Selective Enhancement and Suppression of Frog Gustatory Responses to Amino Acids

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ABSTRACT Properties of the receptor sites for L-amino acids in taste cells of the bullfrog (*Rana catesbeiana*) were examined by measuring the neural activities of the glossopharyngeal nerve under various conditions. (*a*) The frogs responded to 12 amino acids, but the responses to the amino acids varied with individual frogs under natural conditions. The frog tongues, however, exhibited similar responses after an alkaline treatment that removes Ca^{2+} from the tissue. The variation in the responses under natural conditions was apparently due to the variation in the amount of Ca^{2+} bound to the receptor membrane. (*b*) The responses to hydrophilic L-amino acids (glycine, L-alanine, L-serine, L-threonine, L-cysteine, and L-proline) were of a tonic type, but those to hydrophobic L-amino acids (L-valine, L-leucine, L-isoleucine, L-methionine, L-phenylalanine, and L-tyrptophan) were usually composed of both phasic and tonic components. (*c*) The properties of the tonic component were quite different from those of the phasic component: the tonic component was largely enhanced by the alkaline treatment and suppressed by the acidic treatment that increases binding of Ca^{2+} to the tissue. Also, the tonic component was suppressed by the presence of low concentrations of salts, or the action of pronase E, whereas the phasic component was unchanged under these conditions. These properties of the phasic component were quite similar to those of the response to hydrophobic substances such as quinine. These results suggest that the hydrophilic L-amino acids stimulate receptor protein(s) and that the hydrophobic L-amino acids stimulate both the receptor protein and a receptor site similar to that for quinine. (*d*) On the basis of the suppression of the responses to amino acids by salts, the mechanism of generation of the receptor potential is discussed.

INTRODUCTION

The rat and the frog have been widely used as experimental animals for studying vertebrate gustatory receptor mechanisms because they are easily available and respond well to various chemical stimuli. To stimulate their taste receptors, salts, acids, sugars, and quinine have been used as representative stimuli for fundamental taste qualities. L-Amino acids are contained abundantly in foods and hence must be important gustatory stimuli, but few studies have been performed on the responses to amino acids in these animals

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(Halpern et al., 1962; Tateda, 1967). It is not certain whether amino acids stimulate any of the receptors for the fundamental taste qualities or whether there are specific receptors for amino acids. It is also not known how amino acids induce the receptor potential in the taste cell.

In the present study, we found that the frog gustatory receptors respond to various L-amino acids. However, there are rather large individual variations in the frog gustatory responses to L-amino acids: some frogs respond well to L-amino acids, but others respond poorly. We suggest that the responses of the frog to L-amino acids are highly controlled by the membrane-bound Ca^{2+} and that the variation in the frog taste response could be attributable to the difference in the amount of Ca^{2+} bound to the receptor membrane. The responses of the frog to L-amino acids are also greatly affected by the presence of Na, K, and Mg salts. On the basis of the results of our experiments, we discuss the mechanism of generation of the receptor potential in response to L-amino acids. Amino acids are roughly classified into hydrophobic and hydrophilic molecules. Our findings indicate that hydrophobic L-amino acids stimulate a receptor site similar to that for bitter stimuli as well as the receptor protein(s) for L-amino acids, whereas hydrophilic L-amino acids stimulate only the receptor protein(s).

MATERIALS AND METHODS

Measurements of Gustatory Responses

Adult bullfrogs, *Rana catesbeiana*, were used in the present experiments. Animals were anesthetized with urethane (0.3 g/100 g body wt). The exposure of the glossopharyngeal nerve and the recording of the nerve activities employed here are carried out as described in a previous paper (Kashiwagura et al., 1980). The nerve impulses were summated with an electronic integrator with a time constant of 0.3 s.

Stimulating solutions were applied to the tongue for ~30 s with a flow rate of ~2 ml/s. The tongue was adapted to distilled water for 10 min before the L-amino acid dissolved in distilled water was applied. When the stimulating amino acid solution contained salts, the tongue was preadapted for 10 min to a solution containing the same concentration of the salts as the solvent. The stimulating solutions were prepared by dissolving L-amino acids of analytical grade in distilled water or salt solutions. pH values of solutions of neutral amino acids were ~6.0, and those of the solutions of acidic or basic amino acids were adjusted to ~6.0 with NaOH or HCl, respectively.

