



Carp horizontal cells in culture respond selectively to L-glutamate and its agonists

(neurotransmitter/intracellular recording/membrane resistance/cell culture)

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ABSTRACT Horizontal cells were enzymatically isolated from the carp retina and maintained in culture for 2–7 days. Cultured horizontal cells typically had resting membrane potentials of -50 to -70 mV and input resistances of 100 – 150 m Ω . The cells were treated with a number of neurotransmitter agents and their analogues. Significant responses were evoked only by 3,4-dihydroxyphenylalanine (dopamine), L-glutamate, and certain glutamate analogues. The responses to dopamine were inconsistent; most often, the membrane hyperpolarized and input resistances increased. However, highly characteristic responses to L-glutamate and its analogues, quisqualate and kainate, were observed in virtually all of the cells tested. The responses consisted of an initial graded depolarization accompanied by a resistance increase, followed in most cases by a prolonged (1- to 2-min) regenerative depolarization. The regenerative component of the response appears to be Ca^{2+} dependent, while the underlying graded potential may be due to a decrease in K^{+} conductance of the membrane.

Horizontal cells are second-order neurons believed to mediate lateral inhibitory effects in the outer plexiform layer of the retina (1–3). They respond to light with sustained graded potentials (4); *in situ*, these neurons have never been observed to generate action potentials. In the dark-adapted retina, horizontal cells are maintained in a partially depolarized state by neurotransmitter continuously released by the photoreceptors (5, 6). During illumination, horizontal cells hyperpolarize because transmitter flow from the receptors decreases. Some horizontal cells respond differentially to color, however; certain wavelengths hyperpolarize the cells whereas other wavelengths depolarize them (4, 7, 8).

The identity of the photoreceptor transmitters has not been established. It has been found that the acidic amino acids, L-glutamate and L-aspartate, depolarize horizontal cells in several species and block light-evoked responses (9–11). A recent study in the carp found L-aspartate to be more potent than L-glutamate in this regard and showed that an aspartate antagonist, α -amino adipic acid, blocked both the effects of the natural photoreceptor transmitter and exogenously applied aspartate on many horizontal cells (12). However, in most studies, high concentrations (5–50 mM) of either aspartate or glutamate were used to elicit responses in horizontal cells, and this has led several authors to question whether either of the substances could be the natural photoreceptor transmitter (9–11, 13).

The horizontal cells of fish are especially large, which has made them particularly useful for physiological and pharmacological studies. Fish horizontal cells can also be readily isolated by enzymatic dissociation of the retina, and this technique has been used for biochemical studies of these cells (14, 15). As

yet, few physiological studies of isolated horizontal cells have been reported (16–18), and no information concerning the pharmacological properties of isolated horizontal cells has been provided. Such studies are of particular significance because horizontal cells in the retina are tightly coupled electrically (19, 20). This coupling severely compromises measurements of input membrane resistance of these cells *in situ* and prevents accurate determination of the conductance changes that take place in the cells in response to neurotransmitter agents. We describe here methods for maintaining carp horizontal cells in culture and the responses of these cells to a number of transmitter agents. We have found that these cells respond in a highly specific and selective way to low concentrations (5–50 μM) of L-glutamate and its agonists and that these responses can be partially blocked by α -methylglutamate, an L-glutamate antagonist.

MATERIALS AND METHODS

Retinas were dissected from dark-adapted carp (*Cyprinus carpio*). The isolated retinas were incubated at 17°C for 1.25 hr in modified Leibowitz's tissue culture (L-15) medium containing dispase (25 mg/ml; Boehringer) or, on occasion, for 15 min, in cysteine-activated papain (0.35 mg/ml; Worthington). The modified L-15 medium consisted of standard L-15 (GIBCO) culture mixture to which was added distilled H_2O (280 ml/liter), D-glucose (450 mg/liter), Hepes (10 mmol/liter; Sigma), and penicillin/streptomycin solution (20 ml/liter; GIBCO). The retinas were washed twice with modified L-15 medium and gently broken up by repeated passage through a serological pipette. As the retinas were dissociated, the suspension was serially pipetted into eight 30-mm plastic tissue culture dishes (Falcon) containing ≈ 2.5 ml of modified L-15 medium/10% fetal bovine serum (GIBCO). The suspension was vigorously pipetted up and down in the last few dishes to cause greater dispersion of the cells. The last four dishes were saved and maintained for 1–7 days before recordings were made. The cultures were stored in an incubator at 17°C in 99% air/1% CO_2 .

