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Enhancement of Salt Responses in Frog Gustatory Nerve by Removal of Ca^{2+} from the Receptor Membrane Treated with 1-Anilinonaphthalene-8-Sulfonate

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Summary. The frog tongue was incubated in 1-anilinonaphthalene-8-sulfonate (ANS) solution and the responses of the glossopharyngeal nerve to various chemical stimuli were measured after the ANS solution was washed out. The responses to galactose, quinine and distilled water were unchanged by the ANS treatment. On the other hand, the responses to the salts, except for $CaCl_2$, were enhanced in greater or lesser degree after the ANS treatment. The order of relative magnitude of the enhanced response to 100 mM salts of monovalent cations was $Na^+ > NH_4^+ > K^+ > Li^+$, while that before the treatment was $NH_4^+ > K^+ > Na^+ > Li^+$. The enhancement of the salt responses was also observed after the tongue was treated with 6-*p*-toluidinonaphthalene-2-sulfonate or 1,2-cyclohexanediaminetetraacetic acid solution.

The enhanced responses to the salts were suppressed to the original level before the ANS treatment by addition of $CaCl_2$ or $SrCl_2$. The suppression curve satisfied the Langmuir adsorption isotherm when the suppression was postulated to be responsible for the binding of Ca^{2+} or Sr^{2+} to the receptor membrane treated with ANS. The apparent binding constants for Ca^{2+} and Sr^{2+} in the presence of 100 mM NaCl were obtained to be $1.2 \times 10^4\ M^{-1}$ and $6.7 \times 10^3\ M^{-1}$, respectively.

The ANS treatment modified the temperature dependence of the salt responses. For example, 100 mM KCl solution of low temperature induced a large response after the ANS treatment, while that of 20 °C induced only small response.

It was concluded that the removal of Ca^{2+} from the gustatory receptor membrane in the frog, which was brought about by the ANS treatment, led to the enhancement of the salt responses. The mechanism on the enhancement of the salt response by the Ca^{2+} removal was discussed.

The role of divalent cations in regulation of the membrane function has become a matter of increasing interest [6, 7, 16]. In some excitable membranes, reducing the concentration of divalent cations in the external medium leads to removal of divalent cations from the membranes and, in turn, brings about a low threshold, slow accommodation or repetitive

firing. On the other hand, application of a solution free of divalent cations to the gustatory organs does not bring about any particular effect on the function of the gustatory receptor membrane. Probably, the divalent cations are so tightly bound to the gustatory receptor membrane that reducing the concentration of divalent cations in the external medium does not lead to the removal of divalent cations from the receptor membrane. At present, we have very little information as to the role of divalent cations in the gustatory receptor membrane. In the present study, we have found that the application of 1-anilinonaphthalene-8-sulfonate (ANS) to frog tongue removes Ca^{2+} from the gustatory receptors, which results in enhancement of the salt response.

Various species of animals respond quite differently to various inorganic salts. For example, the rodents respond well to NaCl and LiCl compared to KCl, whereas the opposite is true with the carnivora [4]. The frog responds similar to the carnivora to these salts of monovalent cations. It is, however, shown in the present paper that the ANS treatment of the frog tongue changes the order of relative magnitude of the response to various salts.

Materials and Methods

Adult bullfrogs, *Rana catesbeiana,* in autumn, winter and spring seasons were used in the present experiments. The enhancement of the salt response by the ANS treatment was relatively small with the frog in summer season compared with the frog in other seasons. The preparation of the tongue with the glossopharyngeal nerve and the recording method of nerve activity employed here were the same as those described in the previous paper [11, 12, 13]. The nerve impulses were amplified with a CR-amplifier (Iwatsu Electric Co. Type HP-606) and integrated with an electronic integrator (Nihon Kohden Co. Type RFJ-5) with a time constant of 0.3 sec.

The ANS treatment of the frog tongue was carried out as follows. The tongue was incubated in 1 mM ANS solution at 5 °C for 2 min and the ANS solution was washed away by flowing 5 mM NaCl solution at 20 °C on the tongue surface for 2 min with a flow rate of 2 ml per sec. A stimulating solution was applied to the tongue with the same flow rate. After each application of the stimulating solution, the tongue was rinsed with Ringer's solution. About 15 min were interposed between each successive stimulation. The ANS treatment was carried out before each stimulation.

