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Reconstituted Ion Channels of Frog Fungiform Papilla Cell Membrane

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ABSTRACT—We identified a Cl⁻ channel, two K⁺ channels and a cAMP-gated channel which were isolated from bullfrog fungiform papilla cell membranes and incorporated into phospholipid bilayers using the tip-dip method. The 156 pS channels were inhibited by 100 µM 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and displayed the reversal potential identical to the equilibrium potential of Cl⁻, it was identified as a Cl⁻ channel. Two types of K⁺ channel had unitary conductances of 79 and 43 pS, which may correspond to those of Ca²⁺-activated and cAMP-blockable K⁺ channels observed in isolated intact frog taste cell membranes, respectively. These results suggest that the tip-dip method is useful for stable investigation of the properties of ion channels already identified in the taste cell. Furthermore, the 23 pS channels were newly found and were activated directly by internal cAMP as cyclic nucleotide-gated (CNG) nonselective cation channels established in olfactory receptor cells. Thus, our results suggest the possibility that besides Cl⁻ and K⁺ channels, the cAMP-gated channels contribute to taste transduction.

INTRODUCTION

A variety of ionic channels has been suggested to be directly related to the taste transduction processes (Sato, T. et al., 1994; Lindemann, 1996) but few analyses of the single channels (Avenet et al., 1988; Cummings et al., 1992; Fujiyama et al., 1993, 1994a, b; Miyamoto et al., 1996) have been done. The difficulty with stable single channel recordings is mainly due to the high density of ionic channels in a focal area but the extremely low density in other areas (Cummings et al., 1992; Fujiyama et al., 1994). In addition, the formation of stable single channel recordings is generally difficult because taste cells are relatively small and fragile cells, whose surface are covered with blunt microvilli even after enzyme treatment (Richter et al., 1988).

If the receptors or ion channels isolated from the taste cell membrane would be stably incorporated into the artificial membrane, one could not only characterize the properties of receptors or ion channels in more detail, but also take cue for the development of an artificial taste cell, which may be useful as a gustatory sensor. Therefore, we attempted to isolate the membrane frac-

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tions containing ion channels from enzymatically dissociated frog taste cells, and incorporated them into phospholipid bilayers. The same method was successfully employed in single channel analysis of L-arginine-gated channels isolated from the cutaneous taste epithelium of the catfish (Teeter et al., 1990; Kumazawa et al., 1998) and cAMP-gated channels isolated from the olfactory cilia (Labarca et al., 1988; Bruch et al., 1989).

In the present experiment, we confirmed the presence of a Cl⁻ channel and two K⁺ channels, which may be identical to those observed in the frog taste cell membrane and some of which are known to directly play an important role for the gustatory transduction (Avenet et al., 1988; Miyamoto et al., 1993; Fujiyama et al., 1993, 1994a, b; Okada et al., 1998). Furthermore, we found a novel cAMP-gated channel that has never been reported in the taste cell so far, whereas the presence of a cyclic-nucleotide suppressible cation conductance has been reported (Kolesnikov and Margolskee, 1995; Tsunenari et al., 1996).

MATERIALS AND METHODS

Preparation of membrane fraction

Adult bullfrogs (Rana catesbeiana) weighing 150–300 g were decapitated and pithed, and the tongues were dissected out. A few hundreds of fungiform papillae were removed from the isolated tongues with a pair of fine scissors in an ice-cold divalent ion-free Ringer (DFR) solution containing (mM): 110 NaCl, 3.5 KCl, 5 HEPES-NaOH, 2
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EDTA, pH 7.4. The fungiform papillae were incubated in the DFR solution containing 15 U/ml papain (Sigma) activated by 10 mM cysteine (Sigma) for 15 min at 22 – 24°C. The tissues were then rinsed with a normal Ringer (NR) solution (mM): 110 NaCl, 3.5 KCl, 1.0 CaCl₂, 1.6 MgCl₂, 5 HEPES-NaOH, pH 7.4. The cell suspension prepared by gentle trituration was homogenized. After centrifugation at 1,500 × g and 4°C for 30 min, the supernatant was centrifuged at 20,000 × g and 4°C for 30 min again. The sediment containing the membrane fraction was ultrasonicated for 30 s and stored in the ice-cold NR solution.

