The Combination Effect of L-Arginine and NaCl on Bitterness Suppression of Amino Acid Solutions

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The purpose of the present study was to quantify the degree of suppression of the bitterness of two amino acids (L-isoleucine (L-Ile), and L-phenylalanine (L-Phe)) which could be achieved by the addition of various test chemicals, and to examine the mechanism of this bitterness suppression. The test chemicals used were two sweeteners (sucrose, aspartame), NaCl, various acidic (L-aspartic acid, L-glutamic acid), or basic (L-histidine, L-lysine and L-arginine) amino acids, tannic acid and phosphatidic acid. The combination of L-arginine (L-Arg) and NaCl together was the most effective in reducing the bitterness of 100 mM L-Ile and L-Phe solutions in human gustatory sensation tests. Even in bitterness of 0.1 mM quinine solution, L-Arg was also successful in reducing the bitterness. This bitterness-suppression effect was specific to L-Arg and not to the other basic amino acids. No comparable taste-masking effect was observed for the acidic amino acids. The artificial taste sensor failed to predict completely the bitterness-suppressing effect of L-Arg. It seems likely that the bitterness-suppressing effect of L-Arg is mediated not only by binding at the receptor site, but also elsewhere in the process of bitterness perception, such as a direct effect on the sodium channel. It is conjectured that the guanidinium group of L-Arg may interact with sodium channels in taste bud membranes.

Key words L-isoleucine; L-phenylalanine; L-arginine; NaCl; bitterness-suppressing agent; sodium ion channel

The taste-masking of bitterness in pharmaceutical medicines is an important component of the drive to improve patient compliance. We have previously evaluated quantitatively the bitterness of quinine using gustatory sensation tests, binding studies, and an artificial taste sensor.[1] In these studies, we have used quinine as a standard compound of known bitterness and examined the taste-masking effect of bitterness-suppressing agents such as sweeteners, NaCl, tannic acid (TA), and phosphatidic acid (PA).

In the present study, we focused on taste-masking of the bitterness of amino acid solutions. Amino acids are constituents of many beverages, such as sports drinks, and are also used in many kinds of elemental diets. Elemental diets contain high concentrations of amino acids (especially branched-chain amino acids such as isoleucine, leucine, and valine, which are extremely bitter-tasting), in order to improve Fischer's rate.[2,3] Patients with liver failure may be administered such elemental diets or nutritional supplements for long periods, and their bitterness may not only cause noncompliance but also have an adverse effect on the quality of life of such patients due to their unpleasant taste. Physical taste-masking techniques such as film coating are not adequate, given the large amino acid dosages involved. Therefore, it would be a great advantage to be able to mask the bitterness of amino acids in elemental diets by the addition of bitterness-suppressants. Although many articles have been published on the taste of amino acids,[4—6] reports on the suppression of their bitterness are less common.[7]

In the present study, we focussed on two nonpolar amino acids, L-phenylalanine (L-Phe) and L-isoleucine (L-Ile), as we have previously demonstrated that these are among the most bitter-tasting amino acids.[8] The ability of various known bitterness-suppressing agents, such as sweeteners (sucrose, aspartame), TA and PA, to suppress the bitterness of 100 mM solutions of L-Ile, and L-Phe, as well as a 0.1 mM quinine solution, was investigated using human gustatory testing, binding studies, and the artificial taste sensor.

Basic amino acids have been reported to enhance the saltiness of NaCl,[9] so we decided to also test three basic amino acids, L-arginine (L-Arg), L-lysine (L-Lys), and L-histidine (L-His), as well as two acidic amino acids, L-aspartic acid (L-Asp) and L-glutamic acid (L-Glu), for their bitterness-suppressing ability. Finally, we tested the bitterness-suppressing ability of a combination of L-Arg and NaCl.

Experimental

Materials Quinine hydrochloride was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.), and dissolved and diluted to 0.1 mM with purified water. L-Ile and L-Phe were from Wako Pure Chemical Industries (Osaka, Japan), and dissolved and diluted to 100 mM with purified water. L-His, L-Asp and L-Glu were donated by Kyowa Hakko Co. Ltd. (Tokyo, Japan). L-Arg, L-Lys were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sucrose, aspartame, and tannic acid (TA) were purchased from Nacalai Tesque Co. Ltd. (Kyoto, Japan). Phosphatidic acid (PA) (BMI-40®), a commercial bitterness-suppressing agent, was supplied by Kao Chemical Co. Ltd. (Tokyo, Japan). All other reagents were of special reagent grade.