The chemicals used in the present study were analytical grade. ANS and 6-*p*-toluidinonaphthalene-2-sulfonate (TNS) were purchased from Eastman Kodak Co. and 1,2-cyclohexanediamine-tetraacetic acid (CyDTA) and ethylenediamine-tetraacetic acid (EDTA) from Dojindo Laboratories. The water used to prepare all solutions was distilled in a Barnstead still and then passed through an ion exchange column. Concentration of Ca^{2+} contaminated in the water thus prepared was less than 2×10^{-6} M, which was determined by the calcein method [1]. All solutions were stored in polyethylene bottles.

The composition of Ringer's solution was: 112 mM NaCl, 3.4 mM KCl, 2.5 mM $CaCl_2$ and adjusted to pH 7.1 with 2.5 mM $NaHCO_3$/HCl buffer.

Results

The gustatory responses to various chemical stimuli were suppressed in the presence of 1 mM ANS, which is consistent with the result obtained by Nomura and Kano [14]. In the present study, simulating solution was applied to the tongue after the ANS solution was washed away from the tongue. Records *1* and *2* in Fig. 1 represent the summated responses of the frog glossopharyngeal nerve to 300 mM NaCl before and after the frog tongue was treated with 1 mM ANS solution, respectively. It is seen in the records that the ANS treatment exhibits no effect on the spontaneous activities of the nerve but leads to enhancement of the salt response. The enhancement of the salt response was also observed after the tongue was treated with TNS (*see* record *3*). However, the response to the salt after the TNS treatment declined more rapidly compared with those shown in records *1* and *2*. In the following experiments, ANS was used to obtain the enhancement effect on the salt reponses. The result shown by record *4* will be discussed later in this paper.

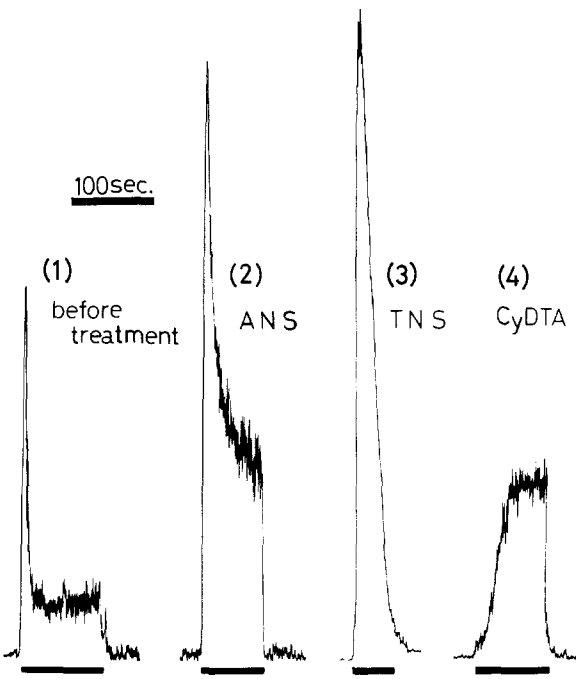

Fig. 1. The summated responses of the frog glossopharyngeal nerve to 300 mM NaCl before (*1*) and after the tongue was treated with 1 mM ANS (*2*), 1 mM TNS (*3*), and 10 mM CyDTA (*4*). The bar at the bottom of each record represents duration of application of the stimuli

