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Psychophysical Investigations of Cetylpyridinium Chloride in Rats: Its Inherent Taste and Modifying Effects on Salt Taste

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Salts are transduced by at least 2 mechanisms: (a) antagonized by amiloride and (b) antagonized by cetylpyridinium chloride (CPC). The authors report on 4 behavioral experiments in rats that characterize the orosensory properties of CPC itself as well as its effect in suppressing the intensity of NaCl and KCl taste. Experiments 1 and 2 indicated that CPC has a quinine-like taste quality. Experiments 3 and 4 demonstrated that the recognition of KCl, but not NaCl, is modestly reduced by mixture with CPC. However, control experiments call into question the mechanism of the salt suppression of CPC, because both CPC–salt and quinine–salt mixtures had similar effects. The relevance of these studies for understanding salt and bitter taste coding is discussed.

Rats, like humans, can discriminate sodium from nonsodium salts on the basis of taste (Erickson, 1963; Morrison, 1967), despite the fact that different salts (such as NaCl and KCl) share transduction mechanisms in taste receptor cells. Current evidence suggests that sodium salts are transduced by at least two distinct transduction mechanisms; nonsodium salts are transduced by these two mechanisms in addition to a third (Boughter & Gilbertson, 1999; DeSimone et al., 2001; Lyall et al., 2004).

The predominant transduction mechanism for sodium salts (in rats) is the influx of the sodium ion through apically located epithelial sodium channels. These channels are more permeable to sodium and lithium salts than other cations and are inhibited by specific antagonists applied to the tongue, such as amiloride (Brand, Teeter, & Silver, 1985; DeSimone & Ferrell, 1985; Heck, Mierson, & DeSimone, 1984; Herness, 1987). The evoked response to midrange concentrations of NaCl in the whole chorda tympani nerve (CT) is reduced by ~60%–70% at asymptotic amiloride concentrations; thus, the gustatory signal evoked by NaCl can be dissociated into amiloride-sensitive and amiloride-insensitive components (Brand et al., 1985; DeSimone & Ferrell, 1985; Ye, Heck, & DeSimone, 1993). It is important to note that amiloride-sensitive and amiloride-insensitive sodium signals remain segregated in fibers of the afferent nerves (Hettinger & Frank, 1990; Ninomiya & Funakoshi, 1988) and the central nervous system (Boughter, St. John, & Smith, 1999; Giza & Scott, 1991; Nishijo & Norgren, 1997; D. V. Smith, Liu, & Vogt, 1996; St. John & Smith, 2000), suggesting that these transduction mechanisms have different functional roles in guiding salt-related behavior.

Amiloride has many features that make it an ideal tool for probing the functional role of the amiloride-sensitive system. First, it is tasteless to rats (Markison & Spector, 1995). Second, amiloride's effects are rapidly reversible, allowing responses to amiloride-adulterated and unadulterated solutions to be assayed in the same subjects (DeSimone & Ferrell, 1985). Third, amiloride is a competitive inhibitor (DeSimone & Ferrell, 1985), allowing parametric dose–response studies (Spector, Guagliardo, & St. John, 1996; St. John & Smith, 2000). Behavioral studies have concluded unequivocally that the amiloride-sensitive system is necessary for the recognition of the taste quality of NaCl (Bernstein & Hennessy, 1987; Geran & Spector, 2004; Hill, Formaker, & White, 1990; Kopka, Geran, & Spector, 2000; McCutcheon, 1991; Roitman & Bernstein, 1999; Spector et al., 1996) and for the detection of low NaCl concentrations (Eylam & Spector, 2003; Geran & Spector, 2000a, 2000b; Kopka & Spector, 2001).

The necessity of this system for the detection or discrimination of NaCl does not, of course, rule out a role for the amiloride-insensitive system. Until recently, however, specific antagonists of this system have not been available. A report by Kloub, Heck, and DeSimone (1998) suggested that high concentrations of $CaCl_2$ inhibited the amiloride-insensitive salt transduction mechanism(s), but the inherent taste of $CaCl_2$ posed interpretive difficulties in behaviorally assessing its effect on NaCl recognition. A more promising candidate was recently discovered by DeSimone and colleagues (DeSimone et al., 2001; Lyall et al., 2004), who showed that the integrated response of the CT to NaCl was completely inhibited by a combination of amiloride and cetylpyridinium chloride (CPC).

Like amiloride, CPC's properties make it a potentially useful tool in behavioral studies aimed at understanding the taste quality coding of salts (DeSimone et al., 2001; Lyall et al., 2004). First, CPC's effects on an ongoing salt response are virtually immediate, and its effects are also rapidly reversible. Second, low concentrations of CPC facilitate salt responses (with a maximal enhancement at 0.25 mM) whereas high concentrations inhibit it (with an

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asymptote at about 2 mM) so that CPC can be used both as an agonist and antagonist of the amiloride-insensitive system. Third, CPC has a large effect on responses evoked by nonsodium salts, whereas amiloride has a much larger effect on sodium than nonsodium salts.

Understanding the contribution of the amiloride-insensitive system to the discrimination of sodium and nonsodium salts could potentially shed light on some very old issues in taste quality coding (Erickson, 2000; Frank, 2000; Geran & Spector, 2004; D. V. Smith & St. John, 1999; D. V. Smith, St. John, & Boughter, 2000; St. John & Smith, 2000). Because of the potency of amiloride in eliminating the distinctive taste quality of NaCl, the amiloride-sensitive system may represent a *labeled-line* for saltiness in rats. Such a conclusion, however, remains premature until the contribution of the amiloride-insensitive system is assessed behaviorally (Frank, 2000; D. V. Smith et al., 2000).

The goal of the current series of studies was to evaluate the potential of CPC as a tool for understanding the taste quality coding of salts. We first demonstrated that, unlike amiloride, CPC has an inherent, quinine-like taste. Using quinine as a control stimulus, we investigated the effect of CPC on the recognition of NaCl and KCl in two different paradigms.

Experiment 1: The Orosensory Properties of CPC

As an initial approach to determining whether CPC alone has significant orosensory properties, thirsty rats were presented with a range of CPC concentrations in a commercially available lickometer. Rats were tested both before and after single or combined transection of the CT and glossopharyngeal nerve (GL) to assess the lingual (especially the lingual gustatory) contributions to any avoidance behavior (i.e., suppression of licking CPC relative to water).

Method

Subjects. Thirty-three naive, male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 243–520 g were subjects of the experiment. Three of the rats died shortly after surgery, and a 4th rat was removed from the experiment after it lost a tooth (final $N = 29$). Rats were housed individually in plastic cages in a room where temperature and lighting (13:11-hr light–dark cycle) were automatically controlled. All manipulations were performed during the lights-on portion of the cycle. Food (Harlan Teklab 8604, Madison, WI) and tap water were available ad libitum except where noted.

In this and in other experiments detailed in this article, rats were motivated to lick taste solutions by water restriction. Typically, rats receive all of their fluid in training or test sessions (typically >10 ml of fluid in total) and are often tested for several days in succession. When on a water restriction schedule, rats are weighed daily so that supplemental fluid can be delivered as needed (i.e., if body weight drops below 85% of the free-drinking value). In the experiments reported herein, such interventions were never necessary. All behavioral procedures reported in this article were approved by the Reed College Institutional Animal Care and Use Committee and conform to the principles published by the American Psychological Association (2002).

Apparatus. Rats were tested daily in an automated lickometer referred to as the "Davis Rig" (Davis MS-160, DiLog Instruments, Tallahassee, FL). The Davis Rig allowed the presentation of up to 16 different taste stimuli within a single behavioral session, with the duration and order of stimulus presentation at the control of the experimenter (Rhinehart-Doty, Schumm, Smith, & Smith, 1994; J. C. Smith, 2001). The test chamber consisted of a plastic rectangular cage (30 cm × 14.5 cm × 18 cm) with a wire mesh floor; an oval opening centered in the front wall allowed access to taste solutions contained in leak-proof sipper tubes. Fluid access was restricted by a computer-operated shutter.

Trials began with the opening of the shutter and ended 4 or 8 s after the rat made its first lick on the drinking spout (see the *Procedure* section). Licks were counted with a high-frequency AC contact circuit. Failure to initiate a lick within 60 s also ended a trial (although such a "zero-lick trial" was ignored in analyses of lick rate, as the failure to initiate licking could not be an orosensory-based behavior). In between trials, a platform on which the stimulus tubes were mounted was driven to a new location. Although the time that the motor was activated was variable, the intertrial interval was always constant.

Finally, a small house fan was directed across the sipper tubes during the Davis Rig sessions. This was done in response to a previous report (Rhinehart-Doty et al., 1994) that indicated that rats can smell sucrose when presented in the Davis Rig but that the use of a fan eliminated this cue. The report by Rhinehart-Doty et al. (1994) suggested that olfactory cues do not affect lick rate in the Davis Rig but rather affect the latency to initiate a trial. This procedure may have minimized the olfactory contribution to appetitive behavior, but there was evidence that rats showed a longer latency to initiate trials for the highest CPC concentration (see the *Results* section).

Stimuli and experimental design. Each iteration of the experiment lasted 4 weeks. During the first week, rats were habituated to the Davis Rig and had access to deionized water. During the second week, we assessed unconditioned licking responses to an array of CPC concentrations (0.03, 0.1, 0.3, 1.0, 3.0, and 10.0 mM) and water. The CPC was made fresh prior to each week of testing and was obtained from Sigma (St. Louis, MO). Pilot studies indicated that these concentrations effectively spanned the dynamic range of licking behavior in this task, and this range spans concentrations identified by DeSimone et al. (2001) as having NaCl-enhancing (i.e., 0.25 mM) and NaCl-inhibiting (i.e., 2 mM) effects in rat CT. Rats were given transections of the CT (CTX), GL (GLX), CT and GL (CTX + GLX), or control surgery during the third week, and postsurgical licking responses were assessed during the fourth week. Water was removed 24 hr prior to the first session of each week and was available ad libitum on weekends and during the surgical recovery period.