Concentrations of 100 mM L-Ile and L-Phe were chosen for the bitterness-suppression study, while a 0.1 mM solution of quinine hydrochloride was used for comparative purposes. The concentration of the test substances were as follows: sucrose 30, 150 and 750 mM; aspartame 0.03, 0.15 and 7.5 mM; NaCl 30, 150, and 300 mM; PA 0.001, 0.01, 0.1 and 1.0% (w/v) and TA 0.005, 0.015, 0.05 and 0.15% (w/v). The concentrations of L-Arg, L-Lys, L-His, L-Asp, and L-Glu, were 0.05, 0.10 and 0.15% (w/v), respectively.

Gustatory Sensation Tests The gustatory sensation tests were performed as described in previous reports[10,11] using six fully informed volunteer subjects. The standard quinine hydrochloride concentrations used were 0.01, 0.03, 0.10, 0.30, and 1.00 mM and the corresponding bitterness scores were defined as 0, 1, 2, 3, and 4, respectively. Before testing, the volunteers were asked to keep the above standard samples in their mouths, and were told their concentrations and bitterness scores. After tasting a 5-ml sample of the test drug solutions, they were asked to give the sample a bitterness score. All samples were kept in the mouth for 15 s. After tasting the sample, subjects gargled well and waited for at least 20 min before tasting the next sample.

Evaluation of the Binding of L-Ile, and L-Phe to PA and TA, and L-Arg Solutions of L-Ile and L-Phe (100 mM) containing various concentrations of PA (0.001, 0.01, 0.1, and 1.0% (w/v)), or TA (0.005, 0015, 0.05, 0.1%

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(w/v)), or L-Arg (0.15% (w/v)) were mixed thoroughly, immediately centrifuged (3000 rev/min for 20 min, Hitachi CR5B2, Tokyo Japan), and filtered through a membrane with 0.45-μm pore size. The filtered solutions were diluted 1000 times. The diluent was then derivatized with *O*-phthalaldehyde, as described previously[12] and the prepared sample 10 μl injected on to a chromatograph (Shimadzu LC-10AT, Kyoto, Japan) equipped with a fluorescence detector (Shimadzu RF-10AXL, Kyoto, Japan), an integrator (Shimadzu C-R7A, Kyoto, Japan), and a reversed-phase column (Cosmosil 5C18-AR, 4.6×150 mm, Nacalai Tesque Co., Ltd., Japan). The following mobile phase system was used: A: pH 6.3, 0.1 M sodium acetate/0.1 M citric acid/0.5 mM EDTA–2Na/7% AcCN/3% THF (v/v); B: AcCN; A : B=60 : 40. The flow rate was 0.6 ml/min, the excitation wavelength was set at 330 nm, and the fluorescence wavelength was 440 nm. The procedure was repeated five times for each sample and the binding ratios of L-Ile and L-Phe to PA or TA were calculated. In the case of L-Arg, the binding of Arg with quinine or L-Ile and L-Phe was performed simultaneously. The binding of L-Arg (0.15% (w/v)) to 100 mM L-Ile or Phe solutions was also investigated using this system. The concentrations of unbound L-Ile and L-Phe were determined simultaneously. The binding of L-Arg (0.15% (w/v)) to 0.1 mM quinine was also examined, essentially in the same way as described in our previous paper,[1] and the unbound quinine fraction was determined.

Sensor Measurements The artificial taste sensor used in the present study was essentially the same as that described in previous papers,[13—16] with respect to lipid components, sensor measurements, and data analysis. Various concentrations of L-Arg or L-Lys were added to a 0.1 mM quinine hydrochloride solution or a 100 mM L-Ile solution and the bitterness of the mixture was evaluated using the taste sensor. The output profile for channel 3 of the sensor, which showed the largest output value, was used in the present study.

Physical Properties of Amino Acids Computer software version 4.67 (Advanced Chemistry Development Company, Canada) was used to derive the physical properties of the amino acids used in the present study. The pH changes which occurred when L-Arg and L-Lys were added to 100 mM L-Ile was measured using a pH meter (F-21 Horiba Co. Ltd., Kyoto, Japan).