After each stimulation, the tongue was washed wth adapting solutions and bathed in Ringer's solution.

Alkaline and Acidic Treatments of the Tongue

The frog tongue was treated with an alkaline solution (2.5 mM $NaHCO_3$-Na_2CO_3 buffer, pH 10.0) essentially as described by Kamo et al. (1978), except that the present treatment was applied for ~10 min. After the treatment, the tongue was washed by flowing distilled water over the tongue surface for 2 min. Before each stimulation, the alkaline treatment was applied for ~1 min.

The acidic treatment consisted of flowing Ringer's solution of pH 5.3 over the

tongue surface for 20 min according to Kamo et al. (1978). After the treatment, the tongue was washed with distilled water.

The experiments were performed at 21 ± 1°C.

RESULTS

Response Patterns

The frog tongue adapted to distilled water responded to 12 amino acids (glycine, L-alanine, L-serine, L-proline, L-threonine, L-cysteine, L-valine, L-leucine, L-isoleucine, L-phenylalanine, L-tryptophan, and L-methionine) but did not respond to L-arginine, L-histidine, L-lysine, L-aspartic acid, and L-glutamic acid. Control responses in Fig. 1 represent the summated responses of the frog to 50 mM L-threonine and 50 mM L-leucine. The magnitude of the response to L-threonine varied with individual frogs, but the response patterns of all 50 frogs examined were of a tonic type. Similar response patterns were observed when glycine, L-alanine, L-serine, L-proline, and L-cysteine were applied to the frog tongues. In contrast to hydrophobic amino acids, the above amino acids elicited only tonic-type patterns in all 10 frogs examined. L-Threonine, glycine, L-serine, and L-cysteine are hydrophilic amino acids. L-Alanine and L-proline are usually classified as hydrophobic amino acids when they are incorporated into proteins. However, their free form seems not to be hydrophobic, because they do not have a large hydrophobic side chain. In this paper, all these amino acids are referred to as hydrophilic amino acids for convenience.

The response pattern of the frog to L-leucine varied with individual frogs. The patterns are roughly classified into three types according to the ratio of the response at 10 s after the onset of the stimulation to the peak response. The pattern whose ratio at 10 s to the peak is below 0.2 is referred to as type 1. The pattern of type 1 consists mainly of a large phasic component. The pattern of type 2, whose ratio is between 0.2 and 0.5, consists of a large phasic component and a small tonic component. The pattern of type 3, whose ratio is above 0.5, consists mainly of a large tonic component. The control responses in Fig. 1 *B* show the typical response patterns of types 1, 2, and 3 to L-leucine. Thirty of 50 frogs exhibited the pattern of type 2 to L-leucine. Eleven frogs exhibited the pattern of type 1, and nine frogs exhibited type 3. Hydrophobic amino acids such as L-isoleucine, L-valine, L-phenylalanine, L-tryptophan, and L-methionine elicited response patterns similar to those of L-leucine. Table I presents the numbers of frogs exhibiting the response patterns of types 1, 2, and 3 to the hydrophobic amino acids. More than half the frogs examined exhibit type 2, and the order of the frequencies observed is type 2 > type 1 > type 3.

Effect of Alkaline and Acidic Treatment

In Fig. 1 *A*, the response pattern to 50 mM L-threonine after the frog tongue was treated with an alkaline solution of pH 10.0 is represented. The response is greatly enhanced by the alkaline treatment. The mean magnitude of the

enhanced response with 20 frogs was about 3.5 times that to the responses before the treatment. After the alkaline-treated tongues were incubated in pH 5.3 Ringer's solution containing 2.5 mM $CaCl_2$ (acidic treatment), the responses were diminished to nearly the spontaneous level. Other hydrophilic

