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Glutamate-induced Cobalt Uptake Elicited by Kainate Receptors in Rat Taste Bud Cells

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Abstract

Glutamate-induced cobalt uptake reveals non-*N*-methyl-D-aspartate (non-NMDA) glutamate receptors (GluRs) in rat taste bud cells. However, it is not known which type of non-NMDA glutamate receptors is involved. We used a cobalt staining technique combined with pharmacological tests for kainate or α-amino-3-hydroxy-5-methyl-isoxazole-propionic acid (AMPA) receptors and/or immunohistochemistry against subunits of GluRs to examine the presence of non-NMDA receptors in rat foliate tastebud cells. Cobalt uptake into taste cells was elicited by treating taste buds with glutamate, kainate or SYM 2081, a kainate receptor agonist. Treating taste buds with AMPA or fluorowillardiine did not stimulate significant cobalt uptake. Moreover, 6-cyano-7-nitro-quinoxaline-2, 3-dione significantly reduced cobalt staining elicited by glutamate or kainate receptor agonists, but SYM 2206, an AMPA receptor antagonist, did not. Immunohistochemistry against subunits of GluRs reveals GluR6 and KA1-like immunoreactivity. Moreover, most glutamate-induced cobalt-stained cells showed GluR6 and KA1-like immunoreactivity. These results suggest that glutamate-induced cobalt uptake in taste cells occurs mainly via kainate type GluRs.

Key words: AMPA, cobalt staining, foliate papilla, glutamate receptors, kainate, taste bud

Introduction

Taste buds are composed of several kinds of cells, including taste receptor cells and supporting cells. In gustatory perception, synaptic interactions between taste bud cells may play important roles (Roper, 1992). In rat taste buds, several neurotransmitters have been identified. To date, glutamate (Caicedo *et al.*, 2000b; Kim *et al.*, 2001), serotonin (Herness and Chen, 1997), norepinephrine (Herness *et al.*, 2002) and γ-amino butyric acid (Obata *et al.*, 1997) have been reported in rat taste buds. These neurotransmitters may participate not only in information transfer from receptor cells to afferent neurons, but also among taste bud cells.

Glutamate is a ubiquitous neurotransmitters in the nervous system. There are several types of glutamate receptors (GluRs) including metabotropic and ionotropic. Ionontropic GluRs consist of *N*-methyl-D-aspartate (NMDA), kainate and α-amino-3-hydroxy-5-methyl-isoxazole-propionic acid (AMPA) GluRs. Kainate and AMPA receptors are called non-NMDA GluRs. In rat taste buds, non-NMDA GluRs are present and increase during taste bud maturation (Kim *et al.*, 2001). In the central nervous system, the roles of AMPA and kainate receptors are different. AMPA receptors mediate the majority of rapid excitatory synaptic transmission and participate in synaptic plasticity (Song and Huganir, 2002). In some parts of brain, AMPA GluRs modulate the size of synapses (Passafaro *et al.*, 2003). The kinetics of EPSCs generated at kainate receptors is slower than that of EPSCs from AMPA receptors (Lerma, 2003). A slow, small EPSC such as that mediated by kainate receptors offers the possibility of integrating excitatory inputs over a larger time window (Lerma, 2003). Moreover, gating mechanisms of AMPA and kainate receptors are also different. External ions modulate the amplitude and kinetics of kainate GluRs, but not of AMPA GluRs (Bowie, 2002). Thus it is an important question whether AMPA or kainate GluRs are present in

taste bud cells. The answer may have a bearing on the role(s) of non-NMDA GluRs in taste perception.

To investigate the presence and possible functional significance of iGluRs in taste buds, we have used the cobalt staining technique introduced by Pruss *et al.* (1991) and/or immunohistochemistry against subunits of non-NMDA GluRs. The cobalt staining technique involves the pharmacological stimulation of iGluRs in the presence of $CoCl_2$, which leads to the uptake of Co^{2+} through Ca^{2+}-permeable iGluRs. Subsequently, one can visualize activated cells by precipitating histochemically and intensifying the intracellular Co^{2+}. This technique serves to identify cells that possess Ca^{2+}-permeable iGluRs of the non-NMDA type in taste bud cells (Caicedo *et al.*, 2000b).

