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# Specificity of Glossopharyngeal Nerve Responses to Astringent Compounds in *Xenopus*

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## Abstract

Astringent compounds were applied to oral epithelium of the clawed toad, *Xenopus laevis*, and rapidly rising and highly sensitive responses could be recorded from the whole glossopharyngeal nerve, but not at all from the trigeminal nerve. The response to 10 mM tannic acid decreased progressively with repetitive application. These responses to tannic acid, however, recovered completely by treating with chemicals capable of forming strong hydrogen and hydrophobic bonds. These chemical bondings are generally recognized as a model for polyphenol (tannin)–protein interactions based on physico-chemical measurements *in vitro*. The high affinities of these chemicals for tannic acid may be effective in releasing both bonds in the interaction of tannic acid with the receptor molecules. Our results provide *in vivo* evidence for this model. **Chem. Senses 21: 459–465, 1996.**

## Introduction

Astringent tastes perceived in the mouth upon ingestion of unripe fruits, teas and wines are generally regarded as dry, puckering sensations, which seem more closely allied to the tactile than to the gustatory sense (Joslyn and Goldstein, 1964). On the other hand, perceptual assessments of astringency may be closely linked to bitterness (Lyman and Green, 1990), and there is some electrophysiological evidence that particular gustatory nerves, namely the chorda tympani and glossopharyngeal nerves, transmit the messages for astringency (Kawamura *et al.*, 1969; Schiffman *et al.*, 1991). Very little information, however, is available on the interaction between astringent compounds and gustatory molecules.

The property of astringency may be commonly ascribed to the reversible binding of polyphenols (tannins) with proteins. On the basis of physico-chemical measurements *in vitro*, a model in which the association of polyphenol and protein is composed of a process occurring in two distinct phases, hydrophobic interactions and hydrogen bonding, has been proposed (Spencer *et al.*, 1988).

Our objective in this study is to explore whether or not the gustatory receptors of the clawed toad (*Xenopus laevis*), which has extremely acute gustatory receptors for bitter substances (Yoshii *et al.*, 1982), give some definite information on the gustatory nature for astringent compounds. Also, we provide evidence that the model for polyphenol–protein interactions is available *in vivo*. Some of these results have appeared in abstract form (Yamashita *et al.*, 1993, 1994).

## Materials and methods

Adult clawed toads weighing 30–50 g were deeply anesthetized by i.p. injections of urethan (ethyl carbamate) at doses of 0.25 g/100 g body wt. On the surface of the mandible the skin and adjacent muscles were removed and the glossopharyngeal (IXth) nerve, which innervates gustatory receptors on the ventral epithelium of the oral cavity, was exposed. Neural activities were recorded with Ag–AgCl electrodes connected to an RC-coupled amplifier, integrated with a time constant of 0.5 s, and displayed on a pen recorder. The maximal magnitude of the integrated response was used as a measure of the magnitude of the response and expressed relative to the magnitude of the response to 1 mM L-proline (standard response).

Stimulus solution was delivered to the oral cavity for 5 or 10 s through the flowline system, which consisted of a two-way solenoid-operated valve connected via tubing to a distilled water reservoir and a stimulus reservoir. The flow rate was 1.5 ml/s. The interstimulus interval was 3–5 min and to eliminate tactile responses the oral cavity was continuously rinsed with distilled water until application of the test stimulus. In order to make comparisons with the responses of the glossopharyngeal nerve, the responses of the trigeminal (Vth) and the facial (VIIth) nerves were partly recorded using the same recording and stimulating procedures.

The temporal dilution process was inferred by measuring the change in conductance with a flow of 0.1 M NaCl after distilled water. The stimulus contacted the preparation within 200 ms (188.8 ± 6.9 ms, $n = 10$) after the onset of the two-way valve switch and the concentration of the stimulus attained 63% of the final concentration (0.1 M) within 40 ms (24.7 ± 7.2 ms, $n = 10$) after the contact of the stimulus. Since the most rapid onset time of the impulses that we recorded from single fiber preparations of the *Xenopus* glossopharyngeal nerve was 340.2 ± 55.7 ms ($n = 6$) after the onset of the valve switch (Yamashita *et al.*, unpublished data), it was valid to assume that the stimulus could attain the final concentration before the onset of the response.

All chemicals were purchased from Wako Pure Chemical Industry (Osaka), except for tannic acid, which was purchased from Fluka Chemie AG (Switzerland). All test solutions were made with reagent-grade chemicals and distilled and deionized water. To eliminate thermal responses all stimuli and rinsing distilled water were presented at room temperature (20–22°C).

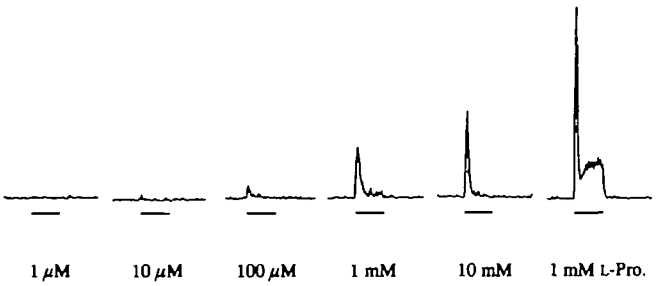



**Figure 1** Integrated glossopharyngeal nerve responses to tannic acids in the clawed toad. The solid bars at the bottom of each response represent the duration of the stimulation (5 s). The response to 1 mM L-proline at the end of the sequence is the standard response.

## Results

Representative glossopharyngeal nerve responses elicited by tannic acid are shown as a function of concentration in Figure 1. Each of the responses to tannic acid is composed of the phasic component alone and increases in amplitude with increasing concentration. The response component and concentration–response function were similar to those in responses to quinine–HCl and HCl at lower concentrations. As seen in Figure 1, the rising time of the integrated response to tannic acid was rapid, and similar to that of the standard response. Although the oral chemoreceptors responded reversibly at concentrations <1 mM, the magnitude of the response progressively decreased from its original value upon repeated stimulation at 10 mM, the highest concentration tested in the present experiment (see also open circles in Figure 3). The activity level took a long time to return to baseline after the stimulus was washed away with water.

The mean concentration–response curves for tannic and gallic acids are shown relative to the standard response in Figure 2 (open circles). Both had threshold concentrations of 10–100 μM, and no saturation of the response was detected even at 10 mM. Although the response to gallic acid was approximately half of that to tannic acid at 10 mM, it decreased from the original magnitude with repeated stimulation, as detected in the stimulation by tannic acid. The response to gallic acid also consisted of the phasic component alone.

Since tannic and gallic acids at high concentrations show low pHs (2.81 and 3.25 at 10 mM), their responses may contain not only those to astringent compounds but also those to acid. To assess the contribution of the pH component to the responses to both compounds we adjusted

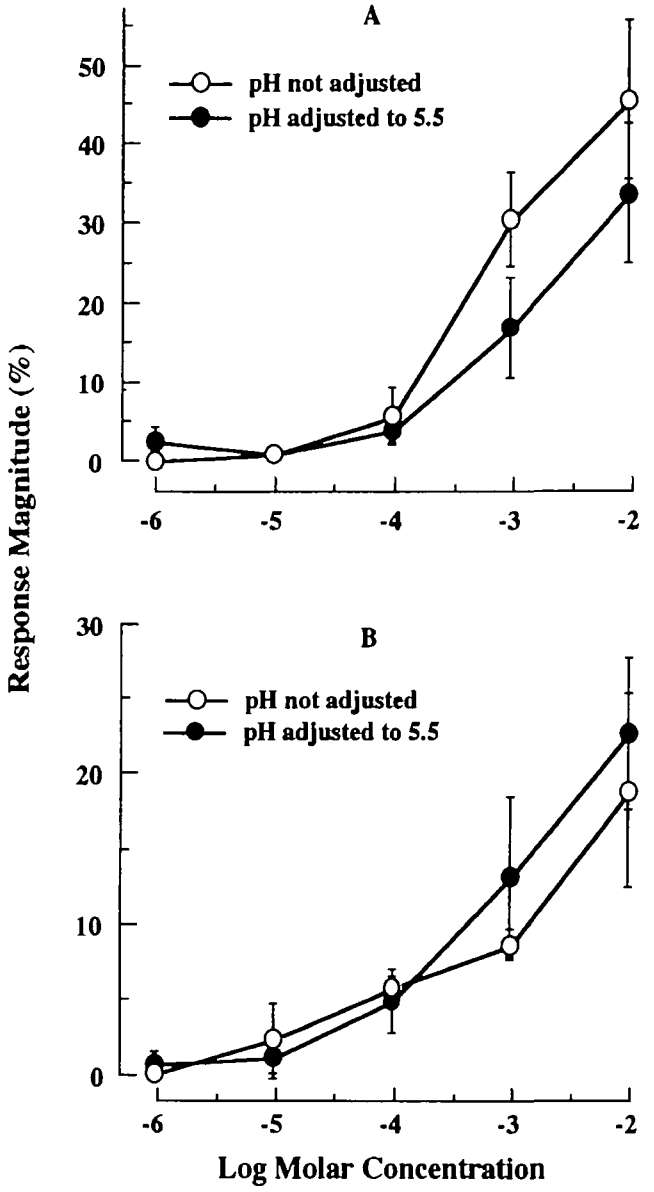



**Figure 2** The mean dose–response curves (open circles) for tannic acid **(A)** and gallic acid **(B)** accompanying those (solid circles) for both acids whose pHs were adjusted to 5.5 by adding NaOH. The responses are given relative to the standard response. Vertical bars at each response represent ±SDs of the means obtained from five preparations.

the pH of the tannic acid solution to 5.5, i.e. close to the pH (5.6) of the rinsing solution, by adding NaOH into the solution, and compared the responses with those to non-pH-adjusted tannic acid solutions (filled circles in Figure 2). It appears that the responses to pH-adjusted tannic acid become smaller in magnitude than the original responses (Figure 2A), whereas those to pH-adjusted gallic acid become greater (Figure 2B), at equivalent concentrations.

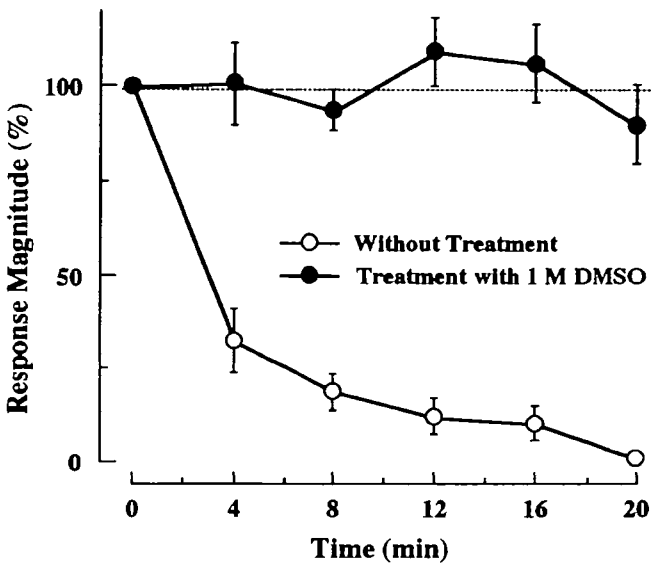



**Figure 3** Depression of the responses to 10 mM tannic acid by the repetitive stimulation for 10 s and recovery of the responses by treatment with 1 M DMSO for 10 s. The treatment was repeated after each application of tannic acid. Vertical bars represent ±SDs of the means obtained from five preparations.

The mean final $Na^+$ concentrations by NaOH added to tannic and gallic acids at 10 mM were 12.67 and 6.37 mM respectively. The dose–response function of NaCl showed that these concentrations of NaCl are not sufficient to elicit the response at pH 5.5. However, no significant differences were obtained between both responses at a given concentration in tannic (Figure 2A) and gallic (Figure 2B) acids, except for those at 1 mM tannic acid where a probably significant difference was estimated ($0.01 < P < 0.05$). Thus, it is suggested that the differences in the responses to tannic and gallic acids at pH 5.5 and pHs around 3 are not sufficient to assess the contribution of the pH component.

Clawed toad chemoreceptors also responded to all the tea catechin compounds in the manner of the integrated response pattern similar to tannic acid. The threshold concentration for epigallocatechin gallate was ~10 μM. The magnitude of response at 10 mM reached nearly 50% of the standard response, while it did not appear to be saturated (data not shown). The definite decrement in the magnitude of the response with repetitive stimulation, as pointed out in the stimulation with tannic and gallic acids, was not observed at 10 mM.

In the present experiment we could eliminate tactile and thermal responses of the glossopharyngeal nerve by using a stimulating apparatus devised to switch from distilled water

**Table 1** Responses of Vth, VIIth and IXth nerves to astringent compounds

| Astringent compound | Nerve | | |
|---|---|---|---|
| | Vth[a] | VIIth[b] | IXth[b] |
| Tannic acid (10 mM) | 0.0 ± 0.0 | 47.4 ± 5.6 | 45.5 ± 10.1 |
| Gallic acid (10 mM) | 0.0 ± 0.0 | 21.2 ± 4.4 | 18.7 ± 6.5 |
| Epigallocatechin gallate[c] (10 mM) | 0.0 ± 0.0 | 48.7 ± 7.8 | 45.7 ± 2.7 |

Data are means ± SD, *n* 5.

[a]Relative response to $CO_2$-saturated water prepared by bubbling $CO_2$ gas into distilled water for 5 min.

[b]Relative response to 1 mM L-proline.

[c]A popular astringent compound contained in green tea.

smoothly over to the stimulating solution of the same temperature. To test further the contributions of the tactile and thermal receptors to the responses to astringent compounds, the responses of the trigeminal and facial nerves were recorded when the oral epithelium was stimulated with the astringent compounds. The results are summarized in Table 1.

The facial nerve that innervates the oral gustatory receptors gave rise to responses similar to those of the glossopharyngeal nerve, while the trigeminal nerve which innervates tactile and thermal receptors in the oral epithelium caused no responses to astringent compounds (Table 1). Thus, it is proven that these responses to astringent compounds originate from gustatory receptors rather than somatosensory receptors.

It is known from physico-chemical studies that astringent compounds at high concentration only reversibly precipitate protein. An abrupt decrease in response was found in the present study, when 10 mM tannic acid (pH 2.80) was applied consecutively to the oral cavity with interstimulus water rinse for 4 min. The responses decreased progressively with the repetition of the application (open circles in Figure 3). Since successive application of 10 mM HCl (pH 2.03) with the same water rinse did not result in any decrease in response, the decrease in response to tannic acid at the highest concentration does not appear to arise from the low pH. These depressed responses to tannic acid, however, recovered completely after treatment with 1 M dimethyl-sulfoxide (DMSO) for 10 s (filled circles in Figure 3; see also Table 2).

Several agents were effective at recovering the response to tannic acid (Table 2). Of the treatment agents tested in the present experiments, 1 M DMSO and 1 M 2-pyrrolidone were very effective and the response to tannic acid recovered completely after treatment for 10 s. Treatment with 0.1 M caffeine and 1 M *N*,*N*-dimethylformamide (DMF) were the next most effective and the response recovered completely with treatment for 15 s. Treatment with 1 M guanidine–HCl and 1 M urea showed higher but incomplete recovery rates after treatment for 20 s. The recovery rate after treatment with formamide was much lower than that of the substituted derivative, DMF.

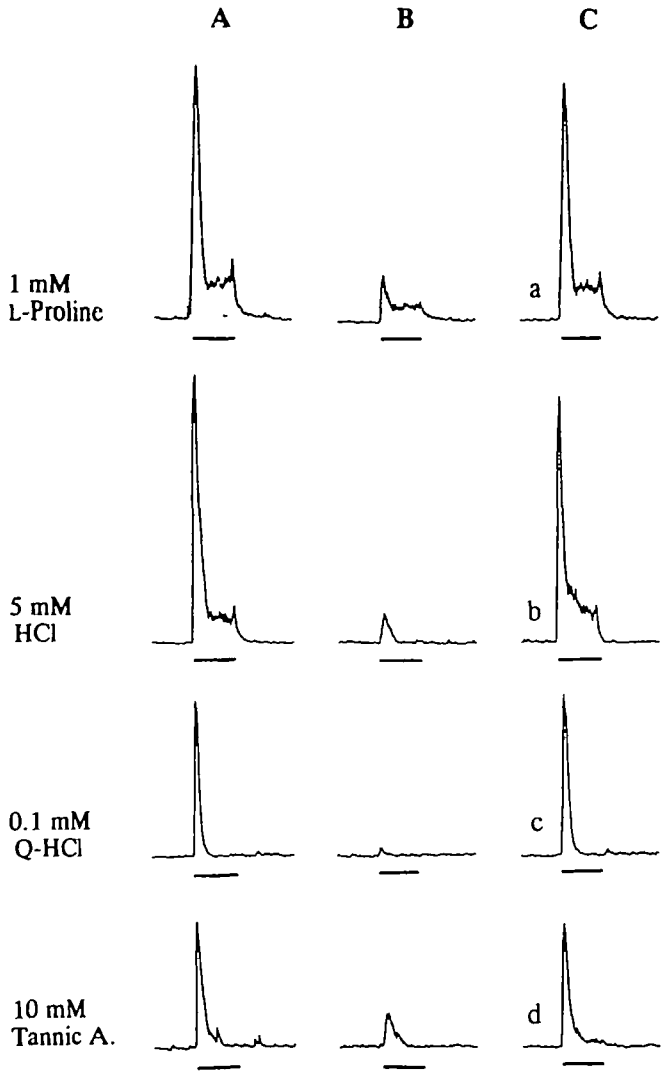
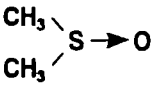
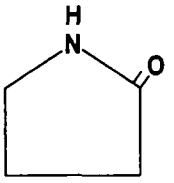
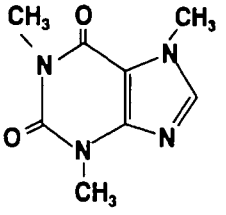
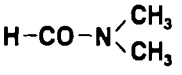
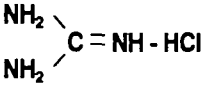
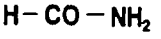



**Figure 4** The integrated responses to various taste stimuli **(A)**, depression of their responses **(B)** caused by the stimulation with 10 mM tannic acid for 10 s, and recovery from the depression **(C)** after treatment with 1 M DMF **(a)**, 1 M 2-pyrrolidone **(b)**, 1 M DSMO **(c)** and 0.1 M caffeine **(d)** for 10–15 s. After each stimulation and treatment, the oral epithelium was rinsed with water for 4 min. The solid bars at the bottom of each response represent the duration of the stimulation for 10 s.

**Table 2** Effects of various agents on the recovery of the response to tannic acid

| Competitor | Structure | Treatment time (s) | Concentration (M) | Recovery rate (%) | Mol. wt | *n* |
|---|---|---|---|---|---|---|
| Dimethylsulfoxide |  | 10 | 1 | 100.8 ± 10.9 | 78.14 | 5 |
| 2-Pyrrolidone |  | 10 | 1 | 102.6 ± 5.6 | 85.11 | 5 |
| Caffeine |  | 15 | 0.1 | 100.6 ± 7.4 | 194.19 | 5 |
| *N,N*-Dimethylformamide |  | 15 | 1 | 101.6 ± 2.6 | 73.10 | 5 |
| Guanidine–HCl |  | 20 | 1 | 85.5 ± 9.0 | 95.53 | 5 |
| Urea |  | 20 | 1 | 82.8 ± 8.7 | 60.06 | 7 |
| Formamide |  | 20 | 1 | 32.0 ± 6.1 | 45.04 | 7 |

Previous application of 10 mM tannic acid caused an abrupt decrease in responses to not only subsequent tannic acid but also all other taste stimuli tested (Figure 4B). These depressions in the taste responses continued for >20 min with usual water rinse. As shown in Figure 4(C), however, application of treatment solutions which bring about recovery of the responses to tannic acid also resulted in the rapid recovery of those to the other taste stimuli.

## Discussion

The properties characteristic of the responses to the astringent compounds tested were as follows: (i) all compounds rapidly stimulate the oral chemoreceptors in a concentration-dependent manner, and the responses are reversible at lower concentrations; (ii) an abrupt decrease in response is often detected with repetitive application even at 10 mM, at which concentration the responses do not appear to be saturated; and (iii) after the application of the astringent compounds at higher concentrations, the activity level takes a long time to return to the baseline level by rinsing with water, and responses to all other taste stimuli tested are also extremely depressed.

Kawamura *et al.* (1969) demonstrated that previous application of 5 or 20% tannic acid to the tongue suppressed the integrated responses of the rat chorda tympani and glossopharyngeal nerve to tannic acid and the other four conventional taste stimuli subsequently delivered. Schiffman *et al.* (1991) reported that the gerbil chorda tympani nerve responded as rapidly to astringent compounds as to other taste stimuli in a concentration-dependent manner, and that the response was reversible at lower concentrations, although consecutive applications of 96 mM tannic acid showed a decrease in activity.

The properties resulting from the present experiment are consistent with these findings, although the effective concentrations needed to cause the depressive phenomena are different in each species. Thus, the present results suggest that the responses of the clawed toad chemoreceptors to the astringent compounds are characteristic of astringent taste. Here, it is interesting that the threshold concentrations measured using a multichannel taste sensor with a lipid membrane, which is a constituent of biomembrane, are 10 μM for gallic acid and 300 μM for tannic acid (Iiyama *et al.*, 1994). These values agree fairly well with those estimated in the present study.

The association of polyphenols (tannins) with proteins is reversible and is largely a surface phenomenon that takes place in two distinct phases: (i) hydrophobic regions of the polyphenol form a hydrophobic bond with hydrophobic sites of proteins; and then (ii) this stage is reinforced by the appropriate deployment of hydrogen bonding between phenolic hydroxyl groups of tannins and the keto-imido or carbonyl groups of proteins (Spencer *et al.*, 1988). It is worth noting that 2-pyrrolidone and caffeine, which were, in the present experiments, sufficiently effective treatment agents for the complete recovery from the repetitive stimulation with tannic acid, have the keto-imido and the keto-amido groups that may act as proton donors and acceptors. These groups have the ability to participate in the formation of strong hydrogen bonds, and contain the pyrrolidine ring and the heterocycle (the purine ring) that may form a hydrophobic bond with proteins. DMSO and DMF also have chemical structures capable of forming these two bonds (Table 2).

Mejbaum-Katzenallenbogen and co-workers (1959, 1962) have demonstrated that caffeine competes effectively with proteins for polyphenolic substrates and that it is possible to regenerate a wide variety of proteins, in a biologically active state, from insoluble protein–tannin complexes by treatment with caffeine. Caffeine precipitates polyphenols from aqueous media by complexation. The interaction with polyphenols may be interpreted, in principle, in terms that are very similar to those of protein with phenolic groups. Thus, the following model can be postulated from their results: the purine ring of caffeine causes hydrophobic interactions with polyphenol molecules, while keto-amido groups of caffeine form hydrogen bonds with the phenolic hydroxyl groups of polyphenols (Spencer *et al.*, 1988). If the decrease in the response caused by repetitive stimulation with tannic acid is due to the complexes of receptor molecules with tannic acid that were not rinsed out by the interstimulus water rinse, these agents may compete with the receptor molecules (proteins) for the residual tannic acid of the complexes so as to regenerate the receptor molecules in an active state.

Hydrogen bonding between hydroxyl and peptide carbonyl is strengthened by alkyl substitution on the amide nitrogen adjacent to the carbonyl (Cannon, 1955). The present result that the substituted derivative, DMF, showed a higher recovery rate than the parent compound, formamide, is due in part to the enhanced strength of the hydrogen bonding. DMF, however, also has two methyl groups showing hydrophobicity, as in DMSO (Table 2). Guanidine–HCl and urea, which were effective to a certain extent for the recovery of the response to tannic acid, can participate in the formation of strong hydrogen bonds but are not intrinsically hydrophobic (Oh *et al.*, 1980).



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