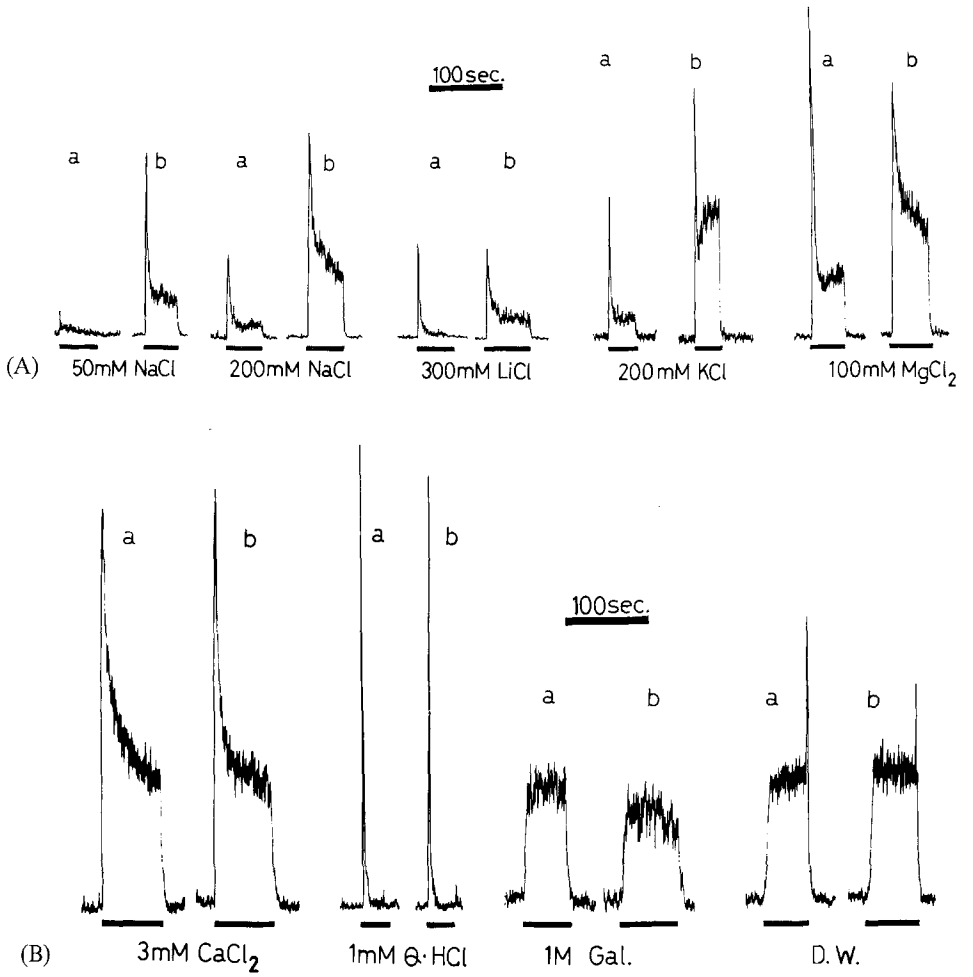

Fig. 2. (*A*): The summated responses of the frog glossopharyngeal nerve to various salts before (*a*) and after (*b*) the ANS treatment. (*B*): The summated responses of the frog glossopharyngeal nerve to $CaCl_2$, quinine hydrochloride, galactose and distilled water (*D.W.*) before (*a*) and after (*b*) the ANS treatment

The enhancing effect of the ANS treatment on the salt response depended on the temperature of ANS solution to be applied to the tongue. When the tongue was incubated in ANS solution below 10 °C, the enhancing effect was much larger than that obtained with ANS solution at 20 °C. In the present experiment, the tongue was treated with 1 mM ANS solution at 5 °C. Further increase of ANS concentration did not lead to further increase of the salt response.

Fig. 2 represents the summated response of the glossopharyngeal nerve to various species of stimuli before and after the ANS treatment.

