Chorda Tympani Responses Under Lingual Voltage Clamp: 
Implications for NH$_4$ Salt Taste Transduction

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Chorda tympani responses under lingual voltage clamp: implications for NH$_4$ salt taste transduction. J. Neurophysiol. 77: 1393–1406, 1997. Rat chorda tympani (CT) responses to NH$_4$Cl, ammonium acetate (NH$_4$Ac), and ammonium hippurate (NH$_4$Hp) were obtained during simultaneous current and voltage clamping of the lingual field potential. Although functional and developmental similarities for gustation have been reported for NH$_4^+$ and K$^+$ salts, we report here that significant differences are discernible in the CT responses to both salts. Unlike neural responses to KCl, those to NH$_4$Cl are voltage sensitive, enhanced by submucosa negative and suppressed by positive voltage clamp. In this regard, NH$_4$Cl responses are qualitatively similar to NaCl responses; however, the magnitude of NH$_4$Cl voltage sensitivity is significantly less than that of NaCl. The concentration dependence of the CT response to NH$_4$Cl manifests a biphasic nonlinear relationship not observed with KCl or NaCl. Below 0.3 M, the CT response increases as if to approach a saturation value. However, beyond 0.3 M an inflection appears in the CT-concentration curve because of an abrupt increase in CT responses. This kinetic profile is Cl$^-$ dependent and is correlated with an increase in transepithelial conductance that displays similar NH$_4$Cl concentration dependence. The biphasic relation to salt concentration is not observed when acetate or hippurate is substituted for Cl$^-$. As with Na$^+$ and K$^+$ salts, less mobile anions than Cl$^-$ (Ac$^-$ and Hp$^-$) lower the CT responses. However, like Na$^+$ salts, but in contrast to K$^+$ salts, the onset kinetics of CT responses to NH$_4$Ac or NH$_4$Hp remain rapid, even under positive voltage-clamp conditions. Amiloride (100 μM) partially suppresses CT responses within the concentration range of 0.05–0.3 M (48–20% suppression). Amiloride also suppresses the voltage sensitivity of NH$_4$Cl CT responses, but does not eliminate that sensitivity as it does for Na$^+$ salts. In conclusion, the data suggest that taste transduction for NH$_4$ salt is mediated over two taste transducing elements. A recent study indicates that K$^+$ salt CT responses are mediated by a single, diffusion-controlled voltage-insensitive transduction mechanism (Ye et al. 1993). K$^+$ salt responses occur via a sub-tight junctional transducer for K$^+$ ions with access limited by anion mobility. In contrast, NaCl CT responses consist of a voltage-dependent (amiloride-sensitive) and smaller voltage-independent (amiloride-insensitive) component (Ye et al. 1993). The amiloride and voltage sensitivity suggests that taste receptors for NH$_4^+$ and Na$^+$ are, at least in part, functionally distinct. A recent study indicates that K$^+$ salt CT responses are mediated by a single, diffusion-controlled voltage-insensitive transduction mechanism (Ye et al. 1993). K$^+$ salt responses occur via a sub-tight junctional transducer for K$^+$ ions with access limited by anion mobility. In contrast, NaCl CT responses consist of a voltage-dependent (amiloride-sensitive) and smaller voltage-independent (amiloride-insensitive) component (Ye et al. 1993). The amiloride and voltage sensitivity suggests that taste receptors for NH$_4^+$ and Na$^+$ are, at least in part, functionally distinct. A recent study indicates that K$^+$ salt CT responses are mediated by a single, diffusion-controlled voltage-insensitive transduction mechanism (Ye et al. 1993). K$^+$ salt responses occur via a sub-tight junctional transducer for K$^+$ ions with access limited by anion mobility. In contrast, NaCl CT responses consist of a voltage-dependent (amiloride-sensitive) and smaller voltage-independent (amiloride-insensitive) component (Ye et al. 1993). The amiloride and voltage sensitivity suggests that taste receptors for NH$_4^+$ and Na$^+$ are, at least in part, functionally distinct.

The main objective of this study is to investigate the transduction mechanisms involved in NH$_4^+$ salt taste perception. This was accomplished by comparing CT responses to NH$_4^+$ salts, with the use of the in situ lingual voltage-clamp method, with the responses to Na$^+$ and K$^+$ salts. The results indicate the presence of two transduction mechanisms for NH$_4$Cl: an apical NH$_4^+$ ion conductance, dominant with NH$_4$Cl concentrations below ~0.3 M, and a mechanism accessible via the paracellular pathway. The latter is especially prominent in the presence of Cl$^-$ and with NH$_4^+$ concentrations >0.3 M.

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INTRODUCTION

NH$_4$Cl has been widely used as a reference stimulus in recordings from different levels of the taste sensory system in various animal models. These include cortical taste areas (Ogawa et al. 1994), the nucleus tractus solitarius (Hill et al. 1983; Nakamura and Norgren 1993), and the chorda tympani (CT) nerve (Elliott and Simon 1990; Hill and Phillips 1994; Hyman and Frank 1980; Somenarain et al. 1992; Ye et al. 1994). Animal (Erickson 1963) and human (van der Klauuw and Smith 1995) psychophysical studies have suggested similarities in the taste quality profiles of NH$_4$Cl and KCl. In addition, functional similarities at the level of nucleus tractus solitarius for NH$_4^+$ and K$^+$ salts have been suggested (Nakamura and Norgren 1993). NH$_4^+$ and K$^+$ ions have similar hydrated radii and ionic conductances in free solution (Knepper et al. 1989). These ions may also have similar properties at the cell membrane level, e.g., NH$_4^+$ has been found to substitute for K$^+$ on transporters in many cell types (Amlal et al. 1994; Kinne et al. 1986; Tsuruoka et al. 1993).

Developmental studies of salt taste in neonatal rats show that CT responses to NH$_4$Cl are fully developed before those to NaCl (Hill et al. 1982; Mistretta and Bradley 1980; Yamada 1980). Recordings from more central loci in the taste neuraxis, in the nucleus tractus solitarius (Hill et al. 1983), and in cortical taste areas (Ogawa et al. 1994) are generally consistent with this. The different maturation rates for NaCl and NH$_4$Cl neural responses suggest that taste receptors for NH$_4^+$ and Na$^+$ are, at least in part, functionally distinct. A recent study indicates that K$^+$ salt CT responses are mediated by a single, diffusion-controlled voltage-insensitive transduction mechanism (Ye et al. 1994). K$^+$ salt taste responses occur via a sub-tight junctional transducer for K$^+$ ions with access limited by anion mobility. In contrast, NaCl CT responses consist of a voltage-dependent (amiloride-sensitive) and smaller voltage-independent (amiloride-insensitive) component (Ye et al. 1993). The amiloride and voltage sensitivity suggests that this Na taste transducing element is an apical membrane ion channel (Avenet and Lindemann 1991; Garty and Benos 1988). The amiloride-insensitive component depends on the presence of Cl$^-$ in Na salt taste (Elliott and Simon 1990; Formaker and Hill 1988; Ye et al. 1993).

