Electrophysiological Characteristics of Rat Gustatory Cyclic Nucleotide–Gated Channel Expressed in *Xenopus* Oocytes

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**INTRODUCTION**

Cyclic nucleotide–gated channels (or CNG channels) are cation-selective ion channels that open in response to the direct binding of intracellular cyclic nucleotides such as cGMP or cAMP. These channels play critical roles in sensory transduction of vertebrate photoreceptors and olfactory neurons (for review, Finn et al. 1996; Li et al. 1997). Serving as downstream targets of the signaling pathways, CNG channels mediate the transduction of sensory stimuli into neuronal activity. CNG channel subtypes differ in their sensitivity for cyclic nucleotides in that olfactory channel can be activated by physiological concentrations of both cAMP and cGMP (Nakamura and Gold 1987; Zufall et al. 1994), whereas the photoreceptor channels are activated only by cGMP (Fesenko et al. 1985; Yau and Baylor 1989). The channel subtypes also differ in their relative permeability to physiological Ca\(^{2+}\) such that the fractional currents carried by Ca\(^{2+}\) in the olfactory channel are greater than that by the rod channel (Friggs et al. 1995).

CNG channel activities were also detected in neurons other than sensory receptor cells (Ahmad et al. 1994; Dryer and Henderson 1993; Kingston et al. 1996; Nawy and Jahr 1990). Subsequently, several groups reported the cloning of CNG channel genes from different tissues in various organisms, strongly suggesting that CNG channels may be involved in other important physiological processes in different tissues (for review, Biel et al. 1999, Finn et al. 1996). Thus far, six different genes encoding CNG channels have been identified in mammals, and these subunits can be classified as \(\alpha\) and \(\beta\) subunits. While the \(\alpha\) subunits (CNG 1–3) can form functional CNG channels expressed in different heterologous expression systems, the \(\beta\) subunits (CNG 4–6) require the co-expression of \(\alpha\) subunits for functional CNG channels.

A complementary DNA (cDNA) of another CNG channel was cloned from rat tongue epithelial tissues where taste reception takes place (Misaka et al. 1997). Composed of 611 amino acid residues and believed as one of the major 5’-splicing variants, the deduced amino acid sequence of the CNG channel (named “gustCNG channel”) shows 50 to 80% similarities to other CNG channels. While the rat gustCNG channel is most homologous to mouse cone CNG channel in its overall amino acid sequence, the predicted amino termini of these two channels show only 58% sequence identity (Gerstner et al. 2000). Expressed in HEK293 cells, gustCNG channel gene resulted in functional CNG channel currents activated by both cAMP and cGMP (Misaka et al. 1997). Based on its specific expression in taste bud cells, it was proposed that this CNG channel might be involved in some types of gustatory signal transduction. Following this report, it was suggested that taste signal transduction by bitter tastants such as caffeine and theophylline may be mediated by CAMP through inhibition of phosphodiesterase (Rosenzweig et al. 1999). This may be different from a G-protein (or gustducin)–mediated pathway that inhibits ion channel activity by decreasing intracellular...
cAMP and cGMP concentration (Kolesnikov and Margolskee 1995). In a recent report, Misaka et al. reported that the gustCNG channel is also expressed in the outer segments of rat cone photoreceptor cells and suggested that this CNG channel is involved in both visual and taste signal transduction (Misaka et al. 1999).

Although the gustCNG channel gene is likely expressed in several different tissues, the functional characteristics of this channel remain to be elucidated in detail. To reveal the functional differences of those closely related CNG channels, it is required to express the channel genes in a heterologous system and to compare their electrophysiological characteristics. In this study, we expressed the cloned gustCNG channel in *Xenopus* oocytes and investigated the functional properties of the channel using electrophysiological methods. We determined the single-channel conductance and the selectivity order of the gustCNG channel. The gating properties of gustCNG channel were examined in both single-channel and macroscopic current levels. From a detailed analysis of single-channel recordings, we were able to elucidate that cGMP activates the channel by increasing an opening rate of the channel. We also examined the blockade of gustCNG channel using a divalent cation, Mg$^{2+}$, and l-cis-diltiazem and compared the effects to two other members of CNG channel family.

**METHODS**

**Materials**

Female *Xenopus laevis* were purchased from *Xenopus* One (Ann Arbor, MI). Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and Boehringer Mannheim (Mannheim, Germany). T7 RNA Polymerase was purchased from Promega (Madison, WI). Collagenase was from Worthington Biochemical (Freehold, NJ). The cyclic GMP, cyclic AMP, and other chemicals were purchased from either Sigma (St. Louis, MO) or Aldrich Chemical (Milwaukee, WI), unless otherwise specified. The l-cis-diltiazem hydrochloride was purchased from Sigma-RBI (St. Louis, MO). Other molecular biological reagents were purchased from GIBCO BRL (Rockville, MD), DIFCO Laboratories (Grayson, GA), or Bio-Rad laboratories (Hercules, CA).

**Subcloning of gustCNG channel gene and in vitro transcription**

The cDNA of gustCNG channel in pUC18 vector was provided by Dr. Keiko Abe of the University of Tokyo, who originally cloned the gene from rat tongue epithelial tissue (Misaka et al. 1997). The entire coding region of gustCNG channel gene was subcloned into a modified pGH expression vector, pGH/NBC, for high-level expression in *Xenopus* oocytes. Several unique sites of restriction enzymes were introduced into the multiple cloning site region of pGH/NBC, which also contains 5′- and 3′-untranslated regions of the *Xenopus β*-globin gene of the original pGH vector (Liman et al. 1992). The cDNAs encoding bovine rod and rat olfactory CNG channels used in this study were described previously (Goulding et al. 1992; Liman and Buck 1994).

