

DESCRIPTION

A method for screening a salty taste modulating substance

Technical Field

The present invention relates to a method for screening a salty taste modulating substance including a substance that alternatively shows a salty taste, or modifies a salty taste. A salt alternative or a salty taste modulating substance are useful in the field of food industry and so forth.

Background Art

Taste is important for detection of nutritional components or harmful components in foodstuffs. Taste perception of mammals is attained with the taste receptor cells contained in the taste buds present in the oral cavity. The received signals are transmitted from the taste receptor cells to taste nerves entering into the taste buds, and to the central nerve system. Tastes sensed by mammals are generally divided into five kinds of fundamental quality of tastes, that is, sweet taste, bitter taste, acid taste, salty taste, and umami taste, and these are called five basic tastes.

The receptors for these five basic tastes are being elucidated in accordance with the progress of researches in recent years (Non-patent documents 1 and 2). Identification and isolation of novel taste receptors enable novel methods of modulating taste perception. For example, search of highly affinitive agonists or antagonists using taste receptors enables screening for taste modulating substances. Such taste modulating substances may provide improvement or refinement of quality of tastes in various consumer products.

Salty taste, one of the five basic tastes, is involved in detection of sodium ions or other inorganic cations, and is very important for maintenance of internal

homeostasis. It is considered that, as salty taste perception pathways, there are the amiloride-sensitive pathway which is inhibited by diuretic amiloride, and the amiloride-insensitive pathway which is not affected by amiloride (Non-patent document 3). Molecules involved in both the salty taste perception pathways exist and function in the taste receptor cells in the taste buds (Non-patent document 2).

It is considered that the amiloride-sensitive pathway is mediated by the epithelial sodium channel (ENaC). ENaC is composed of four kinds of subunits, α , β , γ , and δ , and functions as a hetero-multimer consisting of a combination of α , β and γ , or δ , β and γ (Non-patent document 13). However, although marked inhibition of the salty taste perception by amiloride is observed in rodents, such inhibition is observed in only a part of humans, and presence of a different receptor in the human salty taste perception mechanism is suggested. As described above, a significant part of the salty taste perception mechanism including receptors still remains unknown.

Excessive intake of salt from foodstuffs is considered as one of the risk factors of hypertension or cardiovascular system diseases, and there is movement of restricting salt consumption worldwide, including Japan (World Health Organization, Non-patent document 4).

There have been conventional techniques for decreasing intake of salt, for example, low-salt seasonings and low-salt food using potassium chloride as a substitute for sodium chloride. However, potassium chloride has a problem that it has a bitter taste and an irritating taste. Therefore, taste of food using potassium chloride is markedly inferior. In order to improve this drawback, there have been developed a seasoning composition in addition to potassium chloride, such as a mixture of ammonium chloride, calcium lactate, sodium L-aspartate, an L-glutamic acid salt and/or a nucleic acid type taste substance at a specific ratio

(Patent document 1), a low sodium salty taste seasoning containing ascorbic acid (Patent document 2), a bitterness suppressing method using carrageenan (Patent document 3), and so forth. However, salt reduction techniques do not reach such a level that unpleasant tastes other than salty taste are eliminated, and salty taste intensity equivalent to that of sodium chloride is provided at the same time.

Furthermore, there are salt reduction methods using a salty taste enhancing substance, which does not reduce salty taste intensity even if sodium chloride is reduced. For example, it is known that a basic amino acid, especially L-arginine, has an effect of enhancing salty taste (Non-patent documents 5 and 6). As techniques applying the above knowledge, there have been developed a combination of L-arginine, L-aspartic acid and sodium chloride (Patent document 4), a taste improving agent using neutralized salts of a basic amino acid and citric acid (Patent document 5), and so forth. However, any technique that can sufficiently compensate insufficiency by reduction of sodium chloride has not been developed yet, in view of salt reduction effect, flavor, salty taste intensity and so forth.

Meanwhile, the Kv3 family is known as a family of potassium channel that is originally considered to function in a nerve cell that shows stimulation at high frequency, and release potassium ions when cell membrane potential is depolarized to -20 mV or higher to show outward current (Non-patent document 7). However, for these, only the function as a voltage-dependent potassium channel is known (Non-patent documents 8 to 11), and a function as a cation channel depending on extracellular sodium concentration around the resting membrane potential (about -60 to -80 mV) is not known at all.

In connection with taste, expression of Kv3.1 and Kv3.2 genes confirmed by PCR in taste cells isolated from rat fungiform papillae has been reported (Non-patent document 12). However, functions thereof in the taste

cells are not known at all.

