows typical results for aspartate, glutamate, and carbachol. The drug was first applied to the retina before Co^{2+} exposure, and in each case a transient depolarization was noted. After application of Co^{2+}, the horizontal cells hyperpolarized, and light-evoked responses were suppressed. This is the expected response if synaptic transmission between receptors and horizontal cells were interrupted. During the influence of Co^{2+}, the same concentration of aspartate that previously induced an 18-mV depolarization in the untreated dark-adapted retina now produced a depolarization of 45 mV. Indeed, the level to which the membrane depolarized in the untreated and in the Co^{2+}-treated retina was virtually identical.

The effect of 5 mM glutamate on the horizontal cell after Co^{2+} treatment, on the other hand, was similar to that seen in the untreated retina. Only a small (~5 mV) depolarization was seen in either case. This result suggests that the depolarizing effect of glutamate at this concentration may be a nonspecific or nonsynaptic effect. This will be discussed further below. Finally, the effect of carbachol on the horizontal cell was significantly decreased after Co^{2+} treatment. This result suggests that the main effect of carbachol may be presynaptic (i.e., on the receptor) rather than on the horizontal cell; this too will be discussed below.

Dose-response curves for the depolarization of horizontal cells in the Co^{2+}-treated retina by the three test drugs are shown in Fig. 3. Threshold responses for aspartate occurred with concentrations of about 50 μM and saturating responses were induced by concentrations of about 750 μM. Substantial depolarizing effects were induced by glutamate only with concentrations of about 1 mM or higher; thus, there is at least a 50-fold difference in the effectiveness of aspartate and glutamate in depolarizing horizontal cells in the Co^{2+}-treated retina. Carbachol, on the other hand, was ineffective in the Co^{2+}-treated retina in concentrations up to 10 mM.