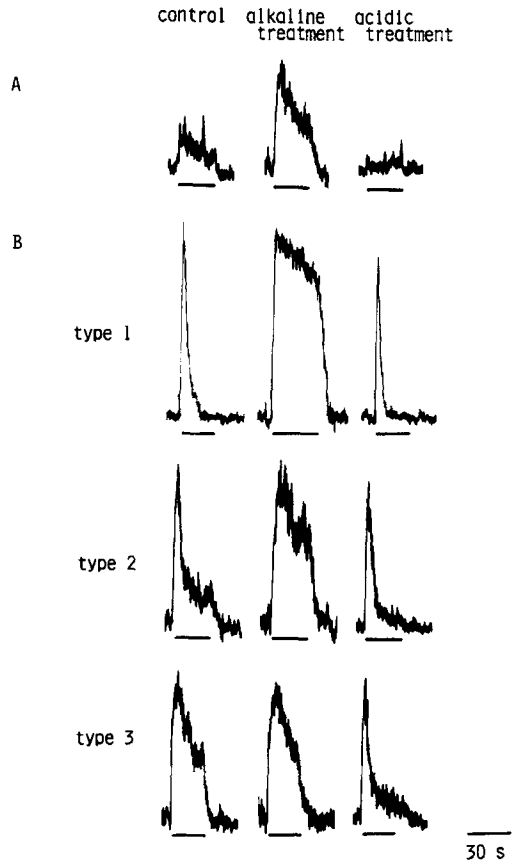

FIGURE 1. The summated responses of the frog gustatory nerve to 50 mM L-threonine (*A*) and L-leucine (*B*) before (control) and after removal of the membrane-bound Ca^{2+} (alkaline treatment) and after binding of excess Ca^{2+} (acidic treatment). The response patterns for L-leucine were classified into types 1, 2, and 3 according to the ratios of the responses at 10 s after the onset of the stimulation to the initial peak responses as follows: type 1, response whose ratio is below 0.2; type 2; response whose ratio is between 0.2 and 0.5.; type 3, response whose ratio is above 0.5.

amino acids also elicited large tonic responses in all the frogs examined (10 frogs) after the alkaline treatment, and the responses to the amino acids in all these frogs were diminished to nearly the spontaneous level after the acidic treatment.

In Fig. 1 *B*, the response to 50 mM L-leucine after the alkaline treatment is represented. Although the response patterns for L-leucine before the treatment varied with individual frogs, all the frogs examined (50 frogs) came to exhibit only the pattern carrying a large tonic component. In other words, the tonic component of the responses (except for type 3, which already had one) was greatly enhanced by the alkaline treatment. After the alkaline-treated tongue was subjected to the acidic treatment, the frogs came to exhibit response patterns carrying a small or no tonic component (types 1 and 2). Similar results were observed with other hydrophobic amino acids. Table II represents frequencies of each response type that occurred after the alkaline and the acidic treatment. All the hydrophobic amino acids elicit only type 3 responses after the alkaline treatment and type 1 and type 2 responses after the acidic treatment.

TABLE I
NUMBERS OF FROGS THAT EXHIBITED THREE RESPONSE TYPES TO HYDROPHOBIC AMINO ACIDS UNDER THE NATURAL CONDITIONS

Amino acids	Type 1	Type 2	Type 3
L-Leucine	11	30	9
L-Isoleucine	4	7	1
L-Valine	3	6	2
L-Phenylalanine	2	6	1
L-Tryptophan	3	6	1
L-Methionine	3	7	1

Fig. 2 shows concentration-response relationships for L-threonine, L-serine, L-leucine, and L-phenylalanine after the tongue was treated with the alkaline solution. Plotted is the peak height of the summated response (R) divided by the response to 50 mM L-threonine and multiplied by 100. The four amino acids give similar response curves. The thresholds of the amino acids are ~1 mM.

Treatment of the Tongue with Pronase E

Fig. 3 illustrates the typical summated response to 50 mM L-threonine and 50 mM L-leucine dissolved in distilled water after the alkaline-treated tongue was treated with 2% pronase E for 20 min. The treatment leads to complete loss of the tonic responses to L-threonine and L-leucine, though the phasic response to L-leucine is not affected. The tonic responses to L-threonine and L-leucine recovered to the original level in ~1 h after the treatment. To check whether pronase E had acted as protease, pronase E inactivated by heating at 80°C for 8 min was applied to the tongue. The inactivated pronase E produced little effect on the tonic responses to L-leucine and L-threonine. The experiments for pronase E treatment were carried out with five frogs in which L-threonine, L-serine, L-leucine, and L-phenylalanine were used as chemical

stimuli. None of the frogs responded to L-threonine and L-serine after pronase E treatment. The treatment led to complete loss of the tonic component of the responses to L-leucine and L-phenylalanine in all the frogs, without affecting the phasic component: the ratios of the responses at 10 s after the onset of the stimulation to the peak responses fell to below 0.2 after the treatment. These results also indicate that the receptor for the phasic response is different from that for the tonic response. This is consistent with the results obtained by the alkaline and the acidic treatment.