Prior Art References

Patent documents

Patent document 1: Japanese Patent Laid-open (KOKAI) No. 11-187841

Patent document 2: Japanese Patent Laid-open No. 1-281054

Patent document 3: Japanese Patent Laid-open No. 4-262758

Patent document 4: U.S. Patent No. 5,145,707

Patent document 5: Japanese Patent Laid-open No. 2003-0144088

Non-patent documents

Non-patent document 1: Chandrashekar, J. et al., Nature, 444:288-294 (2006)

Non-patent document 2: Bachmanov, A.A. et al., Ann. Rev. Nutr, 27:387-412 (2007)

Non-patent document 3: DeSimone, J.A. et al., Am. J. Physiol. Regulatory Integrative Comp. Physiol., 249: 52-61 (1985)

Non-patent document 4: Reducing salt intake in populations - Report a WHO Forum and Technical Meeting, [online], [searched on September 20, 2009], Internet URL: <http://www.who.int/dietphysicalactivity/reducingsalt/en/>

Non-patent document 5: Riha, W. et al., Chem. Senses, 22:778 (1997)

Non-patent document 6: Estrella, N.L. et al., Chem. Senses, 34:A117-8 (2009)

Non-patent document 7: Rudy, B. et al., Trends in Neuroscience, 24:517-526 (2001)

Non-patent document 8: Gutman, G.A. et al., Pharmacological Reviews 57:473-508 (2005)

Non-patent document 9: McCormack, T. et al., Proc. Natl. Acad. Sci. USA., 87:5227-5231 (1990)

Non-patent document 10: HERNANDEZ-PINEDA, R. et al.,

J. Neurophysiol., 82:1512-1528 (1999)

Non-patent document 11: Madeja, M. et al.,
Neuropharmacology, 39:202-210 (2000)

Non-patent document 12: Liu, L. et al., Am. J.
Physiol. Cell Physiol., 289:868-880 (2005)

Non-patent document 13: Stahler, F. et al.,
Chemosensory Perception, 1:78-90 (2008)

Summary of the Invention

Object to be Achieved by the Invention

An object of the present invention is to identify a salty taste receptor protein and a gene encoding the protein, and provide a screening system for searching a compound that enhances or inhibits salty taste perception or a salt alternative substance.

Means for Achieving the Object

The inventors of the present invention conceived that if a salty taste receptor or a molecule controlling such a receptor was used, it became possible to efficiently search and identify a compound that could enhance or inhibit salty taste perception or a salt alternative substance, which can result in development of effective salt reduction method that did not alter tastes, and as a result, enable production of foodstuffs having a low salt concentration with maintaining tastes. Then, they conducted various researches, as a result, succeeded in identifying molecules involved in salty taste perception, and thus accomplished the present invention.

The present invention is as follows.

- (1) A method for screening a salty taste modulating substance, which comprises the step of contacting a test substance with a cell that expresses a Kv3.2 protein, and comparing observed cation influx into the cell with cation influx into the cell observed when the test substance is not contacted with the cell.
- (2) The method according to (1), wherein the cation influx

is measured by measuring cell membrane current of the cell in the presence of sodium ions.

(3) The method according to (1), wherein the cation influx is measured by measuring cell membrane current of the cell in the absence of sodium ion.

(4) The method according to (1) or (2) described above, wherein the salty taste modulating substance is a salty taste enhancing substance or a salty taste inhibiting substance.

(5) The method according to (1) or (3) described above, wherein the salty taste modulating substance is a salt alternative substance.

(6) The method as described above, wherein the Kv3.2 protein has the amino acid sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 40, 47, 49, 51 or 53.

(7) The method as described above, wherein the Kv3.2 protein shows a sequence identity of at least 78% or more to the sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 40, 47, 49, 51 or 53, and is able to constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration.

(8) The method as described above, wherein the Kv3.2 protein has the amino acid sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 40, 47, 49, 51 or 53 including substitutions, deletions, insertions and/or additions of one or more amino acid residues, and is able to constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration.

(9) The method as described above, wherein the Kv3.2 protein is encoded by a DNA shown in the following (a) or (b):

(a) a DNA having the nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 39, 46, 48, 50 or 52;

(b) a DNA which is able to hybridize with the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 39, 46, 48, 50 or 52, or a probe which can be prepared from

the nucleotide sequence under stringent conditions, and codes for a protein that can constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration.

(10) The method as described above, wherein the Kv3.2 protein is selected from Kv3.2 protein homologues derived from human, mouse, rat, frog of the genus *Xenopus*, dog, horse, chimpanzee, rhesus monkey, fowl, opossum, swine or bovine, and a mutant thereof, a fragment thereof, and a chimera protein thereof.

(11) The method as described above, wherein the cell is an oocyte into which a polynucleotide encoding a Kv3.2 protein is introduced in an expressible form.

(12) The method as described above, wherein the cell expresses a Kv3.2 gene isolated from a tissue selected from taste cell, tongue epithelium, adrenal gland, pineal body, thyroid, melanocyte and kidney.

(13) A Kv3.2 protein variant, which is a protein shown in any of the following (a) to (c):

(a) a protein having the sequence of SEQ ID NO: 47, 49, 51 or 53;

(b) a protein showing a sequence identity of at least 78% or more to the sequence of SEQ ID NO: 47, 49, 51 or 53, and being able to constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration;

(c) a protein which has the amino acid sequence of SEQ ID NO: 47, 49, 51 or 53 including substitutions, deletions, insertions and/or additions of one or more amino acid residues, and is able to constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration.

