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TASTE RECEPTOR PROTEINS DIRECTLY EXTRACTED BY LIPOSOME FROM INTACT EPITHELIUM OF BULLFROG TONGUE

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SUMMARY: This work first provides that epithelial membrane proteins can be directly transferred from animal intact tissue to liposome. Bullfrog tongue was treated with a specially modified liposome that contains an artificial boundary lipid. Glossopharyngeal nerve responses of the treated tongue were then measured to five taste stimuli (NH_4Cl, L-Ala, sucrose, L-Leu, and quinine hudrochloride). The liposomal treatment caused remarkable changes of the taste nerve responses. Gel electrophoretic analysis of the treated liposome revealed that the direct transfer of proteins,likely taste receptor,certainly occurred from the tongue epithelium to the liposome. © 1994 Academic Press, Inc.

Generally, investigation and understanding of membrane proteins are much behind compared with those of soluble proteins. One of the reasons is that perfect isolation of membrane proteins from cells is rather difficult without any serious denaturation and/or deactivation. Even if one could somehow isolate them from intact cells by an ordinary method using a surfactant, an organic solvent, or an aqueous salt solution and represent the physiological function or activity to some extent in a reconstitution system, there is no certain evidence whether they are still keeping the native orientation and activity in cell membranes during the procedures. In order for membrane proteins to represent their original function even after the extraction and the subsequent reconstitution, they must exactly keep the native orientation, location, and microenvironment in the cell membrane. To investigate the structure and function of membrane proteins without serious denaturation or deactivation, therefore, the

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perfect extraction of the membrane proteins from intact cells and the subsequent reconstitution into an artificial system such as liposome are essential requirement. For this purpose, one of the present authors (J.S.) and his coworkers have recently proposed an improved method (*1*). The method involved *in vitro* direct transfer of membrane proteins from an intact cell to a liposome that contains an artificial boundary lipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC) (*2*). DDPC has amide bonds instead of ester bonds of usual phospholipids and makes the membrane proteins more stable in the liposomal membranes. This *in vitro* method has been successfully applied to several intact cells, such as human platelet (*3*), human erythrocyte (*4*), B16 melanoma cell (*5*), and BALBRVD tumor cells (*6,7*). In the case of B16 melanoma cell, in addition, this direct protein transfer method was applied to the monolayer culture. In the monolayer culture a specific receptor protein (a-melanocyte stimulating hormone receptor) more effectively transferred to the liposome than others, such as adhesive proteins, accompanied by "*capping*" of cell membrane proteins (*5*). This *in vitro* result strongly encouraged us to apply the methodology also to *in vivo* study such as to epithelium of animal tissue.

Membrane proteins being in tongue epithelium are of special interest relating with the taste transduction. Despite many years of research, molecular biological attempts to purify taste receptors have been unsuccessful, and the receptor mechanisms involved in the sense of taste are still unclear (*8*). Because, their small quantity in the membrane and weak binding constant with the taste substances (*8-10*) make the isolation, purification, and characterization for precise investigation rather difficult. For investigating the taste transduction, a neurophysiological study of the nerve response to taste stimuli as expressed on the surface of an animal tongue is another possible approach. Co-author of this article (K.K.) and his coworkers have dealt well with the neurophysiological measurement to taste stimuli (*11*). Hence, we have started collaborative studies; the liposomal extraction of taste receptor(s) from bullfrog tongue and its effect on the nerve response. To our knowledge, in this sense, this paper reports the very first example showing a possibility that membrane proteins (or receptor(s)) of epithelial cells of an intact animal tissue can directly transfer to a liposome. To understand the mechanisms of taste transduction, in addition, we could directly combine the two methodologies, molecular biological investigation of taste receptor proteins and neurophysiological study of taste stimuli, which have been independently studied so far.

This study is hence consisting of two major parts; namely (i) neurophysiological study of the frog tongue treated with the liposome and (ii) gel electrophoretic analysis of the proteins transferred to the DDPC/DMPC-liposome from the frog tongue.

