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D-Aspartate potentiates the effects of L-glutamate on horizontal cells in goldfish retina

(amino acids/neurotransmitters/vision)

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ABSTRACT **The amino acids L-glutamate and L-aspartate depolarize H1 horizontal cells in the perfused goldfish retina but only at millimolar concentrations. The effects of L-glutamate (but not of L-aspartate) are potentiated approximately 15-fold by exposure to D-aspartate. D-Aspartate blocks acidic amino acid uptake in goldfish retina, so that the potentiation of L-glutamate may be produced by an increase in its effective concentration at the horizontal cell membrane. Because D-aspartate also augments the light responses of horizontal cells, our results are consistent with the possibility that L-glutamate is a neurotransmitter of cone photoreceptors in goldfish.**

Since the demonstration of the effects of amino acids on motoneurons nearly 20 years ago (1, 2), much attention has been given to the possibility that L-glutamate, L-aspartate, and other structurally similar acidic amino acids may function as synaptic transmitters in the vertebrate central nervous system. Exogenously applied L-glutamate has been shown to produce a depolarization or increase in spike firing in neurons from a variety of structures, including the cortex, hippocampus, cochlear nucleus, and thalamus (3). A Ca^{2+}-dependent release of L-glutamate or L-aspartate has been demonstrated in several preparations, and there is evidence for a Na^+-dependent, high-affinity uptake system for acidic amino acids in glia and some neurons (3–5).

In spite of these findings, there is still considerable uncertainty whether L-glutamate or L-aspartate actually functions as a synaptic transmitter in vertebrates. Part of the reason for this is that these substances produce effects only at rather high concentrations and are usually nonspecific (6). Neurons depolarized by L-glutamate or by L-aspartate are usually also affected (and at similar concentrations) by D isomers of amino acids and by a large number of structurally similar analogues (2, 7). It seems possible that these compounds are not all reacting with the same membrane receptor (7, 8), but this notion has been difficult to test critically. Most of the experiments on the effects of amino acids have been done on nonuniform populations of neurons in anatomically complex regions of the nervous system. Furthermore, drugs have been applied in most cases by iontophoresis, so that their concentrations are difficult to quantitate. It is thus difficult to compare the effects of drugs in different preparations or even among cells in the same preparation.

The retina offers several advantages for the study of synaptic transmitter mechanisms. Because it is possible in certain poikilotherms to remove the retina from its surrounding tissue and to maintain it in artificial media, it is possible to add amino acids and other drugs directly to the Ringer's solution. This reduces the uncertainty in estimates of drug concentration at the postsynaptic membrane. Furthermore, the retina is considerably simpler in structure than most of the rest of the central nervous system and has been extensively studied, both anatomically and physiologically (9). This is especially true for the outer plexiform layer, where photoreceptors synapse onto two kinds of second-order cells, the horizontal cell and the bipolar cell (10).

In this study we describe the effects of amino acids on one of the types of horizontal cells in the goldfish retina. This cell, called the H1 (or luminosity-type) cell, receives most of its synaptic input from the cone photoreceptors, predominantly those containing the red-sensitive photopigment (11). The identity of the synaptic transmitter released by the cones onto the H1 cells is as yet unknown. We show, as previously demonstrated by other investigators (12–16), that L-aspartate and L-glutamate depolarize the horizontal cells and thus mimic the photoreceptor transmitter. Though the amino acids produce this effect only at millimolar concentrations, the response to L-glutamate can be potentiated approximately 15-fold by exposure to D-aspartate, a substance that has been shown to produce a competitive inhibition of L-glutamate uptake (17). Because D-aspartate also appears to augment the light responses of horizontal cells, our results are consistent with the possibility that L-glutamate is a synaptic transmitter in goldfish cones.