(14) A DNA encoding a Kv3.2 protein variant, which is a DNA shown in the following (a) or (b):

(a) a DNA having the nucleotide sequence of SEQ ID NO: 46, 48, 50 or 52;

(b) a DNA which is able to hybridize with the

nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 46, 48, 50 or 52, or a probe which can be prepared from the nucleotide sequence under stringent conditions, and codes for a protein that can constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration.

(15) A method for searching a protein having a function of positively or negatively regulating the Kv3.2 activity or a protein that constitutes a salty taste receptor, which comprises identifying a gene showing an expression profile similar to that of the Kv3.2 gene or a gene of which expression is suppressed in a Kv3.2 gene-expressing cell among genes expressed in the Kv3.2 gene-expressing cell.

(16) A method for searching a salty taste modulating substance by identifying a compound that acts on a Kv3.2 protein or a cell expressing the protein.

(17) A method for searching a taste substance or a flavor substance, which comprises identifying a compound that acts on a channel or complex constituted by a Kv3.2 protein and a protein specifically expressed in a taste cell.

(18) An isolated oocyte or taste cell into which a polynucleotide encoding a Kv3.2 protein is introduced in an expressible form.

Effect of the Invention

A salty taste receptor protein identified according to the present invention constitutes a salty taste receptor ion channel expressed in a taste cell. A substance that activates this receptor is useful as a candidate substance of salty taste modulating substance, i.e., salt alternative, salty taste enhancer, or salty taste inhibitor. Moreover, if a cell that expresses the receptor on the cell surface is used, a convenient screening system for salt alternative, salty taste enhancer, or salty taste inhibitor can be provided.

Furthermore, a foodstuff containing a salt alternative substance or a salty taste enhancing substance is effective for suppressing excessive intake of salt, and thus prevention of hypertension or circulatory system diseases.

Brief Description of the Drawings

Fig. 1 shows results of RT-PCR indicating expressions of the Kv3.1, Kv3.2, Kv3.3 and Kv3.4 genes in various mouse tissues (electrophoresis photograph): Lane 1: tip of tongue (including fungiform papillae), Lane 2: vallate papillae region, Lane 3: both side portions of tongue (including foliate papillae), Lane 4: tongue epithelium not including taste buds, Lane 5: without reverse transcriptase, Lane 6: kidney.

Fig. 2 shows change of cell membrane current observed for *Xenopus laevis* oocyte expressing a Kv3.2 protein (human) when extracellular sodium ion concentration was changed. KCNC2 indicates injection of Kv3.2 cRNA.

Fig. 3 shows difference of cell membrane current observed for *Xenopus laevis* oocyte expressing a Kv3.2 protein (human) when extracellular sodium ion concentration was changed from 0 mM to 96 mM.

Fig. 4 shows results of in situ hybridization indicating a Kv3.2 gene expression site in mouse tongue epithelium (microphotography).

Fig. 5 shows cell membrane current observed for *Xenopus laevis* oocyte expressing a Kv3.2 protein (human), which was contacted with the low molecular organic compound A. KCNC2 indicates injection of Kv3.2 cRNA.

Fig. 6 shows cell membrane current observed for *Xenopus laevis* oocyte expressing a Kv3.2 protein (*Xenopus laevis*) when extracellular sodium ion concentration was changed. XlKv3.2 indicates injection of *Xenopus laevis* Kv3.2 cRNA.

Embodiments for Carrying out the Invention

Hereafter, the present invention will be explained in detail.

The inventors of the present invention confirmed expression of genes encoding the ion channels Kv3.1, Kv3.2, Kv3.3 and Kv3.4 belonging to the Kv3 family (Rudy et al., Trends in Neuroscience, 24:517-526 (2001)) in a mouse tongue epithelial tissue containing taste buds, which ion channels are potassium channels originally considered to function in a nerve cell that shows stimulation at high frequency, and shows current when cell membrane potential is depolarized to -20 mV or higher, and found that expression of a human Kv 3.2 gene in a *Xenopus laevis* oocyte could be involved in the salty taste perception in the form of change of cell membrane current according to change of extracellular sodium ion concentration. On the basis of this finding, they found that Kv3.2 functioned as a salty taste receptor ion channel, and could be used for screening a novel salty taste modulating substance. Moreover, they confirmed that a Kv3.2 gene is expressed in the taste buds, found that, in two kinds of mice of which salty taste response sensitivities were different, there was difference of expression level of the Kv3.2 gene according to the sensitivity, and further confirmed that Kv3.2 is a salty taste receptor. Moreover, the inventors of the present invention newly found splice variants of the Kv3.2 protein in the taste buds.

The method of the present invention comprises the step of contacting a test substance with a cell that expresses a Kv3.2 protein, and comparing observed cation influx into the cell with cation influx into the cell observed when the test substance is not contacted with the cell.

The Kv3.2 protein constitutes a potassium channel, which is a member of the Kv3 family, and it was found by the inventors of the present invention for the first time that the Kv3.2 protein has a cation channel activity,

which changes according to change of extracellular sodium ion concentration. The Kv3.2 protein is a polypeptide encoded by the *Kcnc2* gene (also written as KCNC2).