We found that cobalt-stained cells correlated best with kainate GluRs in rat foliate taste bud cells. Parts of these results were presented as an abstract (Chung *et al.*, 2004).

Materials and methods

All experimental protocols were approved by the Animal Care and Use Committee, College of Dentistry, Kangnung National University. The experimental protocol was adopted from procedures published previously (Caicedo *et al.*, 2000; Kim *et al.*, 2001).

Preparation of slices

Tongue slices containing taste buds were obtained from 45- to 60-day-old Sprague–Dawley rats (at least four animals in each group). All animals were weaned at postnatal day 21. Rats were deeply anesthetized with carbon dioxide and decapitated. The tongue was quickly removed and immersed in cold, oxygenated, low sodium, high sucrose uptake buffer containing (in mM): 57.5 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 12 glucose, 139 sucrose, pH 7.4, 305 mOsm. After removing surrounding muscles, blocks of tissue that included foliate papillae were mounted on a tissue chopper (Stoelting, Wood Dale, IL). Tongue slices (500 μm) containing foliate papillae were cut perpendicular to the long axis of the trenches and incubated in oxygenated uptake buffer for 1 h at room temperature. Usually two slices from each rat were analyzed.

Cobalt uptake

Slices were transferred into a six-well dish containing oxygenated uptake buffer. After two 10 min washes in uptake buffer, slices were stimulated for 5 min at room temperature in uptake buffer containing 5 mM $CoCl_2$ plus 1 mM glutamate and/or other drugs. After pharmacological stimulation, slices were rinsed for 5 min in uptake buffer containing 2 mM EDTA to remove non-specifically bound Co^{2+}, followed by 5 min in uptake buffer. Cobalt ions were then precipitated by incubating the slices for 5 min in uptake buffer containing 1.2% $(NH_4)_2S$. Slices were washed again in uptake buffer and then fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4) at 4°C. They were cryoprotected in 20% sucrose in 0.1 M phosphate buffered saline and sectioned at 20 μm on a cryostat (Jung Frigocut 2800N, Leica, Germany). Silver enhancement of cobalt precipitates was carried out on floating sections with the intenSEM kit (Amersham, Arlington Heights, IL). Sections were mounted on gelatin-coated slides, counterstained with methylene blue, dehydrated, cleared and coverslipped. We tested 30–300 μm kainate and SYM 2081, a kainate type receptor agonists, 30–300 μM AMPA and 1–10 μM fluorowillardiine (Patneau *et al.*, 1992), an AMPA type receptor agonist, and 100 μM SYM 2206 (Pelletier *et al.*, 1996), an AMPA type receptor antagonist with or without 100 μM CNQX.

Kainate, AMPA, CNQX, SYM 2081, SYM 2206 and fluorowillardiine were purchased from Tocris (Ballwin, MO). Other reagents were obtained from Sigma (St Louis, MO).

Immunohistochemistry

Sprague–Dawley rats were deeply anesthetized with urethane (1.5 g/kg body wt) and perfused intracardially with 0.1 M phosphate-buffered saline (PBS, pH 7.2) followed by 4% paraformaldehyde in PBS. The tissues blocks containing foliate papillae were excised and fixed in the same fixative solution for 2 h. They were then cryoprotected and sectioned at 20 μm using the same procedure as above. Cryostat sections were treated for 1 h at 4°C with a preincubation solution containing 0.3% Triton X-100, 1% bovine serum albumin (BSA) and 10% normal goat serum in PBS. Immunohistochemistry procedure against GluR1–7 and KA1 and 2 (Santa Cruz Biotechnology Inc. Santa Cruz, CA; Table 1) were performed. Sections were incubated overnight at 4°C with primary antibodies against subunits of GluRs (diluted 1:100) in preincubation solution. Sections were blocked for 1 h at room temperature with 10% normal goat serum and 1% BSA in PBS. Sections were rinsed and labeled by the avidin-biotin-peroxidase method (Vectastain ABC, Vector Laboratories, Burlingame, CA). Peroxidase activity was detected with diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole

Table 1 List of antibodies against subunits of non-NMDA glutamate receptors which were used in present experiments

Type of GluR	Antibodies used
AMPA	anti-GluR1
	anti-GluR2
	anti-GluR3
	anti-GluR4
Kainate	anti-GluR5
	anti-GluR6
	anti-GluR7
	anti-KA1
	anti-KA2

(AEC). Labeling of subunits of GluRs in taste buds was also abolished by omitting the primary antibody (data not shown). For double labeling with cobalt staining, immunohistochemistry procedures against GluR6 or KA1 were performed after cobalt visualization procedure. Other reagents except indicated were obtained from Sigma.

Data analysis

Sections were examined and photographed on a Nikon E-600 microscope (Japan) with bright-field illumination. Specific immuno- or cobalt staining was defined as being evenly distributed intracellularly (Caicedo *et al.*, 2000; Kim *et al.*, 2001). To quantify staining, we determined the numbers of immuno- or cobalt-stained cells per taste bud in two sections from a slice. We examined differences in the amount of Co^{2+} uptake between experimental groups by comparing the incidence of stained cells, that is, the average number of stained cells per taste bud in an individual slice. These values were averaged over the individual slices in each group. The data of each group were compared using the Mann–Whitney *U*-test. All cells counts were done blindly. There were no differences in the cell counts when cells were counted independently by two investigators. Data are expressed as mean ± SEM.

Results

Cobalt uptake in taste cells

As shown in this and previous reports (Caicedo *et al.*, 2000b; Kim *et al.*, 2001), some taste cells in foliate papillae of the tongue showed strong Co^{2+} uptake when stimulated with 1 mM glutamate (3.31 ± 0.54 cells/taste bud, n = 32 number of rats; Figure 1a,f). Cells in surrounding tissues were not stained. Cobalt precipitate was evenly distributed throughout the cytoplasm but was not present in nuclei of taste cells. In the presence of the competitive non-NMDA GluR antagonist CNQX (100 μM), the number of cobalt-stained cells was significantly reduced (1.60 ± 0.51, $n = 34$, $P < 0.001$; Figure 1b,f). Basal cobalt uptake was still observed in the absence of any pharmacological treatment (2.43 ± 0.25, $n = 4$, $P < 0.01$; Figure 1c,f). We believe this residual cobalt uptake represents constitutive stimulation of taste cells by a low level of glutamate endogenous to the slices (Caicedo *et al.*, 2000b). When stimulated