MATERIALS AND METHODS

Preparation and Perfusion System. Adult goldfish (*Carassius auratus*) 20–25 cm in body length were dark adapted for a minimum of 2 hr and the eyes were enucleated in dim red light and hemisected. A portion of the retina dorsal to the optic nerve was placed receptor side up on a piece of Millipore filter in a perfusion chamber (18). The retina was continuously superfused with oxygenated (100% O_2) Ringer's solution at the receptor surface at a rate of 2 ml/min. The chamber volume was 0.1 ml. All experiments were done at room temperature (21–23°C). Solution changes were made with a stopcock placed outside the recording cage. The volume of the tubing between the stopcock and perfusion chamber was a "dead space" in the perfusion line, which produced a delay of 30 sec to 1 min after the solution change was made before the new solution reached the retina. Once the new solution reached the chamber, it exchanged rather quickly: the time for exchange of one volume in the chamber was approximately 3 sec. The normal Ringer's solution, modified from the recipe of Kaneko and Shimazaki (19), had the following composition: 120 mM NaCl, 2.5 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM glucose, and 3 mM Hepes. It was brought to pH 7.8 with NaOH. Amino acids were simply added to the Ringer's solution and the pH was readjusted, when

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necessary. All solutions containing amino acids were made on the day in which they were used, typically 1–2 hr before the beginning of the experiment.

Pipettes and Recording. The ground electrode was a 3 mM KCl salt bridge inserted through the side of the chamber into the perfusion well. Ag/AgCl electrodes were avoided, because they often showed large changes in tip potential when the solution was changed from normal Ringer's solution to one containing an amino acid at the concentrations used in these experiments. Intracellular recordings were made with pipettes pulled on a modified Livingston puller, filled with 2 M potassium acetate, and measuring 100–200 MΩ in resistance. Amplification and recording techniques were conventional.

Light Stimulation. The photostimulator was similar in construction to one previously described (18) and provided two beams whose intensity, spatial configuration, and wavelength could be independently controlled. The absolute calibration of the stimulator was made with a calibrated photodiode (United Detector Technology, Santa Monica, CA), as described (18). Intensity in the two beams was attenuated with neutral glass absorption filters, whose optical densities were calibrated in a Beckman spectrophotometer. Stimulus wavelength was selected with narrow-bandwidth (<10 nm) interference filters (Ditric Optics, Marlboro, MA). The test beam was provided with a series of apertures whose images were focused on the retina. The apertures were mounted on micrometers, so that their images could be moved across the retina.

Identification of H1 Horizontal Cells. A standard battery of light stimuli was used to characterize each cell in normal Ringer's solution prior to the application of amino acid-containing solutions. *First*, we measured the responses to full-field, 200-msec flashes over an intensity range of 10^4 at several wavelengths, in order to determine an abbreviated spectral sensitivity curve and to investigate possible changes in response polarity as a function of wavelength. In initial experiments, we used wavelengths of 460, 500, 575, 621, and 648 nm. In later experiments, only 500 and 621 nm were used. *Second*, we measured the receptive field of the cell by stimulating the retina with a 100-μm-wide, 3-mm-long bar of light at several positions, spanning the width of the recording chamber. The cells described in this report had dark resting membrane potentials of −34 ± 6 mV (mean ± SD), a hyperpolarizing response to all wavelengths tested, a maximal sensitivity to light in the red region of the spectrum, a receptive field at least 1.2 mm in diameter, and responses with rapid onset (time to half-maximal hyperpolarization of about 300 msec). Cells were encountered at 124 ± 30 μm (mean ± SD) below the tissue surface (that is, 124 μm from the tips of the outer segments), suggesting that the cells from which recordings were made lay beneath the photoreceptors, in the inner nuclear layer of the retina. Responses of this description have been shown to originate from the horizontal cells of the H1 class (11) by the dye injection method (20–22), and this identification was confirmed in the present study by iontophoresis of the dye lucifer yellow (23).