Immediately after isolation, the horizontal cells were easily distinguished. Their structure was similar to that observed in a Golgi-impregnated preparation of flat-mounted retinas (21). Over a period of several days, however, this structure changed. The cells typically pulled in their processes and rounded up. However, the cells could be unequivocally identified as horizontal cells because of their large size and cytoplasmic appearance. Freshly isolated cells (1–6 hr old) had low resting potentials (10–20 mV) and did not give sizable responses to drugs, although qualitatively these responses were similar to those elicited from cells in culture for longer periods. Thus, most recordings made were from cells 2–4 days old.

The modified L-15 medium was replaced before recordings were made with oxygenated Ringer's solution (130 mM NaCl/

Abbreviation: dopamine, 3,4-dihydroxyphenylalanine.

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2.93 mM KCl/1.23 mM $MgCl_2$ /2.12 mM $CaCl_2$ /10 mM glucose/10 mM HEPES). Cells were viewed with an inverted phase-contrast microscope and impaled with micropipettes under visual control. The pipettes were filled with 4 M KAc and had resistances of 100–200 m Ω when measured in Ringer's solution. Responses were passed through a unity gain WPI 707 preamplifier (WPI Instruments, New Haven, CT) with low pass filtering (upper cutoff frequency, 3 KHz). The signals were viewed on a Tektronix 5A22N oscilloscope (Tektronix, Beaverton, OR) and recorded on a Brush 220 penwriter (Gould Instruments, Cleveland, OH). Constant-current depolarizing or hyperpolarizing pulses (5–10 pA) were passed through a bridge circuit for measurement of input resistances.

The test agents were dissolved in Ringer's solution and applied by pressure ejection through two triple-barrel micropipettes placed independently near the cell. The drug-containing pipettes were made by pulling fine-tipped pipettes, which were then broken and beveled. The final tips had openings of 10–15 μ m. The drug pipettes were positioned $\approx$ 100 μ m from the cell. The drug-containing Ringer's solution was ejected as a pulse lasting 0.5–1 sec by using a 0.2-ml Gilmont microsyringe (Gilmont Instruments, Great Neck, NY); 0.3 μ l was the standard amount ejected per pulse. We estimate that the test agents were diluted 1:2 to 1:5 before they reached the cell surface. All concentrations reported are those in the drug pipettes.

RESULTS

When horizontal cells maintained 2–4 days in culture were impaled, membrane potentials of -15 to -20 mV were typically recorded initially (Fig. 1a). However, over a period of 1–4 min, the membrane potential usually increased, until it reached a steady level of -50 to -70 mV. As the membrane potential hyperpolarized, the input resistance of the cell also increased, reaching a final value of 100–150 m Ω . After membrane potential and input resistance had stabilized, the responsiveness of the cells was usually tested by ejecting a 0.3- μ l pulse of Ringer's solution/75 mM KCl from one barrel of the pipettes containing the test substances. Most cells rapidly depolarized, with concomitant decrease in input resistance (Fig. 1b). This was followed by slow repolarization over a period of a few minutes. This concentration of KCl routinely depolarized cells by 30–50 mV. The application of 75 mM KCl thus also served for estimating the dilution of the test agents between drug pipettes and impaled cells.

A number of proposed neurotransmitter substances or transmitter agonists were tested on the cultured horizontal cells, including L- and D-aspartate, carbachol (an acetylcholine agonist), cysteine sulfonate, 3,4-dihydroxyphenylalanine (dopamine), γ -aminobutyric acid, L- and D-glutamate, glycine, kainate, ibotenic acid, N-methyl-D-aspartate, quisqualate, and taurine. All of these substances were tried on a minimum of five cells and at concentration ≥ 500 μ M. We found that only dopamine, L-glutamate, quisqualate, and kainate caused significant effects. Of these, dopamine elicited the least-consistent responses. Only $\approx 15\%$ of the cells responded to dopamine, with a 5–30 mV change in membrane potential and, in most cells, concentrations of 1–10 mM dopamine were required to elicit such responses. Approximately 10% of the cells hyperpolarized after dopamine application with a significant increase in input resistance (Fig. 1c), while 5% depolarized with a small decrease or no change in input resistance.