Reconstitution of membrane fraction into artificial lipid bilayer
The procedure of reconstitution was the same as described previously (Coronado et al., 1983; Teeter et al., 1990; Kumazawa et al., 1998). The membrane fraction (50 – 100 µg protein/ml) was suspended in a small (300 µl) teflon chamber filled with NR solution or appropriate salt solutions. A lipid monolayer was formed by carefully adding 30 – 50 µl of azolectin dissolved in hexan to the solution containing the suspension of the membrane fraction in the chamber (Fig. 1A). After allowing 10 min for the solvent to evaporate, high-resistance (>10 GΩ) bilayers were formed on a pipette by repeating the pipette insertion into the solution in the chamber under positive pressure (Fig. 1B and D) and its withdrawal from the solution under releasing pressure (tip-dip method) (Fig. 1C).

Solutions
A stock solution of 4, 4'-disothiocyanostilbene-2, 2'-disulfonic acid (DIDS, Sigma) was dissolved in dimethyl sulfoxide (DMSO, Sigma) at a concentration of 0.1 M, and stored below 0°C. Aliquots of the stock solution were added to the bathing solutions to give the desired final concentration. A concentrated adenosine 3', 5'-cyclic monophosphate (cAMP, 1 mM, Sigma) was added to the bathing solution to provide an appropriate final solution. All pipette and bathing solutions contained 5 mM HEPES-NaOH or -KOH to adjust pH to 7.4.

Electrophysiological recordings
Patch pipettes were fabricated from borosilicate glass capillaries (Narishige, G-1) with an electrode puller (Narishige, PP-83). The electrode tip was heat-polished using a microforge (Narishige, MF-83) so that the resistance was 3–15 MΩ when filled with a pseudointracellular (PIC) solution containing (mM): 12.5 NaCl, 85 KCl, 0.25 CaCl₂, 1.6 MgCl₂, 0.5 EGTA, 5 HEPES-KOH, pH 7.4. Single channel currents were recorded using a patch clamp amplifier (Axon Instruments, Axopatch 200) in the voltage clamp mode. Holding potentials were equivalent to pipette potentials. Current signals were low-pass filtered at 1 kHz, digitized at 125 kHz, sampled at 10 kHz and stored on a computer running pCLAMP software (Axon Instruments). All the experiments were performed at 22–24°C.

RESULTS
The tip-dip method enabled us to record stably the channel activities for more than scores of minutes. When the pipette and bath were filled with the PIC solution and the NR solution respectively, several kinds of channel activities were
observed in 15% of reconstituted membranes (ca. 300 trials) which had a high seal resistance of 10 GΩ. Among them, we identified one type of Cl⁻ channels, two types of K⁺ channels and one type of cAMP-gated channels.

**Chloride channels**

When pipette and bath were filled with 110 mM KCl solution and 110 mM NaCl solution respectively, multiple steps of single-channel events at a holding potential of +60 mV were obtained in 2.5% of the reconstituted membrane (Fig. 2). Three different unitary conductances of single channel events, 156 (155.8±8.6, mean±SD, n=4), 63 (62.6±1.8, n=6) and 28 (27.6±4.2, n=6) pS, were observed in Figure 2. The reversal potentials of 156 pS channel were 0 mV, which was equal to the equilibrium potential of Cl⁻ (ECl). Single channel events of 156 pS channel at different holding potentials and an I-V relationship are shown in Fig. 3A and B respectively. The activity of 156 pS channel was inhibited by a Cl⁻ channel blocker DIDS (Fig. 3C and D). Since 156 pS channels were more frequently observed than other two channels, which always appeared with 156 pS channels as shown in Fig. 2, 63 and 28 pS channels may be subconductances of 156 pS channel.

**Potassium channels**

Activity of channel that had a mean unitary conductance of 79 pS (78.8±4.8 pS, n=4) was observed. Fig. 4A shows activities of this channel at holding potentials of −80, 0 and +40 mV. The reversal potential of this channel (−50 mV) was closer to the equilibrium potential of K⁺ (EK, −69.5 mV) rather than the equilibrium potential of Na⁺ (ENa, 54.8 mV) or ECl (−4.4 mV) (Fig. 4B). The channel activity was obtained immediately after forming of reconstituted membrane on the tip of pipette, and was persistent for more than a few minutes. This type of K⁺ channel was detected in 7.5% of the reconstituted membranes examined.

Channel activities shown in Fig. 5A were obtained when a pipette was filled with 85 mM KCl solution and the bath was perfused with 30 mM KCl solution, where 55 mM KCl was replaced with 55 mM NaCl. This type of channel had a mean unitary conductance of 43 pS (43.2±4.3 pS, n=6). The fact that the reversal potential (−31.0±4.7 mV, n=4) was identical to EK (−26.2 mV) but not to ECl (0.0 mV) (Fig. 5B) suggests that 43 pS channel is involved in a K⁺ channel family. The activity of 43 pS K⁺ channel was found in 5% of the reconstituted membranes. An application of 5 µM cAMP to the bath...
Fig. 3. Single channel recording of 156 pS Cl⁻ channel. (A) Single channel events at different holding potentials. The slope conductance was 156 pS. (B) An I-V relationship of unitary currents. The slope conductance was 156 pS. (C) Blocking of channel activity by 200 µM DIDS. Holding potential: –40 mV. (D) Inhibitory effect of 200 µM DIDS on currents elicited in phospholipid bilayer containing 156 pS channel by voltage ramps from –80 to 80 mV. Insets in this and the other figures show solutions in the pipette and bath.

did not affect the activity of 43 pS K⁺ channel.

cAMP-gated channels

The channel activity induced by cAMP was found in 2.5% of the reconstituted membranes where no channel activity was seen first (Fig. 6). In the case of Fig. 6, 200 µM cAMP was added to the bath containing 110 mM KCl at the beginning of trace 3. Although no channel activity was seen before addition of cAMP (traces 1 and 2), channel activities whose unitary conductance was 23 pS (23.4±4.9 pS, n=7) appeared within 1 s after addition of cAMP (trace 3), and changed to more vigorous opening and closing with time after addition of the drug (traces 4, 5 and 6).

When the pipette contained the same K⁺ concentration as that used in the bath (Inset of Fig. 6), the reversal potential of the 23 pS cAMP-gated channel was approximately 0 mV.
that is equal to $E_K$ (data not shown). However, even when the pipette was filled with NR solution containing 110 mM NaCl and 3.5 mM KCl, and the bath was perfused by PIC solution containing 12.5 mM NaCl and 85 mM KCl, the reversal potential was approximately 0 mV (0.5±3.3 mV, n=4) (Fig. 7), which is close to the equilibrium potential of cations ($E_{cat}$, 4.0 mV). The channel activity was not affected by Cl$^-$ channel blockers such as DIDS and SITS. Because the bilayers usually contained multiple channels gated by cAMP as shown in Fig. 7, it was difficult to obtain the dose-response relationship or the reversal potential from single channel recordings. Therefore, we obtained those data by applying ramp voltage from –40 to 40 mV to the bilayers containing only cAMP-gated channels. As shown in Fig. 6B, the conductance of the bilayer containing only cAMP-gated channels increased dose-dependently.

In bilayers, which displays neither channel activity nor current response to voltage ramps from –40 to 40 mV as shown in control of Fig. 8A, 100 µM cAMP elicited not only vigorous activities of 23 pS channels (Fig. 8A) but also reversible increase in bilayer conductance, whose reversal potential was approximately 0 mV (Fig. 8B). The I-V relationships before µM (control), during (cAMP) and after (wash) application of 100 µM cAMP induced by voltage ramps from –40 to 40 mV were presented in Fig. 8B. The conductance of bilayers before and after application of 100 µM cAMP was 170±130 pS (n=4) and 630±510 pS (n=4), and the reversal potential of the conductance after application of cAMP was 3.3±4.9 mV (n=4). These results suggest that the 23 pS cAMP-gated channel is a nonselective cation channel.
DISCUSSION