In the present invention, examples of the Kv3.2 protein include a protein having the amino acid sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 40, 47, 49, 51 or 53.

As polynucleotides encoding human Kv3.2 proteins, four kinds of nucleotide sequences are registered at gene databases (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/> or Ensembl project, <http://www.ensembl.org/index.html>, NM_139136, NM_139137, NM_153748, AY243473). The nucleotide sequences of SEQ ID NOS: 1, 3, 5 and 7 correspond to the above nucleotide sequences. The amino acid sequences encoded by the nucleotide sequences are shown in SEQ ID NOS: 2, 4, 6 and 8. These amino acid sequences have a common sequence except for the C terminal region (portion corresponding to the positions 1 to 538 in SEQ ID NO: 2), and they are considered to be splice variants.

Moreover, in a similar database, one kind of polynucleotide encoding a mouse Kv3.2 protein is registered (NM_001025581, SEQ ID NO: 9). The amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 9 is shown in SEQ ID NO: 10. Moreover, four kinds of nucleotide sequences are registered as polynucleotides encoding rat Kv3.2 proteins (NM_139216, NM_139217, ENSRNOT00000049943, X62839). These nucleotide sequences are shown in SEQ ID NOS: 11, 13, 15 and 17. The amino acid sequences encoded by these nucleotide sequences are shown in SEQ ID NOS: 12, 14, 16 and 18. These amino acid sequences also have a common sequence except for the C terminal region (portion corresponding to the positions 1 to 593 in SEQ ID NO: 12), and they are considered to be splice variants.

Moreover, as shown in the Examples described later, a Kv3.2 protein gene of *Xenopus laevis* was isolated, and

it was demonstrated that this protein had a cation channel activity which changed according to change of extracellular sodium ion concentration. The nucleotide sequence of this gene is shown in SEQ ID NO: 39, and the amino acid sequence of the Kv3.2 protein encoded by this gene is shown in SEQ ID NO: 40.

Furthermore, as shown in the Examples described later, four kinds of splice variants of mouse Kv3.2 gene were isolated from a mouse tongue epithelial tissue containing the taste buds. The nucleotide sequences of these splice variants are shown in SEQ ID NOS: 46, 48, 50 and 52. The amino acid sequences encoded by these nucleotide sequences are shown in SEQ ID NOS: 47, 49, 51 and 53, respectively. The amino acid sequences of SEQ ID NOS: 10, 47, 49, 51 and 53 have a common sequence except for the C terminus regions (portion corresponding to the positions 1 to 597 in SEQ ID NO: 10).

The Kv3.2 protein used for the method of the present invention includes interspecific homologues, and it may be, for example, a Kv3.2 protein derived from dog, horse, chimpanzee, fowl, opossum, swine or bovine, in addition to proteins having the sequences derived from human, mouse, rat and *Xenopus laevis* as described above. The sequence information of the genes encoding these proteins are registered with the following numbers, respectively: dog: XM_538289, horse: XM_001488185, chimpanzee: XR_020952, fowl: XM_001235254, opossum: XM_001363374, XM_001363455, swine: XM_001926426, XM_001924780, and bovine: XM_590276. Moreover, as for rhesus monkey, anole lizard, marmoset, guinea pig, sloth, armadillo, kangaroo rat, *Echinops telfairi*, hedgehog, three-spined stickleback, gorilla, elephant, wallaby, lemur, microbat, pika, rabbit, galago, orangutan, hyrax, fruit bat, shrew, squirrel, zebra finch, *Takifugu oblongus*, tarsier, tree shrew and dolphin, the sequence information on the genes encoding the Kv3.2 protein of them are registered with the following numbers, respectively: rhesus monkey: ENSMMUG00000012362, anole

lizard: ENSACAG00000007691, marmoset: ENSCJAG00000001261,
 guinea pig: ENSCPOG00000003474, sloth: ENSCHOG00000007167,
 armadillo: ENSDNOG000000013383, kangaroo rat:
 ENSDORG00000000056, *Echinops telfairi*: ENSETEG000000010484,
 hedgehog: ENSEEUG00000002220, three-spined stickleback:
 ENSGACG000000019441, gorilla: ENSGGOG00000003896, elephant:
 ENSLAFG000000031982, wallaby: ENSMEUG00000007789, lemur:
 ENSMICG000000017734, microbat: ENSMLUG000000010813, pika:
 ENSOPRG000000017272, rabbit: ENSOCUG00000004467, galago:
 ENSOGAG00000000768, orangutan: ENSPPYG00000004780, hyrax:
 ENSPCAG000000015808, fruit bat: ENSPVAG000000010964, shrew:
 ENSSARG000000006415, squirrel: ENSSTOG00000004842, zebra
 finch: ENSTGUG00000007354, *Takifugu oblongus*:
 ENSTRUG00000003532, tarsier: ENSTSYG000000010689, tree
 shrew: ENSTBEG00000001105, and dolphin: ENSTTRG000000013226.