Procedure. On the first 2 days of training, rats had access to deionized water for 20 min in the Davis Rig (from a single sipper tube). In a few cases, rats did not lick during the first session, but all rats licked considerably on the second session. On the 3rd day, the rats were given twenty-four 15-s deionized water trials. Although each trial was identical and consisted of deionized water, the water was available from nine sipper tubes. This session thus introduced the rat to the sounds and delays inherent in movement of the shutter and the platform on which the sipper tubes were mounted (see the *Apparatus* section). The fourth and final habituation session was essentially identical to the CPC test sessions except that water was available on every trial.

During presurgical and postsurgical assessment of CPC-evoked licking responses, behavioral sessions lasted for 30 min or 64 trials (whichever came first). *Water-rinse trials* preceded every taste trial; these trials consisted of 4-s access to deionized water and served to rinse the oral cavity. The *taste trials* consisted of 8-s access to deionized water or CPC. Taste trials were presented semirandomly such that each block of eight consisted of the six CPC concentrations and two deionized water trials. Thus, during each behavioral test session, water was available half of the time (a full session was thirty-two 4-s water-rinse trials and eight 8-s water-taste trials [192 s] vs. twenty-four 8-s CPC trials [192 s]). The water-restricted rats were therefore able to rehydrate while rejecting the most aversive concentrations of CPC.

Rats were tested in three squads. Minor procedural changes were made following the first squad (which consisted of 3 CTX rats and 3 GLX +

CTX rats) when more flexible software for the Davis Rig became available. This software allowed sessions to end after a fixed length of time and also allowed the shutter to close only part way (which minimized the chance that the shutter would clip the rat's nose on closing). Thus, for the first squad sessions ended after 64 trials and the shutter closed completely; for the remaining squads the session could end when 30 min elapsed and the shutter closed approximately 75% of the way (preventing access to the sipper tubes). The intertrial interval was 5 s for Squad 1 and 7.5 s for the remaining squads.

Surgery. All rats were deeply anesthetized with 4% chloral hydrate (400 mg/kg, ip). For GLX ($n = 8$), the ventral neck was shaved and prophylactically treated with iodine solution (Betadine). An incision in the skin of the neck permitted access to the GL following dissection of the fascia surrounding the sublingual and submaxillary salivary glands. The GL was visualized infereolateral to the hypoglossal nerve and was cut with microscissors (bilaterally). The incision was closed with surgical staples.

Rats in the CTX group ($n = 7$) received bilateral sectioning of the CT as it passes through the middle ear. The rat was fixed in a custom headholder that permitted access to the ear with the rat's head titled 80° away from the surgeon. One curved #7 microforceps was used to temporarily widen the auditory meatus to allow visualization of the structures of the middle ear and a second forceps was used to remove the tympanic membrane. Deflection of the malleus allowed visualization of the CT, which was severed with the forceps. The malleus was then removed.

Rats in the GLX + CTX group ($n = 7$) received both operations in a single surgical session. Surgical controls ($n = 7$) had the GL exposed (but not handled).

Histology. After the final day of postsurgical testing, rats were deeply anesthetized with 4% chloral hydrate and were perfused with saline and 10% (wt/vol) buffered formalin. The tongue of each rat was removed and stored in formalin. For GLX + CTX, CTX, and surgical control rats, the anterior tongue was soaked in deionized water for 30 min, immersed in 0.5% (wt/vol) methylene blue, and then rinsed with water. The epithelium was removed and pressed between two slides in order to observe the fungiform papilla under a light microscope. The percentage of fungiform papilla containing taste pores was calculated for each rat; a low percentage of papillae with pores indicated a successful bilateral CT transection (Breslin, Spector, & Grill, 1993; Parks & Whitehead, 1998; D. V. Smith et al., 1999; St. John, Markison, & Spector, 1995; Whitehead, Frank, Hettinger, Hou, & Nah, 1987).

For all rats except those in the CTX group, the vallate papilla was embedded in paraffin and sectioned on a rotary microtome (10 μm) through the extent of the papilla. Tissue sections were mounted consecutively on glass slides and were stained with hematoxylin and eosin. The slides were observed under a light microscope; the lack of taste buds in this receptor field indicated a successful bilateral GL transection (Guth, 1957; St. John, Garcea, & Spector, 2003).

The CT and GL together innervate the foliate papillae (Whiteside, 1927). Because of the variability in the number and location of taste buds innervated by one or the other nerve, analysis of the foliate papillae can be ambiguous with regard to whether a nerve was transected or the extent to which it has regenerated. We chose, therefore, to focus our histological analysis on taste bud regions innervated exclusively by one gustatory nerve.

Rats were perfused, at most, 15 days after surgery, an interval shorter than that required for regeneration of lingual taste buds following these manipulations (St. John, Garcea, & Spector, 2003; St. John et al., 1995).

Data analysis. Lick rate during water taste trials provides an assessment of the maximal lick rate on a rat by rat basis. Thus, in order to standardize for differences in lick rate, the primary dependent measure computed for each rat (across all presurgical or all postsurgical sessions) was

$$\frac{\text{average number of licks to CPC}_x}{\text{average number of licks to water}}, \quad (1)$$

where x is a given concentration of CPC. This *taste–water ratio* ranges from a hypothetical zero (complete avoidance of CPC) to one (no difference from water). A taste–water ratio of zero is impossible because zero-lick trials were not counted in this analysis. Concentration–response functions were also fit with a two-parameter logistic function

$$f(x) = 1/(1 + (x/c)^b), \quad (2)$$

where x is the concentration of CPC, c is the concentration of CPC evoking half-maximal avoidance (i.e., a taste–water ratio of 0.5), and b is the slope. One advantage of fitting curves is to provide a single parameter (c) that is sensitive to leftward or rightward shifts in the concentration-response function as a result of surgery.

Finally, the potential role of olfaction was assessed by the *latency to initiate trials*. Evidence that rats differentially delayed initiating trials at higher CPC concentrations was interpreted to mean that the rats were able to sense the identity of the proffered stimulus prior to licking. These cues could be visual, auditory, or olfactory. All variables were analyzed by analysis of variance (ANOVA).

Results and Discussion

Histology. All nerve transections were complete and little or no regeneration was observed. One rat in the GLX group had two taste buds; no other rats in the GLX or GLX + CTX groups had evidence of taste buds in the vallate papilla. Although the number of taste buds of surgical controls was not quantified, previous studies have indicated that surgical controls have > 400 taste buds in the vallate papilla (Hosley & Oakley, 1987; Kennedy, 1972; St. John et al., 2003; e.g., Guth, 1957).

Rats in the CTX group had 6.88% (± 1.70%) and rats in the GLX + CTX group had 8.69% (± 2.06%) of fungiform papillae with methylene-blue-stained taste pores, compared with 95.94% (± 1.10%) in surgical controls. The presence of some pores after even complete CTX (as assessed by methylene-blue staining) is well-documented in the literature (Breslin, Spector, & Grill, 1993; Parks & Whitehead, 1998; D. V. Smith et al., 1999; St. John et al., 1995; Whitehead et al., 1987;). One case in the GLX + CTX group had an unusually high percentage of papillae containing taste pores (i.e., 19.44%, distributed evenly on the right and left halves of the anterior tongue), but this value was far below the lowest percentage for control rats (i.e., 91.06%) and represents, at best, very limited regeneration (St. John et al., 1995). The behavioral effect of surgery in this case was also near the GLX + CTX group mean, and this rat was more severely impaired than any single member of any other surgical group. For these reasons, we did not remove any rats from analysis following histological verification.

Responsivity to CPC. Prior to surgery, all rats responded to increasing concentrations of CPC with decreased licking behavior (see Figure 1A). Thus, CPC appears to be aversive to rats. This aversiveness is clearly evident at 2 mM CPC (see Figure 1A, right arrow), the concentration identified electrophysiologically as maximally suppressing NaCl in the CT (DeSimone et al., 2001). At 250 μM CPC, identified as causing the maximal potentiation of the NaCl response in the CT (DeSimone et al., 2001), rats did not clearly suppress licking (see Figure 1A, left arrow). Because the rats are highly motivated by water deprivation, it could be that rats can differentiate this concentration from water but do not avoid it because it is only mildly aversive. Alternatively, this concentration may be subthreshold.

A sigmoidal curve, accounting for 99.2% of the variance, was fit to these data (Equation 2). The slope (i.e., b) was 1.13 and the