RESULTS

Effects of L-Glutamate and L-Aspartate. Because photoreceptors release transmitter continuously in darkness (9), the effects of amino acids on cells in the dark-adapted retina are complicated by the exposure of these cells to the endogenous transmitter. To simplify the interpretation of our experiments, we therefore sought a means of arresting the release of the photoreceptor transmitter. In previous investigations, two methods have been used for doing this. The more common has been to perfuse the retina with amino acids in the presence of Co^{2+} or

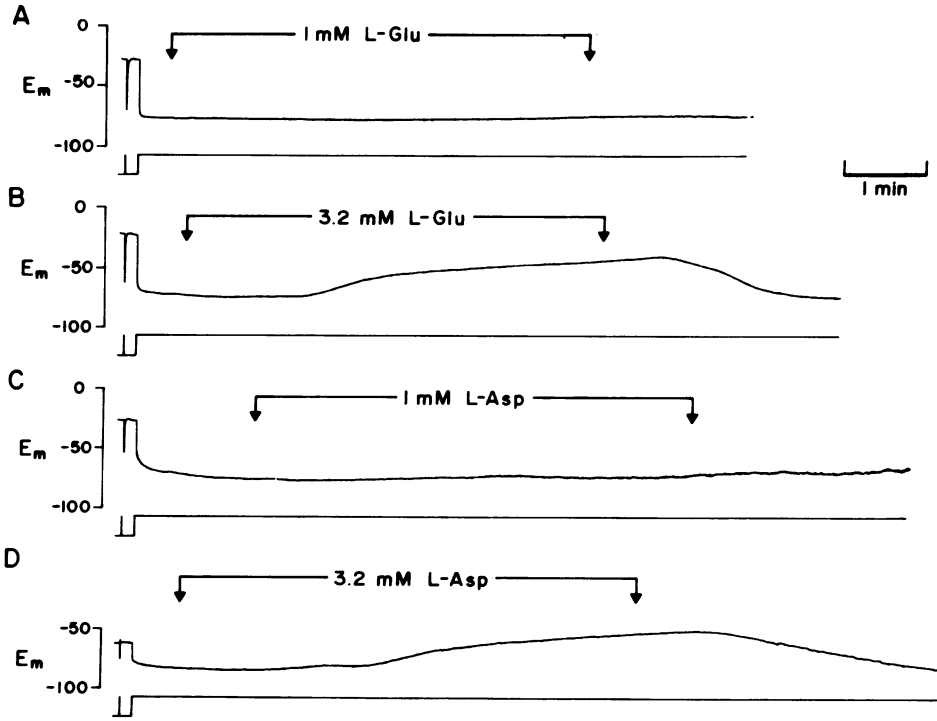

FIG. 1. Effects of 1 and 3.2 mM L-glutamate and L-aspartate on H1 horizontal cell membrane potential E_m in the presence of background light. *A*, *B*, *C*, and *D* have identical formats. The upper trace in each is an intracellular recording of membrane potential from a horizontal cell. The lower trace is a marker for light stimuli. A 200-msec light flash (brief upward deflection of lower trace) preceded the background (maintained upward deflection). Both flash and background were 621-nm illumination at an intensity of 1.1×10^{14} quanta cm^{-2} sec^{-1}. For *A–D*, the dark resting membrane potentials were −30, −22, −27, and −62 mV, respectively; membrane potentials in backgrounds prior to amino acid applications were −80, −73, −75, and −84 mV, respectively. Amino acids were included in the perfusate for the duration indicated between the arrows.