A Cl⁻ channel, 156 pS Cl⁻ channel was identified in the present experiment. We have already observed the presence of the large (200 pS) conductance Cl⁻ channel at the apical receptive membrane of enzymatically isolated bullfrog taste cells using patch clamp technique (unpublished data). Therefore, 156 pS Cl⁻ channel observed in the reconstituted mem-
brane may be identical to 200 pS Cl\textsuperscript{−} channel obtained in isolated taste cells. We also observed 28 pS and 63 pS Cl\textsuperscript{−} channels in the present experiment. However, these lower conductance channels may be subconductances of 156 pS Cl\textsuperscript{−} channels since they were observed with lower probability than 156 pS channels and always appeared with 156 pS channels. The previous intracellular study of the bullfrog taste cells suggested that anion conductances as well as cation conductances at the apical receptive membrane contribute to the generation mechanism of salt-induced responses (Miyamoto et al., 1993) and play an important role in the generation mechanism of water-induced responses (Okada et al., 1993). The Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} channels on the whole taste cell membrane have been reported to be involved in the termination of depolarizing receptor potentials in response to gustatory stimuli (McBride and Roper, 1991; Taylor and Roper, 1994). Recently it has been reported that the Cl\textsuperscript{−} channels play important roles in the generation of the acid-induced responses (Miyamoto et al., 1998) and the maintenance of the resting membrane potentials in mammalian taste cells (Waldkowski et al., 1998). Thus, 156 pS Cl\textsuperscript{−} channels observed in the present experiment may play some roles other than the generation mechanism of water-induced responses.

We found 79 pS K\textsuperscript{+} channel in the reconstituted membrane. The presence of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels that have
similar unitary conductance has been well established in enzymatically isolated frog taste cells (Avenet et al., 1988; Fujiyama et al., 1994a; Miyamoto et al., 1991). This type of channel was concentrated on the apical receptive and dendritic membranes but not on the other parts of the basolateral membrane (Fujiyama et al., 1994a). The reversal potential of −50 mV was considerably more positive than EK. The 80 pS Ca2+-dependent K+ channels recorded from dissociated taste cells showed similar reversal potential (Fujiyama et al., 1994a). Preliminary experiments showed that the 79 pS channel is Ca2+-dependent.

The K+ channels that are blocked by cAMP via protein kinase A (PKA)-mediated phosphorylation in the presence of ATP and have a unitary conductance of approximately 40 pS has been found in frog taste cells (Avenet et al., 1988; Fujiyama et al., 1994a). These channels were distributed over the entire taste cell membrane, but the distribution density was much higher on the apical receptive and dendritic membranes (Fujiyama et al., 1994a). In the present experiment we found 43 pS K+ channels that were not blocked by cAMP alone, suggesting that the simultaneous presence of PKA in the reconstituted membrane and ATP as well as cAMP in the bath is necessary.

In contrast, we found 23 pS channels that were directly activated by cAMP since the activation was induced by only the presence of cAMP without ATP or PKA as reported in the activation of cyclic nucleotide-gated (CNG) channels in photoreceptors (Fesenko et al., 1985) and in olfactory cilia (Nakamura and Gold, 1987). Recent study using molecular cloning techniques suggests the presence of CNG channels, which are homologous to the human cone CNG channels with 82% similarity, in rat taste cell membrane (Misaka et al., 1997), suggesting that the gustatory and visual senses share a common transduction mechanism (Misaka et al., 1999).

On the other hand, it has been suggested that cyclic-nucleotide suppressible cation conductance may contribute to bitter transduction in frog taste cell (Kolesnikov and Margolskee, 1995; Tsunenari et al., 1996) in the context of intracellular cascade involving activation of a taste cell-specific G protein, α-gustducin and phosphodiesterase and consequent reduction of cAMP level (McLaughlin et al., 1992; Wong et al., 1996). Thus, the present study suggests that the 23 pS cAMP-gated channels belong to a CNG channel family, which is a nonselective cation channel, and play an important role in the gustatory transduction other than bitter transduction of frog taste cells.

In the present experiment, we demonstrated that the tip-dip method enables incorporation of the membrane fraction obtained from taste cell containing ion channels into the artificial lipid bilayer and the stable recording of the channel activities. Therefore, through improving this method it may be possible to develop a sensor to detect chemicals such as low concentration of cAMP or a little change of ion concentration.

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