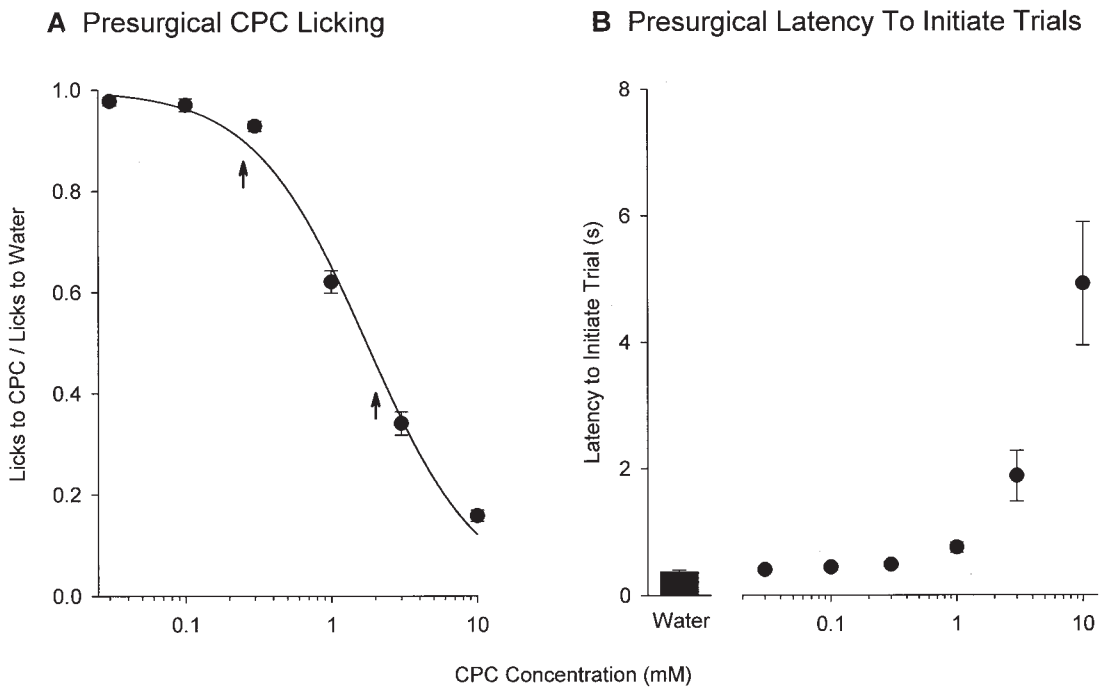

Figure 1. Presurgical behavioral performance of rats licking different concentrations of cetylpyridinium chloride (CPC) in 8-s trials. A: Data are mean (± *SE*) licks to CPC over licks to water. Rats avoided CPC with increasing concentrations, possibly at 0.25 mM (a concentration that maximally enhances the chorda tympani salt response; left arrow) and clearly at 2 mM (a concentration that inhibits maximally the chorda tympani salt response; right arrow). B: Data are means (± *SEs*) of median latencies for each rat. Rats were slower to initiate trials at higher CPC concentrations, suggesting that CPC can be sensed by olfaction as well as taste.

concentration evoking half-maximal avoidance (i.e., *c*) was 1.74 mM CPC. Similar curves were fit to each rat's data; the average ($n = 29$) slope was 1.22 (± 0.04) and the geometric average half-maximal concentration was 1.75 (± 0.033) mM. By comparison, quinine avoidance curves ($n = 59$) obtained in a similar fashion (St. John, Garcea, & Spector, 1994) averaged a slope of 1.21 (± 0.043) and a geometric average half-maximal concentration of 0.21 (± 0.043) mM. In other words, CPC appears to be about tenfold less aversive than quinine.

In addition, rats increased their latency to initiate trials particularly at the highest CPC concentration (see Figure 1B). Mean latencies for a given rat across trials can be misleading because a single long-latency trial can inflate the average. Therefore, mean latencies shown are means of median latencies for individual rats. Because the variance of these values increases as the mean increases, the latencies were logarithmically transformed prior to statistical analysis. A one-way ANOVA on CPC concentration (with water included as a *zero concentration*) revealed a significant main effect, $F(6, 168) = 44.97$, $p < .0001$; post hoc paired *t* tests versus water (with a Bonferroni correction for the six comparisons) of each CPC concentration indicated that latencies were greater than water beginning at 0.3 mM ($df = 28$, all *p* values $<$ .001). The best explanation of this finding is that rats can smell CPC. Although it is possible that the smell of CPC is itself aversive, it is also possible that the smell serves as a cue for the avoidance of aversive orosensory contact with the stimulus.

The effects of nerve transection on lick rate to CPC are consistent with the notion that the aversiveness of CPC is due to an aversive taste (see Figure 2). Although it appears that control rats showed some tolerance of the aversiveness of CPC after sham surgery, the main effect of time was not reliable according to a two-way ANOVA, $F(1, 6) = 5.00$, $p = .067$. There was, however, a significant Time × Concentration interaction, $F(5, 30) = 5.72$, $p = .0008$. Similarly, the main effect of time was not statistically reliable after either GLX, $F(1, 7) = 5.18$, $p = .057$, or CTX, $F(1, 6) = 2.76$, $p = .15$, but there was a significant Time × Concentration interaction in both groups (*F* values > 3.52, *p* values $<$ .011). There was a reliable main effect of time following the combined neurotomy, $F(1, 6) = 73.00$, $p = .0001$.

Because even the sham surgery group showed a reduced aversion to CPC after surgery, it was of interest to compare the effect of surgery across groups. The shift in the concentration–response function was quantified for each rat (see Figure 3) by first fitting sigmoidal curves to the presurgical and postsurgical data (see the *Data Analysis* section) and then deriving the *shift* of the curve for each rat (the difference in the *c* parameter from Equation 2). An ANOVA on the curve shift revealed a reliable effect of group, $F(3, 25) = 16.3$, $p < .00001$. Post hoc comparisons (applying the Bonferroni adjustment for multiple comparisons) revealed that only the GLX + CTX group differed from the controls ($p = .0001$). This group also differed from the other nerve cut groups (both *p* values = .0001), which did not differ from one another. It is interesting that although there was no significant shift in the GLX group, 2 rats in this group were so insensitive to CPC after surgery that the postsurgical half-maximal avoidance parameter had to be extrapolated rather than interpolated (see Figure 3). The

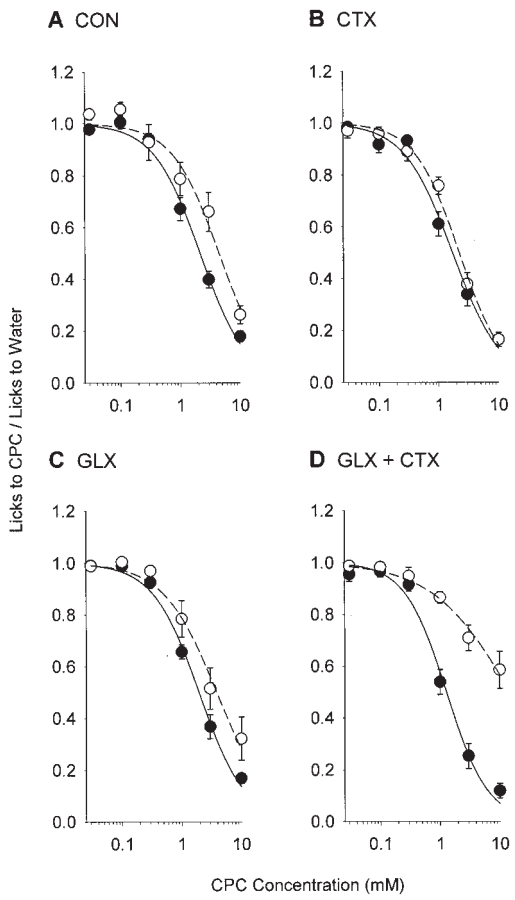

Figure 2. Presurgical (filled circles) and postsurgical (open circles) behavioral performance of rats licking different concentrations of cetylpyridinium chloride (CPC) in 8-s trials. Data are mean (± *SE*) licks to CPC over licks to water. A two-parameter sigmoidal function was fit to the data (see Equation 2). A: Rats given sham surgery (CON). B: Rats given chorda tympani transection (CTX). C: Rats given glossopharyngeal transection (GLX). D: Rats given combined neurotomy (GLX + CTX).

shifts for these 2 rats was greater than any control rat shift. A similar within-group variability has been seen previously with quinine (Markison, St. John, & Spector, 1999; St. John et al., 1994).

In summary, rats find CPC aversive, and this aversiveness is blunted following GLX + CTX, which removes input of about 80% of all rat taste buds. The simplest explanation for this result is that CPC has an aversive taste. It remains possible that CPC produces an aversive somatosensory sensation (pain, astringency, etc.), as both the GL and the CT (particularly the former) contain both somatosensory and gustatory afferents. However, the lingual branch of the trigeminal nerve conveys the bulk of anterior tongue touch information to the brain. The evidence that GLX + CTX reduced avoidance of CPC, whereas GLX alone did not, argues strongly in favor of a gustatory cue for CPC.

Neither single nerve cut altered responses to CPC (relative to the control group) but the combined transection did. This indicates that whereas neither nerve is necessary for the expression of CPC avoidance, both nerves contribute to CPC avoidance. DeSimone et al. (2001) did not report whether CPC alone evoked a response in the whole CT; however, application of 2 mM CPC with NaCl and amiloride produced little or no response. Given this, we had predicted that CT transection would not affect CPC avoidance, but the synergistic effect of CT transection with GL transection suggests that this nerve does indeed respond to CPC itself.

It is interesting to note that a methodologically similar study by St. John et al. (1994) demonstrated that quinine avoidance was not affected by single nerve transection, but that combined GL and CT transection shifted the concentration–response function rightward more than one log unit. Furthermore, in that study, although there was no group effect of transection of the GL alone, half of the subjects given GL transection showed reduced sensitivity to quinine after surgery. A very similar pattern of results occurred in the present experiment (cf. Figure 3).

Experiment 2: The Taste Quality of CPC

Experiment 1 indicated that CPC has an aversive taste to rats. The second experiment was conducted to determine the taste quality of CPC. Because CPC has an inherent taste, any effect CPC might have on behavioral responses to NaCl is difficult to interpret (i.e., effects might be due to CPC's pharmacological properties on salt transduction or to the addition of an unpalatable taste). Determination of CPC's taste quality would allow selection of a control stimulus—a taste compound that tastes like CPC but does not have pharmacological effects on sodium transduction.

As a first step toward classifying CPC's taste quality, we used the conditioned taste aversion generalization paradigm (Nowlis & Frank, 1981; Tapper & Halpern, 1968). In Experiment 2A, a taste aversion was conditioned to 0.5 mM CPC and generalization was assessed in the Davis Rig to a variety of substances. Because Experiment 2A indicated that quinine shares features with CPC, Experiment 2B was conducted to verify that CPC and quinine cross-generalize.

Method

Subjects. Twenty-four naive, male Sprague–Dawley rats weighing between 195 and 405 g at the start of the experiment were housed in conditions identical to Experiment 1.