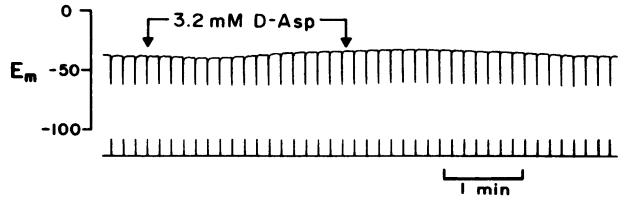

FIG. 2. Effect of D-aspartate on dark-adapted H1 horizontal cell membrane potential and response amplitude. D-Aspartate was included in the perfusate at 3.2 mM during the time indicated between the arrows. Upper trace, intracellular recording of horizontal cell membrane potential. Lower trace, marker for light stimuli. Upper deflections from this trace indicate 200-msec 621-nm flashes of intensity 2.2×10^{13} quanta cm^{-2} per flash.

some other Ca^{2+} channel blocker, to prevent transmitter release (15, 16). This method has the disadvantage that Co^{2+} and many of the other ions of transition metals form complexes with amino acids at relatively low concentrations (24). For example, in a solution containing 1 mM Co^{2+} and 1 mM glutamate, over 85% of the glutamate is in the form of a Co^{2+}–glutamate complex. Though amino acids may still interact with the postsynaptic receptors under these conditions, the interpretation of experiments using amino acids and Co^{2+} (or high concentrations of Mg^{2+}) seemed to us problematic, and we therefore chose a second approach.

When the retina is illuminated, the photoreceptors hyperpolarize and the continuous release of transmitter is reduced. We have therefore examined the effects of amino acids on horizontal cells in the presence of a maintained background light (16). In Fig. 1 we show experiments of this kind for H1 cells at two concentrations of L-glutamate and L-aspartate. In each of the four parts of this figure, the upper trace gives the membrane potential of the cell and the lower, a stimulus marker. After presenting a brief flash of saturating light intensity, we introduced a bright background light at a wavelength of 621 nm and an intensity of 1.1×10^{14} quanta cm^{-2} sec^{-1}. This background maximally hyperpolarized the horizontal cell membrane potential. After the horizontal cell potential stabilized, we switched from normal Ringer's solution to one containing the amino acid. After a brief delay, caused mostly by the dead time in the perfusion system, the horizontal cell depolarized. During the 5–6 min for which we exposed the retina to the amino acid, the horizontal cell continued to depolarize, though the rate of depolarization declined with time. The results in Fig. 1 and those from other experiments show that, for 5- to 6-min exposures, L-glutamate and L-aspartate produce depolarizations of at most a few mV at 1 mM but have significantly larger effects at 3.2 mM. We could detect little difference in the magnitude of the depolarizations produced by L-glutamate and L-aspartate (23), and in this respect our results are similar to those obtained for cells elsewhere in the central nervous system (3).

Effects of D-Aspartate. Although both L-aspartate and L-glutamate depolarize H1 cells and thus mimic the photoreceptor transmitter, Fig. 1 demonstrates that they do this only at rather high concentrations. Because the retina, in common with much of the central nervous system, has an active uptake system for acidic amino acids (25, 26), it seemed possible that the concentrations of L-aspartate and L-glutamate used in the experiments in Fig. 1 did not reflect the actual concentrations at the postsynaptic membrane. To test this notion, we attempted to block uptake by exposing the retina to D-aspartate, which has been shown to be an inhibitor of acidic amino acid uptake in retina (27) and cerebral cortex (17).

The effects of D-aspartate on a dark-adapted H1 horizontal cell are shown in Fig. 2. The resting potential of this cell was −39 mV in darkness, and its responses to a saturating light flash were 24 mV in peak amplitude. In the presence of 3.2 mM D-aspartate, the horizontal cell depolarized to −34 mV and the light responses increased to 28 mV, that is by nearly the same amount as the change in resting potential. Similar effects have been observed in a total of five cells at D-aspartate concentrations between 1.0 and 3.2 mM. These effects are in contrast to those of the L isomers of aspartate and glutamate, which in the dark produce a depolarization of membrane potential and *decrease* in the amplitude of the light response (15, 16, 23).