CNG channels of rat gustatory, bovine retinal, and rat olfactory were expressed in *Xenopus* oocytes for electrophysiological studies. Complementary RNA (cRNA) was synthesized in vitro from an MluI or *Nhe* I-linearized plasmid using T7 RNA polymerase. For transcription of cRNA, 10 μg of recombinant DNA was digested with MluI or *Nhe* I restriction enzymes for 4 h at 37°C. Linearized DNA was extracted once with phenol:sevag (phenol:chloroform:isoamylalcohol = 25:24:1) and subsequently with sevag (chloroform:isoamylalcohol = 24:1). The linearized DNA was added to total 100 μl of reaction mixture containing 1 times transcription buffer, 10 mM dithiothreitol, 100 units RNAsin, 1 mM rNTPs, 1.25 units GpppG, and 40 units T7 RNA polymerase (all reagents were from Promega, except GpppG purchased from Pharmacia Biotech). The mixture was incubated at 37°C for 2 h. After transcription, synthesized cRNA was extracted once with phenol:sevag, and twice with sevag. Purified cRNA was dissolved in ~20–40 μl of RNase-free water [autoclaved nanopure-filtered water containing 0.01% (vol/vol) diethyl pyrocarbonate]. Quality and quantity of cRNA were examined by 1% TAE-agarose gel electrophoresis.

**Expression in gustCNG channels in Xenopus oocytes**

Oocytes harvested from *Xenopus laevis* were incubated in a solution containing (in mM) 82.5 NaCl, 2.5 KCl, 1.0 MgCl$_2$, and 5.0 HEPES, pH 7.6, and 2–4 mg/ml of collagenase. The oocyte preparation was agitated using a rotating shaker for 90–120 min. It was then rinsed thoroughly and stored in ND-96 solution containing 96 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl$_2$, 1.0 mM MgCl$_2$, 5 mM HEPES, and 50 μg/ml gentamicin, adjusted to pH 7.6 with NaOH. Defolliculated oocytes were selected no later than 12 h after collagenase treatment. About 50 ng of cRNA were injected into oocytes for macroscopic, and about 2.5 ng were for single-channel experiments using a micropipette (VWR Scientific, West Chester, PA). Injected oocytes were incubated at 18°C for 3–5 days in ND-96 solution. For single-channel current recordings the expression of channel protein was allowed only for 18–24 h, and cRNA injection was done every 2 days.

**Electrophysiological recordings and data analysis**

Ionic currents through the functionally expressed gustCNG channels were measured with gigaohm-sealed membrane patch-clamp method in excised inside-out and outside-out configurations with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Patch pipettes were fabricated from borosilicate glass (TW150F-4, World Precision Instruments) using a Flaming/Brown micropipette puller (Model P-87, Sutter Instrument). Pipettes were fire-polished with a microforge (MF-83, Narishige Scientific Instrument) to give the pipette a resistance of 3–5 MΩ for macropatch recording and 10–20 MΩ for single-channel recording, respectively. To reduce noise through electrodes, patch pipettes for single-channel recording were coated with beeswax under a stereomicroscope (World Precision Instruments). The amplified analog data were filtered at 1 kHz with 80-dB/decade low-pass bessel filter and digitized with a Digidata 1200 (Axon Instruments). Voltage stimulus was delivered to CV202BU headstage with Digidata 1200 using pClamp6.0 or 7.0 program (Axon Instruments). The data were stored in a pentium computer and analyzed using programs such as pClamp 6.0 or 7.0 and Origin 4.1 (Microcal). Collected data were stored in Axon binary format with pClamp7, which was converted into a relevant format with pClamp6 afterward and analyzed with Origin 4.1 for macroscopic channel current recording and pSTAT for single-channel current recording.

Both intracellular (bath for inside-out and pipette for outside-out) and extracellular (pipette for inside-out and bath for outside-out) solutions contained 130 mM NaOH, 3 mM HEPES, and 0.5 mM Na$_2$-EDTA and were adjusted to pH 7.6 with HCl, unless otherwise specified. Na-cGMP (freely dissolved in water) was added to bath solution for inside-out patch and to pipette solution for outside-out patch before the final pH adjustment. For Mg$^{2+}$ blocking experiments, MgCl$_2$ was added to give the desired free concentration calculated by Martell and Smith (1974). The calculation also took pH into account. Blockade was measured by perfusing the intracellular or extracellular face of the membrane patch with the solutions containing indicated free concentrations of blockers. After the control recordings were obtained in the absence and presence of 10 mM Mg$^{2+}$ (for intracellular side) or 20 mM Mg$^{2+}$ (for extracellular side) to achieve
maximum blockade (Park and MacKinnon 1995), each patch was perfused with various concentrations of blockers to investigate intracellular or extracellular Mg$^{2+}$ effects on channel permeation.

To investigate the selectivity of gustCNG channel, we analyzed current-voltage ($I-V$) relationship under symmetrical bi-ionic conditions. In the test solutions, 130 mM Na$^+$ was substituted with the same concentrations of other monovalent cations, K$^+$, Li$^+$, Rb$^+$, or Cs$^+$. The free acid form of EDTA (Sigma E-6758) was used instead of Na$_2$EDTA to prevent asymmetric Na$^+$ concentration and anomalous mole fraction effect. All the test ions are the hydroxylated forms, which remain symmetric to both intracellular and extracellular solutions after pH adjustment to pH 7.6 with HCl (KOH, Sigma P-5958; LiOH $\cdot$ H$_2$O, Sigma L-4256; RbOH, Aldrich Chemical, 24,389-2; CsOH: Aldrich Chemical 23,204-1). To activate channel currents, 50 µM of cGMP was added into intracellular solutions. Rapid and complete solution changes were obtained by moving a linear array of micorcapillary tubes (1 µL, 64 mm length; Drummond), each containing different concentrations of ligand or blocker sequentially.

To use ramp pulse for macroscopic current recordings, an independent protocol of voltage steps lasting 150 ms was preceded to ensure that the steady state was achieved throughout the ramp. We used only step pulses to record the blocked currents by $l$-cis-diltiazem, since the current did not reach steady-state level until tens of milliseconds after voltage pulses.