The gene encoding Kv3.2 is not limited to a gene having the aforementioned gene information or a gene having a known sequence, a gene having a conservative mutation, such as a homologue or an artificially modified version of such a gene as described above can also be used, so long as the Kv3.2 protein encoded by the chosen gene can constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration. That is, it may be a gene encoding a protein having an amino acid sequence of a known protein including substitutions, deletions, insertions, and/or additions of one or more amino acid residues at one or more positions, or the like. The expression that the Kv3.2 protein "can constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration" means that it has an activity of increasing cell membrane potential induced by influx of cations into the cell when the cell expressing the protein is contacted with sodium ions. Examples of the cation include sodium ion, calcium ion, magnesium ion, lithium ion, ammonium ion, and so forth, and sodium ion is preferred.

Although the number of the "one or more" amino acid residues referred to herein may differ depending on the position in the three-dimensional structure of the protein or the types of amino acid residues, specifically, it may be preferably 1 to 20, more preferably 1 to 10, still more preferably 1 to 5, particularly preferably 1 to 3. A typical example of the conservative mutation is conservative substitution. The conservative substitution is a mutation wherein substitution takes place mutually among Phe, Trp, and Tyr, if the substitution site is an aromatic amino acid; among Leu, Ile and Val, if it is a hydrophobic amino acid; between Gln and Asn, if it is a polar amino acid; among Lys, Arg and His, if it is a basic amino acid; between Asp and Glu, if it is an acidic amino acid; and between Ser and Thr, if it is an amino acid having a hydroxyl group. Substitutions considered conservative substitutions include, specifically, substitution of Ser or Thr for Ala, substitution of Gln, His or Lys for Arg, substitution of Glu, Gln, Lys, His or Asp for Asn, substitution of Asn, Glu or Gln for Asp, substitution of Ser or Ala for Cys, substitution of Asn, Glu, Lys, His, Asp or Arg for Gln, substitution of Gly, Asn, Gln, Lys or Asp for Glu, substitution of Pro for Gly, substitution of Asn, Lys, Gln, Arg or Tyr for His, substitution of Leu, Met, Val or Phe for Ile, substitution of Ile, Met, Val or Phe for Leu, substitution of Asn, Glu, Gln, His or Arg for Lys, substitution of Ile, Leu, Val or Phe for Met, substitution of Trp, Tyr, Met, Ile or Leu for Phe, substitution of Thr or Ala for Ser, substitution of Ser or Ala for Thr, substitution of Phe or Tyr for Trp, substitution of His, Phe or Trp for Tyr, and substitution of Met, Ile or Leu for Val. The aforementioned amino acid substitutions, deletions, insertions, additions, inversions or the like may be a result of a naturally-occurring mutation or a variation due to an individual difference or difference of species of a microorganism from which the genes are derived (mutant or variant).

Such genes can be obtained by, for example, modifying a known nucleotide sequence of a gene by site-specific mutagenesis so that the amino acid residues at the specific sites of the encoded protein include substitutions, deletions, insertions, or additions of amino acid residues.

Furthermore, such genes having a conservative mutations as described above may be a gene encoding a protein having a homology of 78% or more, preferably 80% or more, more preferably 90% or more, still more preferably 95% or more, further preferably 97% or more, particularly preferably 99% or more, to the entire encoded amino acid sequence and having a function equivalent to that of the wild-type Kv3.2 protein. In this specification, the term "homology" may mean "identity".

Moreover, codons in the gene sequences may be replaced with other codons which are easily used in the host into which the genes are introduced.

The gene having a conservative mutation may be a gene obtained by a method usually used for mutation treatment, such as treatment with a mutagen. Furthermore, the Kv3.2 gene may contain one or more introns, so long as the Kv3.2 protein can be expressed when the gene is introduced into a host cell.

Examples of the DNA encoding Kv3.2 (henceforth also referred to as "Kv3.2 gene") include a polynucleotide that is able to hybridize with a nucleotide sequence complementary to a known gene sequence, or a probe which can be prepared from the complementary sequence under stringent conditions, and codes for a protein having a function equivalent to that of a known Kv3.2 protein.

The "stringent conditions" are conditions under which a so-called specific hybrid is formed, and a non-specific hybrid is not formed. Although it is difficult to definitely define the conditions with numerals, examples of the stringent conditions include those under which highly homologous polynucleotides hybridize to each

other, for example, polynucleotides not less than 78% homologous, preferably not less than 80% homologous, more preferably not less than 90% homologous, still more preferably not less than 95% homologous, particularly preferably not less than 97% homologous, hybridize to each other, and polynucleotides less homologous than the above do not hybridize to each other, or conditions of washing once, preferably 2 or 3 times, at a salt concentration and temperature corresponding to washing of typical Southern hybridization, i.e., 1 x SSC, 0.1% SDS at 60°C, preferably 0.1 x SSC, 0.1% SDS at 60°C, more preferably 0.1 x SSC, 0.1% SDS at 68°C.

As the probe, a part of the sequence which is complementary to the gene can also be used. Such a probe can be prepared by PCR using oligonucleotides prepared on the basis of a known gene sequence as primers and a DNA fragment containing the nucleotide sequence as a template. For example, when a DNA fragment having a length of about 300 bp is used as the probe, the washing conditions of hybridization may be 50°C, 2 x SSC and 0.1% SDS.