MATERIALS AND METHODS

Liposome preparation. Multilamellar liposomes (MLVs) were prepared in 10.0 ml of 10 mM Hepes buffer (pH 7.4) containing 150 mM NaCl and protease inhibitors (0.2 mM Pefabloc SC (Merck, Rahway, NJ) and 1.0 mM EDTA (Wako, Tokyo)) using an appropriate mixture of 1,2-dimyristoylphosphatidyl-choline (DMPC, Sigma Chemical Co., St. Louis, MS) (48.8 mg, 60.0 mol%) as the matrix lipid and DDPC (*2*) (Dojindo Laboratories, Kumamoto) (32.4 mg, 40.0 mol%) as an artificial boundary lipid (*12*). A liposomal suspension so obtained was

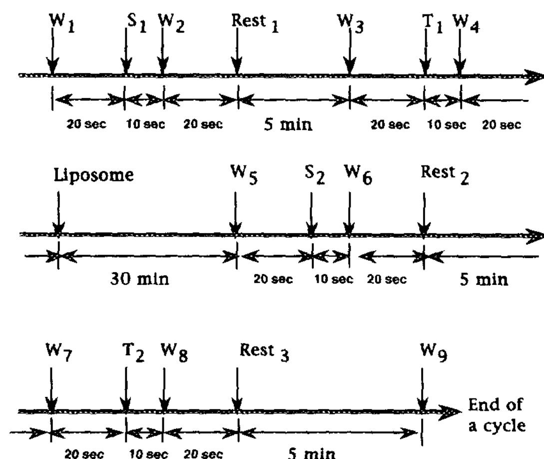

Fig. 1. Flow chart of the liposomal treatment of the frog tongue and the measurement of nerve response of the tongue against taste stimuli; **W** stands for washing with pure water; **S** for stimulation with an aqueous NH_4Cl solution as the standard; and **T** for stimulation with an aqueous solution of a taste stimulus such as L-alanine, sucrose, L-leucine, or quinine hydrochloride.

extruded (The Extruder, Lipex Biomembrane Inc., Vancouver) by passing through polycarbonate membrane filters sequentially decreasing the pore size from 1.0 mm through 0.1 mm (Nuclepore Corp., Pleasanton, CA.). MLVs so obtained were rather monodispersive with the mean diameter of 100 nm (DLS-70, Photal Otsuka Electronics, Hirakata). The final concentration of the liposomal suspension (10.0 ml) was adjusted to 5.0×10^{-3} M as the lipid concentration. Our previous *in vitro* studies (*3-7*) have indicated that conventional DMPC-liposome without DDPC was less effective to transfer membrane proteins from cells to the liposome, while the liposome bearing more than 60 mol% of DDPC somewhat caused damages to cells. Therefore, in this work, we employed 40 mol% DDPC-60 mol% DMPC liposome.

Neurophysiological study. Figure 1 shows the flow chart of the protein extraction with the liposome and the subsequent measurement of the nerve response of the tongue. After a healthy adult bullfrog, *Rana catesbeiana*, (body weight, 180 - 250 g, n = 3) was anesthetized by *i.p.* injection of 4.0 ml of an aqueous 20% urethane solution, the glossopharyngeal nerve of the frog was dissected from the surrounding tissues and was cut in proximity (*13*). To eliminate bacteria and other contamination as possible, the tongue was first washed by 20 ml of pure water. The water washings were carefully inspected microscopically by trypan blue and Giemsa dying that none of bacteria or other microorganisms were contaminated.

A given concentration of an aqueous solution of taste stimulus (100 mM NH_4Cl at S_1 and S_2, 250 mM L-alanine, 1.0 M sucrose, 100 mM L-leucine, and 0.1 mM quinine hydrochloride respectively in this sequence) was then showered at T_1 for 10 sec at 1.7 ml per sec, and the nerve response was measured. According to the method established previously (*13*), we decided the sequence of the taste stimulation and the reasonable concentration of the stimuli. Before and after each stimulation, the tongue was thoroughly washed again by deionized water for 20 sec (W_{1-8} in Fig. 1). In addition, a suitable rest was interposed for 5 min between each taste stimulation and a Ringer's solution was showered on the tongue during the period. Even if the period of the rest was elongated 10 min or so longer, no significant difference was observed. The cycle between W_5 and W_9 was repeated four or five times to ascertain the recovery of the nerve response after the liposomal treatment and the reproducibility of the measurement. The nerve impulses were amplified with an AC-amplifier and integrated using an electronic integrator at the time constant of 0.3 seconds. To evaluate the nerve responses of the frog, the initial peak (the phasic wave) height was integrated during 10 sec after each taste stimulation (*14*). The response to an aqueous 100 mM NH_4Cl solution was measured always before and after each taste stimulus as control (*13*). The procedure between S_1 and W_4 was repeated for all the four taste stimuli in the case without the liposomal treatment (the very first flow of Fig. 1).