Experimental design. The rats were run in two phases (Experiment 2A and Experiment 2B). In Experiment 2A, the generalization of a taste aversion to 0.5 mM CPC was assessed. (In pilot studies, we found that 2 mM CPC was so aversive that some rats would not sample it prior to aversion conditioning, necessitating the use of a milder concentration.) Experiment 2B was planned as a cross-generalization experiment: The stimulus that showed the strongest generalization in Experiment 2A would be used as the conditioned stimulus (CS) in Experiment 2B. The four groups and their abbreviations are given in Table 1. The saline-injected rats in each experiment were combined into a single control group (SAL).

Procedure. Generalization of the taste aversion was assessed by delivering multiple taste stimuli during three sessions in the Davis Rig (see Experiment 1). Prior to conditioning, the rats were trained in the Davis Rig in a manner similar to Experiment 1. Following overnight water deprivation, rats were given 20-min access to a single tube of deionized water for 2 days. The next three sessions were designed to mimic the generalization test except that only water was available. These sessions were a maximum of 30 min in length, during which the rat could initiate up to 50 trials. Trials were 6 s in length (timed from the first lick); water was delivered from 1 of 10 leak-proof sipper tubes on a pseudorandom basis. Failure to initiate a trial within 2 min terminated the trial and began the next.

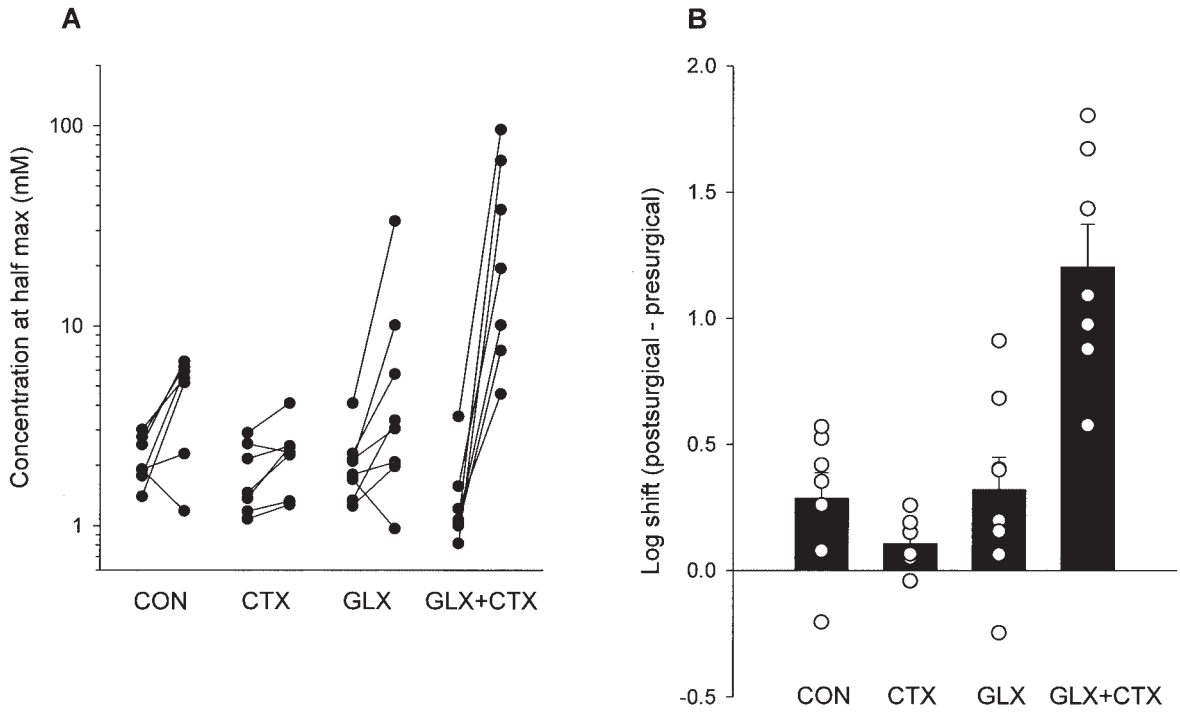

Figure 3. A summary of the effects of chorda tympani transection (CTX), glossopharyngeal transection (GLX), combined neurotomy (GLX + CTX), and sham surgery (CON) on avoidance of the taste of cetylpyridinium chloride. A two-parameter sigmoidal curve (see Equation 2 and Figure 2) was fit to lick–concentration data. A: Concentration causing a 50% reduction of licking relative to water from the presurgical (left circles) and postsurgical (right circles) curves for each rat in each surgical group. B: Mean (± *SE*) curve shift (the difference in the postsurgical and presurgical parameters shown in A) is displayed for each surgical group. Curve shifts for each rat in each group are also represented (open circles). Positive shifts represent less avoidance after surgery.

On the day following the final Davis Rig training session, rats were habituated to receiving fluid access in the home cage twice daily. Fluid was delivered in 50-ml glass centrifuge tubes fitted with solid amber silicone stoppers (Thomas Scientific, Swedesboro, NJ) and standard stainless steel sipper tubes. The morning fluid access was 15 min in duration, and the afternoon fluid access was 1 hr in duration (beginning 5 hr after the beginning of the morning access period). Fluid intake was estimated by weighing bottles (to the nearest 0.1 g) before and after the access period. Rats typically drank about 30 ml over the two sessions.

Deionized water was the only fluid delivered during the afternoon sessions and was also the stimulus during the majority of the morning sessions. On the fifth and seventh sessions, however, the water was replaced with the CS (see Table 1). If a rat failed to drink at least 2 ml of the CS, an additional 1 ml was infused into the rat's mouth via syringe (as was true for every LiCl-injected rat on the second conditioning trial). Within 15 min of the end of the drinking session, all rats were injected with the appropriate unconditioned stimulus (US; see Table 1). Following the second conditioning trial, rats were maintained on this restricted fluid access schedule for 2 more days. On the final day, the afternoon drinking session was omitted.

Table 1
Treatment of Rats in Experiment 2

Group	Conditioned stimulus (CS)	Unconditioned stimulus (US)	*n*	Experiment
CPC+	0.5 mM CPC	LiCl	8	2A
QUI+	0.2 mM quinine-HCl	LiCl	8	2B
CPC−[a]	0.5 mM CPC	NaCl	4	2A
QUI−[a]	0.2 mM quinine-HCl	NaCl	4	2B

Note. Each US was delivered by intraperitoneal injection at a dose of 2 mEq/kg and a concentration of 0.15 M. CPC = cetylpyridinium chloride; QUI = quinine.
[a] Groups CPC− and QUI− were combined for some analyses and are referred to as Group SAL (saline-injected controls).

Generalization testing occurred in three daily sessions in the Davis Rig. These sessions were identical to the final days of Davis Rig training, except that only one of the 10 tubes was filled with deionized water. Five of the remaining nine stimuli were: 0.5 mM CPC, 0.2 mM quinine-hydrochloride, 20 mM citric acid, 0.2 M NaCl, and 0.2 M sucrose. In addition, because we were concerned that the aversive stimuli might be so unconditionally aversive that it would not be possible to evaluate the generalization of a conditioned aversion, we also included the potentially aversive stimuli (CPC, quinine, citric acid, and NaCl) in mixture with palatable sucrose (all concentrations as in the single stimuli). This precaution proved to be unnecessary, but the data are shown for completeness.

Data analysis. As in Experiment 1, rats were expected to lick water at their maximal rate, so all data were converted to taste–water ratios (cf. Equation 1) in order to control for individual differences in lick rate. However, a rat might produce a low taste–water ratio (e.g., 0.5) either because the stimulus resembles the CS (a conditioned effect) or because the stimulus is unpalatable (an unconditioned effect). Therefore, in some analyses, the taste–water ratio is divided by the average taste–water ratio

for that stimulus in the SAL group. This metric, which we call the *aversion index,* ranges from virtually 0 (complete suppression relative to the SAL group) to 1 (no difference from the SAL group). As an example, a LiCl-injected rat might produce a taste–water ratio of 0.5 to citric acid, indicative of avoidance. If the SAL rats also show a taste–water ratio of 0.5, then the aversion index (0.5/0.5 = 1) properly indicates that the lick suppression is not due to similarity to the CS. In contrast, if the SAL rats average 0.75, then the aversion index (0.5/0.75 = 0.67) indicates a conditioned generalization.

Results and Discussion

The LiCl-injected rats in Group CPC+ strongly avoided the CS relative to the SAL group (see Figure 4A). Strikingly, these rats did not avoid any other single test stimulus except 0.2 mM quinine. Statistical support for these conclusions are based on multiple one-sample *t* tests (with Bonferroni corrections) for each group testing whether the aversion index for single stimuli differed from 1.0; only comparisons for CPC, $t(7) = 14.10$, $p < .001$, and quinine, $t(7) = 9.50$, $p < .001$, in the CPC+ rats were significant. Interestingly, there was not a significant difference in aversion index for CPC and quinine in Group CPC+, paired *t* test, $t(7) = 0.96$, $p > .37$. Although it did not prove necessary to resort to the sucrose mixtures (because none of the single stimuli were strongly avoided by the thirsty SAL rats), the results for the mixtures confirm those for single test stimuli. That is, CPC+ rats avoided both CPC- and quinine-containing mixtures, but to a lesser extent than the single-stimulus test stimuli. This result is consistent with demonstrations that rats will avoid a CS present in a mixture (D. V. Smith & Theodore, 1984).