The main objective of this study is to investigate the transduction mechanisms involved in NH$_4^+$ salt taste perception. This was accomplished by comparing CT responses to NH$_4^+$ salts, with the use of the in situ lingual voltage-clamp method, with the responses to Na$^+$ and K$^+$ salts. The results indicate the presence of two transduction mechanisms for NH$_4$Cl: an apical NH$_4^+$ ion conductance, dominant with NH$_4$Cl concentrations below ~0.3 M, and a mechanism accessible via the paracellular pathway. The latter is especially prominent in the presence of Cl$^-$ and with NH$_4^+$ concentrations >0.3 M.

METHODS

Surgical preparation

The surgical procedure has been described in detail (Heck et al. 1989; Ye et al. 1993). Sprague-Dawley rats weighing 180–240 g were preanesthetized with ether and then given an intraperitoneal injection of pentobarbital sodium (65 mg/kg). Additional injections were administered as needed during the experiment. Rats were placed on an isothermal pad to maintain their body temperatures. The trachea was cannulated, and the head was immobilized with a nontraumatic head holder (Erickson 1966). The left CT nerve was surgically exposed, cut caudally, and placed on a platinum electrode. Petroleum jelly was placed around the CT and a platinum reference electrode was positioned nearby.

Stimulation chamber and recording

The stimulation chamber allowed delivery of stimulus solution to a 7 mm diam section of the anterior tongue containing an average of 25 fungiform papillae (Miller 1976). Aliquots (3 ml) of stimulating and rinse solutions were injected into the chamber at 1 ml/s, and the solutions were kept in the chamber for 1 min. The whole CT neural activity was detected with a battery-operated differential amplifier (Ye et al. 1993). The amplified signal was recorded on a modified Toshiba DX-900 VCR, filtered by a band-pass filter (cutoff frequencies 40 Hz to 3 kHz), and fed to an oscilloscope. To obtain integrated CT responses, the signal was full wave rectified and integrated with a time constant of 1.0 s and displayed on a Linseis TYP 7045 strip chart recorder.

Current and voltage clamp

Transepithelial voltage or current clamp was maintained with a model VCC600 voltage-clamp amplifier (Physiological Instruments, San Diego, CA). An Ag/AgCl current-passing electrode and a voltage-sensing salt bridge were placed noninvasively beneath the tongue. A second Ag/AgCl current-passing electrode, positioned inside the stimulating inflow tubing, was in electrical contact with solutions at all times and acted as virtual ground. A second voltage-sensing salt bridge was placed in the chamber. The clamp could be operated in either voltage- or current-clamp mode. In voltage-clamp mode, the clamp drove sufficient current so that the differential voltage ($V_m$) matched a programmed reference voltage. All voltages were referenced to the mucosal side, and the direction of positive current was taken as the direction of the cation flow from mucosa to submucosa. The potential at zero current clamp ($V_{m0}$) yielded the equivalent of an open circuit potential. The voltage-clamp values in our experiments were measured relative to the current-clamp potential ($V_{cm}$; $\Delta V = V_{m0} - V_{cm}$). A periodic (15 s) bidirectional pulse of either $1 \mu A$ (current clamp) or 20 mV (voltage clamp) was generated to measure the transepithelial resistance and transepithelial conductance (TC).

Data analysis

Integrated CT responses were analyzed off-line as previously described (Ye et al. 1993). The area under the integrated CT response curve for 1 min from the onset of chemically evoked neural activity was used as the numerical value of an integrated CT response. Areas were calculated with the use of the computer software AutoCad (Autodesk, Sausalito, CA). To detect changes in the responsiveness of the CT, 0.1 M NaCl was applied at the beginning and end of each experiment as a reference. Only preparations that maintained a stable baseline throughout the experiment were used. A series of neural responses was included for analysis only when the initial and final NaCl responses differed by <20%. In any given experiment the initial/final NaCl response difference was used to correct all other responses by assuming that the rate of change in neural activity during the course of the experiment was linear. All responses for a given animal were normalized to that of 0.1 M NaCl. Phasic responses were observed to be sensitive to nonchemosensory factors, such as stimulus flow rate, and therefore were not analyzed in this study. In experiments with amiloride, 0.3 M KCl was used as a second reference, because NaCl CT responses are amiloride sensitive and recover slowly after repeated amiloride application (DeSimone and Ferrell 1985). To quantify the effect of the amiloride on NH4Cl CT responses, the normalized CT responses for a given concentration of NH4Cl were compared in the presence and absence of the drug, as illustrated

\[
\begin{align*}
[\text{NH}_4\text{Cl}] & \quad [\text{NH}_4\text{Cl} + \text{amiloride}] \\
1 \text{ min Rinse} & \quad 1 \text{ min Rinse}
\end{align*}
\]

Statistics

Numerical results are expressed as means ± SE. Statistical significance was determined by paired Student’s $t$-test or by one-way analysis of variance. $P$ values < 0.05 were considered statistically significant.

Solutions and chemicals

STIMULUS SALTS. NaCl, KCl, NH4Cl, NH4Ac, and NH4Hp were obtained from Mallinkrodt Chemical (Paris, KY). All chemicals were reagent grade and were prepared in distilled water. A 1-min rinse consisting of 15 M KHCO3 and 15 mM KCl (pH 8.3) preceded and followed each test stimulus. To maintain stable recording conditions, NaCl-depleted Krebs-Henseleit buffer was periodically applied to the tongue as an artificial saliva. The buffer consisted of (in mM) 6 KCl, 2 CaCl2, 1.2 MgSO4, 1.3 NaHPO4, 25 NaHCO3, and 5.6 glucose, pH 7.5. Test stimuli had pH values between 5.1 and 7 depending on the stimulus and its concentration. Test stimuli had pH values between 5.1 and 7 depending on the stimulus and its concentration (NH4Cl, 0.05–0.6 M, pH 5.92±5.1; NH4Ac, 0.1±1 M, pH 6.5±7; NH4Hp, 0.1–0.5 M, pH 5.49–6). To test for a specific effect of pH on the CT response, 0.5 M NH4Cl solutions were adjusted to pH 5.1, 5.3, 5.6, and 5.8 with tris (hydroxymethyl) aminomethane (tris). A trachea was cannulated, and the head was immobilized with a nontraumatic head holder (Erickson 1966). The left CT nerve was surgically exposed, cut caudally, and placed on a platinum electrode. Petroleum jelly was placed around the CT and a platinum reference electrode was positioned nearby.