Effect of Salts

The tonic responses to amino acids were suppressed in the presence of low concentrations of salts in the stimulating solution. The effect of NaCl on the

TABLE II

NUMBERS OF FROGS THAT EXHIBITED THREE RESPONSE TYPES TO HYDROPHOBIC AMINO ACIDS AFTER THE TONGUE WAS SUBJECTED TO THE ALKALINE AND ACIDIC TREATMENTS

	After alkaline treatment			After acidic treatment		
Amino acids	Type 1	Type 2	Type 3	Type 1	Type 2	Type 3
L-Leucine	0	0	50	8	22	0
L-Isoleucine	0	0	10	3	7	0
L-Valine	0	0	10	2	8	0
L-Phenylalanine	0	0	8	3	7	0
L-Tryptophan	0	0	9	2	5	0
L-Methionine	0	0	8	2	4	0

response was examined with the alkaline-treated tongue (Fig. 4). The middle records in Fig. 4 *A* and *B* illustrate the suppressive effect of 10 mM NaCl on the tonic responses to 50 mM L-threonine and 50 mM L-leucine, respectively. The response to L-threonine and the tonic response to L-leucine are suppressed completely by the addition of 10 mM NaCl, but the phasic response to L-leucine is not affected. The suppressive effect of 10 mM NaCl on the responses to 12 amino acids that were stimulative for the frog gustatory receptors was examined with at least three frogs for each amino acid. The tonic responses to all the amino acids were suppressed, whereas the phasic responses to the hydrophobic amino acids were not affected.

The tonic response suppressed in the presence of a low concentration of salts reappeared at high salt concentration. The right-hand records in Fig. 4 *A* and *B* show the response of the alkaline-treated tongue to 50 mM L-threonine and 50 mM L-leucine, respectively, in the presence of 200 mM NaCl. The effect of 200 mM NaCl on the responses to 12 amino acids was examined with at least three frogs for each amino acid. All amino acids elicited the tonic response: the ratios of the responses at 10 s to the peak responses were >0.2. The effect of NaCl on the responses of untreated tongue to 12

amino acids was examined with at least two frogs for each amino acid. The tonic responses were similarly suppressed in the presence of a low concentration of NaCl, and the responses reappeared in the presence of high NaCl concentration, whereas the phasic responses to the hydrophobic amino acids were not affected in the presence of NaCl.

The effects of salts on the responses to the amino acids were studied systematically in the following experiments. Fig. 5 shows the response to 50 mM L-threonine plotted as a function of the ionic strength of the stimulating solution. The magnitude of the response of the alkaline-treated tongue to 50

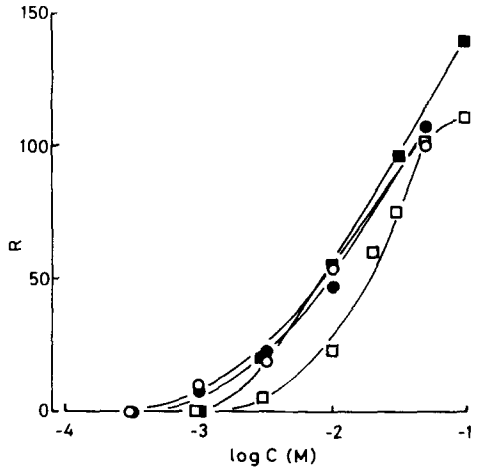

FIGURE 2. Relative magnitude of responses (R) of the alkaline-treated tongue to amino acids as a function of log stimulus concentration ($log\ C$). The peak height of the summated response was taken as the magnitude of the response. Plotted responses were calculated relative to the response to 50 mM L-threonine and multiplied by 100. Each point in the figure is a mean value of data obtained from three frogs. ○, Thr; ●, Ser; □, Leu; ■, Phe.

mM L-threonine dissolved in distilled water is taken as the standard (100). The suppressive effects of the Na and K salts can be illustrated with a single curve as a function of ionic strength. The suppression appears when the ionic strength exceeds 10^{-4}. The response decreases with increasing ionic strength and is completely suppressed at the ionic strength of 10^{-2}. The suppressive effect of Mg salts is stronger than that of both Na and K salts. The effect of the Mg salts appears at the ionic strength of 10^{-5}, and the response is completely suppressed at $\sim 10^{-3}$. The responses appear again when the ionic strength rises to 10^{-1} and increase with increasing ionic strength.