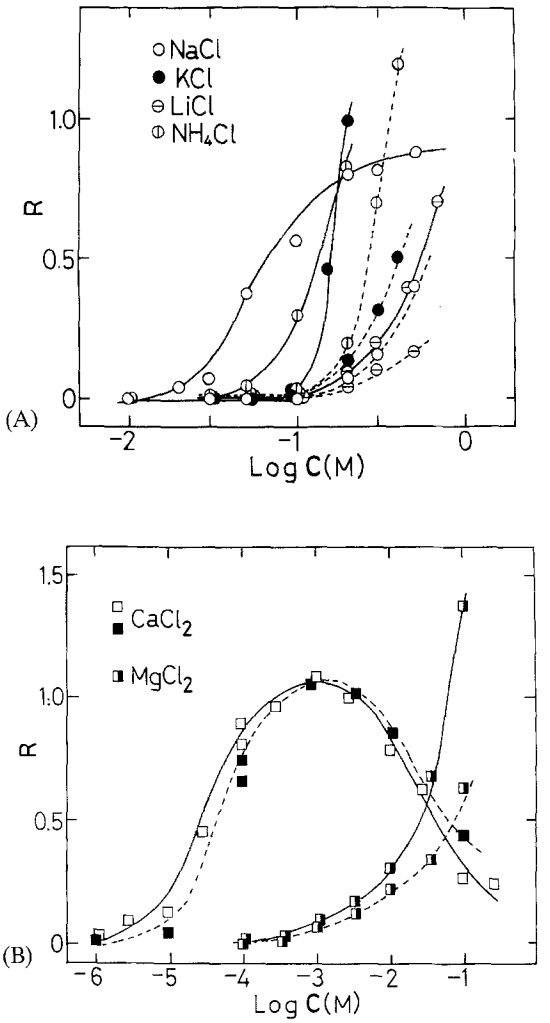

Fig. 3. Magnitude of the summated response (R) to various salts as a function of logarithm of concentration. The dotted and solid lines represent the responses before and after the ANS treatment, respectively. Magnitude of the response to 3 mM $CaCl_2$ is taken as a unit of the ordinate. (A): Salts of monovalent cations; (B): Salts of divalent cations

The figure demonstrates that the responses to NaCl, LiCl, KCl and $MgCl_2$ are enhanced in greater or lesser degree after the ANS treatment. On the other hand, the response to $CaCl_2$, quinine, galactose (galactose induced the largest response in the frog glossopharyngeal nerve among various sugars [13]) and distilled water was not affected by the ANS treatment.

In Fig. 3 magnitude of the tonic response to various salts is plotted

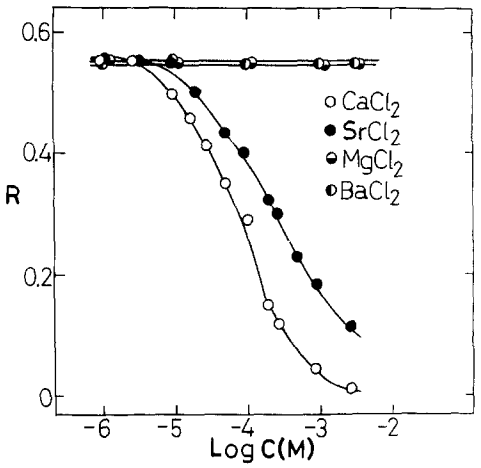

Fig. 4. Effect of divalent cations on the enhanced response to 100 mM NaCl after the ANS treatment. After the tongue was treated with 1 mM ANS, 100 mM NaCl solutions containing divalent cations of varying concentration were successively applied to the tongue

against logarithm of salt concentration. Dotted and solid lines represent the responses before and after the ANS treatment, respectively. The most pronounced effect of the ANS treatment can be seen in the effect on the NaCl response; the threshold concentration for NaCl was shifted from 100 mM to 10 mM by the ANS treatment. The threshold for NH_4Cl was also shifted by the ANS treatment. In the cases of KCl, LiCl and $MgCl_2$, the thresholds were not appreciably changed, but the enhancing effect was observed at relatively high concentration of the salts. On the other hand, any enhancing effect was not observed with the $CaCl_2$ response in the whole range of concentration examined.

The enhanced response to the salts after the ANS treatment was suppressed by the presence of $CaCl_2$ and $SrCl_2$. Open and closed circles in Fig. 4 represent the response to 100 mM NaCl in the presence of $CaCl_2$ and $SrCl_2$ of varying concentrations, respectively. Note that 1 mM $CaCl_2$ or $SrCl_2$ alone induces a large response in the frog glossopharyngeal nerve [12], but the response (tonic response) is suppressed in the presence of 100 mM NaCl [11]. As seen from the figure, addition of $CaCl_2$ or $SrCl_2$ to 100 mM NaCl returned the enhanced response to the original level before the ANS treatment. The enhanced responses to other salts such as KCl, NH_4Cl, LiCl and $MgCl_2$ also returned to the original level by addition of 3 mM $CaCl_2$. On the other hand, $MgCl_2$ and $BaCl_2$ exhibited practically no effect on the enhanced responses to the salts.