RESULTS

pH effects

As stated in the METHODS, changes in the NH4Cl concentration over the range of 0.05–0.6 M were accompanied by a monotonic decrease in pH from 5.9 to 5.1. We tested the effect of pH over this range with a fixed concentration of ammonium ion to determine the extent of contribution of pH to the observed CT response. The integrated CT responses to 0.5 M NH4Cl at pH 5.1, 5.3, 5.6, and 5.8 did not differ significantly from each other (data not shown). pH changes
perturbation (Fig. 1, middle). As previously reported (Ye et al. 1994), the absence of the voltage-dependent modulation of the CT response to KCl is probably caused by the absence of an apical membrane K⁺ transduction site. Figure 1, bottom, also shows a voltage-dependent modulation of the CT response to 0.2 M NH₄Cl. Clamping at negative voltage enhanced the CT response; positive voltage suppressed it. In this regard, NH₄Cl responses are qualitatively similar to NaCl responses, but the voltage sensitivity for NH₄Cl is less than that for NaCl. The sensitivity of NaCl, KCl, and NH₄Cl CT responses to voltage perturbation is apparent when expressed as the voltage sensitivity index (VSI), defined as

\[ \text{VSI} = R(c, -50) - R(c, +50) \]

where \( R \) is the CT response to a salt stimulus at a concentration \( c \) and clamp voltage \( V \) (here, ±50 mV). Table 1 shows that VSI for 0.1 M NaCl is significantly greater than for NH₄Cl. Although the VSI for NH₄Cl at a higher concentration increased (e.g., VSI for 0.2 M NH₄Cl in Table 1), it never exceeded that for 0.1 M NaCl. VSI values for KCl were ≤ 0.1 and did not vary significantly with concentration (Ye et al. 1994). One can summarize the voltage sensitivity of NaCl, KCl, and NH₄Cl as follows

\[ \text{VSI} (\text{NaCl, 0.1 M}) > \text{VSI} (\text{NH}_4^+ \text{Cl, } c) > \text{VSI} (\text{KCl, } c) \]

**CT responses and TC**

A typical CT recording for a series of NH₄Cl concentrations (0.05–0.5 M) under zero current clamp is shown in Fig. 2, bottom. The concentration–CT response relation shows an unusual nonlinearity. Below ~0.3 M the CT response increased with concentration in a manner suggesting saturation kinetics. However, above 0.3 M an abrupt increase in CT response appeared. In fact, this nonlinearity of the CT responses as a function of concentration was observed in every experiment in which a range of NH₄Cl concentration was used. Although the concentration at which the abrupt increase in CT response was observed varied somewhat among animals, the main inflection point occurred ~at 0.3 M. Figure 3A shows a plot of a concentration–CT response function for three individual experiments and the mean responses for all experiments conducted (Fig. 3B). The biphasic character of the concentration–CT relation was a dominant feature in all animals studied. This also implies that a small change in one variable (NH₄Cl concentration) produces a large nonlinear change in the other variable (CT response). In fact such an interactive correlation is a characteristic of a positively cooperative process (Fromm 1975).

**Voltage sensitivity of NH₄Cl CT responses**

Figure 1, top, shows the CT response to 0.1 M NaCl at zero current clamp and ±50 mV. In accordance with earlier results, positive voltage perturbation of the lingual epithelium caused marked suppression and negative voltage perturbation enhanced the CT response (Ye et al. 1993). Modulation of the CT response through voltage perturbation follows from the conduction properties of the amiloride blockable epithelial Na⁺ channels in taste cell apical membranes (Avenet and Lindemann 1991; Ye et al. 1993). In contrast, CT responses to 0.25 M KCl were insensitive to voltage within this range are evidently not a factor in the magnitude of the ammonium response (see also DISCUSSION).

**Table 1. VSI**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>VSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M NaCl</td>
<td>1.13 ± 0.09</td>
</tr>
<tr>
<td>0.25 M KCl</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>0.1 M NH₄Cl</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>0.2 M NH₄Cl</td>
<td>0.54 ± 0.02</td>
</tr>
</tbody>
</table>

Values for voltage sensitivity index (VSI) are means ± SE (n = 5). Note that the VSI for NH₄Cl is lower than that for NaCl and never reached a value greater than that for 0.1 M NaCl. Note also the low VSI for KCl relative to NaCl and NH₄Cl VSIs.
FIG. 2. Typical experiment showing the integrated CT and voltage responses, under zero current clamp, for a series of NH₄Cl concentrations ranging from 0.05 to 0.5 M. Pulses superimposed on the potential response were used as a measure of resistance. Voltage pulses were in response to a periodic (15 s) bidirectional current pulse (1 μA). Note how the neural responses (bottom) appear to reach a saturation limit at 0.3 M. Note also the abrupt increase in the CT response above 0.3 M. These manifestations in the CT response are also seen in the transepithelial resistance (top) at the same concentrations of NH₄Cl.

Further demonstration of this phenomenon can be obtained by the use of Scatchard plot analysis. Figure 3C shows a Scatchard plot over NH₄Cl concentration ranging from 0.05 to 0.7 M. Analysis of this plot indicates the presence of two process: one describes the CT responses <0.7 (which corresponds to 0.3 M NH₄Cl concentration), with a linearity suggesting a single Michaelis-Menten-type process in this region. The other process is characterized by a pronounced convex geometry (Hill type). The latter finding is considered to be diagnostic of positive cooperativity (Boeynaems and Dumont 1975; Fromm 1975). The rising phase of this convex is expected with the Hill coefficient >1, and the falling phase is also expected when the final saturation region has been reached. At saturation, a further increase in one variable (NH₄Cl concentration) produces no change in the other variable (CT response), and therefore the ratio diminishes.

Monitoring changes in TC along with the CT response provides some insight into the origin of the unusual NH₄Cl CT response changes with concentration. As shown in Fig. 2, top, the abrupt increase in CT response at >0.3 M coincides with a sudden decrease in transepithelial resistance (i.e., an increase in TC). Figure 4 shows a plot of the normalized concentration-conductance functions for three individual experiments (top) and the mean normalized conductance for all experiments analyzed (bottom). The similarities between the two functions (concentration-CT and concentration-conductance) suggest a correlation between TC and NH₄Cl-evoked neural responses. We note, however, that the CT response function saturates at lower NH₄Cl concentrations than the TC function. This probably reflects a limitation on further increases in CT response by the cellular processes that determine maximum rate of firing of taste nerves. The latter play little if any role in determining the conductance of transepithelial ion shunts.