The effects of D-aspartate in the light-adapted retina depend upon the intensity of the background light. In bright backgrounds, 3.2 mM D-aspartate appeared to have little or no effect on the horizontal cells. However, in the presence of a moderate illumination, this concentration always produced a depolarization of membrane potential and enhancement of the light responses, much like that in the dark. These effects can be seen in the initial sections of the records in Fig. 3. This enhancement occurs only during exposure to D-aspartate and does not take place if the horizontal cell is simply left for long periods of time in normal Ringer's solution in the presence of the background. We have consistently observed that the light responses grad-

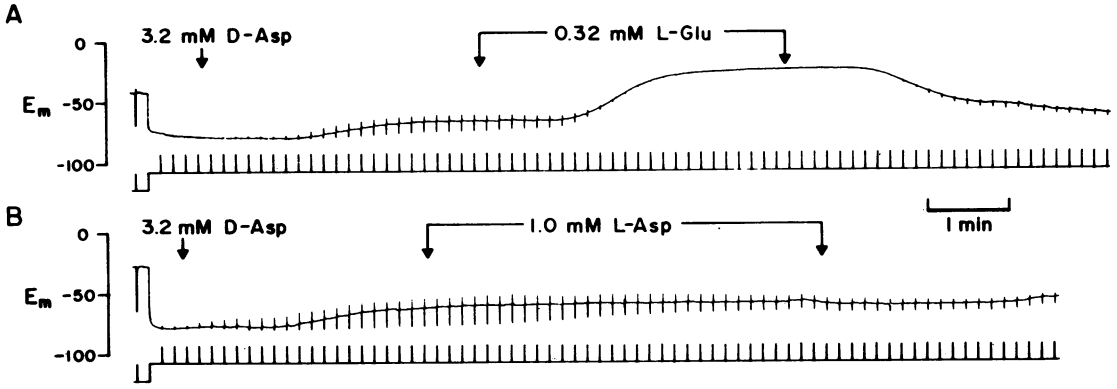

FIG. 3. Potentiation of L-glutamate (but not of L-aspartate) by exposure to D-aspartate. The format of the figure is the same in *A* and *B*: upper traces, intracellular recording of horizontal cell membrane potential; lower traces, marker for light stimuli. A single light flash was presented before the presentation of a maintained background. Thereafter, a series of flashes at regular intervals was presented, superimposed upon the background. All flashes were 200 msec in duration at 621 nm and of an intensity of 1.0×10^{14} quanta cm^{-2} per flash. Maintained backgrounds were 1.1×10^{14} quanta cm^{-2} sec^{-1}. After the membrane potential had stabilized in the presence of background, 3.2 mM D-aspartate was added (single arrows); it remained in the perfusate for the duration of the recording. (*A*) L-Glutamate included in the perfusate at 0.32 mM for the time indicated between arrows. (*B*) L-Aspartate included in the perfusate at 1.0 mM for the time indicated between arrows.

ually decrease in amplitude during prolonged (>6 min) exposure to D-aspartate. The reason for this is unknown.

Effects of L-Glutamate and L-Aspartate in the Presence of D-Aspartate. Because D-aspartate appears to augment the responses of horizontal cells, we examined its effects on exogenous applications of amino acids. Typical results are shown in Fig. 3. The experiments in this figure are similar in protocol to those of Fig. 1, except that the retina was exposed to D-aspartate before and during the time the L amino acids were added. For the cell of Fig. 3A, a 0.32 mM concentration of L-glutamate produced a reversible 44-mV depolarization of the horizontal cell membrane potential. In the absence of D-aspartate, this concentration produced no detectable effect. In Fig. 3B, we show the results of a similar experiment using L-aspartate instead of L-glutamate. Though it is possible to detect a small depolarization after the addition of the L-aspartate, the change in potential was too small to be clearly distinguished from electrode drift. We have done similar experiments in 10 cells at L-aspartate concentrations between 0.32 and 1 mM, and we were unable to obtain any evidence that the response to L-aspartate is potentiated by exposure to D-aspartate. We were also unable to detect potentiation of L-aspartate responses by 1–3 mM D-glutamate.

Dose–Response Curves for L-Glutamate. The magnitude of the potentiation of the response to L-glutamate can best be estimated from dose–response curves in the presence and absence of D-aspartate. In Fig. 4 we have plotted the amplitude of the horizontal cell depolarization as a function of L-glutamate concentration. We have included only those cells for which the drug-induced depolarizations were reversible. For the L-glutamate responses in the absence of D-aspartate, the magnitude of the depolarization was measured 6 min after switching to the solution containing the amino acid. This choice was to some extent arbitrary because, as we have shown, the membrane potential continues slowly to depolarize for as long as we expose the horizontal cell to the amino acid. This slow creep was not observed when L-glutamate was added in the presence of D-aspartate (Fig. 3A). In any event, the responses to L-amino acids were measured at approximately the same times after the onset of background lights in all experiments—i.e., regardless of whether D-aspartate was included in the perfusate.

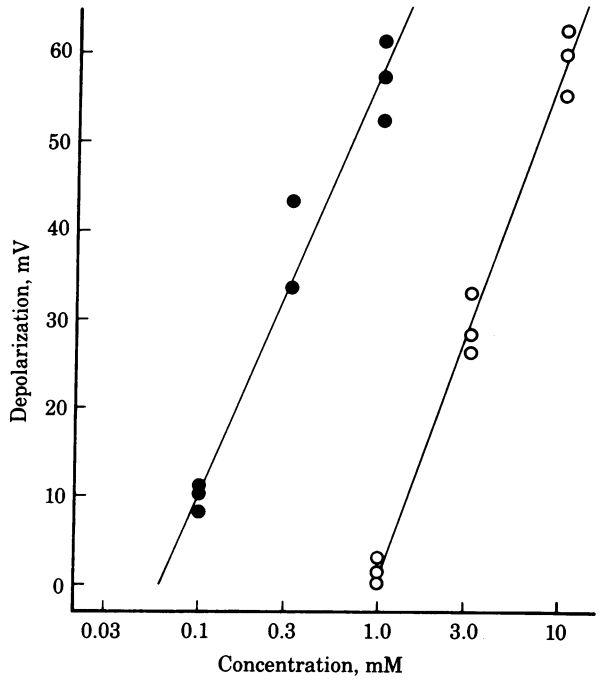

FIG. 4. Dose–response curve for L-glutamate in presence and absence of D-aspartate. All measurements were made in the presence of backgrounds as in Figs. 1 and 3. ○, Depolarization recorded 6 min after switching to a solution containing L-glutamate. ●, Steady-state depolarization produced by L-glutamate in the presence of 3.2 mM D-aspartate. L-Glutamate concentrations indicated are total concentrations added to Ringer's solution and have not been corrected for complexing of the glutamate to the Ca^{2+} and Mg^{2+} in the solution [less than 10% of the glutamate was in the form of a Ca^{2+} or Mg^{2+} complex at the cation concentrations in our solutions (24)].

DISCUSSION

L-Glutamate and L-Aspartate. We have shown that millimolar concentrations of the acidic amino acids L-glutamate and L-aspartate superfused onto the goldfish retina in the presence of bright background illumination produce a depolarization of the horizontal cell membrane potential. Our results are substantially similar to those of Kaneko and Shimazaki (15), who applied the amino acids in the presence of Co^{2+} or Mg^{2+} to arrest synaptic transmission. In contrast, Wu and Dowling (16) have reported that L-aspartate is considerably more potent than L-glutamate on carp horizontal cells when the amino acids are sprayed onto the isolated (but unperfused) retina with an atomizer. This discrepancy is difficult to understand. It is likely that, with their technique, the amino acids were in contact with the postsynaptic membrane for only a brief period of time. Aspartate might then have seemed more potent because it reacted with the postsynaptic receptors more quickly. However, this hypothesis is not supported by our data, because the time course of membrane depolarization in the perfused retina was usually the same for the two amino acids, though in some cases aspartate was *slower* than glutamate (see Fig. 1). The dose–response curves of Wu and Dowling are difficult to compare with our own, because the estimates of effective concentrations produced by their atomizing system cannot easily be related to the concentrations in our superfusate.