Statistical Analysis In gustatory sensation data, the bitterness scores are expressed as mean±S.E.M. Statistical analysis was performed with one-way analysis of variance followed by two-sample *t*-test. $^{**}p<0.01$, $^{*}p<0.05$ were considered as statistically significant.

Results and Discussion

Bitterness Suppression of L-Ile and L-Phe in Human Volunteers The taste-masking effect of sucrose, aspartame, and NaCl on the bitterness of L-Ile and L-Phe solutions was measured in human gustatory sensation tests, as had been done in our previous study with quinine.[1] Figure 1A shows the effects of sucrose and aspartame on the bitterness scores of 100 mM L-Ile and L-Phe solutions, compared with their effects on a 0.1 mM quinine solution (data from previous study). The actual concentration of aspartame was 1/100th of that depicted. The bitterness scores decreased dramatically with increasing amounts of sucrose and aspartame, as they had with quinine. Figure 1B shows the effects of NaCl on the bitterness score of 100 mM L-Ile, and L-Phe solution; data from the earlier study with 0.1 mM quinine are also shown for comparative purposes. The bitterness scores of the L-Ile and L-Phe solutions decreased with increased amounts of NaCl, as they had with quinine, even though the amino acids are hydrophilic while quinine is hydrophobic. It is interesting that the taste-masking effects of the two sweeteners and NaCl were similar in gustatory sensation tests.

Figure 1C shows the effects of PA or TA on the bitterness scores of 100 mM L-Ile and L-Phe solutions and 0.1 mM quinine solution. On the addition of 0.001—1.0% (w/v) PA, the bitterness score of L-Ile was reduced by about 30% (*e.g.*, from 1.68±0.14 to 1.15±0.23 in the presence of 1.0% PA). In the case of L-Phe, the bitterness score was reduced by only about 20% (*e.g.*, from 2.62±0.20 to 2.08±0.25 in the presence of 1.0% PA). This result was unexpected, as the addition of 1.0% PA to 0.1 mM quinine solution had resulted in 82% bitterness suppression. The discrepancy between the magnitude of the effect of PA on bitterness suppression of quinine and the amino acids may be explained in several ways. It may be due to differences in the bitterness receptors for quinine and amino acids. Recent articles have reported that there are specific taste receptors for amino acids,[7] and it is possible that different characteristics of quinine and amino acid receptors might account for this discrepancy. Differences in the

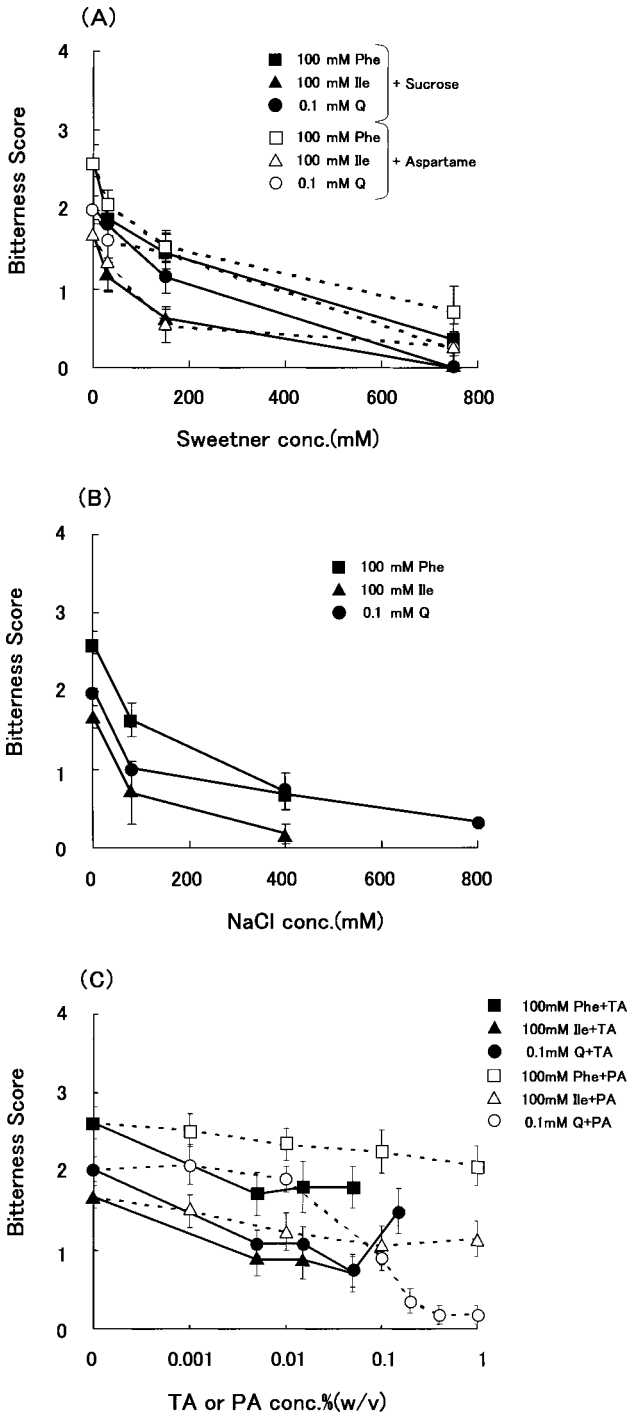

Fig. 1. The Relationship between Human Gustatory Bitterness Scores and the Added Concentrations of (A) Sweetener (Sucrose, Aspartame), (B) NaCl, (C) Phosphatidic Acid (PA) or Tannic Acid (TA)

The data represent the mean±S.E.M. (n=6). The actual concentration of aspartame is 1/100th of the theoretical concentration shown.

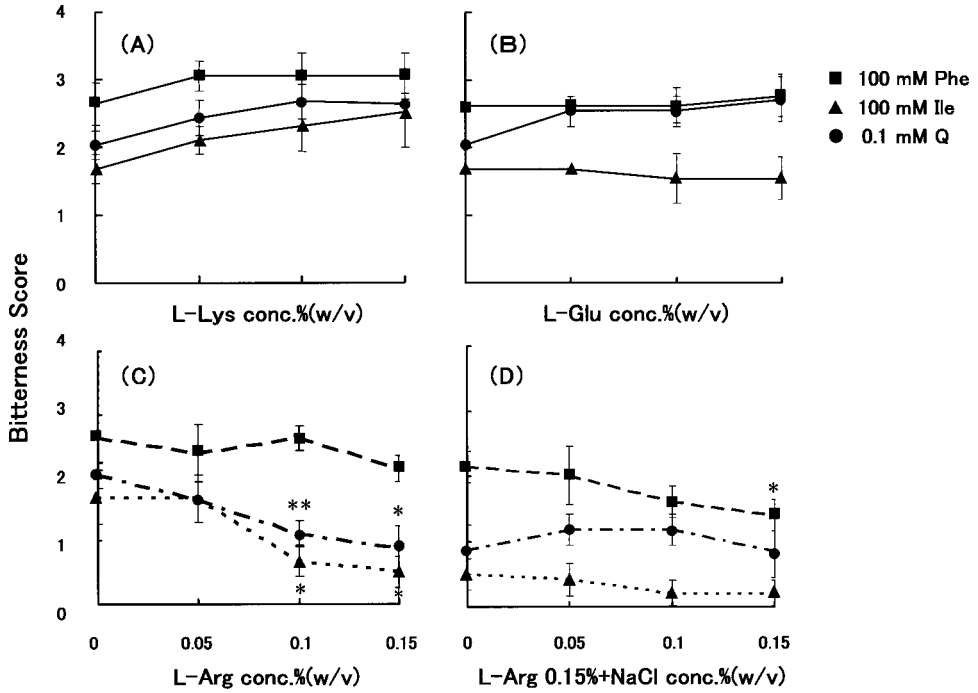

Fig. 2. The Relationship between Human Gustatory Bitterness Scores and Increasing Concentrations of (A) L-Lys, (B) L-Glu, and (C) L-Arg, and in (D) L-Arg 0.15% (w/v) Plus Increasing Concentrations of NaCl

The data represent the mean±S.E.M. (n=6). ** $p<0.01$, * $p<0.05$ compared with control (in the absence of bitterness suppressant).

sample concentrations may also play a role. In this experiment, we used concentrations of 0.1 mM quinine, and 100 mM L-Ile and L-Phe, while PA was used at the maximum concentration of 1.0% (w/v). Quinine is extremely bitter even at low concentrations of 0.1 mM, while the amino acids induce comparable bitterness only at the much higher concentration of 100 mM. Thus, while the PA was able to compete with quinine at the receptor site at concentrations of 0.1 mM quinine, it could not compete with the amino acids as their concentrations were so much higher.