The mean CT responses as a function of NH₄Ac concentration under zero current clamp are shown in Fig. 5A. The NH₄Ac neural response is nearly a linear function of concentration. The absence of the biphasic character in the CT responses (Fig. 5A) and in the normalized conductances for NH₄Ac (Fig. 5B) suggests that these responses differences are Cl⁻ dependent; the biphasic response profile seen with NH₄Cl was observed neither for NH₄Ac nor for NH₄Hp (not shown). The similarities between the functional profiles of TC and CT response for NH₄Cl suggest that the increase in CT response at >0.3 M is caused by a significant increase in TC. Because the paracellular ion pathways comprise a major part of the TC, it would appear that the NH₄Cl-induced conductance increase is a highly cooperative process that takes place when the concentration of NH₄⁺ ion exceeds a critical value (see DISCUSSION). Achieving the critical NH₄⁺ ion concentration is evidently coion limited, because the biphasic conductance is observed for NH₄Cl but not for NH₄Ac or NH₄Hp. The obvious correlation of the respective TC changes with the CT responses for NH₄Cl and NH₄Ac suggests that transport of ammonium salts through the paracellular shunts plays a role in taste transduction for NH₄ salts, especially for NH₄Cl at concentrations >0.3 M.

NH₄Cl response under voltage perturbation

Analysis of the biphasic character of the concentration–CT response, Scatchard Plot, and concentration-conductance functions suggests the presence of two types of processes: a Michaelis-Menten type and a Hill type. On this basis, and on the basis of other data presented latter in this paper, we have constructed a model that describes the two regimes of the CT-concentration response function and each of its transformations (see APPENDIX). The theoretical lines in Fig. 6A are derived from that model, and when the transepithelial potential is considered, the same model describes the CT-clamped voltage function at constant NH₄Cl concentration (Fig. 6B). Figure 6A shows the change in CT response for a series of NH₄Cl concentrations under zero-clamp and ±50-mV voltage-clamp conditions. When the concentration series was run at ±50-mV voltage clamp, CT responses were generally suppressed and biphasic responses, seen under zero current clamp, were not observed. At ±50-mV voltage clamp, responses were enhanced as indicated by a shift of the whole concentration–CT response relation to the left along the concentration axis. The ±50-mV curve also showed evidence of saturation at >0.4 M. A more detailed plot of the CT response as a function of clamp voltage is shown in Fig. 6B. Here we display the voltage dependence of the response for two concentrations, one lower (0.2 M) than the inflection concentration (0.3 M) in the biphasic response curve and one higher (0.5 M). At 0.5 M, negative voltage perturbation does not result in a significant increase in CT response, i.e., true saturation of the NH₄⁺ CT response has been achieved at or slightly above 0.5 M under zero current-clamp conditions. At some lower concentrations, sufficient negative voltage perturbation can result in response enhancement up to
FIG. 3.  A: 3 individual experiments showing the normalized CT response as a function of NH₄Cl concentration. Each experiment consists of a concentration series; the response to 0.1 M NaCl was assigned a value of 1 and all other responses were normalized to it. B: normalized mean CT responses as a function of NH₄Cl concentration. Each point represents the mean ± SE (n = 6–12 except for the last 2 points, where n = 3). Note that in A and B CT responses exhibit biphasic profiles with a mean inflection point at ~0.3 M. C: Scatchard plot for NH₄Cl concentrations ranging from 0.1 to 0.7 M. Note that the plot contain evidence of 2 processes: noncooperative and cooperative.

The CT responses to NH₄Cl at 0.2 M exceeded those to NH₄Ac and NH₄Hp under zero current clamp at the same concentration (Fig. 7, top). The values of Vₑ for NH₄Cl were typically less than those for the same concentration of NH₄Ac and NH₄Hp (Fig. 7, top). Figure 7 also shows that the TCs for NH₄Cl exceed those for NH₄Ac and NH₄Hp, consistent with the higher mobility of Cl⁻ than Ac⁻ or Hp⁻. Consistent with previous results for NaCl and KCl (Ye et al. 1993, 1994), the Cl⁻ salt gave the highest CT response at zero current clamp among the NH₄⁺ salts (Fig. 7, top). At sufficiently high negative voltage clamp (in this case –75 mV for 0.2 M salts; Fig. 7, middle), all responses were enhanced and differences were completely compensated. At +75 mV (Fig. 7, bottom) the response for NH₄Cl was significantly suppressed, whereas those to NH₄Ac and NH₄Hp were not. This probably reflects the fact that 0.2 M NH₄Ac and NH₄Hp responses were small in current-clamp mode; the responses show measurable suppression at higher concentrations (not shown). At +75 mV, the Cl⁻ salt continued to give the largest response, as it did under current-clamp conditions. In this respect the NH₄⁺ salts behave like the Na⁺ salts; responses to Cl⁻ exceed those to other anions. This is also true for K⁺ salts, but NH₄⁺ and Na⁺ salt responses share similar temporal characteristics that are different from K⁺ salt responses. Both Na⁺ and NH₄⁺ salt responses show a rapid phasic first component in their CT responses irrespective of the anion. However, large anions such as gluconate eliminate the phasic response to K⁺ salts altogether and overall have a more profound inhibitory effect (Ye et al. 1994).
Effect of various channel blockers