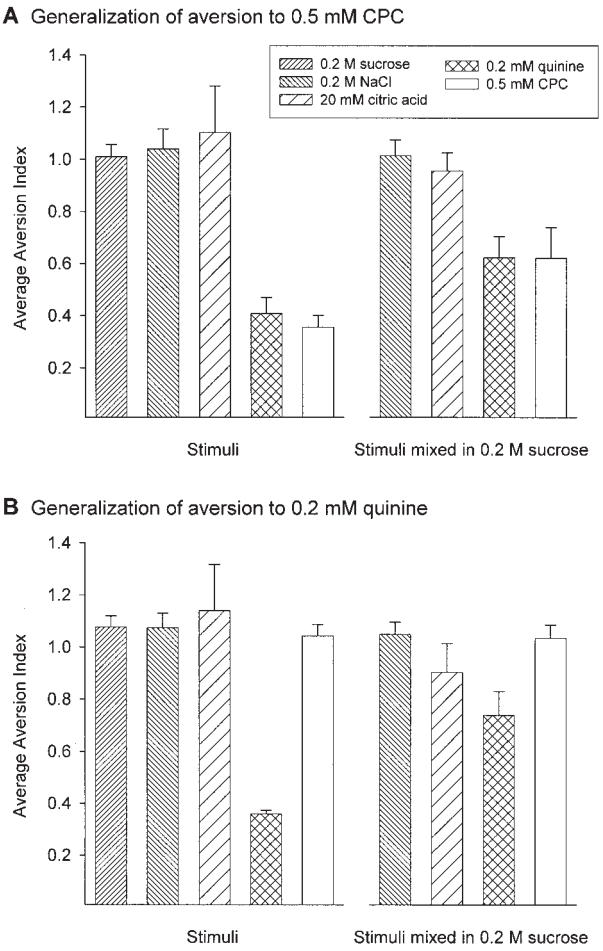

Figure 4. Mean (+ *SE*) aversion index, which ranges from 0 (complete conditioned avoidance) to 1 (no conditioned avoidance; see *Experiment 2, Data Analysis* for details), for several taste stimuli for rats conditioned to avoid 0.5 mM cetylpyridinium chloride (CPC; A) and 0.2 mM quinine (B). Generalization of the aversion was tested for the conditioned stimulus and four other taste stimuli (left panels) and for aversive stimuli mixed in sucrose (right panels).

Surprisingly, aversions conditioned to 0.2 mM quinine did not cross-generalize to CPC (see Figure 4B), although all rats showed a strong aversion to the CS. In other words, only the aversion index for quinine differed from 1.0 in Group QUI+, $t(7) = 45.60$, $p < .001$. We suspected that the failure to find cross-generalization was due to intensity differences between quinine and CPC. If 0.2 mM quinine is perceptually stronger than 0.5 mM CPC, then one would not expect avoidance of CPC even if CPC and quinine are qualitatively similar. Comparing the avoidance curves generated for CPC in the current study (see Figure 1) with those generated in a similar study for quinine (St. John et al., 1994), 0.2 mM quinine is strongly avoided (taste–water ratio < 0.5), whereas 0.5 mM CPC is only mildly avoided (taste–water ratio ~0.8).

Taste aversions generalize fully to stronger concentrations of the CS but show a decrement for weaker concentrations (Spector & Grill, 1988; Tapper & Halpern, 1968). To assess whether perceived intensity differences explain the failure of cross-generalization, we tested the rats in the QUI+ and QUI− groups for responses to an extended concentration series of CPC and quinine. First, the aversion to 0.2 mM quinine was strengthened with two conditioning trials (because the 3 test days represent extinction trials). Rats were first returned to the morning and afternoon fluid presentation schedule (Days 1–3), conditioned on Days 4 and 6 as described previously (with a day of water only in between). These reconditioning sessions may have been unnecessary, as intake of the CS on the first reconditioning trial (see Figure 5A, Trial 3) was less than that on the previous conditioning trial (see Figure 5A, Trial 2) for all rats in the QUI+ group. In any event, all rats displayed a strong aversion to quinine after this reconditioning period (i.e., voluntary intake of the CS was less than 1 ml for all QUI+ rats on the final trial).

Following this trial, rats were given 2 more days of water only (Days 7–8) and then were given a single "warm-up session" in the Davis Rig similar to the test sessions, except that only deionized water was available. Over the next 2 days, rats were tested in sessions identical to the earlier generalization test sessions except that the 10 stimuli were: deionized water (Tubes 1–4), quinine (0.02 mM, 0.06 mM, 0.2 mM), and CPC (0.5 mM, 1 mM, 2 mM). Essentially, quinine responses to the CS and lower concentrations were assessed, as were responses to the initially used CPC concentration (0.5 mM) and two higher concentrations.

Although avoidance behavior was somewhat weak to both the CS and the highest concentration of CPC, rats in the QUI+ group clearly avoided 2 mM CPC relative to the QUI− group (see Figure 5B). Separate Group × Concentration analyses of variance for each stimulus indicated a main effect of group on quinine licking, $F(1, 10) = 29.30$, $p < .0005$, and a significant Group × Concen-

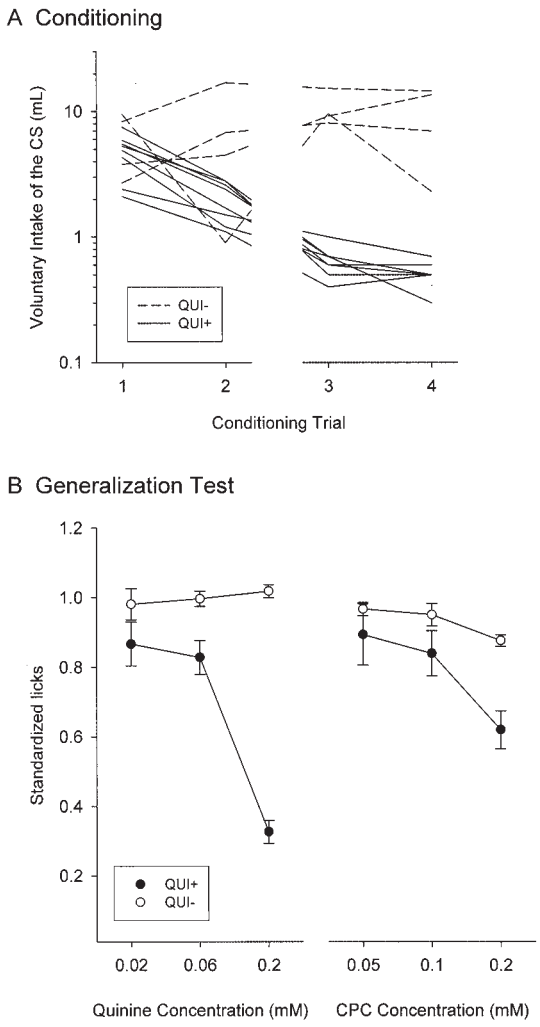

Figure 5. A: Voluntary intake of 0.2 mM quinine (QUI) on four conditioning trials for each rat in a conditioning group (QUI+; solid lines) and a saline-injected control group (QUI−; dashed lines). Generalization of the aversion (see Figure 4) was tested after the second trial. Generalization to an extended range of stimulus concentrations was tested after the fourth trial. Although the test after Trial 2 represents a potential extinction trial, rats in the conditioning group continued to strongly avoid the conditioned stimulus (CS) in Trials 3 and 4. B: Mean (± *SE*) licks for both groups to both quinine (left panel) and cetylpyridinium chloride (CPC; right panel).

tration interaction for CPC licking, $F(2, 20) = 4.10$, $p < .05$. Independent, one-tailed t tests for each stimulus (with a Bonferroni correction for 6 comparisons) revealed that the LiCl-injected groups licked less only for 0.2 mM quinine, $t(10) = 14.00$, $p < .0001$, and 2 mM CPC, $t(10) = 3.20$, $p < .05$. It is interesting to note that, under these conditions, rats did not avoid lower but detectable (Koh & Teitelbaum, 1961; Shaber, Brent, & Rumsey, 1970; St. John & Spector, 1996; Thaw & Smith, 1994) concentrations of the CS. As it appears from Experiment 2A that quinine and CPC share a taste quality, the failure of rats to avoid CPC in Experiment 2B (see Figure 4) can be rationalized if one assumes that 0.5 mM CPC tastes like a weaker concentration of quinine (e.g., 0.06 mM quinine). Support for this interpretation comes from that fact that, under these conditions, rats did not avoid weaker concentrations of the CS when an extended array of quinine concentrations was tested (see Figure 5B, left panel). Furthermore, QUI+ rats did show suppression of a higher concentration of CPC (2 mM) relative to unconditioned (QUI−) rats (see Figure 5B, right panel). In fact, 2 mM CPC and 0.2 mM quinine, the only test stimuli avoided in the follow-up experiment, appear to be isohedonic based on the avoidance curves reported in previous research on quinine (St. John et al., 1994) and in Experiment 1.

Experiments 1 and 2 together support the notion that CPC, a potent antagonist of salt transduction, itself has a taste, and that taste is perceptually similar to quinine. In the following experiments, quinine is used as a control stimulus for CPC—one that produces a similar taste without having effects on salt transduction. In these experiments, the concentration of quinine was chosen to be isohedonic to the concentration of CPC on the basis of Experiment 1 and previous research (St. John et al., 1994).

Experiment 3: The Effect of CPC on Salt Recognition

The primary interest of these studies was to determine whether CPC affected behavioral responses to salts. Two experiments were conducted to establish whether CPC adulteration reduced the recognition of either NaCl (Experiment 3A) or KCl (Experiment 3B) following the establishment of a conditioned taste aversion.

Method

Subjects. Subjects were naive, male Sprague–Dawley rats housed in conditions identical to Experiments 1 and 2. Sixteen rats were used in Experiment 3A and 12 rats were used in Experiment 3B. The rats weighed 248–330 g at the start of the experiment.