RESULTS

Macroscopic current-voltage relationship of gustCNG channels

The cDNA of gustCNG channel was transcribed in vitro and functionally expressed in *Xenopus* oocytes. Ionic currents through gustCNG channels in excised membrane were investigated in symmetrical 130 mM Na$^+$ conditions using various concentrations of cGMP perfused onto the intracellular side of the membrane to activate channels. The membrane was held at 0 mV in the bath solution containing cGMP and stepped from −100 to 100 mV with 20-mV increments (Fig. 1). As the concentration of intracellular cGMP ([cGMP]$_{\text{int}}$) was increased from 0.1 to 100 µM, the currents evoked by cGMP were also increased. However, the channel currents were saturated at around 100 µM, and the higher [cGMP]$_{\text{int}}$ did not further increase the channel currents significantly. The channel currents were rapidly activated and reached steady-state current levels on stepping to various voltages. No apparent inactivation or desensitization was observed during the 150-ms voltage pulses. We thus used a ramp pulse protocol to study $I-V$ relationship of this channel in subsequent experiments.

The membranes containing many channels (usually 1,000 – 2,000 channels) were applied with a ramp pulse from −100 to 100 mV, and currents evoked by the voltage pulse were recorded (Fig. 2A). The macroscopic $I-V$ relationship was roughly linear in most of the voltage range. At extreme depolarizing membrane potentials, slight outward rectifications were observed in some recordings. From five such experiments of macroscopic current recordings, the dose-response relationship and the effects of membrane voltage on the activation of gustCNG channels were analyzed. Since the membrane potential of rat taste cell is usually around −70 mV or even more negative under physiological conditions (Akabas et al. 1988) and becomes positive when excited, the dose-response rela-

FIG. 1. Macroscopic currents of gustatory cyclic nucleotide–gated (gustCNG) channels activated at various [cGMP]$_{\text{int}}$. The macroscopic currents of gustCNG channel activated by indicated concentrations of intracellular cGMP were recorded in inside-out patch configuration. Membrane voltage was held at 0 mV and stepped from −100 to 100 mV for 150 ms in 20-mV increment and returned to 0 mV. Bath solution (intracellular) and pipette solution (extracellular) contained 130 mM NaOH, 3 mM HEPES, 0.5 mM Na$_2$-EDTA adjusted to pH 7.6. Na-cGMP of indicated concentrations were added to the bath and perfused onto the intracellular surface of the membrane to activate channel currents. In the above representative traces, the leak currents in the absence of cGMP were subtracted.
tionships were analyzed at two different membrane voltages, −80 and 80 mV. After partially activated currents were normalized to fully activated currents at 100 μM cGMP, the data were plotted against cGMP concentrations and fitted to the Hill equation (Fig. 2B). The results show no significant difference in the concentration for half-maximal activation, $K_{1/2}$, and the Hill coefficient, $n$, both at −80 mV, 3.3 ± 0.1 (SE) μM cGMP and 1.4 ± 0.1, and at 80 mV, 2.9 ± 0.2 μM cGMP and 1.4 ± 0.1, respectively. The $K_{1/2}$ value of 3.3 μM at −80 mV is consistent with that of the previous report obtained from gustCNG channel expressed in HEK293 cells (Misaka et al. 1997). In Fig. 2C, $K_{1/2}$ values were plotted against membrane voltages. Although $K_{1/2}$ values show slight voltage dependence, i.e., higher affinity at more positive voltages, the tendency is not significant enough to argue that the cGMP-dependent channel activation is voltage dependent. We also examined the activation of gustCNG channel by intracellular cAMP (Fig. 3). $K_{1/2}$ for cAMP was 250 ± 20 μM at −80 mV with $n$ of 1.61 ± 0.1. Fully activated currents by 10 mM cAMP was about 90% of the currents by a saturating concentration of cGMP at 100 μM.

**Single-channel characteristics of gustCNG channels**

To investigate the detailed characteristics of gustCNG channel, we obtained single-channel currents of gustCNG channel at various membrane voltages and cGMP concentrations. Figure 4 shows a continuous current recording of a single gustCNG channel at −80 mV in the presence of 3 μM of cGMP. Several single-channel characteristics of gustCNG channels were evident. Single-channel current amplitude was ~2.2–2.3 pA at −80 mV, and stochastic long closed states along with fast open-close transition were observed. Consistent with the macroscopic current recordings, single-channel activity of gustCNG channel did not show any desensitization or inactivation even at 100 μM cGMP (data not shown). Since the long gaps observed in single-channel recordings did not seem to have any particular pattern in its frequency and duration, we
excluded those events from the analyses of single-channel characteristics (see DISCUSSION).

The effects of cGMP on channel gating were investigated at the single-channel level under various cGMP concentrations. Since we already showed that the gating of gustCNG channel was not strongly influenced by membrane voltages, the single-channel currents were measured at a constant membrane voltage, $-80$ mV. As shown in Fig. 5A, the frequency of the channel opening was increased as a function of cGMP concentration. Open probability ($P_{\text{open}}$) of a single channel was analyzed as following, and the results provided relative times during the channel spent in the open state: $P_{\text{open}} = t_o / t_i$ (where, $t_o$, total open time for the level under consideration; $t_i$, total interval over which $P_{\text{open}}$ is measured).

As we expected, the analysis result showed that open probabilities of this channel followed sigmoidal curve when the data were plotted against cGMP concentrations and the curve was fitted to the Hill equation (Fig. 5B). The two parameters, $K_{1/2}$ of $3.9 \pm 0.7 \mu$M and $n$ of $1.3 \pm 0.3$, obtained from single-channel analysis, were in good agreement with those values obtained from macroscopic dose-response relationship at $-80$ mV. These results strongly support the idea that the cGMP-evoked macroscopic currents of gustCNG channel are due to the increase in the open probability and cGMP is a direct agonist of the channel.

**Single-channel conductance of gustCNG channel**

To determine the single-channel conductance, we measured the single-channel current amplitudes at different membrane voltages. The raw traces of Fig. 6A show the current levels of a single gustCNG channel increase as membrane potentials increase from $-80$ to $20$ mV. Precise current levels of single-channel currents were determined by analyzing the histogram of current amplitudes. The total areas under the histogram were normalized to give a unity, and the amplitude histograms were fitted to the sum of two Gaussian functions (Fig. 6B). Increases in current amplitudes following membrane potentials can be clearly seen. The peaks of the Gaussian functions representing the closed level were used to subtract the leak currents so that the closed level at each membrane potential could be lined.

From several single-channel recordings, average single-channel currents at various membrane potentials were obtained, and the current values were plotted against membrane voltages (Fig. 6C). The $I$-$V$ relationship shows a linearity throughout the membrane voltages tested, consistent with the macroscopic $I$-$V$ relationship shown in Fig. 2A. The linear slope in Fig. 6C gave rise to the single-channel conductance of 28 pS.

**Single-channel kinetics of gustCNG channels: two exponentially fitted open and closed events**

Answering the question regarding how gustCNG channel may open and close in response to cGMP requires the kinetic analysis of open and closed events. In an expanded time scale as shown in Fig. 7A, two distinct populations of dwell-times,
short and long, were observed in both open and closed events of single gustCNG channels. In addition, the channels also exhibited flickering openings in most of membrane voltages. When the closed dwell-time and open dwell-time were measured and distributed in an exponential time scale, the sum of two decaying exponential functions well described the histograms of both dwell-times. In Fig. 7B, both open and closed events are represented with log dwell-time function to show more clearly the two exponentially distributed components of a single-channel current trace recorded with 3 μM cGMP at −80 mV. The opening and closing rates can be obtained from the reciprocal of closed and open time constants (τ), respectively. After analyzing the single-channel dwell-times, opening and closing rate constants were obtained and plotted against cGMP concentrations in Fig. 7C. The analysis of closed events shows that there are two opening rates: one is fast and independent of [cGMP]_{int}, 2 × 10^3 ~ 3 × 10^3 s^{-1}, and the other is slower and changes in response to [cGMP]_{int}, from 10 s^{-1} at 1 μM cGMP to 300 s^{-1} at 100 μM cGMP. Open event analysis shows that both of the fast and the slow closing rates are insensitive to [cGMP]_{int}, about 2 × 10^3 s^{-1} and 10 ~ 50 s^{-1}, respectively. Thus the gustCNG channel goes to open state with high propensity as cGMP concentration increases without much effects on the closing process.

Selectivity of gustCNG channel among monovalent cations

Cyclic GMP–gated channels are nonselective cation channels (Bastian and Fain 1982; Capovilla et al. 1983; Hodgkin et al. 1984; Woodruff et al. 1982; Yau et al. 1981) permeating both Na^+ and K^+. These channels are also highly selective for divalent cations as permeant blockers (Capovilla et al. 1983; Hodgkin et al. 1984; Yau et al. 1981). Although the similarity

![FIG. 5. Open probabilities of single gustCNG channels with various intracellular cGMP. A: representative current traces of single gustCNG channel recorded with various intracellular cGMP, [cGMP]_{int}. Single-channel currents were recorded in inside-out patch configuration with membrane containing a single gustCNG channel. Indicated concentrations of cGMP were perfused onto the intracellular side of the membrane. The membrane was held at −80 mV, and the data were sampled at 25 kHz. The compositions of pipette and perfusion solutions were same as described in Fig. 1. Control trace without cGMP shows no endogenous channel activity under this recording condition. The current levels of closed channel (C) were indicated using arrows, and individual openings were shown as downward deflections. The single-channel currents at −80 mV were estimated as −2.2 pA. B: comparison for open probability and macroscopic dose-response curve of gustCNG channel. Open probabilities of single gustCNG channels measured with different [cGMP]_{int} at −80 mV (□) was co-plotted with the macroscopic dose-response relationship against cGMP previously shown in Fig. 2B (●). Data were fitted using Hill equations {I/I_{max} = (1 + K_{1/2}[cGMP]_{int})^{-n}}. K_{1/2} and Hill coefficients of single-channel and macroscopic current recordings were estimated as 3.9 ± 0.7 μM (n = 1.3 ± 0.3) and 3.3 ± 0.1 μM (n = 1.4 ± 0.1), respectively. Each data point represents the mean ± SE from at least 5 raw traces for both macroscopic and single-channel currents.}
of amino acid residues known to form ion-conducting pathway is high among CNG channels, their selectivity for monovalent cations can be different as revealed in the case of CNG channels of bovine rod and catfish olfactory neurons (Goulding et al. 1993). We examined the selectivity of gustCNG channel among monovalent cations. Under bi-ionic conditions, i.e., pipette solution contains 130 mM Na⁺ and bath solutions contain 130 mM Na⁺, K⁺, Li⁺, Rb⁺, or Cs⁺, reversal potentials are determined in accordance to their permeability. Then we can obtain the relative ratios of the permeability coefficients according to the GHK-derived equation. From the four independent experiments, we obtained reversal potential values of gustCNG channel under bi-ionic condition, as follows, in millivolts (Fig. 8): Na⁺, −0.4 ± 0.5; K⁺, 3.1 ± 0.7; Li⁺, 10.2 ± 1.4; Rb⁺, 10.0 ± 1.9; Cs⁺, 20.4 ± 2.6.

After liquid junction potential was taken into account (Barry and Lynch 1991; Neher 1992), the selectivity order of gustCNG channel was determined that Na⁺:K⁺:Rb⁺:Li⁺:Cs⁺ equals 1:0.95:0.74:0.63:0.49. As a control, the same experiments were performed with the α subunit of bovine rod CNG channel expressed in Xenopus oocytes and the selectivity order was obtained as Na⁺ ~ K⁺ > Rb⁺ > Li⁺ > Cs⁺, identical to that of the previous report (Goulding et al. 1993).

**Effects of Mg²⁺ ions on gustCNG channel permeation from intracellular sides**

Magnesium ion is a ubiquitous and abundant divalent cation in both intra- and extracellular milieu and affects the permeation of several different ion channels. CNG channels are known to be blocked by both intracellular and extracellular Mg²⁺ at a physiological concentration range. We thus investigated the effects of Mg²⁺ on gustCNG channel using inside-out and outside-out patch configurations. In the inside-out patch configuration, 100 μM cGMP was used to fully activate gustCNG channels, and different concentrations of Mg²⁺ were perfused to block the channel currents (Fig. 9A). Intracellular Mg²⁺ blocked the outward currents with higher affinity than the inward currents. Due to the voltage-dependent blockade, 10 mM Mg²⁺ almost completely blocked the outward currents, while large inward currents are still detected in an identical Mg²⁺ concentration. In Fig. 9B, the fraction of unblocked
currents normalized to fully activated currents ($I/\text{I}_\text{o}$) were plotted as a function of Mg$^{2+}$ concentrations and fitted to the Langmuir isotherm. The Mg$^{2+}$ concentrations inhibiting the channel currents to one-half ($K_i$) were quite different at different voltages such as 8.2 ± 1.5 mM at −70 mV and 360 ± 40 μM at 70 mV. As the membrane voltage was increased from −70 to 70 mV, the affinity of intracellular Mg$^{2+}$ was increased about 23-fold. The Hill coefficient ($n$) of Mg$^{2+}$ binding near unity (e.g., 1.1 at 70 mV and 0.7 at −70 mV) suggests that a single Mg$^{2+}$ may be enough to block the channel currents.