BaCl$_2$ (5 mM), MIA (1 $\mu$M), and amiloride (100 $\mu$M) were tested as possible inhibitors of CT responses to NH$_4$Cl. The tongue was first rinsed for 1 min in a solution containing either BaCl$_2$ or MIA, and then a stimulus solution of either 0.2 or 0.5 M NH$_4$Cl containing the same test agent was applied. No significant differences in CT responses were observed between control presentations and BaCl$_2$- or MIA-treated cases (data not shown). However, a significant reduction in CT responses was observed with amiloride treatment. Further investigation of the amiloride effect on NH$_4$Cl CT responses revealed that it suppressed CT responses in a concentration-dependent manner. Figure 8 shows CT responses as a function of NH$_4$Cl concentration at zero current clamp with and without amiloride (100 $\mu$M). The application of amiloride did not change the biphasic profile for NH$_4$Cl CT responses. Furthermore, the inhibitory effect of amiloride was nonuniform over the two domains of the NH$_4$Cl CT responses. The percent suppression decreased with increasing NH$_4$Cl concentration, with the largest percent suppression at <0.3 M. The percent suppression ranged from 48% for 0.05 M to 4% for 0.5 M NH$_4$Cl. As previously shown (Ye et al. 1993), the voltage-sensitive component of NaCl CT responses is amiloride sensitive, i.e., amiloride eliminated the voltage-dependent modulation of NaCl CT responses. Because NH$_4$Cl CT responses were also voltage sensitive, we tested the effect of amiloride on the voltage-sensitive component of the NH$_4$Cl CT response. Figure 9 shows the CT response to 0.2 M NH$_4$Cl under voltage clamp with or without 100 $\mu$M amiloride. This concentration was chosen as a representative of the lower concentration domain of NH$_4$Cl CT responses, where the amiloride sensitivity is higher. After the application of amiloride, the CT responses were significantly less voltage sensitive. However, amiloride treatment did not completely eliminate the voltage sensitivity of NH$_4$Cl CT responses, as it does for NaCl CT responses (Ye et al. 1993). Figure 9 shows that the suppression of the CT response by amiloride is also voltage dependent. The percent suppression is larger at negative voltage than at zero current clamp or positive voltage clamp (see DISCUSSION). On the other hand, the application of amiloride did not significantly affect the CT response for 0.5 M NH$_4$Cl under any voltage-clamp condition (Fig. 9). These results suggest
that the voltage-sensitive component of the high concentration domain is amiloride insensitive.

Amiloride suppression of the CT response of NH₄Ac under zero current-clamp condition was also observed (data not shown). Amiloride suppressed the CT response to 0.5 M by 33%. This is a higher percent suppression than was observed for 0.5 M NH₄Cl (4%, Fig. 8). This is probably because NH₄Ac responses show less of the relatively amiloride-insensitive higher

![Graph](image1)

**FIG. 5.** A: normalized mean CT responses as a function of NH₄Ac concentration ($n = 4$). B: normalized mean conductances at 0 current clamp as a function of NH₄Ac concentration. Note the absence of the biphasic profile in the CT and conductance curves, in contrast to NH₄Cl responses.

![Graph](image2)

**FIG. 6.** A: CT responses over NH₄Cl concentration ranges from 0.05 to 0.5 M under 0 current clamp (●) and −50-mV (■) and +50-mV (▲) voltage clamp. Each experiment represents the mean ± SE ($n = 6–12$). The theoretical lines were obtained with the use of Eq. A5 (see APPENDIX). The best fit was obtained with the following parameter values: $R_{\text{in}} = 1.17$, $K_{\text{in}} = 183 \text{ mM}$, $R_{\text{pm}} = 1.10$, $K_{\text{pm}} = 435 \text{ mM}$, and $n = 10.6$. Additional parameters were added under voltage-clamp conditions: at −50 mV $\delta = 0.25$, $\gamma = 0.12$, $n = 4$; at 50 mV $\delta = 0.25$, $\gamma = 0.14$, $n = 13.8$. B: CT responses to 0.2 M NH₄Cl (a concentration below the CT response inflection at 0.3 M NH₄Cl, ●) and 0.5 M NH₄Cl (a concentration above the CT response inflection at 0.3 M NH₄Cl, ▲) under voltage clamp. Each point represents the mean ± SE ($n = 6–12$). Note that clamping at negative voltage enhances the CT response for 0.2 M NH₄Cl, but not for 0.5 M NH₄Cl, whereas clamping at positive voltage suppresses the CT responses for NH₄Cl at both concentrations, with the largest suppression for 0.5 M NH₄Cl. The theoretical lines were obtained with the use of Eqs. A5 and A6 (see APPENDIX). The best fit was obtained with the following parameter values: for 0.2 M NH₄Cl concentration $\delta = 0.25$, $\gamma = 0.19$; for 0.5 M $\delta = 0.25$, $\gamma = 0.13$. 

![Graph](image3)
FIG. 7. CT and electrical responses to 0.1 M NaCl and 0.2 M NH₄Cl, NH₄Ac, and NH₄Hp. Top: CT responses under 0 current clamp. Middle: responses obtained under voltage clamp at −75 mV. Bottom: responses obtained under voltage clamp at +75 mV. Current pulses were in response to a periodic voltage pulse (20 mV). Note the similarities to Na⁺ salt CT responses.

concentration domain, i.e., 0.5 M NH₄Ac responses appear qualitatively similar to the lower concentration domain of NH₄Cl responses.

DISCUSSION

Ionic transport of NH₄Cl

Ammonium permeation across most cell membranes has been assumed to occur mainly by diffusion of uncharged NH₃ through the lipid phase of the membrane (nonionic diffusion). Recently, however, cell types have been observed that acidify immediately on NH₄Cl exposure and thus appear to be more permeable to the charged NH₄⁺ than to the uncharged NH₃ (Amlal et al. 1994; Burckhardt and Fromter 1992; Grandin and Charbonneau 1989; Hall et al. 1992; Kikeri et al. 1989; Lee and Steinhardt 1981; Wall and Koger 1994). Our data indicate that taste receptor cells (TRCs) have high permeability to NH₄⁺ ion. Observations in support of this are as follows.

Changing the NH₄Cl extracellular pH over the range encountered with NH₄⁺ salt did not affect the neural response. This could occur if NH₃ and NH₄⁺ enter the taste cells at the same rate, thereby preserving the ratio of NH₃ concentration to NH₄⁺ concentration, keeping the intracellular pH constant.

FIG. 8. CT responses as a function of NH₄Cl concentration at 0 current clamp with or without 100 μM amiloride treatment. Each point represents the mean ± SE (n = 6). Note that the amiloride treatment suppresses CT responses in a concentration-dependent manner. Suppression decreases with increasing concentration, with the largest suppression at <0.3 M NH₄Cl (see inset).

Ionic transport of NH₄Cl

Ammonium permeation across most cell membranes has been assumed to occur mainly by diffusion of uncharged NH₃ through the lipid phase of the membrane (nonionic diffusion). Recently, however, cell types have been observed that acidify immediately on NH₄Cl exposure and thus appear to be more permeable to the charged NH₄⁺ than to the uncharged NH₃ (Amlal et al. 1994; Burckhardt and Fromter 1992; Grandin and Charbonneau 1989; Hall et al. 1992; Kikeri et al. 1989; Lee and Steinhardt 1981; Wall and Koger 1994). Our data indicate that taste receptor cells (TRCs) have high permeability to NH₄⁺ ion. Observations in support of this are as follows.