On the other hand, the application of single 0.5- to 1-sec pulses of Ringer's solution containing low concentrations of L-glutamate, quisqualate, or kainate to the cultured horizontal cells resulted in large (60–80 mV) long-lasting (1- to 2-min) depolarizing responses in 98% of the tested cells. Fig. 2a shows

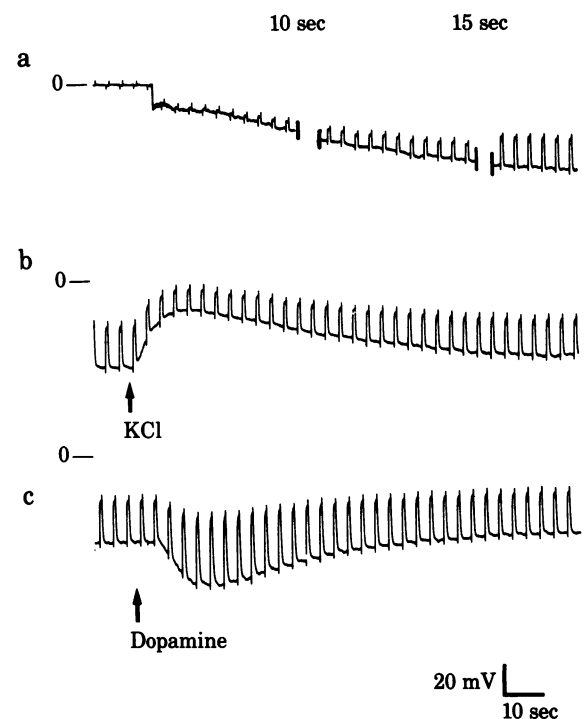


FIG. 1. Typical recordings from a horizontal cell maintained in culture for 2 days. (a) Initial penetration of the cell and subsequent slow increase in resting membrane potential. Depolarizing current pulses injected into the cell showed that, as the membrane potential developed, input resistance increased to an unusually high resting value (≈ 200 m Ω). Times given above the trace are breaks in the record. (b) Continuation of the record in a, illustrating the effect of application of 75 mM KCl. The cell was transiently depolarized by 35 mV and there was a decrease in input resistance. This was followed by a slow return of membrane potential and input resistance to resting levels. (c) Continuation of the record in b, illustrating the response of the cell to 10 mM 3,4-dihydroxyphenylalanine (dopamine). The response consisted of a transient hyperpolarization accompanied by a substantial increase in input resistance. Ascorbate was added to the Ringer's solutions containing dopamine to retard oxidation of the drug. Drug-application pulses ($\uparrow$) lasted 0.5 to 1 sec and typically had a vol of 0.3 μ l.

a record in which Ringer's solution/500 μ M L-aspartate, L-glutamate, and D-glutamate were applied to a cell. The L-aspartate and D-glutamate produced no significant responses; however, the L-glutamate rapidly depolarized the cell to almost 0 mV. The cell remained highly depolarized for ≈ 40 sec and then repolarized rather rapidly over the next 20–30 sec. The input resistance of the cell also showed characteristic changes. Initially, little change in overall input resistance occurred, but then input resistance gradually increased, becoming dramatically higher (by a factor of 4 or 5) during repolarization of the cell. During repolarization, while input resistance was high, the cell membrane often showed increased noise levels (see also Fig. 3b). After repolarization, input resistance and membrane noise returned to their initial resting levels.

Quisqualate and kainate induced similar large (60–80 mV) responses in cultured horizontal cells at concentrations as low as 10–30 μ M. Fig. 2b shows the effects on a cell of 50 μ M quisqualate and 200 μ M glutamate; the resulting responses were remarkably similar. In this cell, the repolarization phase was particularly rapid, which was characteristic of many of the cells studied. Of the three active agents studied, we found the order of potency to be quisqualate > kainate > glutamate, with quisqualate being ≈ 10 times more potent than glutamate.