For more detailed analysis of voltage-dependent Mg$^{2+}$ blockade on gustCNG channel currents, $K_i$ values at various membrane voltages were obtained from five independent experiments and plotted as a function of membrane voltages (Fig. 9C). The linear relationship appears in negative membrane voltages, and thus data points were fitted to the equation, $K_i = K_i(0 \text{ mV}) \exp(z\delta FV/RT)$. The slope gave a $z\delta$ value of 1.04, which indicates that the electrical distance of internal Mg$^{2+}$ binding site is about 52% across the membrane potential from inside. At positive voltage range, this voltage dependency disappeared. Voltage dependency observed in negative membrane potential indicated that the internal Mg$^{2+}$ binding site might be located in the conducting pathway as suggested for another CNG channel in a previous study (Root and MacKinnon 1993). At highly depolarized voltage range, Mg$^{2+}$ “pops through” the channel, and the voltage dependence of Mg$^{2+}$ blockade would deviate from the above relationship to membrane voltages. The affinities ($K_i$) and the electrical distances ($\delta$) of rat gustatory, bovine rod, and rat olfactory CNG channels were compared in Fig. 9D. Three different channels showed very similar $K_i$ values, 6.6 ± 0.4 mM for gustatory ($n = 5$), 8.8 ± 2.6 mM for rod ($n = 4$), and 8.9 ± 1.9 mM for olfactory ($n = 4$) at −60 mV and $\delta$ values, 0.52 for gustatory, 0.64 for rod, and 0.52 for olfactory CNG channels. These

![Image](http://jn.physiology.org/)

**FIG. 7.** Gating characteristics and kinetic analysis of single gustCNG channel. A: typical single-channel current traces of gustCNG channel. Single-channel currents were recorded at −80 mV in the presence of 10 μM cGMP, and the characteristics of channel gating were shown in 2 different time scales. Individual openings were shown as downward deflections. B: representative dwell-time histograms of single gustCNG channels for open and closed events. The histograms of both closed (left) and open (right) dwell-times obtained from more than 5,000 transitions were plotted in log bin time scale and fitted by 2-exponential functions. Dotted lines represent the sum of 2 individual exponential functions of time constant ($\tau_1$, $\tau_2$). The time constants ($\tau$) obtained from the closed dwell-time histogram were 0.16 ms for short and 24.57 ms for long events, while the time constants from the open dwell-time histogram were 0.35 ms for short and 11.18 ms for long events, respectively. C: effects of [cGMP]$_\text{int}$ on opening and closing rates of single gustCNG channels. Channel currents of single gustCNG channels were recorded at −80 mV in the presence of different concentrations of cGMP. Dwell-time histograms of both open and closed events were well fitted to 2-exponential functions of time constants, $\tau_1$ and $\tau_2$. The reciprocals of time constants ($1/\tau$) were plotted against cGMP concentrations for closed events (left) and open events (right). The $1/\tau$s of short closed events or faster opening rate (□) were estimated as (2.55 ± 0.31) × 10$^5$, (2.71 ± 0.35) × 10$^5$, (2.84 ± 0.31) × 10$^5$, and (3.24 ± 0.26) × 10$^5$ per second (s$^{-1}$), and the $1/\tau$s of long closed events or slower opening rate (●) were 9.57 ± 3.38, 12.2 ± 5.26, 37.6 ± 21.4, and 351 ± 208 in 1, 3, 10, and 100 μM cGMP, respectively. The $1/\tau$s of short open events or faster closing rate (○) were estimated as (1.27 ± 0.24) × 10$^3$, (0.97 ± 0.13) × 10$^3$, (2.15 ± 0.75) × 10$^3$, and (1.69 ± 0.91) × 10$^3$ s$^{-1}$, and the $1/\tau$s of long open events or slower closing rate (■) were 9.39 ± 3.69, 34.5 ± 11.6, 48.59 ± 21.2, and 29.2 ± 8 in 1, 3, 10, and 100 μM cGMP, respectively.

![Image](http://jn.physiology.org/)