When various Kv3 family proteins were compared for homology, the Kv3.2 protein homologues of the aforementioned various animals showed a homology (identity) of 78% or more, but when the Kv3.2 protein and other Kv3 family proteins were compared, the highest homology was 74%.

A Kv3.2 gene can be obtained by designing appropriate primers or probe on the basis of information on nucleotide sequence of a known Kv3.2 gene or a novel Kv3.2 gene disclosed in this specification, such as a polynucleotide encoding a polypeptide having the amino acid sequence shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 40, 47, 49, 51 or 53, and performing polymerase chain reaction (PCR), Southern hybridization, or Northern hybridization by using the primers or probe and a sample (for example, total RNA, mRNA fraction, cDNA library) derived from an objective organism, for example, a mammal

(e.g., human, mouse, rat, dog, horse, chimpanzee, rhesus monkey, fowl, opossum, swine, bovine, etc.).

The Kv3.2 gene may be a fragment or may be a gene encoding a chimera protein of the Kv3.2 protein or a fragment thereof and other protein, so long as the gene codes for a protein that can constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration.

For example, there are splice variants of the Kv3.2 protein as described above, and it is highly possible that the regions on the C-terminal side of the common portions (for example, the positions 1 to 538 of SEQ ID NO: 2) may be deleted or replaced with another sequence.

By introducing a polynucleotide encoding a Kv3.2 protein into an appropriate host cell in an expressible form, and allowing expression of the Kv3.2 protein, a cell having a salty taste receptor ion channel can be obtained. For example, by introducing a linear DNA encoding a Kv3.2 protein or a vector containing a sequence encoding a Kv3.2 protein into a host cell, a Kv3.2 protein can be expressed. Examples of the sequence in an expressible form include a sequence prepared on the basis of the DNA information and comprising a sequence encoding a Kv3.2 protein and sequences required for transcription and translation upstream of the sequence encoding a Kv3.2 protein, so that the Kv3.2 protein can be produced. Furthermore, by injecting a cRNA encoding a Kv3.2 protein into a host cell, a cell having a salty taste receptor ion channel can also be obtained. In this case, the cRNA contains a sequence required for translation on the 5' end side of the cRNA. Examples of the sequence required for transcription include expression control sequences such as promoter and enhancer. Furthermore, it may contain a transcription terminator sequence. Examples of the sequence required for translation include a ribosome-binding site. Furthermore, it may contain, for example, a processing information site, such as RNA splice site, a

polyadenylation site, and so forth, as required. Examples of the promoter include promoters derived from immunoglobulin gene, SV40, adenovirus, bovine papilloma virus, cytomegalovirus, and so forth.

As the cell into which the polynucleotide encoding a Kv3.2 protein is introduced, an animal cell, an insect cell, or yeast are preferred, and an animal cell is particularly preferred. For example, a fraction in which taste cells are concentrated, an isolated taste cell, a tissue isolated from a tissue selected from tongue epithelium, adrenal gland, pineal body, thyroid, melanocyte, and kidney, and so forth may be used. Specific examples of cell, which is considered to enable temporary functional expression, when a recombinant vector that expresses a polynucleotide encoding a Kv3.2 protein is introduced, include *Xenopus laevis* oocyte, Chinese hamster ovary cell (CHO), human embryonic kidney (HEK) cell, Sf-9 insect cell, and so forth. The present invention provides a cell introduced with such a polynucleotide encoding a Kv3.2 protein in an expressible form. As the cell, an oocyte or a taste cell is preferred, and a taste cell is especially preferred for use in screening a salty taste modulating substance.

As the method for introducing a polynucleotide encoding a Kv3.2 protein into such cells, a known method can be used. Techniques required for the operations such as introduction of a polynucleotide into a cell are described in Sambrook, J., Fritsch, E.F. and Maniatis, T., "Molecular Cloning A Laboratory Manual and Second Edition", Cold Spring Harbor Laboratory Press (1989), etc.

As shown in the Examples, a cell that expresses the Kv3.2 protein has a cation channel activity which changes according to change of extracellular sodium ion concentration. Therefore, the Kv3.2 protein expressed in a cell can constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration. Although it is presumed that the Kv3.2

protein constitutes a cation channel of which activity changes according to change of extracellular sodium ion concentration as a multimer, the Kv3.2 protein may be monomer or multimer, so long as the cell that expresses the Kv3.2 protein has the cation channel activity which changes according to change of extracellular sodium ion concentration. The "cation channel" means a channel that allows flow of cations such as sodium ion, calcium ion, and potassium ion into or out of cells.

Furthermore, as shown in the Examples, the gene encoding the Kv3.2 protein is expressed in the taste buds of the tongue, which are taste receptors, and since the expression level thereof is higher in a mouse strain showing higher salty taste perception sensitivity as compared with a mouse strain showing lower salty taste perception sensitivity, it is supported that the Kv3.2 protein is a salty taste receptor protein that controls salty taste perception sensitivity.