As shown in Fig. 1C, the bitterness scores gradually decreased with increasing quantities of PA up to 1%. This effect was greatest with respect to quinine. It is possible that a greater effect might be seen at higher concentrations of PA, but we were unable to examine this in human gustatory sensation tests due to the unpleasant taste and smell of PA.

The inhibitory effect of TA on the bitterness of the two amino acids and quinine was similar, as shown in Fig. 1C. At concentrations of 0.005—0.05% (w/v) TA caused a decrease in the bitterness of quinine, while at 0.15% (w/v) TA gave rise to a significant increase of astringency (astringency score not shown). A similar result was observed with the amino acids. While the addition of 0.005% (w/v) TA to L-Phe slightly decreased the bitterness score (from 2.62±0.20 to 1.72±0.27), at concentrations over 0.15% (w/v), the bitterness score dramatically increased such that gustatory sensation tests could not be carried out. Thus, we propose that the astringency of TA tends to suppress the bitterness of drugs at low concentrations, while increasing the concentration of TA up to 0.1% (w/v) causes a significant enhancement of bitterness. While we are not able to test this proposal directly, bitterness enhancement by TA seems to exert its main effect centrally not peripherally.

Bitterness Suppression of L-Ile and L-Phe by Basic and Acidic Amino Acids Figures 2A—C show the effect of L-Lys, L-Glu, and L-Arg on the bitterness of L-Ile, L-Phe and quinine in human gustatory sensation tests (Neither L-Asp nor L-His could mask the bitterness of any of the three solutions; data not shown).

L-Arginine was so successful in reducing the bitterness of all three solutions (Fig. 2C), and this bitterness suppression was enhanced by the further addition of up to 0.15% (w/v) NaCl (Fig. 2D). Even in single usage of L-Arg, the bitterness of quinine and L-Ile was significantly decreased the bitterness as increased concentration of L-Arg as shown in Fig. 2C. In the case of L-Ile, the initial bitterness score of 1.68±0.43 was also significantly reduced by 0.15% (w/v) L-Arg (to 0.50±0.25), and the subsequent addition of up to 0.15% (w/v) NaCl reduced it still further (to 0.21±0.20), as shown in Fig. 2D. In the case of L-Phe, the bitterness score of 100 mM of L-Phe (2.62±0.37) was only slightly reduced by 0.15% (w/v) L-Arg (to 2.14±0.20), the subsequent addition of up to 0.15% (w/v) NaCl resulted in a further significant decrease in the bitterness score (to 1.41±0.25). (This value of 1.41 was significantly smaller compared with values of 2.14 ($p<0.05$) and 2.62 ($p<0.01$), respectively). Whereas in the case of 0.1 mM quinine, the initial bitterness score of 2.03±0.20 was also significantly reduced by 0.15% (w/v) L-Arg (to 0.88±0.37). Nevertheless the subsequent addition of up to 0.15% (w/v) NaCl did not work (to 0.88±0.37), as shown in Fig. 2D. The above discrepancy in effect of NaCl among drugs was not cleared yet in the present study and will be dissolved in near future.

The combination of L-Arg+NaCl is only effective and useful in the concentration range 0.05—0.15% (w/v). At concentrations over 0.2% (w/v) L-Arg has an unpleasant smell, while at over 2.0% (w/v) NaCl, the volunteers complained of excessive saltiness. The degree of suppression reached by

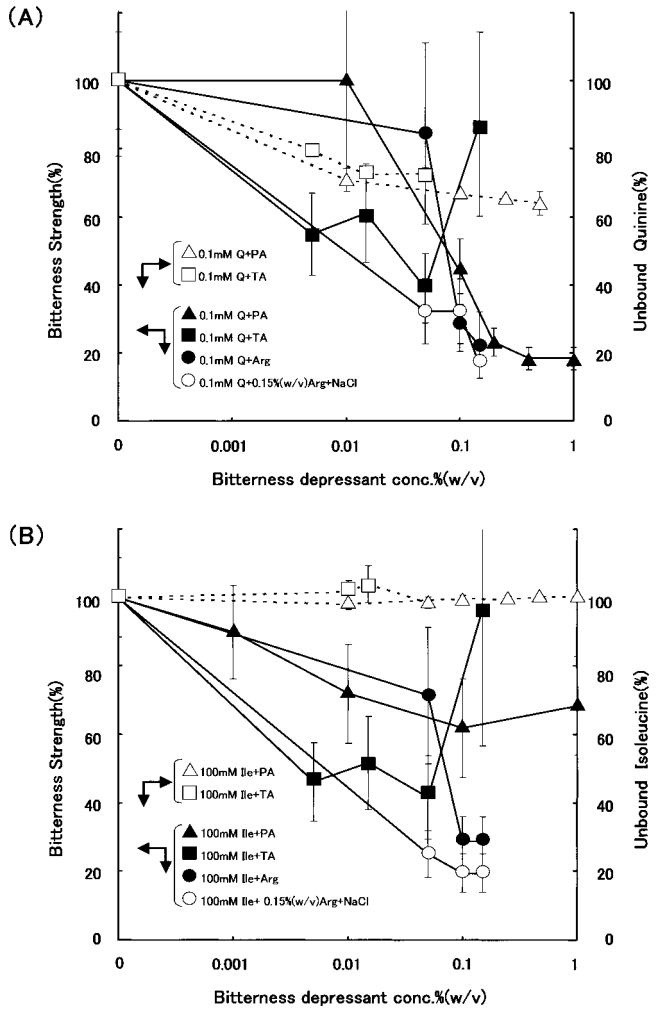

Fig. 3. The Relationship between the Concentrations of Added TA, PA, L-Arg, and L-Arg+NaCl, and the Bitterness Strength (Left Axis, Solid Lines, Expressed as Equivalent Quinine Concentrations) or Unbound Fraction (Right Axis, Dotted Lines, Expressed as a %), of Quinine Hydrochloride (A) and L-Ile (B) Solutions

In related to samples containing 0.15% (w/v) Arg plus NaCl (open circles in Figs. 3A, B), the bitterness depressant conc.% (*x*-axis) represents NaCl concentraton itself. For further explanation, see text.

synergy between L-Arg+NaCl was greater than that of any of the other bitterness-suppressing agents, including TA and PA.

In order to evaluate the bitterness-suppressing effect of the L-Arg+NaCl combination on L-Ile and quinine in more detail, the gustatory sensation data were expressed as equivalent quinine concentrations and compared quantitatively with the binding study data. The data for 0.1 mM quinine and 100 mM L-Ile are summarized in Fig. 3 (A and B, respectively), in which the left axis represents the bitterness strength from the human gustatory sensation tests expressed as equivalent quinine concentrations (relative values), while the right axis represents the unbound fraction % calculated from the binding data.

Figure 3A shows the relationship between the concentrations of TA, PA, L-Arg, and L-Arg+NaCl, and bitterness strength of quinine on the left axis, or the percentage of unbound quinine on the right axis. There was some binding of TA and PA to quinine, but no binding of L-Arg. However, 0.15% (w/v) L-Arg was almost as effective in reducing the bitterness of 0.1 mM quinine as 1.0% (w/v) PA. The combination of L-Arg and NaCl enhanced this inhibitory effect and reduced the bitterness strength of quinine still further.

As shown in Fig. 3B, L-Ile did not bind to PA or TA, so the unbound fraction of L-Ile was almost 100%. A similar result was obtained for L-Phe (data not shown). This result can be accounted for by the fact that L-Ile and L-Phe are amphoteric electrolytes and not likely to adsorb the PA and TA. In terms of bitterness suppression, the addition of L-Arg was more effective than PA or TA. When 0.15% (w/v) L-Arg, alone or in combination with 0.15% (w/v) NaCl, was added to the 100 mM L-Ile solution, bitterness scores were decreased to 28.6% and 19.4%, respectively. These values are smaller than those obtained with either PA or TA.

Using quantitative data from the binding studies and bitterness scores from human gustatory tests, the proposed corresponding taste-masking mechanisms are presented schematically in Figs. 4A (quinine) and B (L-Ile). The inhibitory effects of 0.05% (w/v) TA or 1.0% (w/v) PA on quinine were derived from a combination of adsorption and masking of the receptor site binding, in contrast to those of L-Arg or L-Arg+NaCl, which were derived solely from receptor site binding.

Neither TA nor PA was very effective in reducing the bitterness of 100 mM L-Ile solution, and binding of TA or PA to L-Ile could not be demonstrated. Binding between L-Ile and L-Arg could not be demonstrated either, however, so that the main inhibitory effect of L-Arg on the bitterness of L-Ile appeared to be due to interaction between L-Arg and the bitterness receptor site for L-Ile.

Evaluation of Bitterness Suppression by the Taste Sensor The mechanism by which L-Arg interferes with bitterness perception was investigated using the artificial taste sensor. L-Arg and L-Lys are very similar in structure, but quite different in their taste-masking abilities. We therefore examined the electrical characteristics of the two amino acids by looking at the effect of the addition of L-Arg and L-Lys to solutions of quinine or L-Ile on sensor output values.

As shown in Figs. 5A and B, the addition of both L-Arg and L-Lys decreased the sensor output of quinine and L-Ile solutions. These data suggest that L-Arg and L-Lys have a similar effect at the level of the membrane. However, the two amino acids were quite different in their abilities to mask the bitter taste of either L-Ile or quinine in human gustatory sensation tests. Thus, the sensor output did not fully reflect the bitterness-suppressing effect of L-Arg. We therefore looked at the structural differences between L-Lys and L-Arg.

Figure 6 summarizes the physicochemical properties of various acidic, basic, and nonpolar amino acids. Although their physical properties such as pK_a value and isoelectric point may be similar, their side-chains are structurally quite different. For example, it is clear that the presence of the guanidinium group has improved the reactivity of L-Arg. In recent articles in the catfish, the arginine, especially the guanidinium group, has been reported to interact with the sodium channel.[6,17,18] As the sodium channel is known to be intimately involved in bitterness perception, a similar interaction between L-Arg and the sensory receptor may occur in humans.