**FIG. 8.** Single-channel currents of rat gustatory, bovine rod, and rat olfactory CNG channels. Single-channel currents were recorded at −80 mV in the presence of 10 μM cGMP, and the characteristics of channel gating were shown in 2 different time scales. Individual openings were shown as downward deflections. A: representative dwell-time histograms of single gustCNG channels for open and closed events. The histograms of both closed (left) and open (right) dwell-times obtained from more than 5,000 transitions were plotted in log bin time scale and fitted by 2-exponential functions. Dotted lines represent the sum of 2 individual exponential functions of time constant ($\tau_1$, $\tau_2$). The time constants ($\tau$) obtained from the closed dwell-time histogram were 0.16 ms for short and 24.57 ms for long events, while the time constants from the open dwell-time histogram were 0.35 ms for short and 11.18 ms for long events, respectively. B: effects of [cGMP]$_\text{int}$ on opening and closing rates of single GustCNG channels. Channel currents of single GustCNG channels were recorded at −80 mV in the presence of different concentrations of cGMP. Dwell-time histograms of both open and closed events were well fitted to 2-exponential functions of time constants, $\tau_1$ and $\tau_2$. The reciprocals of time constants ($1/\tau$) were plotted against cGMP concentrations for closed events (left) and open events (right). The $1/\tau$s of short closed events or faster opening rate (□) were estimated as (2.55 ± 0.31) × 10$^5$, (2.71 ± 0.35) × 10$^5$, (2.84 ± 0.31) × 10$^5$, and (3.24 ± 0.26) × 10$^5$ per second (s$^{-1}$), and the $1/\tau$s of long closed events or slower opening rate (●) were 9.57 ± 3.38, 12.2 ± 5.26, 37.6 ± 21.4, and 351 ± 208 in 1, 3, 10, and 100 μM cGMP, respectively. The $1/\tau$s of short open events or faster closing rate (○) were estimated as (1.27 ± 0.24) × 10$^3$, (0.97 ± 0.13) × 10$^3$, (2.15 ± 0.75) × 10$^3$, and (1.69 ± 0.91) × 10$^3$ s$^{-1}$, and the $1/\tau$s of long open events or slower closing rate (■) were 9.39 ± 3.69, 34.5 ± 11.6, 48.59 ± 21.2, and 29.2 ± 8 in 1, 3, 10, and 100 μM cGMP, respectively.
FIG. 8. Selectivity of gustCNG channel for monovalent cations. \( I-V \) relationships of macroscopic gustCNG channels were obtained under bi-ionic conditions by ramp pulses. The pipette solution contained 130 mM NaOH, 3 mM HEPES, and 0.5 mM EDTA (free acid form) adjusted to pH 7.6 with HCl. Perfusion solutions were identical to the pipette solution except the test ions (K\(^+\), Li\(^+\), Rb\(^+\), or Cs\(^+\)) were substituted for Na\(^+\). Cyclic GMP was added in intracellular solutions (bath) to 50 \( \mu M \) for current activation. The membrane was held at 0 mV and ramped from \(-100\) to \(+100\) mV during 150 ms.

Results also reflect the high similarity in their amino acid sequence of the pore-forming region (see DISCUSSION and Fig. 12). In addition, six or seven negatively charged amino acids are also found in the putative intracellular vestibule of all three different CNG channels, further suggesting that the local electrostatic environment of channel entryways for the binding of intracellular Mg\(^{2+}\) may be quite similar.

**Effects of Mg\(^{2+}\) ions on gustCNG channel permeation from extracellular sides**

The effects of extracellular Mg\(^{2+}\) on the permeation of gustCNG channel were studied in outside-out patch configuration. To determine the effects of Mg\(^{2+}\) from the extracellular side, 100 \( \mu M \) cGMP was used to activate gustCNG channel and indicated concentrations of Mg\(^{2+}\) were applied to block the channel currents from the outside surface (Fig. 10A). The inward currents were blocked almost completely in 1 mM Mg\(^{2+}\), while the outward currents were not blocked completely even at 20 mM in extremely positive voltage ranges. The current blockade was also voltage dependent in that the inward currents were more sensitive to Mg\(^{2+}\) than the outward currents. Under physiological conditions, retinal CNG channel shows a strong outward rectification due to largely voltage-dependent blockades by divalent cations (Yau and Baylor 1989), and the blockade is known to reduce the signal-to-noise ratio of the rod cells. The gustCNG channel seems to share these characteristics. The fractions of unblocked currents were plotted against Mg\(^{2+}\) concentrations and fitted with a Langmuir function in Fig. 10B, where \( K_i \) was 1.1 \( \pm \) 0.3 \( mM \) at 70 mV and 20 \( \pm \) 14 \( \mu M \) at \(-70\) mV, an almost 50 times higher affinity at \(-70\) mV than at 70 mV. \( K_i \) values obtained from seven independent experiments for external Mg\(^{2+}\) at various membrane voltages were plotted as a function of membrane voltages (Fig. 10C). The linear relationship appearing in the positive voltage range resulted in the slope, \( z \) value, of 0.94. In other words, the electrical distance of external Mg\(^{2+}\) binding site across the membrane potential is about 47% from the outside. The affinity of external Mg\(^{2+}\) on macroscopic currents among gustatory, rod, and olfactory CNG channels showed somewhat different results (Fig. 9D). While the voltage dependence of external Mg\(^{2+}\) blockade are almost identical among three different channels leading to the same electrical distance of external Mg\(^{2+}\) binding site of about 0.47, the apparent affinity of Mg\(^{2+}\) shifted in parallel where olfactory CNG channel showed the highest affinity and gust CNG channel the lowest. Their half-maximal blocking concentrations at 60 mV were 760 \( \pm \) 190 \( \mu M \) for gust (\( n = 7 \)), 540 \( \pm \) 30 \( \mu M \) for rod (\( n = 5 \)), and 370 \( \pm \) 30 \( \mu M \) for olfactory (\( n = 5 \)).

**Effects of intracellular l-cis-diltiazem on gustCNG channels**

A benzothiazepine, l-cis-diltiazem, has been known to block many types of CNG channels from intracellular side of an excised patch in a voltage-dependent manner (Haynes 1992; McLatchie and Matthews 1992; Stern et al. 1986; Yau and Baylor 1989). Since the affinity of intracellular Mg\(^{2+}\) to gustCNG channel was similar to the other two CNG channels, we wondered whether l-cis-diltiazem also blocks gustCNG channel with a similar affinity compared with other CNG channels. Assuming that l-cis-diltiazem blocks the channel current by occluding the ion conduction pathway from intracellular side, the binding site of l-cis-diltiazem within gustCNG channel may remain similar to other CNG channels. Three different CNG channels were fully activated using 500 \( \mu M \) cGMP, and 100 \( \mu M \) l-cis-diltiazem was used to block the channel currents. Since it took tens of milliseconds to reach the steady-state current levels after step-pulse to positive voltages (Fig. 11A), the blockade was studied using step-pulse protocols instead of voltage ramps. The significant blockade of gustCNG channel currents by 100 \( \mu M \) l-cis-diltiazem was detected only at depolarizing voltage steps >80 mV. Quite different from gustCNG channel, the steady-state currents of rat olfactory CNG channels were greatly reduced by 100 \( \mu M \) of intracellular l-cis-diltiazem, especially in positive voltage range. The potency of l-cis-diltiazem for bovine retinal channel seemed to be lower than that of rat olfactory but higher than gustatory CNG channels. From several such experiments, we obtained a normalized \( I-V \) relationship where blocked currents were normalized to fully activated currents at indicated membrane voltages (Fig. 11B). The inward currents of gustCNG channel did not show any current blockade, and a slight reduction of channel currents was observed in extreme positive voltages (\( n = 4 \)). The steady-state outward currents of bovine rod CNG and rat olfactory CNG channels, however, showed significant reductions in all voltages. The blockade by 100 \( \mu M \) l-cis-diltiazem was highly voltage dependent, and more than one-half of the currents by rod and olfactory CNG channels were blocked by 100 \( \mu M \) at 100 mV (\( n = 5 \) for each channel).