Therefore, by identifying a compound that acts on a Kv3.2 protein or a cell expressing it, a salty taste modulating substance can be searched for.

Identification of such a compound can be attained by, in addition to the aforementioned methods, for example, measuring binding between a purified Kv3.2 protein and a test substance. In addition, a compound that acts on the Kv3.2 protein can be efficiently searched by estimating stereostructure thereof with a computer with reference to stereostructure information on related proteins (for example, Kv1.2, Chen et al., Proc. Natl. Acad. Sci. USA, 107:11352-11357 (2010)), and choosing a compound showing affinity to a site that may affect the ion channel activity with a computer.

By contacting a test substance with a cell expressing a Kv3.2 protein obtained as described above, and comparing observed cation influx into the cell with cation influx into the cell observed when the test substance is not contacted with the cell, a salty taste

modulating substance can be screened for.

If the cation influx observed when a test substance is contacted with a cell that expresses the Kv3.2 protein is larger than cation influx into the cell observed when the test substance is not contacted with the cell, it is judged that the test substance activates the salty taste receptor channel, and if the cation influx observed when a test substance is contacted with a cell that expresses the Kv3.2 protein is smaller than cation influx into the cell observed when the test substance is not contacted with the cell, it is judged that the test substance inactivates the salty taste receptor channel.

Furthermore, by identifying a gene showing an expression profile similar to that of the Kv3.2 gene or a gene of which expression is suppressed in a Kv3.2 gene-expressing cell among genes expressed in the Kv3.2 gene-expressing cell, a protein having a function of positively or negatively regulating the Kv3.2 activity or a protein constituting a salty taste receptor can also be searched for.

The Kv3.2 activity means a function of the Kv3.2 protein for constituting a cation channel which changes in a cell that expresses it according to change of extracellular sodium ion concentration. Moreover, the Kv3.2 activity is also an activity of the Kv3.2 protein as a salty taste receptor protein.

The gene showing an expression profile similar to that of the Kv3.2 gene refers to a gene of which tissue specificity of gene expression is similar to tissue specificity of Kv3.2 gene expression. As one of the methods for identifying a gene showing a similar expression profile, a comprehensive gene expression analysis method such as a DNA microarray method can be exemplified. Specifically, such a gene can be identified by extracting RNAs from various tissues and searching them for a gene showing tissue specificity for gene expression similar to that of the Kv3.2 gene using a comprehensive

gene expression analysis method such as a DNA microarray method.

The gene of which expression is suppressed in a Kv3.2 gene-expressing cell refers to a gene of which expression is suppressed in a cell in which Kv3.2 gene is expressed, and of which expression can be confirmed in a cell in which Kv3.2 gene is not expressed. As one of the methods for identifying such a gene of which expression is suppressed, a comprehensive gene expression analysis method such as a DNA microarray method can be exemplified. Specifically, such a gene can be identified by extracting RNAs from various tissues and searching them for a gene of which expression is not observed in a Kv3.2 gene-expressing tissue using a comprehensive gene expression analysis method such as a DNA microarray method.

Such a gene as identified as described above is a gene encoding a protein having a function of controlling the Kv3.2 activity or a protein that constitutes a salty taste receptor, and it is estimated that such a protein constitutes a channel or complex together with the Kv3.2 protein, and is involved in taste perception.

Furthermore, by identifying a compound that acts on a channel or complex constituted by the Kv3.2 protein as a taste receptor and a protein specifically expressed in a taste cell, a taste substance or a flavor substance can also be searched for. The flavor substance is a substance that modifies tastes, and it can be used for foodstuffs as a flavor additive. As the taste substance or flavor substance, specifically, a salty taste modulating substance can be exemplified.

It is known that a substance that activates a taste receptor promotes excitation of a taste receptor cell, and thereby shows a taste or enhances a taste. Therefore, by using a cell in which a salty taste receptor channel is expressed on the cell membrane, a substance that activates or inactivates a salty taste receptor channel, i.e., a salty taste modulating substance, can be screened for.

That is, a substance that activates a salty taste receptor channel can be expected to promote excitation of a salty taste receptor cell in which a salty taste receptor ion channel is originally expressed and thereby enhance salty taste perception. Therefore, a substance that activates a salty taste receptor channel in the absence of sodium ion is useful as a candidate substance of a salt alternative substance, and a substance that activates a salty taste receptor channel in the presence of sodium ions is useful as a candidate substance of a salty taste enhancing substance. Therefore, a cell that expresses the Kv3.2 protein itself can be used for screening for a salt alternative or salty taste enhancing substance. Moreover, a substance that inactivates a salty taste receptor channel is useful as a candidate of a salty taste inhibiting substance.

Cation influx into a cell that expresses the Kv3.2 protein can be measured by suspending the cell in a solution containing cations, and directly or indirectly measuring amount of cations flowing into the cell.