If lower concentrations of glutamate or these agents were

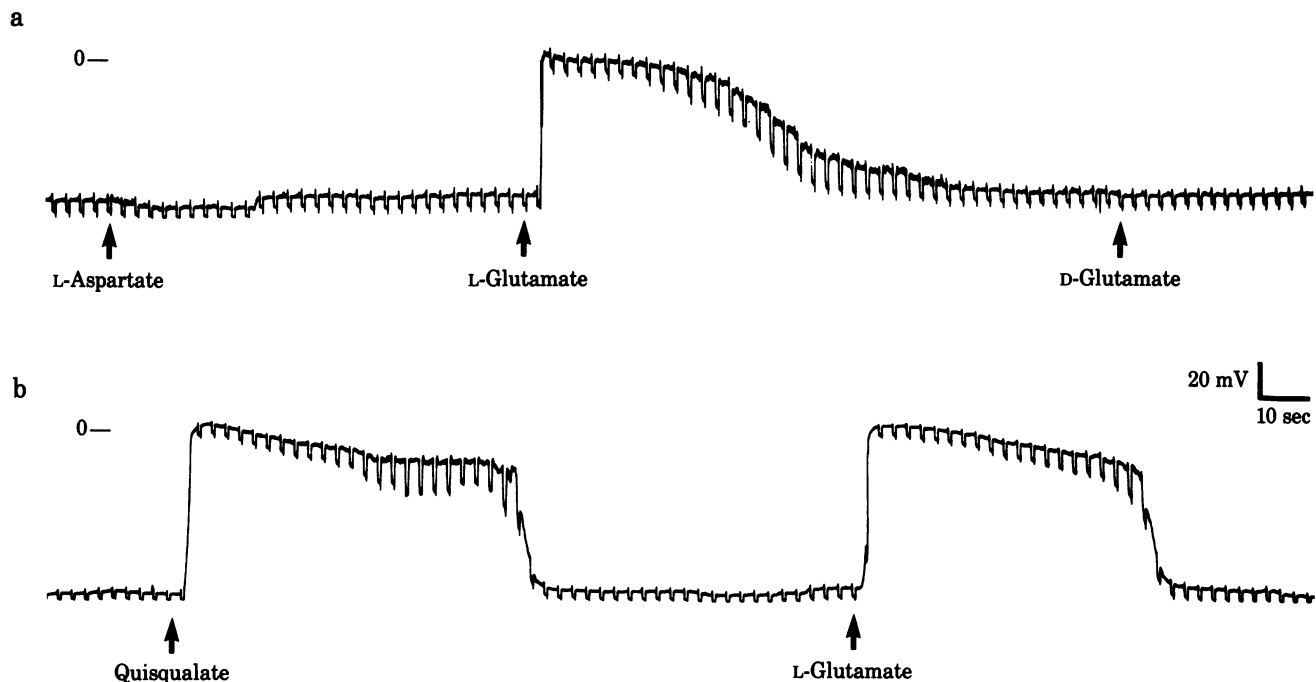


FIG. 2. (a) Effects of single 0.5- to 1-sec pulses of Ringer's solution containing 500 μ M L-aspartate, L-glutamate, or D-glutamate on cultured horizontal cells. Only L-glutamate evoked a significant response, which consisted of a large rapid depolarization lasting about 1 min. Note the significant increase in membrane resistance as the membrane potential returned to resting levels. The small hyperpolarization to aspartate was probably a movement artifact. (b) Responses to pulses of Ringer's solution containing 200 μ M L-glutamate or 50 μ M quisqualate in another cell. Cultured horizontal cells were typically more sensitive to quisqualate than to glutamate.

used (5–50 μ M), the response of the cell was variable. Sometimes no response was seen, but often a small (5–30 mV) transient depolarization occurred (Fig. 3a). These relatively small transient depolarizations were invariably accompanied by a significant increase in input resistance that appeared concomitant with the membrane potential change. If slightly more drug was applied, a maximal (60- to 80-mV) response was invariably evoked. Increasing the drug concentration further did not increase the amplitude of response but always increased the duration of the response. Responses as long as 3–5 min could occasionally be evoked by applications of 200–500 μ M quisqualate or kainate.

These observations suggested that the responses of the isolated horizontal cells to all but minimal concentrations of glutamate, quisqualate, and kainate consisted of at least two components: an initial graded depolarization accompanied by an input-resistance increase, followed by a very fast, usually long-lasting, depolarization of an all-or-nothing regenerative nature accompanied by an input-resistance decrease. Evidence for such a sequence of events was obtained on occasion using concentrations of drug at just the threshold for eliciting the regenerative component. For example, Fig. 3b shows the response of a cell to a pulse of Ringer's solution/6 μ M quisqualate. The initial depolarization of the cell was accompanied by a large input-resistance increase. The rapid depolarization that followed was accompanied by a substantial resistance decrease so that, at the peak of the response, the input resistance of the cell was similar to the resting input resistance. The regenerative component of the response lasted for $\approx$ 10 sec, at which point the membrane potential rapidly fell to an intermediate level, where the input resistance was once again substantially higher than that at rest. The response subsequently subsided with a return of input resistance to resting values.

The nature of the regenerative component evoked by glutamate or its analogues was examined by applying tetrodotoxin

(30–300 μ M) to cultured horizontal cells and subsequently applying 50 μ M quisqualate or 100 μ M glutamate to the cells. No effect of tetrodotoxin on the response was noted, indicating that a voltage-sensitive Na^+ channel was unlikely to be involved in the generation of this potential. On the other hand, decreasing the Ca^{2+} concentration in the Ringer's solution had profound effects on the response. For example, note the effect of 20 μ M quisqualate on a cell bathed in Ringer's solution lacking Ca^{2+} (Fig. 3c). Only a relatively slow transient depolarization of $\approx$ 45 mV was evoked in the cell and, for the duration of the response, the input resistance was substantially increased. In low- Ca^{2+} Ringer's solution, no prolonged regenerative component could be elicited unless very large quantities of stimulating drug were applied.

A number of glutamate and aspartate antagonists were tested for their ability to block the response to glutamate, quisqualate, or kainate. The agents tested included D- α -amino adipic acid, L-glutamic acid diethyl ester, DL- α -methylglutamate, and pimilic acid (22). Of these only, DL- α -methylglutamate had significant effects on the responses. The effect of two applications of 500 μ M DL- α -methylglutamate on the responses of a cell to 100 μ M quisqualate is shown in Fig. 4. After application of the antagonist, the amplitude of the response to quisqualate was unchanged; however, the duration of the response was dramatically decreased—from $\approx$ 70 to 10 sec. In such experiments, the duration of the response recovered 2–3 min after the last application of antagonist.

DISCUSSION

Horizontal cells from the carp retina that were maintained in culture for 2–4 days responded to two types of neurotransmitter substances. About 15% of the cells responded to dopamine, a catecholamine, while virtually all of the cells responded to L-glutamate, an acidic amino acid, or some of its analogues. It is known that many of the horizontal cells in carp receive two syn-

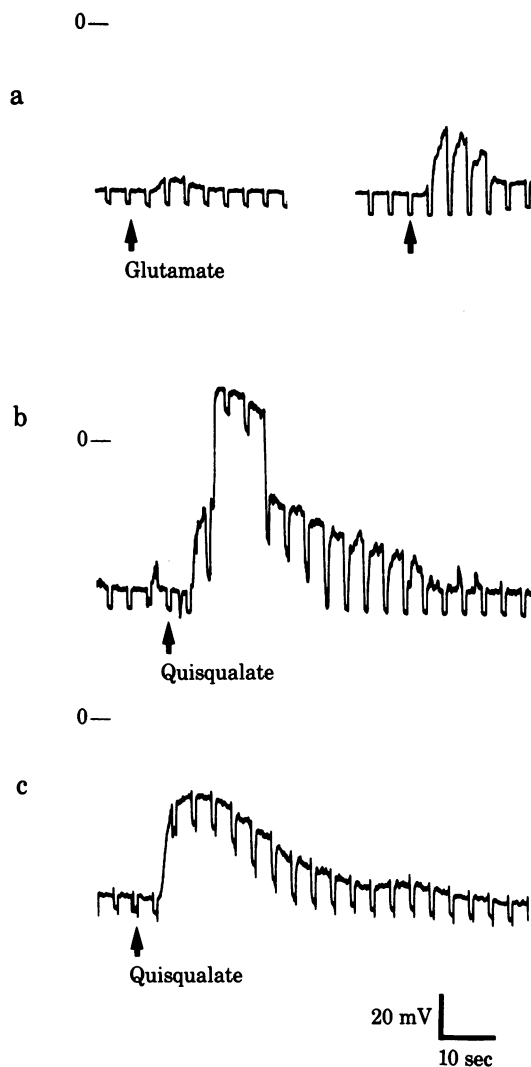


FIG. 3. (a) Two responses of a cultured horizontal cell to 50 μ M glutamate. There was evoked typically, as here, variably sized depolarizations accompanied by large increases in input resistance. (b) Response of a cell to an amount (6 μ M) of quisqualate just above threshold for the regenerative component. There was an initial depolarization and a resistance increase, followed by a relatively brief all-or-none response. (c) Response of a cell to 20 μ M quisqualate in Ringer's solution lacking Ca^{2+} . The depolarization evoked was relatively slow and no regenerative component occurred, even though the cell was depolarized >40 mV.

aptic inputs—one from the interplexiform cells, which use dopamine as their neurotransmitter (23), and the other from photoreceptors which, it has been proposed, may use glutamate or aspartate as their neurotransmitter (12, 13). However, the responses of the cultured cells to both kinds of agent were not as expected, based on earlier results obtained from studies on carp horizontal cells *in situ*. For example, in the intact retina, dopamine depolarizes most of the cells on which it has effects (24). It does hyperpolarize a small percentage of cells (10–20%) (24–26), but we have found that about two-thirds of the cultured cells that respond to dopamine do so by hyperpolarizing. Furthermore, in the intact retina, the majority of recorded horizontal cells respond to dopamine while, in culture, only $\approx 15\%$ of the cells were dopamine sensitive.

There is evidence, however, that the horizontal cells in carp that connect exclusively to rods do not have dopaminergic input (23) and that the membrane potential of horizontal cells that respond differentially to color (C cells) is not usually affected

by dopamine (24). It is possible, therefore, that the rod or type C horizontal cells (or both) survive preferentially in culture and that this is the reason for our findings. As yet, we cannot differentiate the various kinds of horizontal cells once they have been in culture for more than a day.

Another possibility is that dopamine does not directly affect the membrane potential of most horizontal cells *in situ*. For example, it is known that dopamine activates adenylate cyclase in many carp horizontal cells, which results in the synthesis of cAMP in the cells (27). If the cAMP served to modify the coupling between certain horizontal cells (25, 28), the membrane potential of many of the cells might alter because of changes in input resistance. In isolated cells, on the other hand, no change in coupling could occur and no such potential changes would be observed.

The finding that virtually all of the horizontal cells in culture respond dramatically to L-glutamate and its analogues, but not to aspartate, was also unexpected. Previous work had shown that, in the intact retina, L-aspartate is ≈ 50 times more effective than L-glutamate in depolarizing the cone-related type L horizontal cells (12). Cone-related type C horizontal cells are also depolarized more effectively by L-aspartate than by L-glutamate, while no information is currently available concerning the pharmacological specificity of rod horizontal cells *in situ*. However, a recent study has indicated that rod photoreceptors in goldfish take up L-glutamate 500 times more effectively than they take up L-aspartate (29), which suggests that rods in Cyprinids may use glutamate as their transmitter. The red and green cones in goldfish take up L-glutamate and L-aspartate about equally, which indicates that either substance could be used as a transmitter by these cells. It is possible that the intact Cyprinid retina has much more effective mechanisms in general for the uptake of L-glutamate than for L-aspartate. Thus, the increased sensitivity of the cone-related horizontal cells for exogenously applied L-aspartate as compared with L-glutamate might reflect a differential uptake system in the retina for these two amino acids. However, it is difficult to understand why the cultured cells seldom responded at all to L-aspartate, and never with a large response, and why α -amino adipic acid, which blocks both the natural cone photoreceptor transmitter and exogenously applied aspartate in the intact retina, appears to be without effect on cultured cells. Again, it is possible that rod horizontal cells preferentially survive in culture. Another possibility is that enzymatic dissociation and culturing of cells somewhat alters the properties of the membrane receptors.

It is of considerable interest that the isolated horizontal cells responded so selectively to L-glutamate. In virtually all preparations that show sensitivity to the excitatory acidic amino acids, there are some potency differences between glutamate and aspartate and between the D- and L-forms; however, all of these agents usually produce effects (30, 31). From the cultured horizontal cells, we have not been able to evoke significant responses with D-glutamate or L- or D-aspartate, even at concentrations up to 500 μ M. L-glutamate, on the other hand, induced significant responses at 30–50 μ M. Furthermore, the actual concentration of L-glutamate inducing responses in these cells was probably $\frac{1}{2}$ to $\frac{1}{3}$ the nominal value because of the dilution of the test agents between the drug-containing pipettes and the cells. It thus appears that the receptors for L-glutamate on cultured horizontal cells are much more specific than those of other neurons.

Another surprising result was the nature of the cultured horizontal cell response to L-glutamate and its analogues. All but the lowest concentrations of these agents induced a depolarization of the cells of 60–80 mV that often lasted for 1 to 2 min (Fig. 2). This response appeared to be made up of at least two

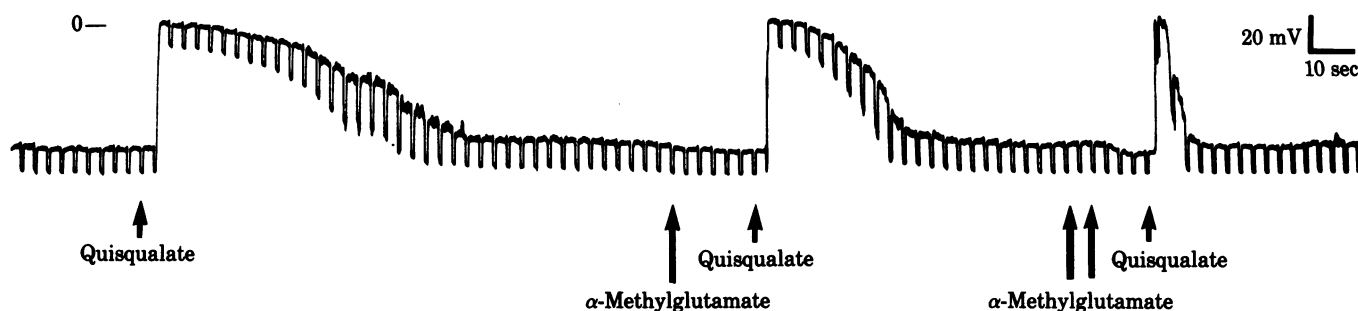


FIG. 4. Antagonism by 500 μ M α -methylglutamate of the responses of a cultured horizontal cell to 100 μ M quisqualate. On the left is shown the control quisqualate response. After recovery, a single pulse of Ringer's solution/ α -methylglutamate was applied to the cell followed by the same dose of quisqualate. Although the amplitude of the response was unaffected, the duration was less than half. After recovery, a second application, consisting of two consecutive pulses of Ringer's solution/ α -methylglutamate, resulted in a 90% decrease in response duration.

components, an initial depolarization accompanied by a resistance increase followed by a prolonged regenerative response accompanied by a resistance decrease. The regenerative component was found to be sensitive to the presence of Ca^{2+} in the Ringer's solution but not to the presence of tetrodotoxin. These data suggest that the regenerative component of the response is an extremely prolonged Ca^{2+} action potential (17, 18).

The initial depolarizing component of the response of the cultured horizontal cells to L-glutamate and its analogues appeared to be insensitive to alterations in the concentration of Ca^{2+} in the bathing Ringer's solution (Fig. 3c). Preliminary experiments suggested that varying the Cl^- concentration in the Ringer's solution also has little effect on the response. This depolarization is accompanied by a substantial input-resistance increase, and thus a plausible explanation for the response is that L-glutamate and its analogues cause a decrease in K^+ conductance of the cells.

Much more work is needed to substantiate the above ideas and to analyze the membrane potential and complex input-resistance changes that occur in cultured horizontal cells in response to glutamate and its analogues. As noted above, horizontal cells have not as yet been shown to generate regenerative potentials *in situ*. Thus, the significance of the regenerative response of cultured horizontal cells to L-glutamate and its analogues remains an open question. On the other hand, the initial graded depolarization observed in these cells in response to these agents is similar to that expected from the action of the naturally occurring photoreceptor transmitter, and this response may represent the fundamental event occurring in many horizontal cells on stimulation by the photoreceptor transmitter.

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