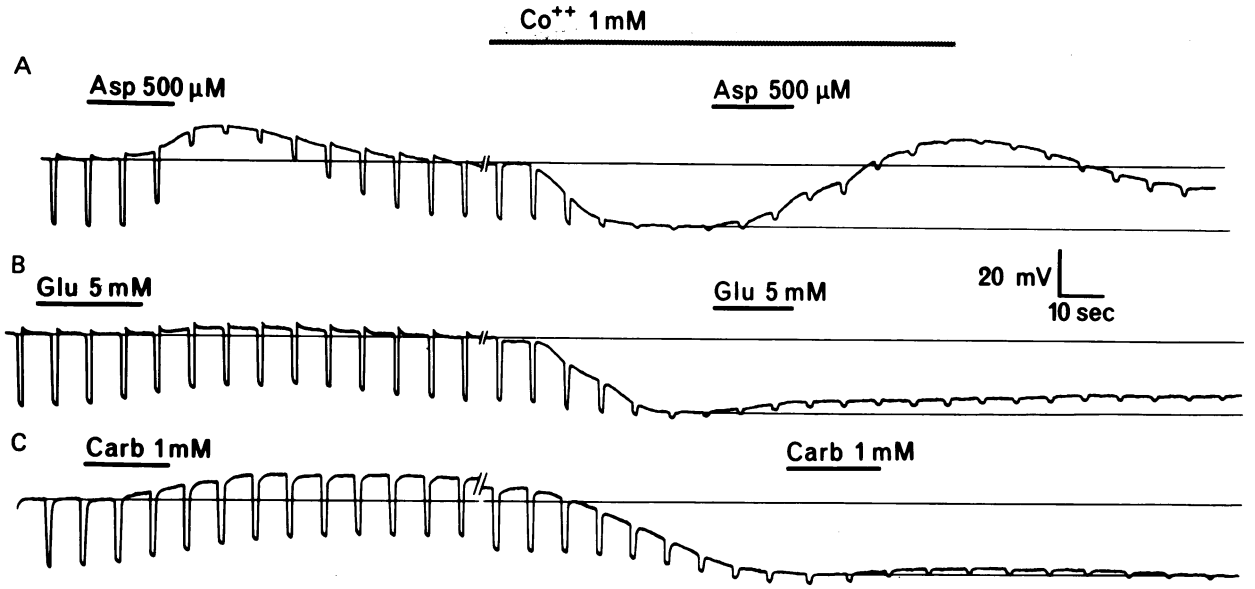

FIG. 2. Effects of L-aspartate, L-glutamate, and carbachol on the L-type horizontal cells in the carp retina before and after Co^{2+} treatment. The left part of each trace is the control response to each of these chemicals. Co^{2+} (1 mM) applied to the retina for about 2 min hyperpolarized the membrane potential by 30–40 mV and abolished the light-evoked responses. Note that only aspartate depolarized the membrane potential to the same level after Co^{2+} treatment as it did before Co^{2+} treatment.

Light Adaptation. Release of neurotransmitter from the receptors can also be suppressed by light. Fig. 4 shows the effects of aspartate, glutamate, and carbachol on horizontal cells in the partially light-adapted retina. As in Fig. 2, the depolarizing effect of the three substances was first tested in the dark-adapted retina. Then, a background light was turned on to hyperpolarize the cells by 20–35 mV. The adapting light also decreased significantly the light-evoked responses of the cells. In the light-adapted retina, 500 μM aspartate had an effect similar to that seen in the Co^{2+}-treated retina—that is, aspartate depolarized the cell by about 40 mV, or to the same level that was reached in the dark-adapted or untreated eye. The effect of 5 mM glutamate on the light-adapted retina was also similar to that seen in the Co^{2+}-treated retina. Only a small depolarization was seen in response to the substance at 5 mM under both light- and dark-adapted conditions.

Carbachol, however, caused in this instance a depolarizing effect similar to that obtained in the dark-adapted retina. This result contrasts with its effect in the Co^{2+}-treated retina, in which it was ineffective. These results suggest further that carbachol's main effect is presynaptic rather than postsynaptic. This is because, in the light-adapted retina, as contrasted to the Co^{2+}-treated one, presynaptic effects may be transmitted and expressed postsynaptically.

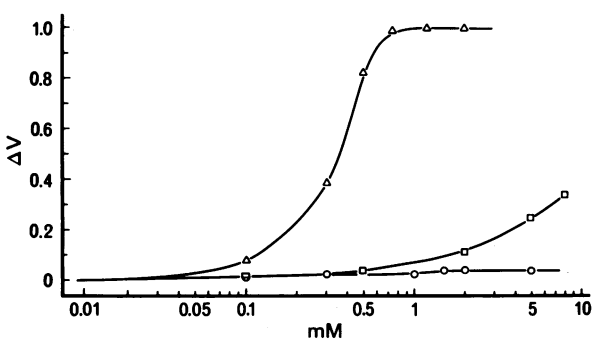

FIG. 3. Dose-response curves of L-aspartate (△), L-glutamate (□), and carbachol (○) on the L-type horizontal cells in the carp retina after Co^{2+} treatment. Data points are averages from the responses of six typical cells. The ordinate is the normalized dark membrane potential change (ΔV) of the horizontal cells from the maximum hyperpolarization (0) induced by 1 mM Co^{2+} to the maximum depolarization (1) caused by a saturating dose of aspartate applied in each case subsequent to the test. The abscissa is the concentration of applied drug on a logarithmic scale.

A summary of the effects of all of the substances tested is presented in Table 1. L-Aspartate was the most effective substance tried under all conditions. The only other substance that approached aspartate in effectiveness was cysteine sulfinic acid which, like aspartate, has a negatively charged acidic group linked to the β-carbon atom. Other amino acid analogues tested were much less effective (glutamate) or totally without effect in the dose range used (taurine, asparagine, β-alanine, glycine, and D-aspartate); others produced inappropriate potential changes (GABA). Carbachol, the other possible transmitter agonist tested, depolarized dark-adapted elements, but had no effect on horizontal cells isolated from the receptors by Co^{2+}.