Stimuli and experimental design. In Experiment 3A, aversions were conditioned to 0.2 M NaCl and generalization of the aversion was tested for the following stimuli: water, NaCl (0.05, 0.1, and 0.2 M), 0.3 M KCl, and 0.2 M NaCl mixed in 30 μM amiloride, 2 mM CPC, and 0.2 mM quinine hydrochloride. Amiloride has previously been shown to affect the recognition of NaCl in taste aversion generalization, salt appetite and operant discrimination paradigms (Bernstein & Hennessy, 1987; Geran & Spector, 2000a; Hill et al., 1990; Kopka et al., 2000; Kopka & Spector, 2001; McCutcheon, 1991; Roitman & Bernstein, 1999; Spector et al., 1996) at concentrations that are tasteless to rats (Markison & Spector, 1995). We chose the concentration of CPC that maximally suppressed NaCl and KCl responses in the CT in electrophysiological experiments (DeSimone et al., 2001), and the quinine control stimulus was chosen by comparison of avoidance curves for CPC (Experiment 1) and quinine (St. John et al., 1994) generated using similar methodologies. Eight rats received taste aversions (NaCl+) and 8 rats served as saline-injected controls (NaCl−).

In Experiment 3B, aversions were conditioned to 0.3 M KCl and generalization of the aversion was tested for the following stimuli: water, KCl (0.1, 0.3 M), 30 μM amiloride, 1 mM CPC, and 0.1 mM quinine, as well as 0.3 M KCl adulterated with 30 μM amiloride, 1 mM CPC, and 0.1 mM quinine. Because KCl itself may have a quinine-like taste, we were concerned that KCl may itself generalize to the CPC or quinine portion of the mixture, which necessitated testing each of the adulterants in isolation (unlike Experiment 3A). This concern also prompted lowering the CPC concentration (and quinine concentration) in Experiment 3B; this concentration is less aversive than 2 mM CPC (cf. Experiment 1) but still significantly suppresses the CT response to KCl (DeSimone et al., 2001). Seven rats received taste aversions (KCl+) and 5 rats served as saline-injected controls (KCl−).

Procedure. Experiments 3A and 3B were designed to be identical where possible and, thus, will be described together. Differences are noted.

Rats were first trained to lick in the Davis Rig prior to taste aversion conditioning (similar to Experiment 2). On the 1st day, water was removed from the home cage. On the next 2 days, rats had access to a single tube of deionized water in the Davis Rig for 30 min. On Day 4, the 30 min session presented 22 trials from 11 tubes containing water. Trials terminated 15 s following a lick or 2 min without a lick. On Days 5 and 6, the Davis Rig sessions reflected the forthcoming test sessions except that only water was presented. Sessions were 30 min long and presented up to 80 total trials. Trials were of two types: test trials, which were 6 s long, and rinse trials, which were 4 s long. The first trial was always a rinse trial and rinse and test trials alternated throughout the session. The rationale for these two trial types is given below.

Following Davis Rig training, taste aversion conditioning began. Over the next 3 days, rats were habituated to the twice-a-day fluid access schedule described previously. On the following day, the CS (0.2 M NaCl in Experiment 3A and 0.3 M KCl in Experiment 3B) was provided instead of water in the morning fluid period. Rats in the taste aversion groups (NaCl+ and KCl+) received a 2 mEq/kg injection of 0.15 M LiCl ip, immediately following the fluid availability; rats in the control group (NaCl− and KCl−) received a 2 mEq/kg injection of isotonic saline. Two identical conditioning trials followed; each was preceded by 2 days of water only during the same twice-a-day fluid access schedule. On conditioning trials, 1 ml of the CS was fed to the rat by syringe if the rat failed to voluntarily drink at least 2 ml (this was true of all LiCl−injected rats in Experiment 3A and all but one in Experiment 3B on the final conditioning trial). Two water-only days followed the final conditioning trial; the afternoon water availability was omitted on the final day.

Two Davis Rig test sessions followed. Test sessions were identical to the final days of training except for the identity of the stimuli. Each stimulus was presented once in a block of 10 trials, except for water, which was presented three (Experiment 3A) or two (Experiment 3B) times per block. The rinse trials (deionized water) preceded every test trial and served three functions: to rinse the tongue of the previous solution, to provide a relatively uniform adaptation state for each trial, and to ensure that the rat had ample access to nonaversive stimuli during the session.

Results and Discussion

Rats in the NaCl+ and KCl+ groups had strong taste aversions to their respective CS (see Figure 6). The aversion index was less than 0.4 for all NaCl concentrations in the NaCl+ group (see Figure 6A) and was less than 0.2 for both KCl concentrations in the KCl+ group (see Figure 6B). The NaCl+ rats avoided all solutions except 0.3 M KCl and 0.2 M NaCl mixed with amiloride; KCl+ rats avoided all solutions except 0.03 mM amiloride (one-tailed t test vs. a hypothesized mean of 1.0, with a Bonferroni correction applied for multiple tests). The only stimuli that the NaCl+ rats treated differently than the 0.2 M NaCl CS were 0.05 M NaCl, 0.3 M KCl, and 0.2 M NaCl mixed with amiloride (paired t test, Bonferroni-adjusted $\alpha = .0083$). That is, CPC did not affect responses to 0.2 M NaCl. For KCl+ rats, amiloride, CPC, quinine, and KCl mixed with CPC were all avoided less than the 0.3 M KCl CS alone (paired t test, Bonferroni-adjusted $\alpha = .0071$). Thus, CPC did reduce the avoidance of KCl, albeit only slightly (see Figure 6B).

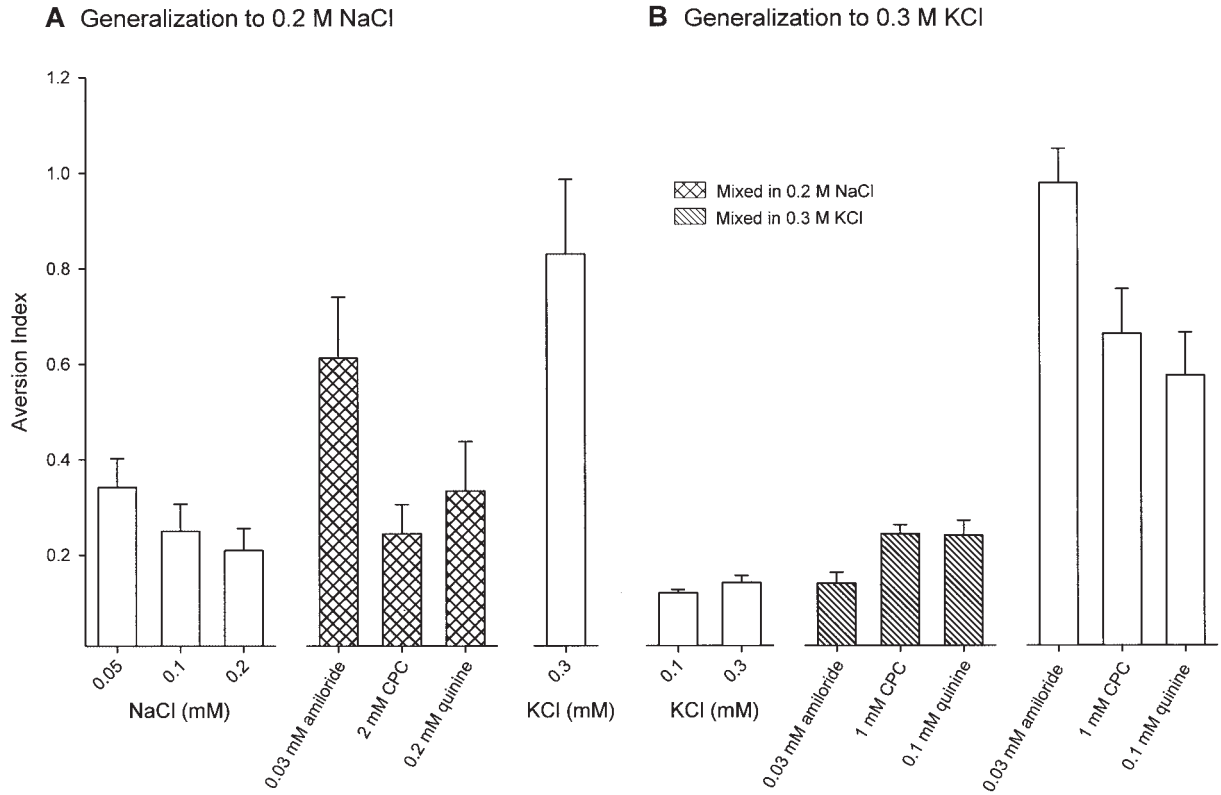

Figure 6. The mean (+ *SE*) aversion index, which ranges from 0 (complete conditioned avoidance) to 1 (no conditioned avoidance; see *Experiment 2, Data Analysis* for details), for several taste stimuli for rats conditioned to avoid 0.2 M NaCl (A) and 0.3 M KCl (B). CPC = cetylpyridinium chloride.

Thus, consistent with other reports, NaCl taste aversions do not generalize strongly to KCl, and rats fail to recognize NaCl when it is mixed in 30 μM or higher concentrations of amiloride (Bernstein & Hennessy, 1987; Hill et al., 1990; McCutcheon, 1991; Roitman & Bernstein, 1999; Spector et al., 1996). Unlike amiloride, CPC did not block avoidance of the NaCl CS. Likewise, neither CPC nor amiloride prevented recognition of KCl. It is interesting that although the rats did not appear to taste amiloride on its own (cf. Markison & Spector, 1995), the aversion indices for CPC and quinine alone were significantly less than 1.0. This result is consistent with the implication from Experiment 2 that quinine and CPC taste similarly and also suggest that these compounds share perceptual features with KCl, as is the case in humans (van der Klaauw & Smith, 1995).

The finding that CPC reduces recognition of KCl relative to KCl alone is consistent with the notion that CPC affects the taste quality of KCl pharmacologically, particularly since responses to KCl and KCl mixed with quinine were not statistically different. However, there is reason for caution. First, the difference in lick rate of the CS and the CS mixed with CPC is small (cf. Figure 6B). Second, although quinine did not statistically reduce avoidance of the CS, the comparison was close to reaching significance despite the conservative correction for multiple comparisons, $t(6) = 2.72$, $p = .017$. Finally, a two-tailed, paired t test of the aversion index for KCl mixed with CPC versus KCl mixed with quinine failed to provide evidence to reject the null hypothesis that rats treated these two stimuli similarly, $t(6) = 0.10$, $p = .92$; the aversion index for these solutions is virtually identical. Given these considerations, the conclusion that CPC affects recognition of KCl through a pharmacological mechanism is not particularly compelling.

In summary, CPC had a small effect on the recognition of KCl, but not of NaCl, in a taste aversion generalization paradigm. Similar deficits in recognition, however, were seen when isohedonic quinine was mixed with KCl. Unless quinine also blocks amiloride-insensitive salt transduction, it would be parsimonious to conclude that these results are not due to CPC blocking an apical cation channel in taste buds.

Experiment 4: The Enhancement of Salt Taste by CPC

As a final attempt to assay the effect of CPC on salt taste, we examined its effect on the recognition of NaCl in a salt-appetite paradigm. When deprived of sodium, either chronically or acutely, rats show a robust appetite for NaCl even at concentrations that would normally be avoided (Richter, 1936, 1956; e.g., Breslin, Kaplan, Spector, Zambito, & Grill, 1993). In addition, the appetite is specifically tuned to sodium salts; nonsodium salts are not preferred by sodium-deprived rats (Breslin, Spector, & Grill, 1993; Krieckhaus & Wolf, 1968; Markison, St. John, & Spector, 1995; Nachman, 1962).

Furthermore, sodium-deprived rats show a heightened preference for all concentrations of NaCl. Normally, rats prefer isotonic NaCl to hypertonic or hypotonic salt solutions, producing an inverted-U shaped preference–concentration function in brief access tests (Breslin et al., 1993). Salt-deprived rats show this same function in a brief access licking test, but behavioral responses are heightened dramatically across the concentration range (Breslin et al., 1993). The psychophysical effect of introducing an adulterant to NaCl in sodium-deprived rats presents three distinct predictions. Adulterants that reduce the intensity of NaCl should shift the inverted-U function rightward; rats should treat some hypertonic concentration as if it were isotonic. Adulterants that enhance the intensity of NaCl (as micromolar CPC appears to do in electrophysiological studies of the CT nerve; DeSimone et al., 2001) should shift the inverted-U function leftward; rats should treat a hypotonic solution as if it were isotonic. Finally, an adulterant that altered the taste quality of NaCl without affecting its perceived intensity should reduce the amplitude of the preference function or change its shape altogether.

Method

Subjects. Twenty-three experimentally naive, male Sprague–Dawley rats weighing 229–292 g at the start of the experiment were subjects of the experiment. Rats were housed in stainless steel, wire-mesh hanging cages and provided food (Harlan Teklad 8604) and tap water ad libitum except were noted. The lighting was automatically controlled (13:11-hr light–dark cycle), and all manipulations were performed during the lights-on portion of the cycle.

Stimuli and experimental design. The experiment was run in two phases. In the first phase, 11 rats were made sodium deficient by injection of furosemide and were tested the next day for their licking responses to several concentrations of NaCl (0.028, 0.05, 0.089, 0.158, 0.281, 0.5, and 0.89 M) and deionized water. For half of the rats, the NaCl was adulterated with 250 μM CPC, the concentration of CPC identified electrophysiologically as causing a maximal enhancement of the NaCl response of the CT nerve (DeSimone et al., 2001). After 6 days, the rats were given a second sodium deprivation treatment and were tested on the following day with either NaCl or NaCl mixed with CPC (whichever treatment the rat had not received previously).

In the second phase, 12 naive rats were subjected to the identical procedure, except that the two test sessions contained either NaCl or NaCl mixed with 0.029 mM quinine. This concentration of quinine is above the detection threshold for quinine in rats (Koh & Teitelbaum, 1961; Shaber et al., 1970; St. John & Spector, 1996; Thaw & Smith, 1994), and is isohedonic to 250 μM CPC based on previous research (St. John et al., 1994) and Experiment 1.

Procedure. The procedure used was adapted, with relatively minor changes, from that used by Breslin and colleagues (Breslin et al., 1993). On Day 1, water was removed from the home cage, and Davis Rig training occurred over the next 5 days. In addition, because rats would be maintained on a sodium-deficient chow during the deprivation period, rats were accustomed to this food (Harlan Teklad 90228) during the training phase beginning on the second day of spout training. This food was presented in a separate hopper next to the standard lab chow.

On the first 2 days of training, rats had access to a single tube of water in the Davis Rig for 20 min. Following the second session, rats were provided with 20 ml of water on the home cage for a 2-hr access period. On the 3rd training day, the rats had access to 0.3 M sucrose in the Davis Rig for 20 min. The intent of this session was to encourage rats to sample the drinking spout when not motivated by water deprivation. Following this session, rats were returned to ad libitum water in the home cage.

The final two training sessions were 30 min in duration, during which the rat could initiate up to 100 trials. Trials were 15 s in duration. In our previous experiments, a trial could end without a lick if 2 min elapsed; in this experiment, the only way to progress to the next trial was by taking at least one lick on the spout. Trials were presented in randomized blocks of two; stimuli were water and 0.3 M sucrose.

After a day off, the rats were subjected to acute sodium depletion by subcutaneous injection of 2 mg furosemide. This injection procedure results in near-maximal sodium appetite without producing food aversion (Lundy, Blair, Horvath, & Norgren, 2003). Rats were returned to the home cage after injection, but prior to their return, the cages were wiped clean

with a wet sponge to remove any salt deposits, and the standard sodium-replete chow was removed, leaving the sodium-deplete chow as the only dietary alternative. Tap water was replaced with deionized water.

On the following day, rats were given their first 40-min test session, during which rats had access to up to one hundred twenty 15-s trials. The eight solutions were presented in randomized blocks, such that each block of eight trials included each stimulus once in an unpredictable order. Immediately following this session, normal chow was returned to the home cage and rats were given a full 6 days with both diets available to allow rats to recover from sodium deprivation. Following this repletion period, rats were again depleted of sodium with furosemide. On the following day, rats were given the second test session.

Results and Discussion

Sodium-depleted rats showed the typical inverted-U shaped lick–response functions across a range of NaCl concentrations, with the most preferred stimulus in the array near the isotonic concentration (Breslin et al., 1993). The functions generated by rats in both phases were similar (see Figure 7). The adulterants, CPC and quinine, essentially had identical effects: The lick-concentration functions shifted rightward by about a quarter of a log unit, most notably at hypotonic and isotonic concentrations. Such a shift is indicative of a reduction in perceived intensity of the NaCl, which is the opposite that would be predicted if 0.25 mM CPC caused an enhancement in the perceived intensity of NaCl.

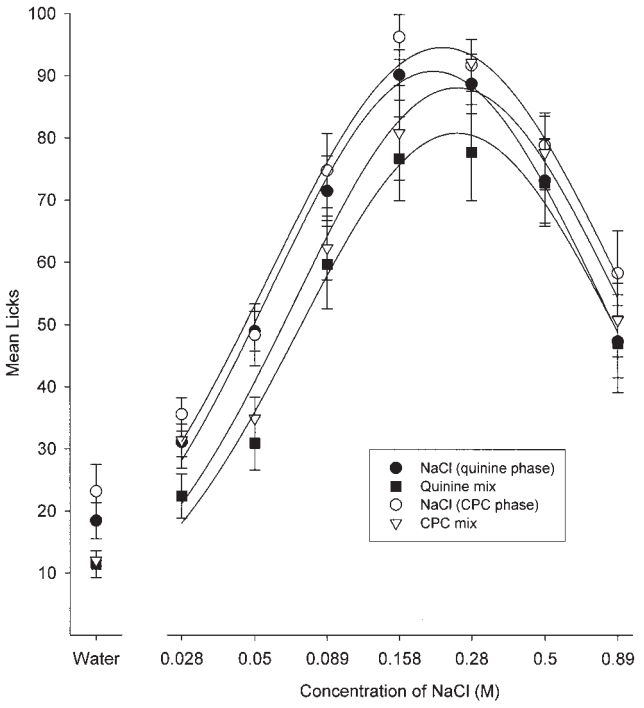

Figure 7. Mean (± *SE*) number of licks in 15-s trials for sodium-deprived rats. Filled symbols are data from rats tested with NaCl (circles) and NaCl mixed with quinine (squares) in separate sessions, counterbalanced for order; open symbols are data from rats tested with NaCl (circles) and NaCl mixed with cetylpyridinium chloride (CPC; triangles) in separate sessions, counterbalanced for order. A three-parameter log normal function was fit to the data in order to make the response patterns more apparent (all fits accounted for >94.5% of the variance).

However, a two-way ANOVA (Adulterant × Concentration) conducted separately for the CPC phase and the quinine phase failed to detect an effect of the adulterant: for CPC, $F(1, 10) = 4.55$, $p = .058$, Adulterant × Concentration interaction, $F(6, 60) = 1.54$, $p = .18$; for quinine, $F(1, 11) = 3.25$, $p = .099$, Adulterant × Concentration interaction, $F(6, 66) = 1.21$, $p = .31$. With regard to the trend toward a significant effect of the two adulterants on responses to NaCl, it is instructive to note that responses to water (which were also adulterated) were significantly reduced by the adulterant (two-tailed paired *t* test): for CPC, $t(10) = 2.54$, $p = .03$; for quinine, $t(11) = 2.32$, $p = .04$. Thus, any alteration in behavior caused by the adulterant might best be ascribed to the addition of a small aversive taste to the salt solutions. Consistent with this, a Group × Concentration ANOVA did not reveal differences between responses to NaCl + CPC and NaCl + quinine mixtures, $F(1, 21) = 1.01$, $p = .33$, Group × Concentration interaction, $F(6, 126) = 0.44$, $p = .85$. In summary, CPC did not enhance the taste of NaCl. Furthermore, CPC and quinine appeared to equivalently affect responses to NaCl.