The results described above indicated that Ca^{2+} or Sr^{2+} has a suppressive effect on the enhanced response to various salts. This suggests that the binding of Ca^{2+} or Sr^{2+} to the sites in the gustatory receptor membrane treated with ANS leads to the suppression. Assuming that the Langmuir adsorption isotherm is applicable to the binding of Ca^{2+} or Sr^{2+} to the sites in the receptor membrane treated with ANS, we obtain:

$$n=\frac{NKC}{1+KC} \tag{1}$$

where n stands for number of sites occupied by Ca^{2+} or Sr^{2+}, N, total number of binding sites, K, equilibrium constant which is the reciprocal of the dissociation constant, and C, molar concentration of Ca^{2+} or Sr^{2+} in a bulk solution. If the suppression of the enhanced response is linearly related to the number of sites occupied by Ca^{2+} or Sr^{2+} (this assumption does not always imply that Ca^{2+} or Sr^{2+} binds competitively to the receptor site for Na^{+} in the membrane treated with ANS), then:

$$R_0-R=an \tag{2}$$

$$R_0=aN \tag{3}$$

where R_0 stands for magnitude of response to 100 mM NaCl in the absence of Ca^{2+} or Sr^{2+}, R, magnitude of response to 100 mM NaCl in the presence of Ca^{2+} or Sr^{2+} of varying concentrations and a, the proportionality constant. Inserting Eqs. (2) and (3) into (1) and rearranging the equation, we obtain:

$$(R_0-R)/C=-K(R_0-R)+KR_0. \tag{4}$$

In Fig. 5, $(R_0-R)/C$ is plotted against (R_0-R) according to Eq. (4). The figure indicates that the data shown in Fig. 4 follows Eq. (4), that is, the enhanced response to NaCl after the ANS treatment is suppressed by the Langmuir type binding of Ca^{2+} or Sr^{2+} to the sites in the gustatory receptor membrane. From the slope of straight line shown in Fig. 5, the apparent equilibrium constants for Ca^{2+} and Sr^{2+} were obtained to be 1.2×10^{4} M^{-1} and 6.7×10^{3} M^{-1} respectively.

The results shown by Figs. 4 and 5 indicated that the binding of Ca^{2+} or Sr^{2+} to the sites in the gustatory receptor membrane treated with ANS returned the enhanced salt response to the original level before the ANS treatment, while Mg^{2+} and Ba^{2+} gave no effect on the enhanced response. These results suggest that the ANS treatment removed Ca^{2+}

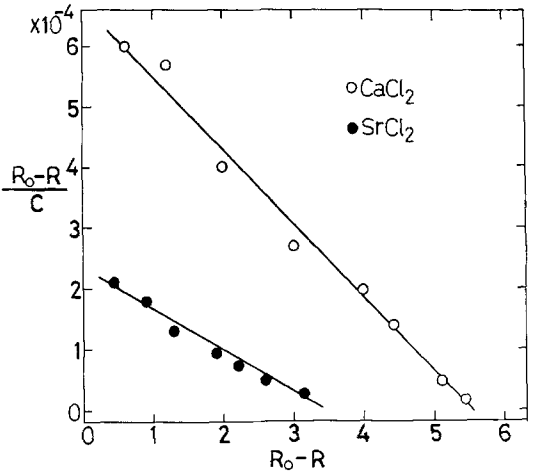

Fig. 5. Plots of $(R_0-R)/C$ against (R_0-R) according to Eq. (4). The data were taken from Fig. 4. Details are described in the text

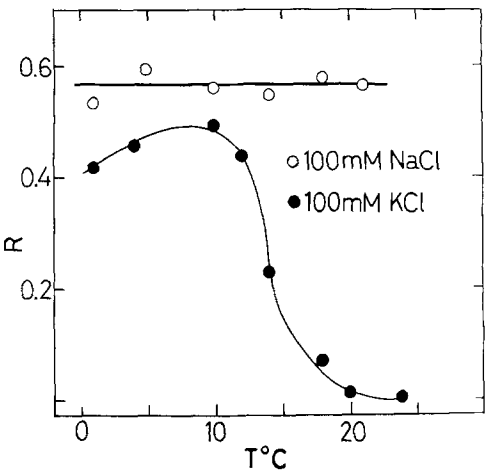

Fig. 6. Temperature dependence of the enhanced response to 100 mM NaCl and 100 mM KCl after the ANS treatment. After the tongue was treated with ANS, 5 mM NaCl solution at varying temperature was flowed on the tongue for 2 min, and then stimulating solution at the same temperature as the 5 mM NaCl solution applied previously was applied