Drug Antagonists. An important test for a suspected neurotransmitter is to show that its actions can be blocked by a specific antagonist. One of the difficulties in demonstrating that amino acids are neurotransmitter substances has been the lack of selective antagonists. Recently, however, two blocking agents, one specific for aspartate (D-α-aminoadipic acid or DL-α-aminoadipic acid) (17, 18) and another for glutamate (L-glutamic acid diethyl ester) (19, 20) have been described. Fig. 5 shows the effect of DL-α-aminoadipate (the pure D form is unavailable at the present time) on a horizontal cell. Shortly after the application of 100 μM α-aminoadipate, the horizontal cell hyperpolarized by about 20 mV and the light-evoked responses were substantially decreased. In some cases, α-aminoadipate caused a small initial depolarization before the onset of the hyperpolarizing effect. This small initial effect may be caused by the weak excitatory action on the cell membrane of the L stereoisomer (17). In all cases (12 cells), however, a significant hyperpolarization of the horizontal cell was observed after α-aminoadipate exposure.

The application of 750 μM aspartate while the horizontal cell was fully hyperpolarized by α-aminoadipate caused no change in membrane potential—i.e., the aspartate effect was completely blocked. With time, however, the cell gradually recovered (depolarized) and aspartate was once again effective

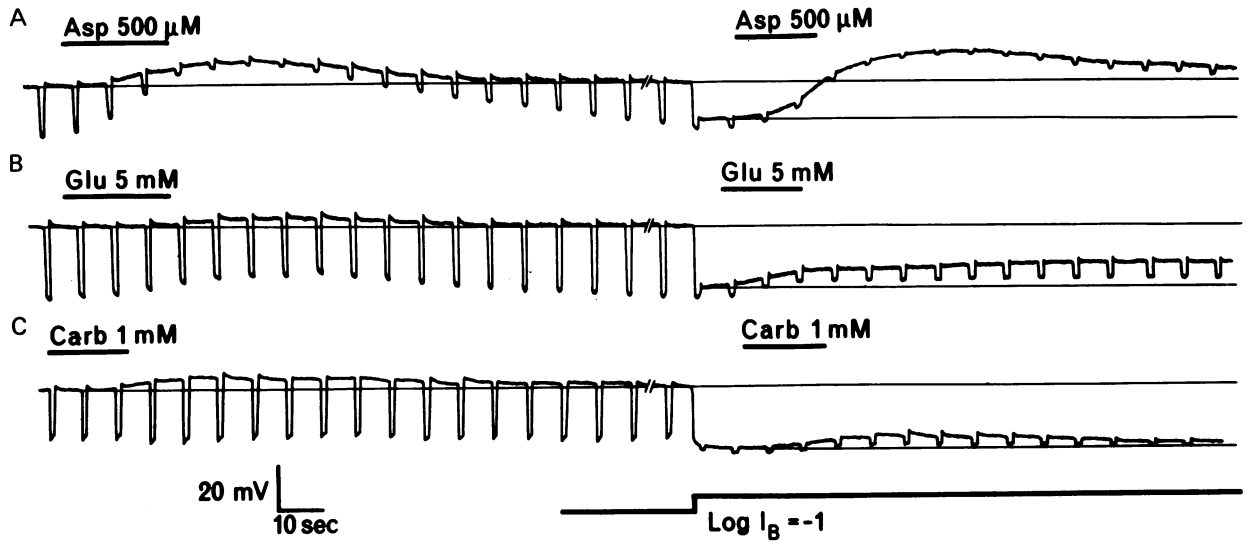

FIG. 4. Effects of L-aspartate, L-glutamate, and carbachol on the L-type horizontal cells in the carp retina with and without background illumination. The left-hand side of each trace is the control response of dark-adapted horizontal cells to each of these chemicals. Background illumination caused a 20- to 30-mV hyperpolarization. Only aspartate depolarized the HBC to the same level with background light as it did without the background illumination.