Cation influx into a cell can be measured by, for example, measuring electrophysiological characteristics of the cell, for example, cell current, in the presence of extracellular cations. For example, by contacting a cell expressing the Kv3.2 protein with a test substance in the presence of sodium ions, and detecting change in cell membrane potential or membrane current, or intracellular cation concentration, a salty taste modulating substance such as a salty taste enhancing substance or a salty taste inhibiting substance can be screened for. Moreover, by contacting a cell expressing the Kv3.2 protein with a test substance in the absence of sodium ion, and detecting change in cell membrane potential or membrane current, or intracellular cation concentration, a salt alternative substance can be screened for.

Specific methods for screening for a salty taste modulating substance using a cell expressing the Kv3.2

protein are exemplified below.

(1) A *Xenopus laevis* oocyte is allowed to express cRNA encoding the Kv3.2 protein, and a ligand that acts on a salty taste receptor ion channel is searched for on the basis of increase or decrease of the salty taste receptor ion channel current according to the two electrode voltage clamp method. For example, if inward current increases when a test compound is added to an extracellular fluid of a cell of which membrane voltage is clamped at a holding potential of -60 to 80 mV, as compared with the case where the test compound is not added, it can be judged that the test compound is a substance that activates the polypeptide of the present invention. Alternatively, the extracellular sodium ion concentration may be changed stepwise or continuously, and inward current may be measured.

(2) With a cell where a salty taste receptor ion channel is expressed on the cell surface, a ligand that acts on a salty taste receptor ion channel is searched for on the basis of increase or decrease of the salty taste receptor ion channel current with a membrane potential clamped according to the voltage clamp method, especially the whole cell membrane voltage clamp method. For example, if inward current increases when a test compound is added to an extracellular fluid of a cell of which membrane voltage is clamped at a holding potential of -80 mV in the presence of sodium ions, as compared with the case where the test compound is not added, it can be judged that the test compound is a substance that activates the polypeptide of the present invention, i.e., a salty taste enhancing substance.

(3) A cell where a salty taste receptor ion channel is expressed on the cell surface is allowed to incorporate a membrane potential sensitive dye beforehand, then change

of fluorescence intensity of the dye in the cell is analyzed (namely, measured or detected) when a test compound is added in the presence of sodium ions, and thereby whether the Kv3.2 protein is activated or not is analyzed. This method uses a property of the membrane potential sensitive dye that it enables optical detection of change of the membrane potential in connection with the opening of the ion channel. More specifically, as the membrane potential sensitive dye, DiBAC [bis-(1,3-dibutylbarbituric acid)trimethineoxonol, Molecular Probes] or a derivative thereof, Membrane Potential Assay Kit (Molecular Devices) etc. can be used.

(4) A cell where a salty taste receptor ion channel is expressed on the cell surface is allowed to incorporate a cation sensitive dye (for example, SBFI, CoroNa Green, Sodium Green (Molecular Probes), etc.) beforehand, and a salty taste enhancing or inhibiting substance is searched for on the basis of change of fluorescence intensity ratio (intracellular cation concentration) at the time of contacting a ligand candidate compound and a salty taste receptor ion channel expressing cell for a certain period in the presence of sodium ions.

(5) By performing the methods of (2) to (4) described above in the absence of sodium ion, a salt alternative substance can be searched for.

Examples

Hereafter, the present invention will be specifically explained with reference to examples. However, the examples do not limit the scope of the present invention. Unless otherwise indicated, the procedures of the examples were carried out by known methods (Maniatis, T. et al., "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory, NY, 1-1982; and Hille, B., Ionic Channels of Excitable Membranes,

2nd Ed., Sinauer Associates Inc., MA, 1992).

Example 1: Expression analysis of mouse Kv3 family channel genes

Expression distribution of the Kv3 family channel gene encoding a protein having the amino acid sequence shown in SEQ ID NO: 10 in the tongue epithelium was analyzed by RT-PCR according to the following procedures using a mouse tongue epithelial tissue.

The epithelium was isolated from the tongue of mouse, and a tongue tip portion containing fungiform papillae, a region containing vallate papillae (center rear portion of the tongue), both side portions of the tongue containing foliate papillae, and the epithelium not containing taste buds were further excised, respectively. Separately, the kidney was excised and pulverized.

RNA was extracted from each tissue using an RNA extraction kit (Absolutely RNA Microprep Kit, Stratagene). The extracted RNA was reverse transcribed using SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) to synthesize a first strand cDNA. By using the obtained first strand cDNA as a template, PCR was performed with the combinations of primers shown in Table 1 to amplify cDNAs of the genes of the Kv3.1, Kv3.2, Kv3.3, and Kv3.4 proteins.

Table 1

Gene	Forward primer SEQ ID NO:	Reverse primer SEQ ID NO:
Kv3.1	21	22
Kv3.2	23	24
Kv3.3	25	26
Kv3.4	27	28

PCR was performed by the hot start method using FastStart Taq DNA polymerase (Roche Applied Science). The

nucleotide sequences of the aforementioned primers are specific to a mouse Kv3.1 gene (NM_001112739), mouse Kv3.2 gene (NM_001025581, SEQ ID NO: 9), mouse Kv3.3 gene (NM_008422) and mouse Kv3.4 gene (NM_145922), and allow amplification of the DNA fragments of Kv3.1 (241 bp), Kv3.2 (574 bp), