INTRODUCTION

Glutamate has been recognized as an important intercellular signal molecule for neurotransmission (Tanabe et al., 1992; Wo and Oswald, 1995; Nakanishi, 1992). Perhaps not as well appreciated is that glutamate also serves as an important environmental cue for many different organisms. Lobsters (Carr and Derby, 1986; Fine-Levy et al., 1987), fish (Caprio et al., 1993), and mammals including humans (Yamaguchi, 1987) can detect, that is, taste and/or smell, glutamate among other amino acids. Mammalian taste has the added interesting aspect of synergism of glutamate with 5′ ribonucleotides, known as ‘umami’ taste (Ugawa and Kurihara, 1994; Faurion, 1991). Therefore, it is not unusual that paramecia detect and are attracted to glutamate, which could very well indicate that bacteria, their food, are at hand. Preston and Usherwood (1988) described attraction to glutamate and specific binding sites on cilia. Likewise, in our studies, glutamate is an attractant to P. tetraurelia, albeit at higher concentrations than used by Preston. The attractant responses are consistent and robust, as shown in this paper.

Attractants of Paramecium tetraurelia tend to be small molecules that probably indicate the presence of bacteria. Not only glutamate, but also acetate, extracellular cyclic AMP, biotin, folate, and NH₄Cl are among the stimuli that we have identified (Van Houten, 1978; Van Houten and Preston, 1987; Bell and J. L. Van Houten, unpublished observations). They have in common that they all hyperpolarize cells and cause relatively fast and smooth swimming. Such swimming behavior, with adaptation, causes the accumulation of cells by a biased random walk (Van Houten, 1990).

There are at least three different signal transduction pathways, or at least alternate versions of pathways, and the attractant stimuli acetate, NH₄Cl, and glutamate each stimulate a different pathway. The pathways epitomized by acetate and glutamate are cell surface receptor-mediated, and that epitomized by NH₄Cl involves intracellular pH modulation probably without receptor involvement.

Hyperpolarization by altering membrane potential (Vm) through lowering extracellular K levels has long been demonstrated to elevate Paramecium cyclic AMP levels, and both hyperpolarization and cyclic AMP correlate with increased ciliary beating and rapid swimming (Machemer, 1989; Pech, 1995). Here we show that hyperpolarization of the cells induced not only by altering extracellular K, but also by receptor-mediated processes raises intracellular cAMP levels. What is even more interesting is that this second messenger response is specific to the glutamate pathway; other attractant stimuli that hyperpolarize do not appear to elevate cAMP.
We also report here that protein kinase inhibitors interfere specifically with attraction to glutamate, which is consistent with the demonstration in this paper that glutamate alone among the attractants tested increases intracellular cyclic AMP. Additionally, we demonstrate that the increase in cyclic AMP occurs rapidly enough to be consistent with a role in the signal transduction pathway as opposed to or perhaps in addition to a role in slower processes such as adaptation. To accomplish the rapid kinetic measurements, we utilized a custom rapid mixing apparatus in order to rapidly stimulate and keep whole cells intact (Knoll et al., 1991, 1992a,b). Other rapid mixing protocols would require broken cells or membranes.

MATERIALS AND METHODS

Culturing

Stocks of Paramecium tetraurelia 51-S (sensitive to killer) were grown in wheat grass medium as previously described (Sasner and Van Houten, 1989). Cells for assays of cyclic nucleotides were grown in a supplemented wheat medium (described by Wright et al., 1992).

Slow time-course assays of cyclic AMP

In general, early stationary stage cells were harvested by centrifugation and washed by centrifugation in basic buffer: 1 mM CaCl₂, 1 mM MOPS (3-[N-morpholino] propane sulfonic acid), 1 mM IBMX (1-isobutyl-1-methyl xanthine), 0.01 mM EDTA with various salts indicated, adjusted to pH 7.2 with Tris base. (IBMX is added to decrease phosphodiesterase activity. We have previously shown that IBMX does not interfere with the cAMP or cGMP assay by comparing standard curves with and without IBMX.) After the last wash, the cells were resuspended in control solution (basic CaCl₂/MOPS/IBMX/EDTA buffer with KCl or NaCl appropriate for that particular control). After 30 minutes, aliquots of the cells were pelleted, and the pellet was transferred by Pasteur pipet and rapidly dispersed into the same control buffer or the basic buffer with stimulus. For example, a pellet of approx. 10⁵ cells from control 5 mM KCl in buffer would be transferred to buffer with 5 mM KCl as control for mechanical stimulation or to 5 mM K-L-glutamate as test stimulus.

For a time course beginning at 30 seconds, EDTA was added to aliquots of cells (10 mM EDTA, final concentration, pH 7.2) to stop cyclic AMP production during a subsequent 2 minute centrifugation. (We determined that this method of stopping the reaction is comparable to rapidly mixing the cells with acid.) The pellet was then homogenized in 10 mM EDTA buffer; two aliquots were taken for protein assays (Pierce Assay using bovine serum albumin as standard). 1 ml was boiled for 4 minutes, and centrifuged at 10,000 g for 2 minutes. 50 µl duplicate aliquots of the supernatant were assayed by the Amersham RIA assay kit and ³H-cyclic AMP counts were determined in 3 ml Ready Protein scintillation fluid in a Beckman 2000 scintillation counter.

For time points at 1 second to 30 seconds, cells were not pelleted and transferred but rather were mixed with either more control buffer or stimulus buffer, and rapidly stopped by addition of trichloracetic acid (TCA) to the cells (6% final concentration). Cells were then vortexed for 1 minute after protein samples were removed. Cell debris was removed by centrifugation at 300 g for 1 minute and the supernatant was neutralized by washing with water-saturated ether several times until a neutral pH was achieved. Alternatively, the mixture was neutralized by the addition of concentrated 1.5 M KOH and 60 mM Hepes buffer to pH 7.0 and centrifuged to remove debris. Duplicate 50 µl aliquots of the aqueous layer were assayed by the Amersham RIA kit.

The alternative mixing method was used to simulate the rapid kinetic measurements below, in which cells in control buffer were rapidly mixed with more control buffer or with stimulus. This has an impact on the degree of hyperpolarization that can be achieved in the hyperpolarization control.

Assays were done three times in duplicate and data points were an average of 3 ± one standard deviation or error of the mean, as specified.

Rapid kinetic measurements

For the rapid kinetic time course, a rapid mixing apparatus was used (Knoll et al., 1991, 1992a,b). Cells were prepared and washed as above and incubated for 30 minutes in the control buffer. 2 ml of cells (10⁵/ml) cells were rapidly mixed with either 2 ml of control buffer or with 2 ml of stimulus buffer. Cells were then rapidly stopped by quenched-flow when they were shot into 20% ice-cold perchloric acid after 30, 100, 300, 1,000 or 5,000 mseconds. The mixture was then homogenized and neutralized with cold 1.5 M KOH/60 mM Hepes to pH 7.0. The mixture was left on ice until the salt precipitated. Half of the supernatant was removed, dried, and stored at –70°C. For assays of cyclic AMP, the sample was dissolved in 0.5 ml of 4 mM Tris/EDTA buffer (from the Amersham kit) for 2 hours, centrifuged at 10,000 g for 2 minutes. 50 µl duplicate aliquots were removed for assays.

Assays of cyclic GMP

Preparation of cells for assays of intracellular cyclic GMP were similar to those for cyclic AMP, with the exception that 100 µl duplicate samples of supernatant were taken for assays of cyclic GMP by the Amersham kit protocol. Positive controls also differed, as described in the Results.

T-maze assays of chemoreactivity

Attraction to glutamate was assayed by T-mazes (Van Houten et al., 1982). Cells were distributed in glass stopcocks and the numbers of cells in the arm with test buffer relative to those in the arm with control buffer were used to calculate an index of chemokinesis. Indices >0.5 indicate attraction; <0.5 indicate repulsion. To test for the effects of kinase inhibitors H8 ([2(methylamino)ethyl]-5-isouquinolinesulfonamide) (Sigma) and H7 [1-[5-(isouquinolinesulfonyl)-2-methylpyperazine] (Sigma), cells were washed in 5 mM KCl in chemokinesis buffer (1 mM Ca(OH)₂, 1 mM citric acid, Tris base to pH 7.1 and indicated salt, e.g. 5 mM KCl), incubated in 2 mM H8 or H7 in 5 mM KCl chemokinesis buffer for 30 minutes and immediately assayed in T-mazes for attraction to glutamate or acetate. To assay chemoreponse to NH₄Cl, the control solution was 5 mM NaCl in chemokinesis buffer and the test solution was 5 mM NH₄Cl in chemokinesis buffer. In the T-mazes, solutions in the arms of the T-maze were matched for pH and salts and only one pair of ions, e.g. Cl⁻ vs acetate (OAc⁻), differed between the arms of the T-maze.

RESULTS

L-glutamate stimulation increases intracellular cyclic AMP

Cells stimulated by 5 mM K-L-glutamate show, in relatively slow time courses beginning at 30 seconds, increases in cyclic AMP that decline over time in the stimulus, while cells in control solutions with 5 mM KCl maintain a steady basal level of cyclic AMP (Fig. 1). It has long been known that cells hyperpolarized by changing extracellular cations will increase Paramecium intracellular cyclic AMP (Bonini et al., 1986; Schultz et al., 1984; Majima et al., 1986; Pech, 1995; Schultz and...
Glutamate chemoresponse

Klumpp, 1993). In Fig. 1 there is shown a hyperpolarization control, in which cells are transferred from 10 mM KCl to 1 mM KCl. The cells transferred from 10 mM KCl to 10 mM KCl serve as the control. The hyperpolarization by manipulation of K concentrations results in an increase in cyclic AMP that is in agreement with published observations (Bonini et al., 1986; Schultz et al., 1984).

Other stimuli do not increase intracellular cyclic AMP

Using the slower kinetic protocols, cells treated with glutamate or other stimuli for 30 seconds were assayed for intracellular cyclic AMP (Fig. 2). At these single time points, there is no evidence of increases in cyclic AMP in K-acetate (relative to KCl control) or NH4Cl (relative to NaCl control) (Fig. 2B), although there is a 2- to 3-fold increase in cAMP in cells stimulated by glutamate (Fig. 2A).

Another stimulus, inosine monophosphate (IMP), decreases cyclic AMP by approximately 50% at 30 seconds (Fig. 2C). IMP is a repellent that depolarizes the cells and displaces approximately 60% of 3H-glutamate binding to cells (Yang and Van Houten, 1993). L-glutamate inhibits IMP repulsion but not vice versa (Yang and Van Houten, 1993). Depolarization by high KCl alone also decreases Paramecium cyclic AMP levels by approximately 50% (Fig. 2C), in agreement with observations from other laboratories (Bonini et al., 1986; Schultz et al., 1984; Majima et al., 1986). The increase in KCl in the depolarization control would be expected to depolarize the cells approximately 10 mV and IMP to depolarize cells approximately 8-10 mV (unpublished results).

A glutamate agonist, quisqualate, increases cyclic AMP significantly (by t-test) (Fig. 2D). Quisqualate was tested because it alone, among glutamate analogs tested in 3H-glutamate binding assays, displaced glutamate and interfered with glutamate chemoresponse (Yang and Van Houten, 1993; Yang, 1995).

L-glutamate stimulation increases intracellular cyclic AMP rapidly

In the slow time-course experiments, cells were transferred in a pellet to the stimulus or control solutions. In order to stimulate and stop stimulation more rapidly, we turned to a rapid mixing and quenching protocol. The cells in this experimental design cannot be moved from pure control to pure...
stimulus solutions, because mixing of cells with stimulus solution is required. For example, in the experiment in Fig. 1 in which a pellet is moved from control solution to the same (control) or stimulus solution, the control is 5 mM KCl (squares) and stimulus is 5 mM K-L-glutamate (diamonds). In Fig. 3, cells are in control solution 10 mM KCl and are mixed with 5 mM K-L-glutamate, in the latter to achieve a 5 mM L-glutamate with K+ unchanging at 10 mM. In Figs 1 and 3, the cells are stimulated with the same concentration of glutamate, but in different ionic backgrounds. Also, the hyperpolarization control in Fig. 1 is achieved by moving cells from 10 mM KCl to 1 mM KCl. In Fig. 3, cells experience a dilution by mixing from 10 mM KCl to 5 mM KCl because this is the limit of concentration change that can be achieved by mixing 1:1, and thus the degree of hyperpolarization is not as great in Fig. 3 as in Fig. 1. This is a necessary consequence of mixing as opposed to moving a pellet of cells to a new solution to begin a time course.

Regardless of the difference between the two experimental paradigms for Fig. 1 and Fig. 3, the experimental results can be compared because the levels of cAMP in cells in control 10 mM KCl are similar in both experimental situations. Additionally, it is evident in both Figs that cAMP levels increase in cells stimulated with glutamate or hyperpolarized by lowering KCl. The absolute levels of elevated cAMP differ between Figs 1 and 3, in part because in Fig. 1 we are witnessing only the declining phase of the cAMP stimulation and in part because the background ionic conditions are different as described above.

Fig. 3 demonstrates that cells show increases in cyclic AMP by 30 mseconds in glutamate, and a peak increase by 200 mseconds. The levels in intracellular cyclic AMP decline after this time although the cells remain in L-glutamate.

As discussed, the hyperpolarization control in Fig. 3 differs from that in Fig. 1. Cells are incubated in 10 mM KCl control and mixed with buffer devoid of KCl to achieve 5 mM KCl. Cyclic AMP appears to increase by 30 mseconds and continues to increase from that time, but more slowly than for the L-glutamate treated cells. Nonetheless, there is an increase in cyclic AMP by hyperpolarization by lowering K alone. The 10 mM KCl control serves as control for both the hyperpolarization in 5 mM KCl and in the stimulus 5 mM L-glutamate.

**Table 1. Effects of H7 and H8 on chemorespons**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>K-OAc</th>
<th>K-glutamate</th>
<th>NH4Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>0.75±0.09</td>
<td>0.68±0.11</td>
<td></td>
</tr>
<tr>
<td>H8</td>
<td>0.77±0.13</td>
<td>0.53±0.19*</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>0.71±0.07</td>
<td>0.78±0.06</td>
<td></td>
</tr>
<tr>
<td>H8</td>
<td>0.43±0.13*</td>
<td>0.81±0.04</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>0.74±0.10</td>
<td>0.76±0.08</td>
<td>nd</td>
</tr>
<tr>
<td>H7</td>
<td>0.70±0.14</td>
<td>0.57±0.21*</td>
<td>nd</td>
</tr>
</tbody>
</table>

Cells were incubated in control chemokinesis buffer appropriate for the T-maze or in H7 or H8 in the same buffer for 15-25 minutes and tested in T-mazes. nd, not determined.

T-maze results of tests of 5 mM K-OAc or 5 mM K-L-glutamate vs 5 mM KCl, and 5 mM NH4Cl vs 5 mM NaCl.

Data are averages of 6–12 T-mazes ± one s.d. (representing at least 3 populations of cells). Asterisk indicates statistically significant differences from controls (buffer pretreatment) by the Mann-Whitney U test.

**Protein kinase inhibitors specifically affect chemoresponse to L-glutamate**

The rapid kinetic results imply that cyclic AMP can, indeed, participate in stimulus transduction of L-glutamate. To further probe the role of cyclic AMP in signal transduction, we treated cells with protein kinase inhibitors that are known to affect protein kinase A in addition to other kinases (Hidaka et al., 1984). *Paramecium tetraurelia* has been shown to have protein kinase A (Hochstrasser and Nelson, 1989) and there is the distinct possibility that this enzyme participates in chemosensory signal transduction. Therefore, cells treated with H8 and H7 were tested for chemoresponse to L-glutamate and two stimuli, acetate and NH4Cl, that did not appear to affect cyclic AMP levels. Cells treated with H8 or H7 are selectively defective in attraction to L-glutamate and appear to be normally attracted to K-OAc and NH4Cl (Table 1).

**Cyclic GMP is not affected by L-glutamate**

Cells stimulated with L-glutamate do not statistically significantly change intracellular cyclic GMP levels (Table 2). Depolarization with BaCl2 increases cyclic GMP (Table 2), as expected from and consistent with the results of others (Bonini et al., 1986; Majima et al., 1986; Pech, 1995; Schultz and Klump, 1993; Schultz et al., 1986; Schultz and Schade, 1989). IMP, which depolarizes the cells, might be expected likewise to increase intracellular cyclic GMP. In rapid kinetic measurements, or slower time courses, IMP increases intracellular cyclic GMP only slightly, but statistically significantly (by the Student’s t-test). We found no evidence that acetate affects cyclic GMP at 30 and 100 mseconds (20.5 and 19.0 pmoles cyclic GMP/mg protein, compare to Table 2 KCl controls at 30 and 100 mseconds).
Table 2. Cyclic GMP (pmoles/mg protein)

<table>
<thead>
<tr>
<th>Control</th>
<th>Stimulus</th>
<th>100 msec</th>
<th>1 sec</th>
<th>5 seconds</th>
<th>10 seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>KCl</td>
<td>15.7±0.7</td>
<td>19.8±0.3</td>
<td>18.2±1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KCl</td>
<td>20.1±0.3</td>
<td>18.2±2.7</td>
<td>18.2±1.8</td>
<td></td>
</tr>
<tr>
<td>(B)</td>
<td>KCl</td>
<td>21.2±2.0</td>
<td>21.5±1.0</td>
<td>23.4±0.8</td>
<td>28.6±0.4</td>
</tr>
<tr>
<td></td>
<td>K2IMP</td>
<td>39.0±0.8</td>
<td>36.4±1.8*</td>
<td>40.7±1.9*</td>
<td>28.7±0.7</td>
</tr>
<tr>
<td>(C)</td>
<td>Buffer</td>
<td>14.9±3.5</td>
<td>14.4±1.8</td>
<td>14.4±1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BaCl2</td>
<td>36.7±6.2</td>
<td>45.6±9.6</td>
<td>64.7±9.3</td>
<td></td>
</tr>
</tbody>
</table>

(A) Cells in 10 mM KCl are mixed 1:1 with more 10 mM KCl or with 10 mM K-glutamate. Data are averages of 4 experiments in duplicate ± one s.e.m.
(B) Cells in 2 mM KCl are mixed with more 2 mM KCl or with 1 mM K2IMP yielding a stimulus solution of 0.5 mM K2IMP + 2 mM KCl. Data at 1 and 5 seconds are averages of 4 experiments in duplicate ± s.e.m.; at 100 ms and 10 seconds, data are averages of 2 experiments in duplicate ± range.

The effects of L-glutamate on intracellular cyclic AMP are rapid, robust and reproducible implying a possible role for cyclic AMP in the signal transduction pathway. To probe this idea in preliminary experiments, inhibitors of kinases were used to treat cells before assays of chemoresponse behavior. Responses to L-glutamate were specifically inhibited compared to responses to K-acetate and NH4Cl. While it is understood that inhibitors such as H7 and H8 will have non-specific effects or effects on kinases other than protein kinase A, it is noteworthy that the effects of these inhibitors seem to be stimulus-specific for Paramecium chemoresponse. If there were general effects on ciliary motility, one might expect that all of the stimuli would be affected instead of only L-glutamate.

The coupling between putative L-glutamate binding sites (Yang and Van Houten, 1993) and adenylyl cyclase or phosphodiesterase is not known at this time. One scenario is that the coupling of the glutamate receptor to the adenylyl cyclase is through the hyperpolarization. The cyclic AMP second messenger would then have effects on axononal function and ciliary motility through the activity of specific kinases (see Pech, 1995, for a review). The adenylyl cyclase of Paramecium tetraurelia is thought to be responsive to membrane potential and perhaps is an ion channel itself (Schultz et al., 1992).

The scenario above does not explain the failure of some chemical stimuli that are known to hyperpolarize to increase cyclic AMP levels. Clearly these hyperpolarizations are not sensed by the adenylyl cyclase (or phosphodiesterase) or the order of events in the scenario is wrong. Perhaps the glutamate receptor couples to an adenylyl cyclase and intracellular cyclic nucleotides directly gate an ion channel, for which there is no evidence yet in Paramecium. There are reports of vertebrate metabotropic L-glutamate receptors coupled to adenylyl cyclase, presumably through G proteins that activate the cyclase (Winder and Conn, 1992; Winder and Conn, 1993). However, there is scant evidence for trimeric G proteins in Paramecium at this time (Forney and Rodkey, 1992; Fraga and Hinrichsen, 1994). Alternatively, cyclic AMP could change membrane potential indirectly through the activation of protein kinase A and its action on a substrate such as a channel or ion pump.

Cyclic nucleotides have been found to influence ciliary beating patterns in Paramecium and the levels of the cyclic nucleotides have been shown to be related to membrane...
potential changes (reviewed by Pech, 1995). In general hyperpolarization (for example by lowering KCl), increased intracellular cAMP levels and increased ciliary beating frequency are all correlated. Depolarization (for example achieved by increasing KCl or BaCl2), decreased cAMP, increased cGMP and slow ciliary beating are also correlated. However, there is no consensus as yet for the cause and effect order for the cyclic nucleotide levels and membrane potential changes, but both contribute to ciliary beat pattern (see Pech, 1995). The interesting aspect for the study of chemical stimuli is the imperfect correlation that we have found between hyperpolarization by chemical stimuli and elevation of intracellular cAMP.

In summary, there appear to be at least three pathways for chemosensory signal transduction in Paramecium tetraurelia. Glutamate initiates a pathway that includes cyclic AMP as a second messenger; acetate and NH4Cl initiate pathways that do not include cyclic AMP as a second messenger, at least not on the same time course, yet all three stimuli hyperpolarize the cells. The cyclic nucleotide levels rise rapidly enough after glutamate stimulation to be part of the signal transduction pathway. Studies with inhibitors of protein kinases that include those stimulated with inhibitors of protein kinases that include those stimulated with inhibitors of protein kinases that include those stimulated with inhibitors of protein kinases.

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REFERENCES


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