in depolarizing the cell. This is shown in the lower records. By 150 sec after the beginning of the α-aminoadipate exposure, a measurable aspartate response was seen, and by 250 sec, the dark membrane potential of the cell and its response to aspartate were substantially recovered. We also examined the effects of L-glutamate diethyl ester and atropine on the horizontal cells. Within the dose range tested (0.1–5 mM), neither of these transmitter antagonists affected the horizontal cell membrane potential, the light-evoked responses, or the depolarizing effect of aspartate.

DISCUSSION

The evidence described above indicates that aspartate is a strong candidate for the photoreceptor transmitter. Of all the substances tested, it is effective in the smallest concentrations in depolarizing horizontal cells in both the light- and the dark-adapted retina and in retinas treated with Co^{2+} to block photoreceptor neurotransmission. Furthermore, its effects and the effects of the natural photoreceptor transmitter can be blocked by DL-α-aminoadipate, an aspartate antagonist. Finally, aspartate depolarizes HBC and hyperpolarizes DBC, effects expected of the photoreceptor transmitter (12).

Table 1. Summary of effects of substances tested

Drug	Dark-adapted	After Co^{2+} treatment	Light-adapted
L-Aspartate (300 μM)	++	++++	++++
Cysteine sulfinic acid (300 μM)	++	++++	++++
Carbachol (1 mM)	++	0 or +	++
L-Glutamate (1–5 mM)	+	+	+
Taurine (1 mM)	0	0	0
L-Asparagine (1 mM)	0	0	0
β-Alanine (1 mM)	0	0	0
Glycine (1 mM)	0	0	0
D-Aspartate (1 mM)	0	0	0
GABA (500 μM)	--	+	+
Co^{2+} (1 mM)	----	/	0
DL-α-Aminoadipate (100 μM)	----	0	0
$Glu(OEt)_2$ (1–5 mM)	0	0	0
Atropine (1 mM)	0	0	0

The drugs are listed in descending order of effectiveness in depolarizing the horizontal cells. The concentration required to record measurable effect, or the highest concentration tried when no effect was seen, is shown in parentheses. Under the retinal condition tested, the substance caused a depolarization (+, 1–5 mV; ++, 5–15 mV; +++, 15–20 mV; ++++, 20–50 mV); 0, no effect was observed; −, a hyperpolarization was seen (- -, 5–15 mV; - - - -, 20–50 mV).

It might be argued that one reason aspartate is more effective than other substances in depolarizing horizontal cells is that the retina does not possess an efficient uptake mechanism for aspartate. If this were so, the response of the horizontal cell to aspartate should be prolonged relative to that to other substances. This is not the case; indeed, the response to aspartate is often more transient than that to other substances, suggesting that the retina does possess effective mechanisms to take up aspartate and to terminate its action. Evidence for such an uptake system has also been described: radioactive aspartate