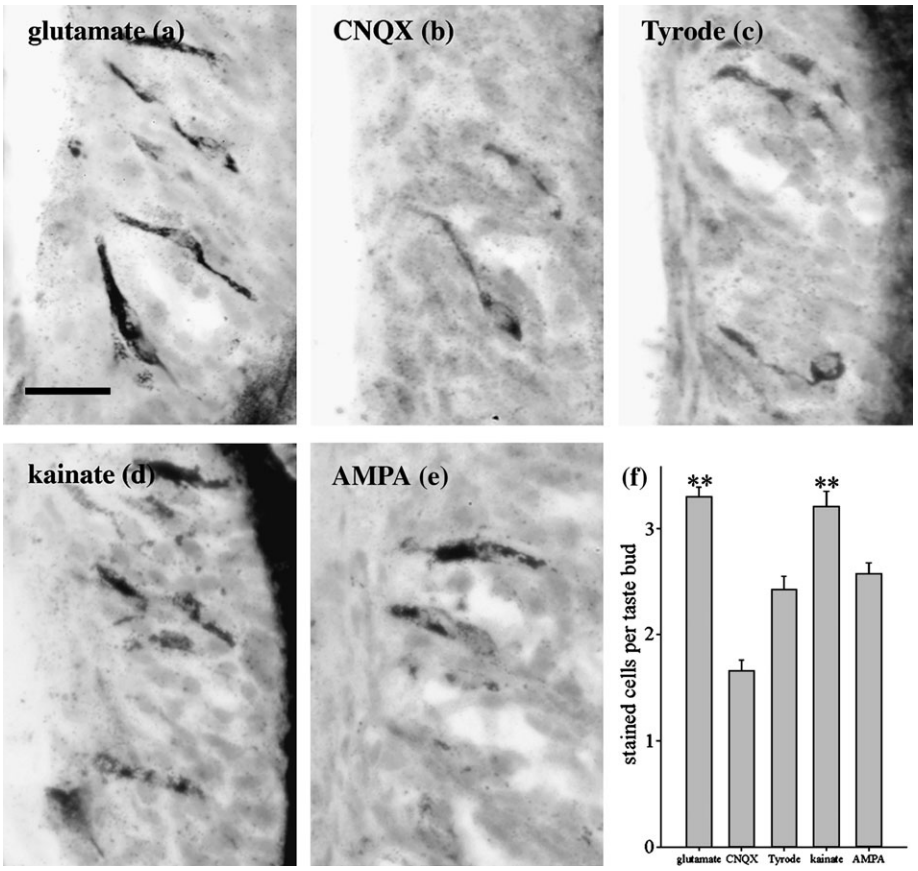

Figure 1 Co^{2+} uptake in foliate taste buds was elicited by glutamate (1 mM) **(a)** and kainate (100 μM) **(d)** but not by AMPA (100 μM) **(e)**. When incubated in Tyrode solution alone, a basal level of Co^{2+} uptake could be seen **(c)**. This basal cobalt uptake was significantly reduced by CNQX (100 μM) **(b)**. These data are summarized in **(f)** (means ± SEM). The mean values for the glutamate and kainate groups were significantly different from the means for the CNQX-, Tyrode- and AMPA groups (**$P < 0.01$). Scale bar = 20 μm.

with 100 μM kainate, all foliate taste buds appeared to contain cobalt-stained cells. The number of stained cells (3.19 ± 0.44, $n = 9$) was significantly higher than those of CNQX-treated or control (absence of pharmacological treatment) slices ($P < 0.001, 0.01$ respectively; Figure 1d,f). In taste buds treated with 100 μM AMPA, cobalt uptake did not differ from slices receiving no treatment (2.58 ± 0.24, $n = 5$, $P = 0.32$, Figure 1e,f). Treating taste buds with 300 μM AMPA stimulated cobalt uptake (3.29 ± 0.35, $n = 8$, $P < 0.01$, Figure 2b).

Pharmacological test of cobalt uptake

To test whether Co^{2+} uptake was mediated by AMPA or kainate receptors, we conducted experiments using agonists and antagonists of kainate or AMPA type GluRs.

Stimulating taste buds with SYM 2081, an agonist of kainate GluRs, increased cobalt staining in a dose-dependent manner. At 300 μM, SYM 2081 elicited almost the same extent of cobalt staining as glutamate- or kainate-stimulated slices (3.10 ± 0.46, $n = 6$, Figure 2a). Moreover, cobalt staining stimulated by SYM 2081 was reduced by adding 100 μM CNQX (Figure 2a). Although SYM 2081 is a potent agonist of kainite receptors, it also rapidly desensitizes the receptors. Thus, we also conducted experiments in the presence of agents that inhibit GluR desensitization (concanavalin A, cyclothiazide) to determine whether this would enhance Co^{2+} loading. Applying 1 mM glutamate or 300 μM SYM 2081 in the presence of concanavalin A (10–1000 μg/ml) or cyclothiazide (1–100 μg/ml) did not influence cobalt staining (data not shown), suggesting that the initial influx of

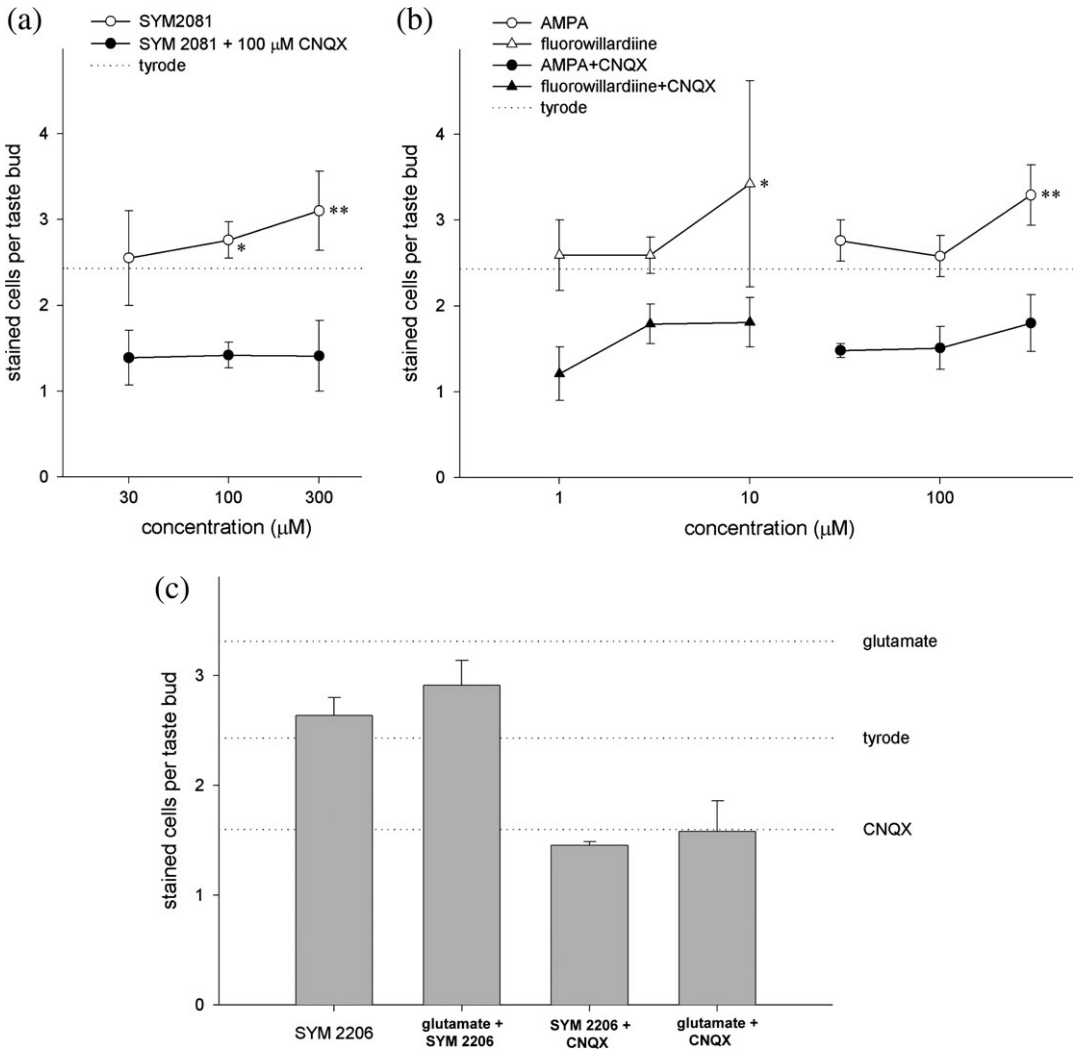

Figure 2 Co^{2+} uptake induced by SYM 2081 was concentration dependent and was inhibited by CNQX **(a)**. AMPA and fluorowillardiine did not elicit Co^{2+} uptake over basal levels at physiological concentrations but do at higher concentrations **(b)**. The dotted lines in (a) and (b) represent basal uptake (Tyrode alone). Symbols are means ± SEM. SYM 2206, an AMPA antagonist, did not significantly reduced basal Co^{2+} uptake nor Co^{2+} uptake induced by 1 mM glutamate (bars = means ± SEM). Dashed lines of glutamate, Tyrode and CNQX indicate the numbers of cobalt-stained cells of the 1 mM glutamate-, Tyrode only- and 100 μM CNQX-treated group respectively **(c)**. $*P < 0.05$, $**P < 0.01$.

Co^{2+} through GluRs when they are maximally stimulated is sufficient to produce complete Co^{2+} staining.

We also conducted experiments using fluorowillardiine, a potent agonist of AMPA GluRs. Cobalt staining over background levels was not apparent around the EC_{50} of fluorowillardiine (Patneau *et al.*, 1992), but higher concentrations (10 μM) revealed cobalt uptake in taste bud cells (Figure 2b), possibly due to non-selective activation of kainate GluRs (Patneau *et al.*, 1992). SYM 2206 (2.63 ± 0.33, n = 4), an antagonist of AMPA receptors (Pelletier *et al.*, 1996), did not reduce basal cobalt uptake (P = 0.56) and addition of SYM 2206 with 1 mM glutamate (2.90 ± 0.26, n = 4) did not significantly reduced cobalt uptake induced by glutamate (P = 0.16, Figure 2c).

Immunohistochemistry against subunits of non-NMDA GluRs

In rat taste buds, we found GluR6 and KA1-like immunoreactivity (Figure 3). Other non-NMDA subunits were not detected. The numbers of GluR6- and KA1-immunostained cells per taste bud were 4.34 ± 0.23 and 6.29 ± 0.24 (n = 5), respectively. Immunostaining against both GluR6 and KA1 was distributed throughout the cytoplasm but was not present in nuclei of taste cells. In double labeling with immunostaining combined with cobalt staining (evoked by 1 mM glutamate, n = 6), there was good correlation between cobalt-staining and immunoreactivity: 97% of cobalt-stained cells revealed GluR6 or KA1-like immuoreactivity (Figure 4). The converse correlation showed somewhat less overlap: 79% of GluR6-positive cells and 60% of KA1-positive cells were cobalt stained by 1 mM glutamate stimulation (Figure 4). Even though there were high incidences of immunostained cells with cobalt staining, the numbers of immunostained cells were not changed by cobalt staining procedure.

Discussion

We used the cobalt staining technique and/or immunohistochemistry against subunits of non-NMDA GluRs to visualize the presence of glutamate receptors in taste bud cells and to determine which kind of GluRs are functional in taste bud cells. Stimulating taste buds with agonists for non-NMDA GluRs in the presence of Co^{2+} revealed the presence of a number of cobalt-stained cells. Cobalt uptake was stimulated by glutamate, kainate or SYM 2081. AMPA and fluorowillardiine only stimulated cobalt uptake at supramaximal concentrations. Immunohistochemistry for GluRs was consistent with these results. GluR6 and KA1-like immunoreactivity, but not immunostaining for AMPA GluRs, was present in rat taste bud cells. We conclude that kainate GluRs are mainly responsible for glutamate-stimulated cobalt uptake in rat foliate taste buds. We cannot exclude a lesser contribution from AMPA receptors that might be present but below our detection level.

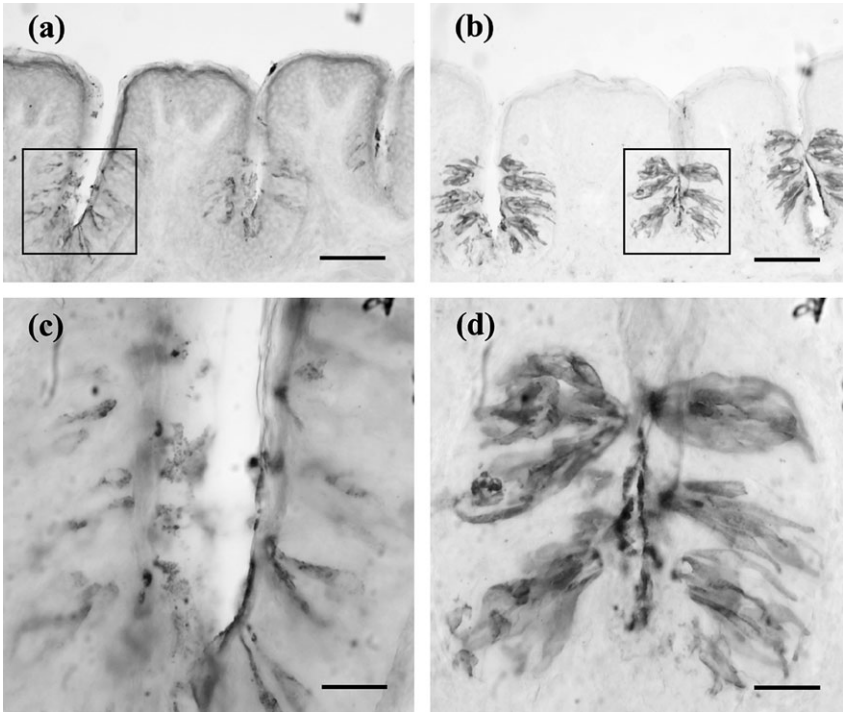

Figure 3 Immunohistochemical staining for kainite receptors in rat foliate taste buds. GluR6 **(a, c)** and KA1-like **(b, d)** immunoreactivities were present. Boxes in (a) and (b) are shown at higher magnification in (c) and (d). Scale bar = 100 μm for (a) and (b), and 25 μm for (c) and (d) respectively.

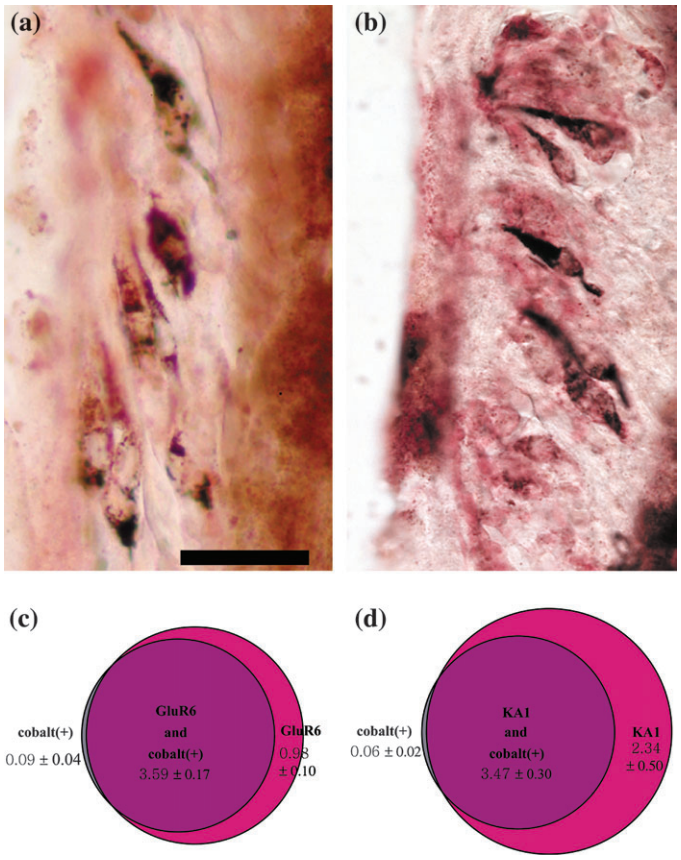

Figure 4 The cells in foliate taste buds labeled immunohistochemically with GluR6 **(a)** or KA1 **(b)** and cobalt staining evoked by 1 mM glutamate. The relative numbers of immuno- and cobalt-stained cells are shown in a Venn diagram in **(c)** and **(d)** respectively. Scale bar = 25 μm.