from the gustatory receptor membrane, since it is unlikely that Sr^{2+} is bound to the biological membrane under the natural condition. This possibility was supported by the following experiment; application of CyDTA, which is a strong chelating agent for polyvalent cations [15], to the frog tongue led to a rather large enhancement of the salt response

(*see* record *4* in Fig. 1), while EDTA led to only small enhancement of the salt response and often to irreversible suppression of the nerve activity. Although CyDTA brought about the enhancement of the salt response, it is not an appropriate agent to obtain the enhancement because the phasic response was eliminated by the CyDTA treatment.

The ANS treatment of the frog modified the temperature dependence of the salt responses. Fig. 6 represents the temperature dependence of the tonic response to 100 mM NaCl and KCl after the ANS treatment. While the response to 100 mM NaCl exhibited no temperature dependence, that to 100 mM KCl exhibited a large temperature dependence. Such a large difference in temperature dependence between the NaCl and KCl responses was not seen in the responses of the frog before the ANS treatment [11].

Discussion

In the present study, it was shown that application of ANS to the frog tongue led to removal of Ca^{2+} from the gustatory receptor membrane. Since ANS is not a chelating agent for Ca^{2+}, it is unlikely that Ca^{2+} is removed from the gustatory receptor membrane by direct interaction between ANS and Ca^{2+} bound to the membrane.

ANS exhibits virtually no fluorescence in water but fluoresces strongly in hydrophobic environments and, hence, has been used as a hydrophobic probe for biological membranes. Recently, Fortes and Ellory [5] suggested that ANS expands the red cell membrane asymmetrically by binding preferentially to the external membrane surface. Based on the above experimental facts on ANS, we speculated a mechanism on the removal of Ca^{2+} from the frog gustatory receptor by the ANS treatment as follows; When ANS is applied to the frog tongue, ANS is adsorbed in the hydrophobic region of the gustatory membrane, which will induce the expansion of the receptor membrane. This expansion of the membrane will lead to cleavage of the salt bridge of Ca^{2+} between two negative sites on the membrane and then Ca^{2+} will be removed from the receptor membrane. Conformational change of the receptor membrane induced by cooling the tongue will be helpful for the removal of Ca^{2+}. The mechanism speculated above should be confirmed by further study.

When the frog tongue was treated at room temperature, only partial enhancement of the salt response was observed. In this case, the incubation of the tongue treated with ANS in Ringer's solution containing Ca^{2+} led to the strong binding of Ca^{2+} to the receptor membrane; Ca^{2+}

was not removed by reducing Ca^{2+} concentration in the external solution, inferring from the result that the NaCl response after the incubation was not enhanced even under the absence of Ca^{2+}. The treatment of the tongue with ANS at low temperature led to a large enhancement of the salt responses and the incubation of the tongue thus treated in Ringer's solution hardly led to the strong binding of Ca^{2+} to the receptor membrane; the enhancement of the salt response was observed without further ANS treatment even after the tongue was incubated in Ringer's solution for 30 min, although the ANS treatment in the present study was carried out every time before each stimulation in order to obtain reproducible data.

Tucker and Shibuya [18] measured the olfactory response and EOG while the nose of box turtle was filled with a perfusing aqueous solution. In this study, they found that reduction of Ca^{2+} concentration in the perfusing solution led to increase of spontaneous activity of the olfactory nerve. Mg^{2+} could not substitute for Ca^{2+}, but Sr^{2+} substituted quite well. In the case of the frog gustatory cell, the removal of Ca^{2+} from the receptor membrane by the ANS treatment did not lead to increase of the spontaneous activity of the gustatory nerve but the effect of divalent cations was quite similar to that in the olfactory receptor cell.