Liposomal treatment. After the measurement of nerve responses as control (after the washing at W_4), 10.0 ml of a DDPC/DMPC-liposomal suspension were showered for 30 min at 25.0 °C. For this purpose, we designed a simple apparatus to shower a liposomal suspension repeatedly onto the tongue, especially the location where a number of taste buds gathers. When the tongue was treated with the liposome, the procedure between S_2 and W_9 was repeated for all the four taste stimuli.

Electrophoretic analysis of proteins extracted by liposome. First, the water washings (W_1) combined were centrifuged at 3000 x *g* and 4 °C for 30 min. Sediments were trace in amount. The supernatant that contains soluble proteins was sonicated three times at 100 W for 20 sec on an ice-water bath, and then submitted to SDS-PAGE (Precast Gel System, IWAKI GLASS Co., Ltd., Tokyo) and detected by silver staining method. The liposomal extracts collected from three frog tongues were first lightly centrifuged at 3000 x *g* and 4.0 °C for 30 min. Sediments of this part also were trace in amount. The supernatant was submitted to ultracentrifugation at 160,000 x *g* and 4.0 °C for 3 hr. To eliminate completely soluble proteins as additionally contaminated during the liposomal treatment, pellets so obtained were redispersed in 2.0 ml of Hepes buffer and sonicated three times at 100 W for 20 sec on an ice-water bath. After dilution of the liposomal suspension using 18.0 ml of Hepes, the suspension was washed repeatedly by ultracentrifugation. If needed, this ultracentrifugation was repeated four or five times. Finally, the liposomal pellets, which contained approximately 1.0 mg of proteins, were again suspended in 10 ml of Hepes and extracted by 15 ml of chloroform-methanol (2 : 1 by vol.) and centrifuged at 2000 x *g* for 20 min to eliminate liposomal lipids as possible before the desalination, lyophilization, and SDS-PAGE.

RESULTS

Nerve response. When the tongue was exposed to the liposome for the period shorter than 30 min, no significant change was observed in the nerve response to NH_4Cl even 90 min after the liposomal treatment. Because it is generally accepted that salty stimuli are transduced by a stimulus influx, the present result suggests that no serious damages occurred on the ion channel for salty stimuli under the conditions. However, when the liposomal exposure was longer than 60 min, a slight decrease in the nerve response to NH_4Cl was obviously observed. Sodium ions diffuse down their electrochemical gradient through Na^+-channels to depolarize taste receptor cells directly (*8-b*).

Figure 2 shows the responses of the frog gustatory nerve to four taste stimuli before and after the treatment with DDPC/DMPC-liposome. Relative nerve response (= $R_{treated}/R_{untreated}$) of each taste stimulus before and after the liposomal treatment (vertical axis in Fig. 3) was calculated as follows; nerve response corrected ($R_{treated}$ or $R_{untreated}$) = (height of phasic wave to taste stimulus)/(averaged height of phasic waves to NH_4Cl before and after liposomal treatment). All the results for three frogs are summerized in Fig. 3. The liposomal treatment largely suppressed the nerve responses to L-Ala, L-Leu, and quinine hydrochloride (70 - 90 %), but partially to sucrose (approx. 30 %). This suppression of the response to sucrose did not recover with time, but that to other three stimuli. Sweet stimuli bind to specific receptor coupled to activation of G-proteins, adenyl cyclase generation of cAMP, activation of protein kinase A, blockade of basolateral K^+ channels, and finally leading to taste cell depolarization (*8*). The present condition might be, therefore, not severe enough to extract the receptor protein of sucrose. The decrease in the response to sucrose just after the liposomal treatment might be some damages in the activation pathway relating with the taste cell depolarization serious in the sweet stimulation but not in the salt stimulation.

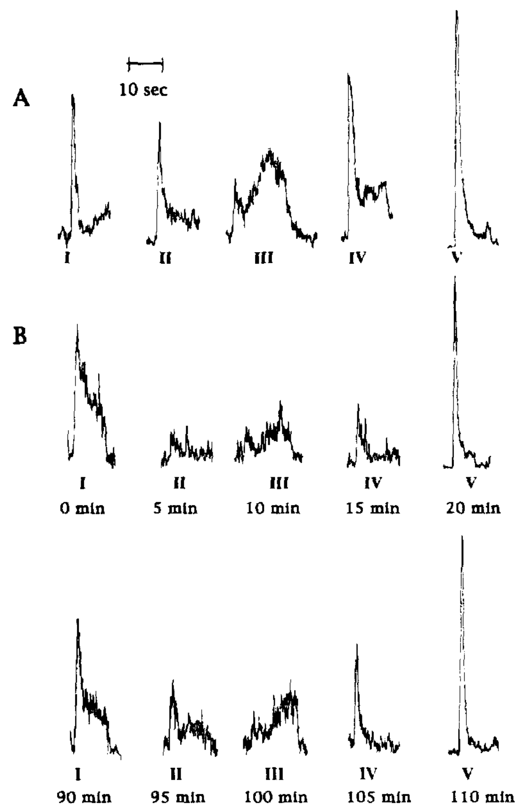

Fig. 2. Integrated gustatory nerve response of a bullfrog tongue to different taste stimuli without the liposomal treatment (**A**) and after the liposomal treatment (**B**). Time given below each nerve response refers the time after the liposomal treatment. **I**, NH_4Cl; **II**, L-alanine; **III**, sucrose; **IV**, L-leucine; and **V**, quinine hydrochloride. The unit of the magnitude of the response is arbitrary.

Most of amino acids taste sweet (e.g., L-Gly) or bitter (e.g., L-Leu) (*8*), and certain amino acids also bind to taste membranes from the catfish (*15*). L-Alanine stimulates both IP_3 and cAMP generation in cell from catfish taste tissue (*16*), presumably by the mechanism coupled to a G-protein. Judging from these previous results, we can consider the present liposome could extract the receptors of L-Ala and L-Leu. The recovery of the responses to amino acids may be some supply of the receptors from either the untreated part of the taste cell membranes by lateral diffusion or from the cytosol. Such the recovery was certainly observed when the receptors of sugars and amino acids in the rat tongue were eliminated by a pronase (*17*).

The response to quinine hydrochloride greatly increased (170 %) 80 min after the liposomal treatment. G-protein activated second-messenger systems have been shown to be involved also in the transduction of bitter compounds. Contrary to the case of sweet stimuli, however, certain bitter compounds decrease levels of intracellular cAMP leading to taste cell hyperpolarization *through* decreased basolateral K^+ channels (*18*). The pathway may concern with the present result.

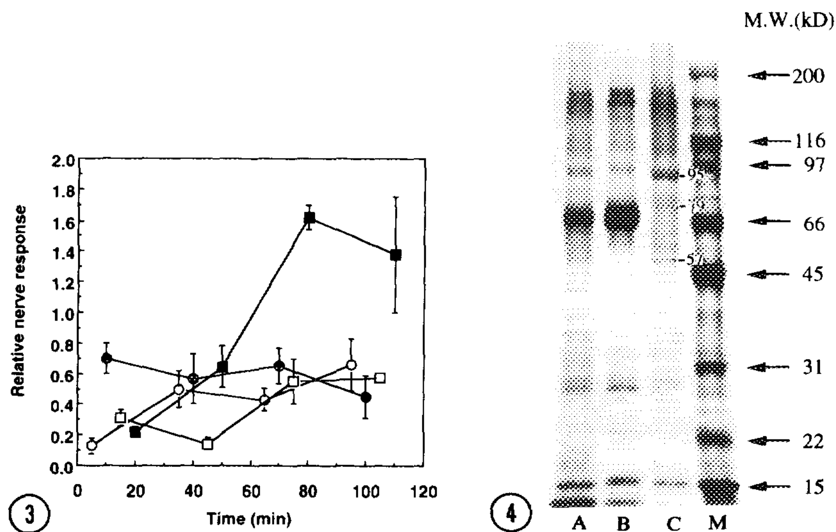

Fig. 3. Relative nerve response of bullfrog tongue for four major taste stimuli as a function of time after the liposomal treatment. -○-, L-alanine; -●-, sucrose; -□-, L-leucine; and -■-, quinine hydrochloride. The ordinate represents ratio of the response to each stimulus after the liposomal treatment to that before the treatment.

Fig. 4. Gel electrophoretic analysis of proteins; lane A is the first water washings before exposure to taste stimuli, lane B is the supernatant in ultracentrifugation of the liposomal extracts, and lane C is membrane proteins of the liposomal extracts. Lane M is marker proteins.