**DISCUSSION**

In this study, we investigated the functional characteristics of a putative gustCNG channel cloned from rat tongue epithe-
The homomeric gustCNG channels were expressed at the level of single and macroscopic currents in *Xenopus* oocytes by injecting cRNA transcribed in vitro. The gating and permeation properties of gustCNG channels were studied using electrophysiological means.

Although the channels can be directly activated using either cGMP or cAMP, cGMP is a better agonist in terms of both affinity and efficacy for channel opening. $K_{1/2}$ value of cGMP for the activation of gustCNG channel was relatively insensitive to transmembrane voltages and was determined as 3.3 ± 0.1 μM. This affinity is similar to that of rat olfactory CNG channel at 1–2 μM (Dhallan et al. 1990) but significantly different from that of bovine rod CNG channel (about 50 μM) under physiological conditions (Nakatani and Yau 1988). Cyclic AMP can activate the gustCNG channel with $K_{1/2}$ of 250 μM and to about 90% of the maximum current level activated...
by saturating cGMP. Both of the affinity and the efficacy of gustCNG channel to cAMP are markedly different from those of homomeric cone CNG channels (Gerstner et al. 2000). Since the amino acid sequence of the cyclic nucleotide binding domain in rat gustatory and mouse cone channels are virtually identical, these differences in gating behavior might come from the differences in their amino-terminal regions. It was shown that the amino-terminal domain participates in the allosteric gating transition in previous studies using other CNG channels (Goulding et al. 1994; Tibbs et al. 1997). It is known that relatively low affinity of rod CNG channel for cGMP is essential for phototransduction by allowing the channels to stay open in the steady presence of cGMP in darkness and to be closed only by light (Yau and Chen 1995).
gustCNG channel for cGMP may reflect the mechanistic similarity between gustatory and olfactory signal transductions, an increase in cGMP concentration on the stimulatory signal, and the opening of CNG channels. Since it is still not clear whether taste receptor cells also express a second (or β) subunit of CNG channel, however, the physiological affinity for cGMP to gustCNG channel may be significantly altered in vivo. We observed that the Hill coefficient for the channel activation of about 1.4 for cGMP, strongly suggesting that the cooperative bindings of more than two molecules of cGMP might be required to activate gustCNG channels. The functional CNG channels are generally believed to be tetramers (Liu et al. 1996; Shammat and Gordon 1999; Shapiro and Zagotta 1998; Varnum and Zagotta 1996). Like other members of CNG channel family (Sunderman and Zagotta 1999), the cloned gustCNG channel does not exhibit any desensitization or inactivation during a prolonged exposure of intracellular cyclic nucleotides both at single and macroscopic current levels.

The I-V relationship of gustCNG channel shows near linearity except at extreme positive voltages. The lack of desensitization allowed us to investigate the gating properties of gustCNG channels in detail using single-channel current recordings. The single-channel gating characteristics were similar to other CNG channels, such as homomeric rod or cone CNG channel expressed in *Xenopus* oocytes. The single-channel currents of gustCNG channel showed rapid transitions between open and closed current levels occasionally interrupted by long closures lasting on the average of about 1 s. The long closures were also observed in single-channel recordings of bovine rod CNG channels expressed in *Xenopus* oocytes, and they were considered as a separate state apart from the typical open and closed transitions (Sunderman and Zagotta 1999). Even at the saturating concentration of cGMP, 100 μM, the gustCNG channels remained open and closed at a constant rate. Two distinct dwell-times, short and long, were readily identifiable in both open and closed events of single gustCNG channels, and the channels also exhibited flickering openings in most membrane voltages. At this concentration of agonist, the bursts of channel openings were often no longer discrete and fused into continuous rapid flickers. As far as single-channel kinetic analysis is concerned, we could not properly fit the dwell time histograms of both open-state and closed-state.

**FIG. 11.** Effects of l-cis-diltiazem on macroscopic currents of rat gustatory, bovine rod, and rat olfactory CNG channel. A: representative current traces of rat gustatory (left column), bovine rod (center column), and rat olfactory (right column) CNG channels in the absence and presence of 100 μM intracellular l-cis-diltiazem. Channel currents were recorded by 150-ms step pulses of −100 to 100 mV in 20-mV increment from the holding voltage of 0 mV. Pipette solution contained 130 mM NaOH, 3 mM HEPES, and 0.5 mM Na-EDTA, and perfusion solutions contained the same composition, along with 500 μM Na-cGMP to activate each 3 kinds of channels (middle traces) and 100 μM l-cis-diltiazem to block fractional current (bottom traces). The leak currents are not subtracted from the each raw trace. B: normalized I-V relationships in the absence and presence of 100 μM l-cis-diltiazem. I-V relationships in the absence (○) and presence (●) of 100 μM intracellular l-cis-diltiazem were shown for rat gustatory (left), bovine rod (middle), and rat olfactory (right) CNG channels. The level of leak currents recorded in the absence of cGMP was also shown in each panel (●). I-V relationships were measured at 100 ± 3 ms after pulse and normalized to maximum currents evoked by 500 μM cGMP. Each data point represents the mean ± SE of more than 4 separate experiments.
using single exponential functions. After the process of various trials for more accurate analysis, we were able to fit both open and closed states with a sum of two exponential functions. In a previous study, the single-channel recordings of olfactory CNG channels in the membrane of the dendrite and soma of isolated Salamander olfactory neurons were best fitted with a single open state and two closed states (Zufall et al. 1991). In another study using the α subunit of the cGMP-gated channel from rod photoreceptor expressed in *Xenopus* oocytes, however, Benndorf et al. reported the kinetic analysis of single channel, which revealed two exponentially distributed open and closed time histograms (Benndorf et al. 1999). Using the single-channel kinetic analysis of gustCNG channel at various concentration of cGMP, we were able to show that the cGMP increases the open probability of the channel by increasing solely the slower component of the opening rate without much effect on the faster opening rate or the components of the two closing rates.