Cobalt staining in taste bud cells is mediated by kainate GluRs

The cobalt uptake procedure for labeling cells that have calcium-permeable GluRs is selective for non-NMDA receptors (Caicedo *et al.*, 2000). We and others have shown that NMDA application or depolarization does not stimulate Co^{2+} uptake in taste cells or brain cells (Caicedo *et al.*, 2000; Pruss *et al.*, 1991). In the present experiments, cobalt staining elicited by kainite receptor agonists in taste bud cells was more pronounced than that elicited by AMPA receptor agonists. Also, we found immunostaining for the kainate receptors GluR6 and KA1, but not the AMPA receptors GluR1–4 in rat taste cells. Caicedo *et al.* (2004) also reported that immunostaining for the AMPA receptor subunits GluR2/3 was principally located in nerve endings in taste buds and was only rarely found in taste cells. Chaudhari *et al.* (1996) reported that KA2 subunits were detected with RT-PCR but not GluR6 or KA1 subunits. RNAs of GluR6 and KA1 might be very unstable and might be destructed during the sample preparation procedure. However, KA2 is a kainate GluR, too. Chaudhari *et al.* (1996) also found no subunits of AMPA GluRs. This could be confirmed by other experiments, such as *in situ* hybridization.

High concentrations of AMPA (300 μM) and the AMPA receptor agonist fluorowillardiine (10 μM) elicited cobalt staining. These high concentrations might nonselectively stimulate kainite receptors (Patneau *et al.*, 1992). Alternatively, Co^{2+} staining induced by these concentrations may reveal a small contribution from AMPA type GluRs, consistent with the much reduced (Caicedo *et al.*, 2004) or absent (this report) immunoreactivity for AMPA GluRs.

Do cells in taste buds communicate with each other?

Taste bud cells consist of at least three morphological cell types, the type I (dark) cells, type II (light) cells, and type III cells (Farbman, 1965; Pumplin *et al.*, 1997). Cobalt- and immuno- stained cells showed same morphological characteristics, the cells had a central, enlarged cell body containing a large oval nucleus and processes extending towards the apical and the basal portion of taste bud. They had clear outline and did not show lateral cytoplasmic projections. Our results suggest that cobalt-stained cells elicited by kainate GluRs may be type II or Type III cells (Caicedo *et al.*, 2000b; Kim *et al.*, 2001). Traditionally only type III cells have unambiguous synaptic connections with primary afferent fibers, and they are considered only true receptive cells in taste bud (Lindemann, 1996). However, type II cells as well as type III cells have apical cytoplasmic extensions into the taste pore (Pumplin *et al.*, 1997). Gustducin, an α-subunit of a G-protein closely related to the transducins (McLaughlin *et al.*, 1992), is expressed only in type II (light) cells (Boughter *et al.*, 1997). Thus, type II cells may respond to taste stimulation and transmit their signals to type III cells. It is highly probable that taste cells communicate with each other.

Nerve cells may be another source of glutamate in taste buds

Axons of primary gustatory neurons are a possible source of glutamate—they are immunoreactive for glutamate (Jain and Roper, 1991) and release glutamate at their central projections (Bradley *et al.*, 1996). Although there is no direct evidence that taste buds have efferent innervations from the central nervous system, efferent inputs are found in other sensory organs such as the cochlea (Raphael *et al.*, 2003). Even though kainate GluRs are mainly located postsynaptically, a number of these receptors have also been identified presynaptically (Chittajallu, 1996). Furthermore, kainate receptors may occur in both pre- and postsynaptic sites (Lerma, 2003). In the present experiments, cobalt-stained cells showed immunoreactivity for GluR6 and KA1. In the hippocampus, GluR6-containing kainate GluRs modulate neuronal activity (Cossart *et al.*, 1998). Thus activation of kainate GluRs in taste bud cells would produce a modulation of the release of the endogenous neurotransmitters. In the central nervous system, kainate GluRs exhibit much

slower EPSCs and integrate excitatory inputs over large time windows (Lerma, 2003). They can mediate excitatory synaptic signals but are they also involved in modulating the presynaptic release of neurotransmitter, and therefore in regulating the strength of synaptic connections (Madden, 2002). In taste bud cells, glutamate receptors, including kainate receptors (this paper) and NMDA receptors (Caicedo *et al.*, 2000a, 2004), might be involved in information transmission from type II cells to type III cells, or efferent glutamatergic control from the central nervous system might modulate communication between taste cells and/or afferent nerve fibers.

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