Changing the NH₄Cl extracellular pH over the range encountered with NH₄⁺ salt did not affect the neural response. This could occur if NH₃ and NH₄⁺ enter the taste cells at the same rate, thereby preserving the ratio of NH₃ concentration to NH₄⁺ concentration, keeping the intracellular pH constant. It is also possible that TRCs have a high-capacity buffering system that eliminates intracellular pH changes due to NH₄Cl and thus any pH-mediated CT responses. If TRC permeability to NH₃ were significantly greater than that to NH₄⁺, NH₄Cl exposure ought to result in transient alkalinization.
ity site. Our data support the concept of two NH$_4^+$ taste receptor mechanisms. This is immediately suggested by the biphasic nature of the CT response curves as a function of the NH$_4$Cl concentration (cf. Fig. 3, A and B) and by Scatchard plot analysis (cf. Fig. 3C). The CT response–concentration curve for NH$_4$Cl can be divided operationally into low and high concentration domains with the concentration at the inflection point (0.3 M) chosen as a convenient boundary.

**LOW CONCENTRATION DOMAIN (<0.3 M).** Below 0.3 M NH$_4$Cl responses appear to be approaching a saturation limit with a half-maximal concentration of ~0.15 M (cf. Fig. 3B), and amiloride significantly inhibited the NH$_4$Cl CT response (cf. Fig. 8), both similar to NaCl responses (Ye et al. 1993). The CT responses in both concentration domains were affected by perturbations in lingual field potential (Fig. 6A). However, the effect of amiloride on the voltage sensitivity is different in the two domains. Amiloride significantly reduced the enhancing effect of negative clamp voltage on the response to 0.2 M NH$_4$Cl (Fig. 9), again reminiscent of the effect of amiloride on the NaCl response. In contrast, amiloride had practically no effect at 0.5 M NH$_4$Cl at negative clamp voltage (Fig. 9). There are two possible sources of voltage sensitivity in the CT response. The first, as with Na$^+$, is that NH$_4^+$ ions enter taste cells through apical membrane ion channels. A second possibility is that NH$_4^+$, like Na$^+$ and K$^+$, can be transported by electrophoresis through the paracellular shunts. Of course, these are not mutually exclusive, as shown by voltage-clamp studies on the NaCl response (Ye et al. 1993, 1994). This appears also to be true for NH$_4$Cl responses, but with different fractions of the response attributable to each pathway compared with NaCl and KCl. This becomes clear in a comparison of the effects of amiloride on the NH$_4$Cl CT response at different clamp voltages (Fig. 9). Amiloride suppressed the CT response of 0.2 M NH$_4$Cl at –50-mV voltage clamp by 35% (cf. Fig. 9), consistent with a partial blockage of an apical membrane conductance. On the other hand, amiloride did not significantly suppress the CT response to 0.5 M NH$_4$Cl under the same conditions (cf. Fig. 9). This suggests that an amiloride-sensitive apical membrane conductance is not a major factor in the NH$_4$Cl response at high concentration, i.e., the voltage sensitivity represents mainly paracellular electrophoresis of NH$_4^+$ ions.

A comparison of these results with NaCl under the same conditions shows that at 0.2 M, amiloride suppressed ~80% of the response at –50 mV and ~75% at 0.5 M (Ye et al. 1993). It seems reasonable to conclude that an amiloride-sensitive apical membrane conductance plays a role in gustatory transduction for NH$_4^+$ ions in the low concentration domain. However, the affinity of the NH$_4^+$ binding site for amiloride is much less than affinity of the Na$^+$ site for this blocker. Whether the different binding affinities for amiloride reflect a population of two channel types, one Na$^+$ conducting and one NH$_4^+$ conducting, or different amiloride binding domains on a single channel is unclear. However, the epithelial sodium channel appears to have a single voltage-dependent amiloride binding affinity (Benos 1982; Kleyman and Cragoe 1988; Palmer 1986). Amiloride blockage of the Na$^+$ conductance of taste cells (Gilbertson et al. 1993) and the NaCl CT response (Ye et al. 1993) is fully consistent

**Two transduction mechanisms for NH$_4$Cl**

The possibility that NH$_4$Cl transduction involves more than a single mechanism was suggested by Beidler (1961) on the basis of the nonlinearity of the Scatchard plot of the CT response over a range of concentration. Beidler concluded that NH$_4$Cl taste sensing involved both a high-affinity/low-capacity receptor site and a low-affinity/high-capac-

![Normalized CT Response](image-url)
with this model. Thus it is unlikely that the taste cell Na\(^+\) conductance has dual amiloride binding sites. A further possibility is the presence of channels inhibitable by amiloride in a voltage-dependent manner but not strictly Na\(^+\) selective. Light et al. (1988) have described an amiloride-sensitive cation channel with these properties in the apical membrane of cells from the inner medullary collecting duct of the kidney. If there is a similar channel in rat taste cells, it must be more selective for NH\(_4^+\) than to Na\(^+\) so that it plays a significant role in NH\(_4^+\) but not Na\(^+\) transduction. In contrast, a nonspecific inhibition by amiloride of canine CT responses to various salts, including NaCl and NH\(_4\)Cl, has been reported (Nakumara and Kurihara 1990). This suggests that a common transduction pathway is utilized for Na\(^+\), NH\(_4^+\), and other cations in the dog, indicating the involvement of a nonspecific cation channel in the transduction mechanism of these salts for the dog. However, in the rat, the rates of recovery from amiloride inhibition of the NaCl and NH\(_4\)Cl CT responses show that NaCl response recovery lags that of NH\(_4\)Cl recovery (unpublished observations). This makes it unlikely that, for the rat, recovery represents the time course of amiloride dissociation from and consequent increase in the conductance of a single channel species.

The major differences observed in rat Na\(^+\) and NH\(_4^+\) CT responses are fully consistent with single-unit recordings (Erickson et al. 1980) and behavioral studies (Nowlis and Frank 1981). Although most of the information the rat receives about Na\(^+\) is through amiloride-sensitive ion channels (Hill et al. 1990; Spector et al. 1996), the larger part of the NH\(_4^+\) response is amiloride insensitive. In this respect the CT responses of K\(^+\) and NH\(_4^+\) show greater similarity to one another. It is perhaps, therefore, not wholly unexpected that single-unit recordings and behavioral studies involving KCl and NH\(_4\)Cl show a high degree of similarity. Yet, certain differences between KCl and NH\(_4\)Cl responses have also been noted in single-unit recordings and psychophysical studies. CT single units responsive to both K\(^+\) and NH\(_4^+\) generally responded more vigorously to NH\(_4^+\) (Erickson et al. 1980). In human psychophysical studies NH\(_4\)Cl responses are of higher intensity and salty quality than KCl responses (van der Klaauw and Smith 1995). These differences are consistent with our findings that NH\(_4\)Cl whole nerve responses are generally greater than those to KCl. Yet, evidence suggests that both salts are similar in that a major part of their responses involves transduction mechanisms depending on ion transport across paracellular shunts in the taste buds. Thus it seems that both similarities and differences as seen in the periphery for NH\(_4\)Cl and KCl responses are translated during taste encoding.