The relative magnitude of the response in the frog to the salts of monovalent cations was $NH_4^+ > K^+ > Na^+ > Li^+$ before the ANS treatment. Beidler [4] pointed out that NH_4^+ is very stimulating for most species of animals and Li^+ acts very similar to Na^+. This rule holds in the frog before the ANS treatment. However, the ANS treatment changed greatly the relative magnitude of the responses to various salts. While the NaCl response was enhanced remarkably, the Li^+ response was small even after the ANS treatment. The relative magnitude of the response after the ANS treatment was $Na^+ > NH_4^+ > K^+ > Li^+$ if the responses were compared at 100 mM.

The temperature dependence of the salt response was also changed by the ANS treatment. Yamashita [20] observed that the NaCl response of the frog was increased with a rise in temperature. After the ANS treatment, the response to 100 mM KCl was decreased greatly with a rise in temperature between 1 °C and 24 °C, while the response to 100 mM NaCl was practically unchanged in the temperature range. The fact that the large difference in the temperature dependence was observed between the NaCl and KCl responses suggest that the temperature dependence of the salt response is not stemming only from the temperature receptors in the gustatory organs. Probably, a conformational change of the gusta-

tory receptor membrane induced by the temperature change affects the structure of the receptor site for KCl.

Beidler [2] proposed that taste stimulation is induced by adsorption of the chemical stimuli to the gustatory receptor membrane. He [3] also pointed out that the generation of taste receptor potentials cannot be explained in terms of permeability change of ions at the microvilli membrane of taste cells in the vertebrates. The reasons why the ionic theory is not applicable to the microvilli membrane were listed in the previous paper [9]. Since most of the reasons still hold in the microvilli membrane treated with ANS, it would not be necessary to take account of the possibility that the permeability of the ions across the microvilli membrane is changed by removal of Ca^{2+} from the membrane.

A mechanism on the enhancement of the salt response by the Ca^{2+} removal is not known to us at the present, but the following results obtained with a primitive organism may offer a hint for understanding the results obtained in the present study. The plasmodia of the true slime mold exhibit chemotaxis against various salts as well as sugars, acids, and nucleotides, and the chemotaxis of the slime mold was closely correlated to the change in the membrane potential [19]. Furthermore, the electrophoretic experiments on the spherical microplasmodia of the slime mold indicated that the magnitude of the change in membrane potential in response to various kinds of chemicals was identical with that of the zeta-potential of the slime mold [8]. This implies that changes in the membrane potential of the slime mold in response to chemical stimulation are mainly stemming from changes in the phase boundary potential at the membrane-solution interface. Recently, it was found that the threshold concentration for NaCl both in the chemotaxis and the membrane potential change was shifted to higher concentration when about 0.1 mM $CaCl_2$ was added to NaCl solution [17]. This result is quite similar to that observed with the frog tongue treated with ANS. The electrophoretic experiments indicated that the surface charge density of the slime mold under the presence of NaCl was changed to negative direction by addition of $CaCl_2$ to NaCl solution no matter how Ca^{2+} has positive charge. The increase of the negative surface charge density seems to be brought about by either or both the following mechanisms: The binding of Ca^{2+} to the surface membrane stabilizes the membrane structure, and hence a conformational change of the receptor domain in response to adsorption of Na^+ is less liable to occur. The binding of Ca^{2+} leads to a conformational change of the surface membrane, and, in turn, the negative charge of the adsorption

site for Na^+ is increased. The increase of the negative surface charge raises the threshold for NaCl in the zeta-potential, and, in turn, the threshold in the membrane potential rises.

In a series of papers [9, 10, 12, 13], we proposed that taste receptor potentials are mainly stemming from changes in the phase boundary potential at the membrane-solution interface. The Ca^{2+} effect on the frog gustatory receptor treated with ANS may also be explained in terms of changes in the surface charge density of the receptor membrane as with the slime mold. In the present study, it was shown that the suppressive effect of Ca^{2+} on the enhanced salt response is attributable to the binding of Ca^{2+} to the ANS-treated membrane, but does not always imply that Ca^{2+} binds competitively to the receptor site for Na^+ in the ANS-treated membrane. The result may be understood provided that the negative surface charge of the gustatory membrane under the presence of NaCl increases with increase of the amount of Ca^{2+} bound to the membrane. However, the speculative mechanism described above should be confirmed by more direct experiments.

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