Fig. 6 shows the response to 50 mM L-leucine and 30 mM L-phenyalanine (hydrophobic amino acids) in the presence of logarithmically increasing concentrations of NaCl. The magnitude of the responses at 10 s after the

beginning of the stimulation is taken as that of the tonic response level. The tonic and phasic responses (R) to L-leucine plotted are calculated relative to the tonic and phasic responses to 50 mM L-leucine dissolved in distilled water, respectively, and multiplied by 100. The response to L-phenylalanine is similarly plotted. The phasic responses are not suppressed within the range of the ionic strength examined. The suppressive effect on the tonic responses is

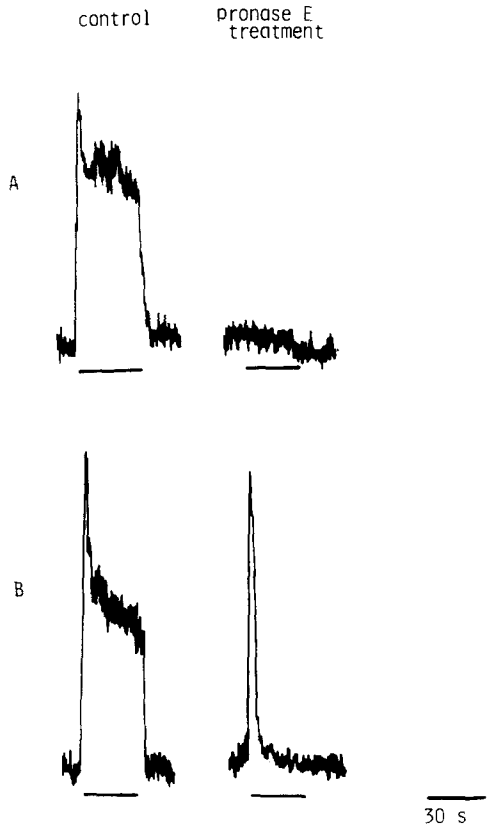

FIGURE 3. Effect of pronase E treatment of the summated responses to 50 mM L-threonine (A) and L-leucine (B). An akaline-treated tongue was treated with 2% pronase E dissolved in Ringer's solution of pH 7.4 for 20 min. After the pronase E treatment, the tongue was washed with distilled water and stimulating solutions were applied.

similar to that for L-threonine (see Fig. 5). These results also indicate that the receptor mechanism for the tonic response is different from that for the phasic response.

DISCUSSION

The responses of the frog to hydrophilic amino acids are enhanced by the alkaline treatment and diminished nearly to the spontaneous level after the

acidic treatment. On the other hand, the hydrophobic amino acids elicit large tonic responses after the alkaline treatment and evoke responses composed of a large phasic component and a small or no tonic component after the acidic treatment. Kamo et al. (1978) showed that the alkaline treatment removed Ca^{2+} from the lingual tissue, whereas the acidic treatment yielded excessive bound Ca^{2+}. Hence, the results described above are interpreted as follows. Removal of Ca^{2+} from the receptor membrane enhances greatly the tonic

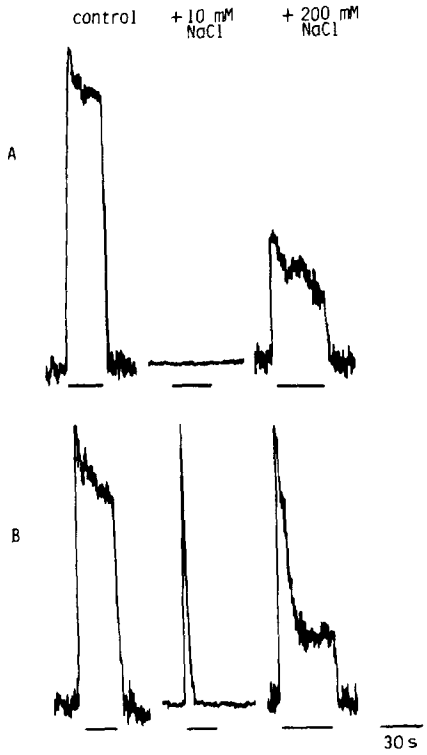

FIGURE 4. Effect of 10 and 200 mM NaCl on the summated responses of the alkaline-treated tongue to 50 mM L-threonine (*A*) and L-leucine (*B*).