A recent mass spectrometry[19] also suggested that arginine

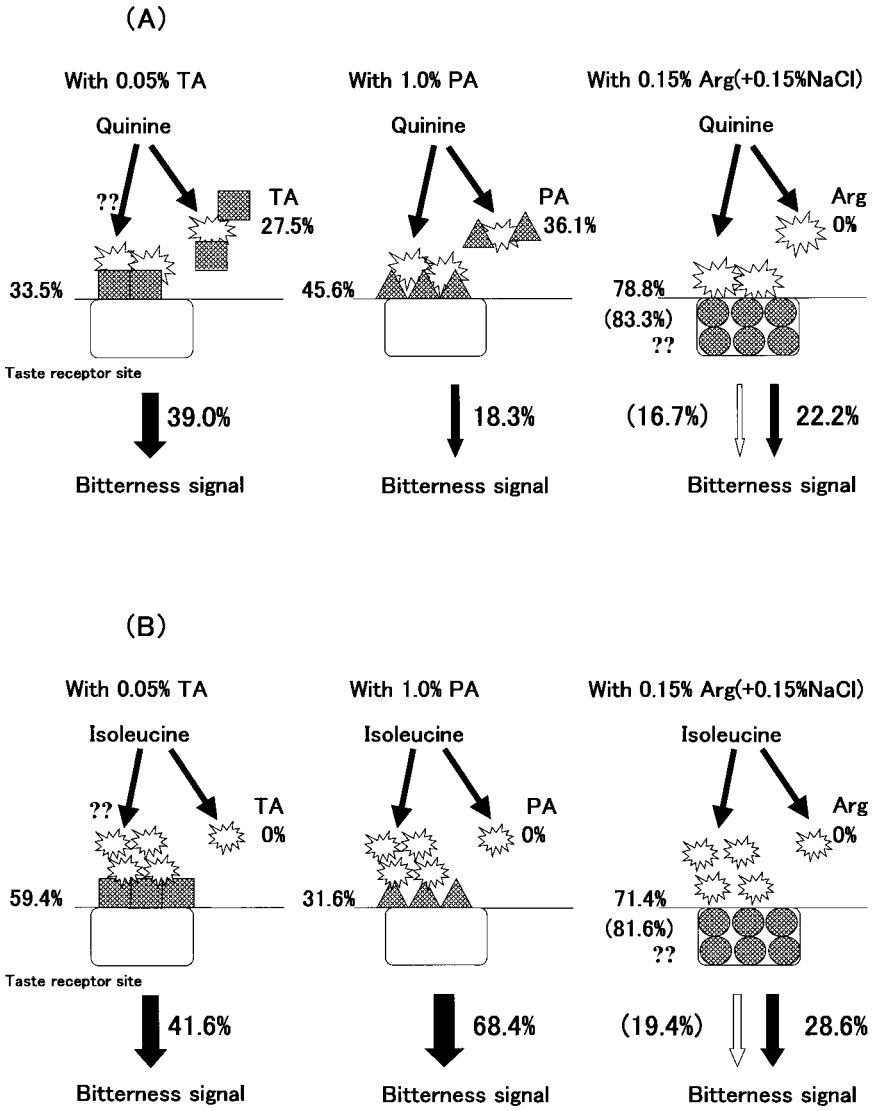

Fig. 4. Proposed Mechanism of Bitterness Suppression of Quinine Hydrochloride (A) and L-Ile (B) by PA, TA, and L-Arg (with or without NaCl), in Man
Figures in parentheses are data from the combination of L-Arg and NaCl. The values are calculated from data shown in Figs. 3A and B.

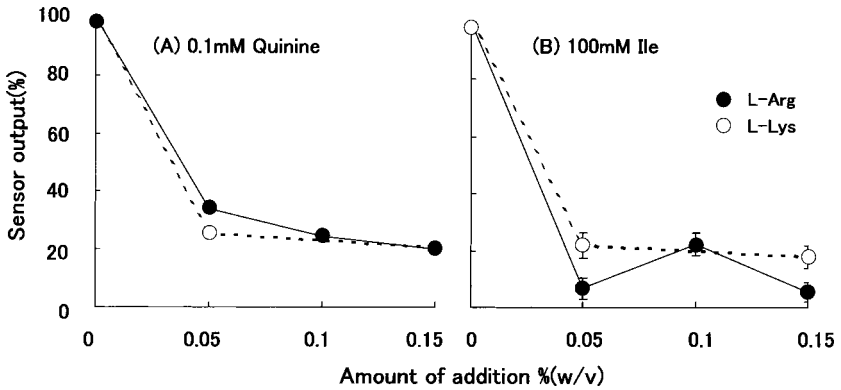

Fig. 5. The Effect of L-Arg and L-Lys on the Sensor Output of 0.1 mM Quinine (A) or 100 mM L-Ile (B)
The sensor data are the mean of values obtained in three experiments.

Class	Amino acid	pKa			isoelectric point	hydropaty index
		carboxyl group	amino group	side chain		
Nonporality	L-Isoleucine	2.36	9.68	-	6.02	4.5
	L-Phenylalanine	1.83	9.13	-	5.48	2.8
Acidic	L-Aspartic acid	2.09	9.82	3.86	2.77	-3.5
	L-Glutamic acid	2.19	9.67	4.25	3.22	-3.5
Basic	L-Histidine	1.82	9.17	6.00	7.59	-3.2
	L-Lysine	2.18	8.95	10.53	9.74	-3.9
	L-Arginine	2.17	9.04	12.48	10.76	-4.5

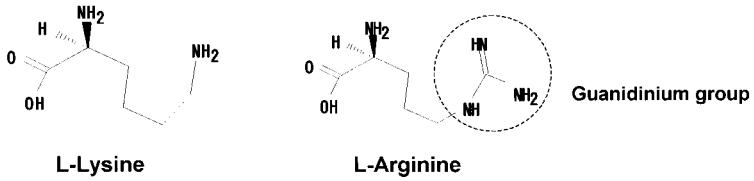

Fig. 6. Physicochemical Properties of the Various Amino Acids and the Chemical Structures of L-Arg and L-Lys

molecules may form stable aggregates, which are themselves more reactive than lysine in certain conditions in the presence of salt. This finding indirectly supports our hypothesis.

These results enable us to draw the following conclusions:

1. L-Arg can suppress the bitterness of quinine hydrochloride, L-Ile and L-Phe solutions and this effect is enhanced by the presence of NaCl in the case of L-Ile, L-Phe.
2. The bitterness-suppressing effect of L-Arg on L-Ile and L-Phe is greater than that of PA. Adsorption is not observed in either case.
3. The polarity of L-Arg is remarkably high. There may be an interaction between the guanidinium side-chain of L-Arg and the sodium channel in the human taste bud, which is greater when NaCl is also present.

We intend to examine the mechanism of the L-arginine effect more closely and also look at the bitterness-suppressing effect of other compounds with a guanidinium group.

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