D-Aspartate. When the dark-adapted retina is perfused with 1–3.2 mM D-aspartate, the H1 horizontal cells depolarize and the amplitude of the light response increases (Fig. 2). D-Aspartate appears to enhance the effect of the natural transmitter, because in this case one would expect an increase in the horizontal cell depolarization produced by the continuous release of transmitter in darkness and a larger hyperpolarization when this release was suppressed by light. In moderate background illumination, the effects of D-aspartate are similar to those in the dark. The horizontal cell depolarizes and the light responses become larger (Fig. 3). However, in brighter backgrounds, D-aspartate appears to be without effect. We interpret these results in the following way. In moderate illumination, the background, though reducing the tonic release of transmitter from the photoreceptor, does not stop it entirely. D-Aspartate could then potentiate the effects of the natural transmitter in the same way as in the dark-adapted retina. In bright background light, on the other hand, the flow of transmitter may be so nearly arrested that this effect of D-aspartate can no longer be observed.

D-Aspartate, in addition to increasing the light responses of the horizontal cells, also produces a 15-fold potentiation of exogenous L-glutamate. This effect is mediated by a nearly lateral translation in the position of the dose–response curve for L-glutamate along the concentration axis, as would be expected if the D isomer were somehow increasing the effectiveness of L-glutamate without changing its mode of action. Because D-aspartate has been shown to block the uptake of L-glutamate in goldfish retina (27), it seems likely that the potentiation of L-glutamate that we observe is produced, at least in part, by an

increase in the effective concentration of L-glutamate at the horizontal cell membrane. However, we cannot exclude the possibility that D-aspartate produces a change in the affinity of the postsynaptic receptors for L-glutamate or has some direct effect upon the photoreceptors, though this latter possibility would be difficult to reconcile with our observation that D-aspartate is without effect upon the horizontal cells in bright background illumination.

The Synaptic Transmitter of Goldfish Cones. Because, in the goldfish retina, D-aspartate potentiates the effects of L-glutamate and seems also to potentiate the effects of the transmitter released by the cones, our results are consistent with the possibility that L-glutamate *is* a cone transmitter. In the presence of D-aspartate, the threshold concentration for L-glutamate in our superfusate is between 10 and 100 μm. The minimal effective concentration at the postsynaptic membrane may be even less than this, because we are uncertain whether the concentration of D-aspartate that we used was sufficient to block glutamate uptake completely. Furthermore, the perfusion of the tissue in our preparation is unlikely to have been uniform, because only one side of the retina was exposed to the Ringer's solution.

The experiment in Fig. 3*B* shows that D-aspartate does not potentiate the effects of L-aspartate. This result is surprising, because L-glutamate and L-aspartate are equally effective in the absence of D-aspartate, and because D-aspartate blocks the uptake of L-aspartate just as effectively as that of L-glutamate in goldfish retina (27). Because, however, L-glutamate is taken up much more effectively by the goldfish retina (27), it seems possible that the effective concentration of L-glutamate at the horizontal cell membrane would be more greatly altered by an uptake blocker than that of L-aspartate. This could explain our failure to observe potentiation of L-aspartate responses. The effective concentration of L-glutamate at the postsynaptic membrane in the absence of an uptake blocker may be much less than that of L-aspartate, even though both depolarize the horizontal cells at nearly identical concentrations (see Fig. 1). Although our results provide no evidence against the notion that L-aspartate is a cone transmitter (16), they also provide no evidence in support of this possibility. A further survey of the effects of amino acids in the presence of a variety of substances that can be shown to block uptake may be necessary in order to decide among these alternatives.

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