component of the responses to both the hydrophilic and the hydrophobic amino acids, and excess binding of Ca^{2+} to the receptor membrane suppressed the tonic component of the responses to both amino acids. On the other hand, the phasic component of the responses to the hydrophobic amino acids is not affected by removal and binding of the membrane-bound Ca^{2+}. The variation in the responses to amino acids under natural conditions could stem from a variation in the amount of Ca^{2+} bound to the receptor membrane.

Under natural conditions, the responses of the frog to the hydrophilic amino acids are of a tonic type and those to the hydrophobic amino acids are usually

composed of phasic and tonic components. The properties of the phasic component are quite different from those of the tonic one: the tonic components are largely affected by the amount of membrane-bound Ca^{2+} as described above, and are suppressed by the presence of a low concentration of salts or by the action of pronase E, whereas the phasic components are unchanged under these conditions.

Among chemical stimuli stimulative for the frog gustatory receptors (e.g., salts, acids, sugars, bitter stimuli, and distilled water), only bitter stimuli and acids elicit a phasic-type response (Kamo et al., 1978) similar to that induced

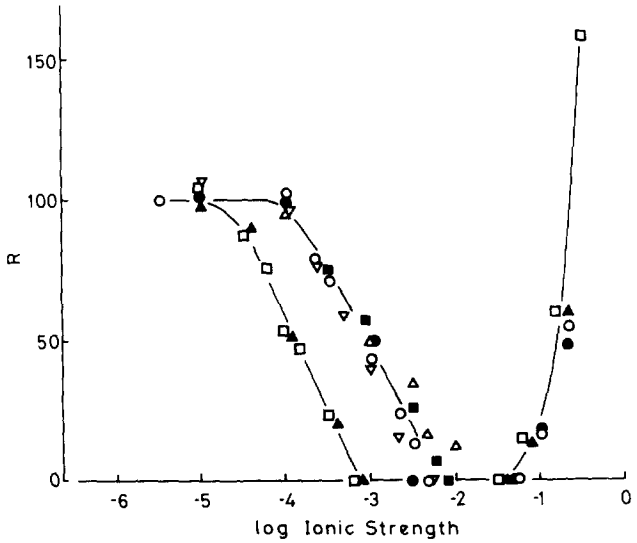

FIGURE 5. Relative magnitude of responses (R) to 50 mM L-threonine solutions containing various kinds of salts as a function of log ionic strength. The peak height of the summated response was taken as the magnitude of the response. Plotted responses were calculated relative to the response to 50 mM L-threonine dissolved in distilled water. Each point in the figure is a mean value of data obtained from three frogs. ○, NaCl; ●, KCl; ▽, CH_3SO_3Na; ■, Na_2SO_4; △, $Na_4Fe(CN)_6$; □, $MgCl_2$; ▲, $MgSO_4$.

by hydrophobic L-amino acids. Most bitter stimuli are hydrophobic substances, and hydrophobic L-amino acids actually elicit bitter taste in humans. In addition, the response of the frog to quinine is not affected by alkaline treatment that removes Ca^{2+} from the tissue (Kamo et al., 1978). It was also confirmed in the present study that the presence of salts and the action of pronase E do not affect the response to quinine. These results indicate that the properties of the response to quinine are quite similar to those of the response to the hydrophobic amino acids. Therefore, hydrophobic amino acids may stimulate a receptor site similar to that for bitter stimuli. On the other hand, the tonic responses to both hydrophobic and hydrophilic amino acids are eliminated by the treatment of the tongue with pronase E. This suggests

that the receptor molecule responsible for the tonic response to amino acids is a protein(s). The tonic responses to L-threonine and L-leucine eliminated by the pronase E treatment recovered to the original level in ~1 h after the treatment. This recovery of the taste responses seems to be brought about by incorporation of new receptor protein into the receptor membrane in that period.