**Positive cooperativity in the TC and CT response**

The CT response–concentration curves for Na\(^+\) and K\(^+\) salts have slopes that decrease monotonically with increasing concentration, approaching zero in the limit (Ye et al. 1994). The CT response concentration for NH\(_4\)Cl is uniquely different. As seen in Fig. 3B, the slope begins by decreasing, but at ~0.3 M it increases sharply again. The same behavior is observed in the TC for NH\(_4\)Cl, suggesting that the critical events responsible for this NH\(_4^+\)-induced increase in NH\(_4^+\) response take place in the paracellular shunts. The necessary conditions that seem to be required for this autoaccelerating process are 1) the presence of NH\(_4^+\), 2) a critical concentration, and 3) the presence of Cl\(^-\).

The Cl\(^-\) requirement may derive from its higher mobility among the anions studied. Because the rate of diffusion through the cation exchanger shunts is controlled by the anion, Cl\(^-\) will confer the highest mobility to NH\(_4\)Cl. This probably allows NH\(_4^+\) ions to achieve the requisite critical concentration in the controlling paracellular region (probably the tight junctions). The question becomes: what does the critical NH\(_4^+\) concentration do to cause an increase in NH\(_4^+\) conductance? Some insight into this is gained from the work of Moreno and Diamond (1975) on the ion permeability of gallbladder tight junctions. Those studies indicate that a cation’s permeability increases with its proton donor ability. Hydrogen bonding solutes (such as NH\(_4^+\)) in an hydrogen bonding microenvironment behave as if they were smaller than their van der Waals radii and therefore smaller than similar-sized cations without hydrogen donor ability. Other cases of a strong correlation between hydrogen bond formation and ion permeability are well established (Hille 1971).
Although the composition of the dense fibrous material of tight junctions is not yet fully characterized (Madara 1992), their ion exchanger properties are well established. When enough hydrogen bonds have been formed in the controlling paracellular regions, i.e., when the local NH$_4^+$ concentration is at a critical level, a conformational change resulting in increased NH$_4^+$ permeability may occur; thus the increase in TC. Although there is no direct evidence that a conformational change occurs in tight junctional protein, some observations on lingual epithelia support the concept of modulation of TC by ion exchange reaction (DeSimone et al. 1995). The junctional polyelectrolytes may normally be relatively condensed because of Ca$^{2+}$ ions. If an ion exchange reaction can occur in which NH$_4^+$ ions displace Ca$^{2+}$ ions, the polyelectrolyte structures could expand (Katchalsky and Oplatka 1971). In fact, they may undergo sudden expansion when they interact with particular electrolytes at a critical concentration. Such structural transformations often resemble phase transitions, or highly cooperative order-disorder transitions (Flory 1956; Katchalsky and Oplatka 1970).

**Chloride versus acetate or hippurate**

The slope of the CT response for NH$_4$Cl decreased markedly at >0.5 M, where the response approaches a second plateau (Fig. 3B). This is the system’s maximal NH$_4^+$ response intensity; responses to 0.5 M NH$_4$Cl at negative voltage clamp were not significantly larger (cf. Fig. 6A). Figure 5A shows that NH$_4$Ac response was still only 50% of the system’s maximal NH$_4^+$ response at 1 M. As might be expected, these submaximal NH$_4^+$ responses can be increased significantly under negative voltage clamp (unpublished observations). The second plateau in the NH$_4$Cl response probably reflects a saturation in the NH$_4^+$ transduction mechanism below the tight junctions, as a consequence of NH$_4^+$ ion reaching a saturating concentration in the intercellular space. This probably explains the ineffectiveness of negative voltage perturbation in increasing the response to 0.5 M NH$_4$Cl beyond its current-clamp value. On the other hand, as little as 25-mV positive voltage clamp suppressed the response by 30% (cf. Fig. 6B). This decline reflects, in turn, a decline in the influx of NH$_4^+$ ions in the current and probably the decline in NH$_4^+$ ion concentration in the tight junctions below the critical value required for the TC to be increased. Both effects acting in concert will significantly reduce the NH$_4^+$ ion available to the submucosal transduction mechanism. A 25-mV positive perturbation has a smaller, graded effect on the CT response to 0.2 M NH$_4$Cl (Fig. 6B). This may be because paracellular transport may not involve cooperative processes under these circumstances. The failure of acetate or hippurate to evoke the paracellular cooperative process required to increase TC means that, for these anions, a smaller proportion of the net NH$_4^+$ CT response will occur through paracellular NH$_4^+$ transport. A larger proportion should occur through the apical membrane transduction route. Consequently a higher proportion of the NH$_4$Ac response at concentrations >0.3 M should be amiloride sensitive relative to NH$_4$Cl. The results support this expectation. This is further confirmation of our conclusion that, as with Na salts, NH$_4$ salt taste transduction involves an NH$_4^+$ conducting channel in the taste cell apical membrane and a submucosal transduction site accessible through the tight junctions.

**Membrane conductive pathways for NH$_4^+$ ion**

The search for an NH$_4^+$ ion conducting channel has been and continues to be concentrated in a system in which ammonium (NH$_4^+$ and NH$_3$) plays an important role in maintaining the acid-base balance, the nephron. Several lines of evidence, in different segments of the nephron, have shown that NH$_4^+$ may be accepted by the K$^+$ site of the luminal Na$^+$/K$^+$-2Cl$^-$ cotransport system, the basolateral Na$^+$/K$^+$ ATPase (Kinne et al. 1986), the K$^+$/H$^+$ antiporter system (Amlal et al. 1994), and the Ba$^{2+}$-sensitive K$^+$ channel (Tsuruoka et al. 1993). On the other hand, there is evidence for NH$_4^+$ conductances distinct from K$^+$ conductances (Biena et al. 1990; Burckhardt and Fromter 1992; Tsuruoka et al. 1993; Volkl and Lang 1991). Amiloride-sensitive pathways mediating NH$_4^+$ ion conductance have been reported (Amlal et al. 1994; Light et al. 1988; Tsuruoka et al. 1993; Volkl and Lang 1991). In the sensory epithelia, the ionic conductance for the guanosine 3’,5’-cyclic monophosphate-gated channels in the rod outer segment is 3 times as high for NH$_4^+$ ion as for Na$^+$ or K$^+$ ions (Kaupp et al. 1989). The higher ammonium conductance is also observed through the adenosine 3’,5’-cyclic monophosphate-gated channels in olfactory receptor neurons (Balasubramanian et al. 1995). Why might NH$_4^+$ have a higher conductance than the usual cations present in a particular physiological system, especially if NH$_4^+$ has no functional and homeostatic role in that system? The functional importance of an ammonium conducting channel in the taste system is not immediately apparent. It is unclear whether this conductance functions as a specific sensor for NH$_4^+$ ion or whether ammonium happens to be a probe ion for a taste cell conductance of as yet unknown physiological significance.