The single-channel conductance of gustCNG channel was determined as 28 pS, which is smaller than those of olfactory CNG channel from the salamander olfactory receptor neuron, 45 pS (Zufall et al. 1991) and the catfish olfactory neuron, 55 pS (Goulding et al. 1992), respectively. However, it is similar to the single-channel conductance of the α subunit of the CNG channel from bovine rod, ~25–28 pS (Kaufp et al. 1989; Nizzari et al. 1993). One of the characteristic gating properties of CNG channels is flickering opening, i.e., open and close transitions are too fast to be analyzed when converted to digitized data. The deviation of current amplitude histogram from the theoretical Gaussian functions seen in the analysis of single gustCNG channel recordings (asterisks in Fig. 6B) may be caused by the flickering openings. Some single-channel current traces showed brief openings at sub-levels of full open state (data not shown). Since this sublevel conductance state was not always seen, however, further experiments are needed to support the preliminary observation using D2O instead of H2O in recording solutions, which markedly slows down the gating (Root and MacKinnon 1994).

CNG channels are nonselective cation channels permeating different monovalent cations. The gustCNG also showed weak selectivity among monovalent cations. The selectivity order of gustCNG channel was determined as Na⁺ > K⁺ > Rb⁺ > Li⁺ > Cs⁺, which was similar to that of bovine retinal CNG channel (Goulding et al. 1993). It is intriguing to find similarities of permeation characteristics between gustCNG channel and rod CNG channel despite a significant difference in the affinity for cGMP.

We probed the structure of ion conduction pathway of gustCNG channel by investigating the effects of CNG channel blockers, Mg²⁺ and l-cis-diltazem, and by comparing the results with two other CNG channels. Both intracellular and extracellular Mg²⁺ blocked Na⁺ currents through gustCNG.

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**FIG. 12.** Amino acid sequence alignment for putative extracellular vestibules and the pore-forming regions of gustatory, rod, and olfactory CNG channels. Negatively charged residues are shown in bold, and the positively charged ones are in italics. Glu363 residue of bovine retinal channel is known to bind extracellular Mg²⁺, and corresponding residues in other CNG channels are underlined. Net charges at neutral pH were estimated as 0 for rat gustatory, −2 for bovine retinal, and −5 for rat olfactory CNG channels.
channel in a voltage-dependent manner. Voltage-dependent blockade and “pop-through” phenomenon at extreme voltages are the characteristics of Mg$^{2+}$ blockade observed in other CNG channels (Root and MacKinnon 1993). Likewise, gustCNG channel also contains Mg$^{2+}$-binding sites located in the conducting pathway, and Mg$^{2+}$ can permeate the conducting pathway by extreme membrane potentials. The electrical distances of Mg$^{2+}$ binding sites ($\delta$) estimated for gustCNG channel, 0.52 from intracellular side and 0.47 from extracellular side, indicate that internal Mg$^{2+}$ and external Mg$^{2+}$ binding sites may be located closely within the electrical field. Comparison of $\delta$ values among rat gustatory, bovine rod, and rat olfactory CNG channels revealed that the binding affinity as well as the electrical location of the intracellular Mg$^{2+}$ binding are quite similar to each other. Although the exact location of the internal Mg$^{2+}$ binding site in CNG channels has not been identified yet, it is not too surprising to find this similarity since the amino acid sequence comprising the ion-conduction pathway (or S5-pore-S6 region) among these channels show a high sequence homology.

On the contrary, however, extracellular Mg$^{2+}$ binding affinities were significantly different among the three CNG channels: the strongest for olfactory CNG channel and the weakest for gustCNG channel with similar $\delta$ values. The blockade of CNG channels by external Mg$^{2+}$ is mediated by a conserved glutamate in the pore-forming region, Glu333 of the catfish olfactory channel (Root and MacKinnon 1993) and Glu363 in the bovine retinal channel (Eismann et al. 1994). The corresponding glutamate residue (Glu369) and adjacent amino acid residues of the pore-forming region are also highly conserved in gustCNG channel. The Mg$^{2+}$ affinity can be altered without too much effect on $\delta$ value by those charged amino acid residues near the Mg$^{2+}$-binding site affecting the local concentration of Mg$^{2+}$. We compared the total net charges in putative extracellular vestibule regions, S5–P linker and P–S6 linker (Fig. 12). It was intriguing to find that the total net charge of homo-tetrameric gustatory, rod, and olfactory CNG channels were 0, −8, and −20, respectively, at a neutral pH. Thus the apparent difference in the affinities of extracellular Mg$^{2+}$ may simply be the result of the differences in local Mg$^{2+}$ concentration influenced by the electrostatic attraction due to net negative charges in the extracellular vestibule. Although the affinity for extracellular Mg$^{2+}$ is lower than that of rod or olfactory CNG channels, the $I-V$ relationship of gustCNG channel expressed in the membrane of mammalian taste bud cells. Therefore the functional properties of cloned gustCNG channel investigated in this study will guide such studies in the future.

In conclusion, we elucidated the electrophysiological properties of the gustatory CNG channel expressed in Xenopus oocytes. While the gustCNG channel shares many common characteristics with other members of the CNG channel family, sever properties of both gating and permeation distinguish the gustCNG channels from the other CNG channels. Since it is still elusive whether any gustatory signaling pathway involves the cyclic nucleotide–mediated increase in cation permeability and this channel is also involved in the visual signal transduction in retinal cone cells, it would be critical to characterize the channel activity in the membrane of mammalian taste bud cells. The authors thank Dr. Keiko Abe (University of Tokyo) for generously providing us with the cDNA of gustatory CNG channel. We also thank the other members of the Neuro-biochemistry Laboratory at K-JIST for timely help throughout the work.

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