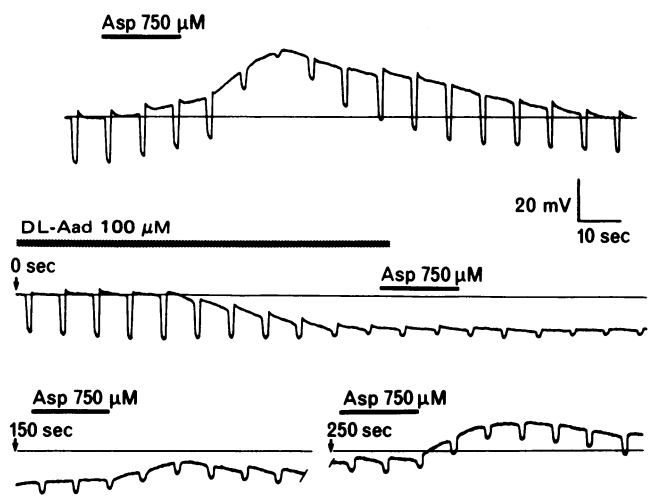

FIG. 5. Effect of DL-α-aminoadipate (Aad) on an L-type horizontal cell in the carp retina. The top trace is the control response to 750 μM aspartate; 100 μM DL-α-aminoadipate was then applied to the retina for about 80 sec. It caused a 20-mV hyperpolarization and virtually abolished the light-evoked responses. The same dose (750 μM) of aspartate caused no depolarization immediately after DL-α-aminoadipate application (right half of the middle trace). With time, the horizontal cell recovered (depolarized) and, once again, aspartate induced a substantial depolarization (bottom traces).

is actively taken up into glial cells in the retina; and the retinal uptake of aspartate is saturable, with high- and low-affinity components (21).

We have noted above that the effect of 1–5 mM glutamate on the horizontal cells appears to be a nonspecific or nonsynaptic effect. That is, a similar small depolarization of the horizontal cell is noted after glutamate application in dark- and light-adapted retinas and after application of Co^{2+}. In the light-adapted or Co^{2+}-treated retina, the flow of the natural transmitter is curtailed; thus, any substance capable of binding to the postsynaptic receptors would have the opportunity to combine with more receptors and be more effective at any given concentration. This is precisely the result obtained with aspartate. The fact that glutamate does not behave this way suggests that its effect under these conditions is not mediated through the postsynaptic receptors. At much higher concentrations, however, glutamate has substantial depolarizing effects and decreases or eliminates light-evoked responses. Under these conditions, glutamate may be acting directly on the postsynaptic receptors.

The effects of carbachol on the horizontal cells are best explained by an action on the presynaptic element, the receptor. The evidence for this is that the depolarizing effect of carbachol disappears in the Co^{2+}-treated retina, when the horizontal cell is isolated from receptor influence. In addition, in both the dark- and partially light-adapted retina, light-evoked responses often increase in amplitude in proportion to the amount of depolarization induced by carbachol. Whether this effect of carbachol has any physiological significance is not clear. Horizontal cells are known to feed back onto cone receptors in a number of species, but no evidence that horizontal cells are cholinergic has been provided.

Our experiments suggest that amino acids that bind and activate the postsynaptic receptors on the horizontal cell membrane must have a specific molecular configuration. The length of carbon chain, the location of specific ionized charge groups, and isomeric configuration all appear to be important for the activity of a substance. Glutamate, with one extra carbon atom in the carbon chain compared with aspartate, depolarizes the horizontal cell only when the dose is high. β-Alanine and L-asparagine, which differ from L-aspartate in lacking a second carboxyl group or substituting an amide group for the β-carboxyl group, have no effect on the horizontal cell. Finally, the D form of aspartate is without effect. On the other hand, cysteine sulfinic acid matches almost all the actions of aspartate on the horizontal cells. This may indicate that the aspartate-sensitive sites can be bound and activated by molecules that consist of an α-carboxylic amino group at one end and a second negatively charged group, either carboxyl or sulfinic, linked to the β-carbon atom.

In conclusion, our evidence suggests that L-aspartate, or a very similar molecule, is likely to be the cone photoreceptor transmitter in the carp retina. This amino acid has been proposed to be an excitatory neurotransmitter elsewhere in the vertebrate nervous system primarily on the basis of its ability to mimic certain synaptic actions (20). In the retina, we have found the same to be true; aspartate closely mimics the natural transmitter substance of the photoreceptors. More rigorous evidence that aspartate is the transmitter of the photoreceptor requires the demonstration that aspartate is released from the photoreceptor in the dark and that light interrupts this release. As yet there is only a single, preliminary report that aspartate is released from the retina in the dark and that light diminishes this release (22). Further evidence on this important point is needed.

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