In conclusion, the data are consistent with the presence of two transduction pathways for NH$_4$Cl in taste cells: an apical NH$_4^+$ ion conductance and one accessible via the paracellular pathway. The latter is especially dominant in the presence of Cl$^-$ and when NH$_4^+$ concentration exceeds 0.3 M.

**APPENDIX**

**Fit of the CT response data to a cooperative model**

The data in Fig. 3, A and B, can be accounted for by a straightforward cooperative model. The biphasic behavior of the NH$_4$Cl CT response may be regarded as arising from the sum of an apical membrane conductance and a paracellular shunt conductance in parallel with it. The apical membrane component will be expected to have the form of the Beidler equation as adapted by Ye et al. (1993, 1994) for an ion, viz.

$$R_i(c_i) = R_{max} c_i / (K_{max} + c_i)$$  

where $R_i$ is the part of the response, $R_i$ due to the apical membrane conductance, $R_{max}$ is the maximum response from the apical membrane component, $K_{max}$ is the concentration at which $R_i$ is half maximal, and $c_i$ is the electrochemical concentration of NH$_4^+$ across the apical membrane conductance. The latter is given by
where $c$ is the stimulating concentration of NH$_4$Cl, $\phi$ is the normalized clamp potential, $F\Delta V/RT$, and $\delta$ is the fraction of the clamp voltage ($\phi$) dropped across the apical membrane conductances.

The contribution of the paracellular shunt is assumed to have a cooperative aspect described by a Hill-type equation, viz.

$$R_e(c_f) = R_{pm}c_f^n/(K_{pm} + c_f^n)$$

(A3)

where $R_e$ is the part of the response, $R$, due to the paracellular shunt conductance, $R_{pm}$ is the maximum response from the paracellular shunt component, $K_{pm}$ is the concentration at which $R_e$ is half maximal, and $c_f$ is the electrochemical concentration of NH$_4$ across the paracellular shunt conductance. The latter is given by

$$c_f = c \exp[-\gamma \phi]$$

(A4)

where $\gamma$ is the fraction of clamp voltage dropped across the barrier to conductance through the paracellular shunt. The Hill coefficient, $n$, should be regarded as a mathematical device permitting the representation of a physical process in which a small change in one variable (CT response) generates with those generated with a free parameter. With the use of $A$. Where $c_f$ turns out to be a function of clamp voltage, $\phi$, as described below.

$R_f$, the overall response, is then given by

$$R_f = R_e + R_p$$

(A5)

Although the number of parameters seems large, there are some constraints that one might reasonably expect. For example, the values of $R_{pm}$, $K_{pm}$, and $K_{pm}$ obtained from fitting the CT response versus concentration for the zero current clamp (i.e., $\phi = 0$) should apply then to all other curves under any voltage-clamp condition. With the use of this strategy we obtained the CT response versus concentration at zero current clamp. The fit required an $n$ value of 10.6, and of course, $\gamma$ and $\delta$ were not involved (a similar $n$ value was found in other systems) (Yonekura et al. 1991). Fitting the CT response–concentration curve for −50-mV voltage clamp with the same $R$ and $K$ parameters required an $n$ value of 4.0, whereas at +50 mV $n$ was 13.8. When $\delta$ was treated as a fit parameter in each of the voltage-clamp conditions, a value of $\gamma$ was observed to be 0.2. The fit produced a $\delta$ of nearly the same value (0.3). To conserve the number of parameters, we chose the average value, $\delta = 0.25$, for use under all voltage-clamp conditions. Similarly the value of $\gamma$ was preserved between the two voltage conditions, i.e., at −50 mV $\gamma = 0.13$, whereas at +50 mV $\gamma = 0.15$. The constancy of $\gamma$ is unexpected and is one test of the internal consistency of the model.

The CT response versus clamp voltage at fixed concentration required a functional representation of the voltage dependence of the Hill coefficient, $n$. In fitting CT response versus concentration we obtained the following pairs, $(\Delta V, n)$: (−50, 4.0), (0, 10.6), (50, 13.8). Assuming that $n$ changes continuously with voltage over the range of −50 to +75 mV, we represented $n$ approximately as follows

$$n = (58\phi + 179.8)/(\phi + 19.1)$$

(A6)

To be consistent we refitted the CT response–concentration curves with the use of $A6$ to represent $n$. The resulting curves are shown in Fig. 6A, and these curves do not differ significantly from those generated with $n$ as a free parameter. With the use of $A6$ we obtained the CT response–clamp voltage curves in Fig. 6B. In these curves the single fit parameter was $\gamma$. For 0.5 M

**Analysis of Scatchard plot**

The same type of analysis for the concentration-response function can be applied for Scatchard plot analysis. Here the total CT response, $R_f$, is plotted as a function of $R_f/C$. With the use of the same parameters in $A1$ and $A3$, we can obtain the value of $C$ in terms of $R_f$, i.e.

$$C = R_fK_{pm}(R_{pm} - R_f)$$

(A7)

Substituting this value of $C$ in Eq. A5, $R_f$ is now defined as follows

$$R_f = R_e + R_p(K_{pm}R_f)^n/[K_{pm}(R_{pm} - R_f)^n + (K_{pm}R_f)^n]$$

(A8)

dividing the value of $R_f$ by $C$, we obtain

$$R_f/C = R_p(K_{pm}R_f)^n/(R_{pm} - R_f)$$

$$[K_{pm}(R_{pm} - R_f)^n + (K_{pm}R_f)^n] + (R_{pm} - R_f)/K_{pm}$$

(A9)

The results of such analysis can be seen in Fig. A1, in which same values of the parameters obtained earlier are used. A comparison between the experimental (Fig. 3C) and the theoretical data (Fig. A1) shows that it is important to include both noncooperative and cooperative processes in representing the data.
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