General Discussion

CPC as a Tool to Study Salt Taste Coding

The primary interest of these experiments was to use CPC as a tool to better understand the contribution of two different transduction mechanisms to the recognition and discrimination of compounds humans describe as salty. Because amiloride rapidly, reversibly, and completely impairs one of these two transduction mechanisms (Brand et al., 1985; DeSimone & Ferrell, 1985; Heck et al., 1984; Hettinger & Frank, 1990; Ninomiya & Funakoshi, 1988), and because this compound is tasteless to rats at effective concentrations (Markison & Spector, 1995), a great deal has been learned about the role of amiloride-sensitive transduction in behavioral responses to salts (e.g., Boughter et al., 1999; Halpern, 1998). Indeed, in rodents at least, the role of amiloride-sensitive systems in human salt taste is apparently quite different (cf. Feldman et al., 2003; Ossebaard, Polet, & Smith, 1997; Ossebaard & Smith, 1995, 1996); this system is clearly critical for the recognition of sodium salts and the discrimination of sodium from non-sodium salts (Bernstein & Hennessy, 1987; Hill et al., 1990; McCutcheon, 1991; Spector et al., 1996). (The role of amiloride-sensitive systems in human salt taste is apparently quite different; cf. Feldman et al., 2003; Ossebaard et al., 1997; Ossebaard & Smith, 1995, 1996.) A strong interpretation of these data and others is that the amiloride-sensitive system represents a labeled line for saltiness (Geran & Spector, 2004; McCaughey & Scott, 1998), although we have cautioned that such a conclusion logically requires empirical establishment of both necessity and sufficiency of the amiloride sensitive system for recognition and discrimination of sodium salts (D. V. Smith & St. John, 1999; D. V. Smith et al., 2000; St. John & Smith, 2000). Such an empirical test, if possible, would provide the best opportunity for coding theorists to address whether the gustatory system (or elements of it) uses pattern coding (in which amiloride-sensitive and -insensitive systems both contribute to the taste quality of sodium salts) or labeled-line coding (in which activation of the amiloride-sensitive system gives rise to saltiness).

This logical necessity has been noted by many authors, including Frank (2000), who noted "pattern theory takes a form that may

be tested empirically by observing the effects of blocking the generalist H-fibers on the taste of NaCl" (p. 57). *Generalist H fibers* is another term for amiloride-insensitive neurons; blocking these neurons would presumably allow for tests of the sufficiency of the spared sodium-responsive system, the amiloride-sensitive system. The report that CPC blocked the amiloride-insensitive portion of the salt signal in the CT (DeSimone et al., 2001) motivated the current series of studies.

We first observed that CPC has an inherent aversive taste (Experiment 1) that was clearly manifest at 2 mM (identified electrophysiologically as causing maximal reduction of the NaCl signal). The effect of CPC alone on the CT nerve was not reported, but DeSimone and colleagues (2001) did report that the addition of amiloride and CPC to an ongoing NaCl response lowered the response rate to the level of the background. Although the signal-to-noise ratio in whole nerve recording could cause small evoked responses to be missed, Experiment 1 assessed behavioral responses to CPC in rats with transection of the GL on the proposition that this nerve might convey the aversive taste of CPC, given that no residual response was seen in the NaCl + amiloride + CPC cocktail in the CT. We found that neither GLX nor CTX substantially reduced the avoidance of CPC but that the combined transection did, implying that both nerves contribute to the aversiveness of this compound. Presumably CPC stimulates the CT only weakly.

Given that CPC has a strong taste at 2 mM, any reduction in behavior toward NaCl in an NaCl–CPC mixture would be causally ambiguous. We next attempted to identify a control stimulus—one that replicated CPC's taste quality without its pharmacological effect on amiloride-insensitive salt transduction. Experiment 2 identified CPC as quinine-like. In order to match CPC and quinine as closely as possible, we used the results of Experiment 1 and a methodologically similar previous study (St. John et al., 1994) to identify concentrations of quinine that were isohedonic to concentrations of CPC used in Experiments 3 and 4. Given the similarity in behavioral responses to quinine and CPC, alone and in mixture, throughout the studies reported here, this strategy appears to be remarkably reliable.

The effects of CPC on salt recognition were not strong. In Experiment 3A, there was no evidence that 2 mM CPC affected the recognition of NaCl in a conditioned taste-aversion paradigm, whereas amiloride virtually abolished recognition of NaCl. In Experiment 3B, there was some evidence that 1 mM CPC reduced the avoidance of KCl following a conditioned taste aversion, but rats behaved similarly toward a KCl–quinine mixture in which the concentration of quinine was selected to be isohedonic to 1 mM CPC. In Experiment 4, we assessed responses to NaCl following sodium depletion in which NaCl was mixed in 0.25 mM CPC—a concentration that was reported to enhance the CT response to NaCl (DeSimone et al., 2001). There was a nonsignificant reduction in responses to NaCl, but this was again matched when isohedonic quinine was mixed in the salt solution. In summary, there was no strong evidence that the pharmacological properties of CPC affected behavioral responses to NaCl or KCl.

In the absence of some clear effect of CPC (i.e., a positive control), these negative results are difficult to interpret. For example, the results for NaCl are on the whole consistent with the labeled line notion that amiloride sensitive neurons signal saltiness. If identification of NaCl following a conditioned taste aversion, or the drive to ingest NaCl following sodium depletion are only dependent on amiloride-sensitive activity, then these negative results are potentially quite meaningful: They indicate that enhancement or inhibition of the other salt-sensitive neurons is irrelevant, as expected. (Though this does gloss over some subtleties; the activity in the other pathway should represent something, producing a more or less unitary saltiness depending on whether other lines are inhibited or enhanced; cf. D. V. Smith et al., 2000.) Unfortunately, the results of Experiment 3B would be more difficult to fit to this interpretation. KCl is much more sensitive to CPC than is NaCl (DeSimone et al., 2001) and must critically depend on amiloride-insensitive activity regardless of whether labeled-line or pattern coding is used to represent its taste.

The failure of CPC to behaviorally affect KCl forces us to consider the possibility that CPC is not effectively impairing salt transduction in the behaving rat. If this failure is concentration dependent, then the fact that 2 mM CPC is already quite aversive indicates that it may not be a useful tool in investigating the behavioral roles of the amiloride-insensitive pathway. However, the recent discovery that CPC inhibits sodium influx through a vanilloid receptor-1 gene product (Lyall et al., 2004) suggests that vanilloid receptor antagonists, such as SB-366791 and capsazepine, should be explored in behavioral paradigms as well.

CPC as an Aversive Stimulus

The positive results in these studies are, in the end, perhaps of more interest. Of sucrose, NaCl, citric acid, and quinine, CPC is clearly the most similar to quinine, despite the fact that these compounds would appear to be structurally quite different from one another. Quinine, and many other compounds that humans describe as bitter, presumably stimulate T2R receptors, a family of $>$ 30 G-protein coupled receptors (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami, Montmayeur, & Buck, 2000). Because these diverse receptors are coexpressed on single taste receptor cells (Adler et al., 2000), structurally diverse molecules (like quinine and CPC) may nonetheless use identical pathways beyond the level of the receptor (cf. Zhang et al., 2003). Indeed, some structurally diverse bitter compounds are indiscriminable to rats (Spector & Kopka, 2002). Of course, some bitter compounds may avail themselves of T2R-independent transduction mechanisms (Caicedo, Pereira, Margolskee, & Roper, 2003; Peri et al., 2000; Spielman, Huque, Whitney, & Brand, 1992; Zhao, Lu, & Herness, 2002), which may in part explain evidence for the discriminability of some bitter compounds from one another (Delwiche, Buletic, & Breslin, 2001; Frank, Bouverat, MacKinnon, & Hettinger, 2004) and the differential sensitivities of taste receptor cells or afferent fibers in electrophysiological studies (Caicedo & Roper, 2001; Dahl, Erickson, & Simon, 1997).

Whether CPC and quinine (at matched concentrations) are truly indiscriminable or merely very similar is a matter for empirical investigation. The rats in this experiment clearly treated the two compounds similarly, whether in a mixture (Experiments 3A, 3B, and 4) or alone (Experiments 1 and 4). If CPC can be considered perceptually similar to quinine, then an intriguing and perplexing pattern is beginning to emerge in the peripheral organization of bitter taste in the rat. Compounds referred to as bitter by humans, such as quinine, generally are very poor stimuli for the rat CT in electrophysiological studies (e.g., Frank, Contreras, & Hettinger,

1983; Ogawa, Sato, & Yamashita, 1968; Pfaffmann, 1955; D. V. Smith & Frank, 1993), but give robust responses in the GL (Yamada, 1966; e.g., Boudreau, Do, Sivakumar, Oravec, & Rodriquez, 1987; Frank, 1991; D. V. Smith & Frank, 1993). Nevertheless, in behavioral tasks that focus on appetitive, hedonic responses, these nerves appear to participate equally in responsiveness to bitter substances (Akaike, Hiji, & Yamada, 1965; Grill & Schwartz, 1992; Grill, Schwartz, & Travers, 1992; Pfaffmann, 1952; St. John et al., 1994; Yamamoto & Asai, 1986). Most studies indicate that behavior is relatively unchanged after the bilateral loss of one nerve, but severe hypoguesia for quinine (and now CPC) occurs after the loss of two nerves. Similarly, detection thresholds for quinine are only marginally affected by single nerve cut but are considerably elevated after combined nerve cut (St. John & Spector, 1996). Most surprisingly, the discrimination of quinine from KCl is unaffected by GLX, but is affected by CTX, and severely affected by GLX + CTX (St. John & Spector, 1998). This result is consistent with electrophysiological investigations of the CT that suggest that although this nerve does not respond strongly to quinine, it does differentiate among aversive substances (Dahl et al., 1997). The current results with CPC, coupled with the apparent failure to demonstrate a substantial electrophysiological response to CPC alone in the CT (DeSimone et al., 2001), suggest that bitter taste is by no means the exclusive purview of posterior lingual receptors, despite electrophysiologically based predictions.

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