The tonic responses to amino acids are suppressed in the presence of low concentration of salts. Similar suppression of response by salts is also observed with the sugar response in the dog (Andersen et al., 1963), the rat (Ozeki and

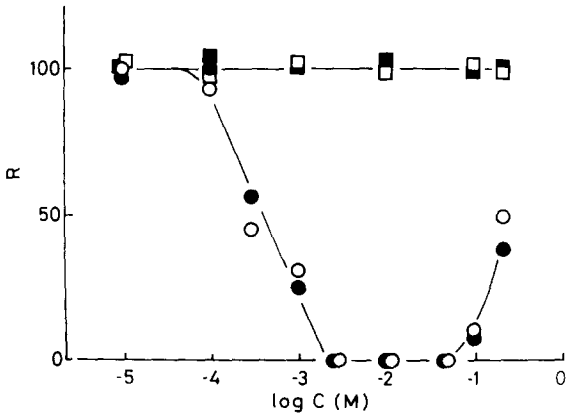

FIGURE 6. Relative magnitude of responses (R) to 50 mM L-leucine and 30 mM L-phenylalanine as a function of log ionic strength. The amino acids were applied to the alkaline-treated tongue. The peak height of the summated response was taken as the magnitude of the phasic response. The magnitude of the summated response at 10 s after the beginning of the stimulation was measured as the tonic response. The magnitude of the phasic or the tonic response to the amino acids dissolved in distilled water is taken as unit (100). Each point in the figure is a mean value of data obtained from three frogs. Phasic response: □, Leu; ■, Phe. Tonic response: ○, Leu; ●, Phe.

Sato, 1972), and the frog (Miyake et al., 1976). It is generally thought that depolarization of the taste cell leads to an increase in the gustatory nerve activities (Akaike et al., 1976). Therefore, the results described above suggest that an increase in salt concentration suppresses the depolarization induced by amino acids or sugars. This was actually demonstrated with the sugar response in the rat (Ozeki and Sato, 1972), where the receptor potential of the rat taste cell in response to sucrose was diminished by 40 mM NaCl. Similar reduction of the receptor potential seems to occur in the case of the response of the frog to amino acids. One might conclude that the receptor potential in response to amino acids is produced by an increase in diffusion potential of cations across the receptor membrane. However, this idea cannot account for

the elimination of the responses to amino acids by increasing the concentration of cations (K^+, Na^+, or Mg^{2+}) in the external media. It is also unlikely that the suppressive effect on the response to amino acids is produced by an increase in the diffusion potential of anions across the membrane, because salts having impermeable anions such as sulfonate or ferrocyanide also suppressed the responses. There is a possibility that the binding of ions on the receptor membrane interferes with the binding of amino acid molecules to the receptor site. However, that the suppressive effect of salts of monovalent cations on the responses to amino acids depends on ionic strengths in the medium cannot be simply explained by a binding of ions to the membrane surface.

The membrane potential is composed of two surface potentials at both sides of the membrane and the diffusion potential across the membrane (Teorell, 1935; Meyer and Sievers, 1936; Chandler et al., 1965). It has been pointed out that the surface potential plays an important role in various chemoreceptor systems (Kamo et al., 1974; Hato et al., 1976; Kurihara et al., 1978). The surface potential has a large value in medium of low ionic strength and decreases with increasing ionic strength. Miyake et al. (1976) interpreted the suppressive effect of the frog sugar response by salts in terms of the surface potential. A similar explanation may be applicable to the suppressive effect of the response to amino acids. That is, an increase in ionic strength may lead to a diminution of the surface potential change produced by the adsorption of amino acids to the receptor sites. In Fig. 5, the magnitude of the response of the frog to 50 mM L-threonine is plotted against ionic strength in the medium, and data fell on respective single curves for salts of monovalent cations and Mg salts. This indicates that the suppressive effect is a function of ionic strength if the valence of cations is fixed. The fact that Mg salts show a stronger suppressive effect may imply that the specific binding of the divalent cation to the membrane surface contributes partially to the suppressive effect.

The responses to amino acids reappear when relatively high concentrations of salts are present in the stimulating solution, as shown in Figs. 5 and 6. One explanation for this phenomenon is that the presence of relatively high salt concentrations decreases the membrane resistance, resulting in the diffusion potential across the membrane that contributes to the total membrane potential. However, the results cannot be explained by this mechanism alone, because Mg^{2+}, which may be less permeable to the receptor membrane than a monovalent cation, shows a larger effect on the generation of the responses to amino acids than a monovalent cation of equal concentration.

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