Electrophoretic analysis. To understand results of above neurophysiological studies, we carried out gel electrophoretic analysis of proteins transferred to the liposome from the tongue. Amount of the soluble proteins in the first washings was approximately 2.8 mg (collected from totally three frog tongues). The result of SDS-PAGE is given in the lane A of Fig. 4. Major bands determined using a densitometer (TIAS-2000S ACI Japan Co., Tokyo) were 149 kd (8.5 %), 75 kd (23.0 %), 27 kd (3.1 %), 15 kd (5.1 %), and 14 kd (9.4 %). From the supernatant of the liposomal extracts, approximately 5.0 mg of soluble proteins were additionally obtained by ultracentrifugation (from three frogs); their major proteins were 150 kd (10.4 %), 73 kd (38.2 %), 27 kd (6.8 %), 15 kd (8.7 %), and 14 kd (5.8 %) (Fig. 4, lane B). They were almost identical with those observed for the very first water extracts before the liposomal extraction (lane A). Major proteins found in the liposomal membrane were 147 kd (12.8 %), 95 kd (6.5 %), 79 kd (4.5 %), 57 kd (2.3 %), 28 kd (3.4 %), and 15 kd (10.4 %) (Fig. 4, lane C). When the liposomal lipids were not eliminated well enough, clear SDS-PAGE was not obtained. When the tongue was exposed to the liposome without protease inhibitors, the total amount of proteins of this part was less and no clear bands were observed. In this case, in addition, many low molecular weight proteins were observed. Judging from the difference between the two cases with and without protease inhibitors, some membrane proteins must be fragmented by proteases released during extraction and/or separation procedures.

DISCUSSION

Stimulation of the gustatory receptors of the tongue has been of interest for many years. At molecular level, however, many ambiguities and questions still remain in the elucidation of the mechanisms (*8,19*). For carp taste epithelium, through investigation of [^{3}H]-labeled amino acid binding to membrane fraction prepared from barbels and lips of carp, it has been proposed that the actual binding of L-Ala, L-Arg, and L-Glu to respective binding site, *i.e.* taste receptor, takes place (*20*). Kalinoski and her coworkers also have revealed that the taste system of the channel catfish contains independent receptor sites for L-Ala and L-Arg (*21*) and that L-Ala receptor acts through G-protein-mediated second messenger mechanisms while the L-Arg receptor is a ligand-gated cation channel (*22*). Recently, G-protein coupled receptors were cloned from the rat (*23*) and the bovine (*24*) taste tissues. Even though these receptors seem to be for sugars, bitter substances, and amino acids, there is no direct evidence to support this notion at present (*8*).

Direct transfer of membrane proteins from mammalian cells to the artificial boundary lipid-containing liposomes has been extensively investigated by Sunamoto and his research group (*3-7*). All the previous studies were, however, carried out *in vitro*. Judging from the obvious reduction of gustatory nerve responses to taste stimuli upon the treatment of tongue epithelium of the frog with the DDPC/DMPC-liposome in this work, it seems reasonable to assume that a sort of taste receptor proteins of tongue is lost or damaged. Plausible candidates are of 147, 95, 79, and 57 kd considering the difference in SDS-PAGE between the two parts of soluble and membrane proteins. Novoselov et al. have isolated glycoproteins, which consist of two subunits (gp98 and gp56), from the olfactory epithelium of skate (*25*). They suggested the glycoproteins (gp98/gp56) could be plausible candidates for olfactory receptors of several amino acids involving L-alanine in fish. Chen and Lancet extracted seven proteins from isolated cilia of frog (*Rana ridibunda*) olfactory epithelium and proposed that gp95 is a most prominent component of the olfactory cilia (*26*). Considering that taste transduction has much in common with olfactory transducton at molecular level (8), our present result is very consistent with their results (*25, 26*).

Unfortunately, however, there is no direct evidence at present to confirm that the proteins extracted by the liposome are certainly from only the taste cells, not from other epithelial cells besides tastebud. To make these problems clearer, we are now doing the direct extraction of the taste receptors of taste cells separated from tastebuds and the confocal laser microscopic study of the cell stimulated by taste substances. More detailed studies for characterization and identification of the proteins extracted by the liposome also are in progress using autoradiography of immunoblots, affinity chromatography and piezoelectronic balance methods. Actual aim of this communication is, however, to demonstrate that the *in vivo* direct extraction of membrane proteins is really possible by liposome even from intact tissue. We believe that this method is powerful and widely applicable to study epithelial cell membrane proteins of animal tissue.

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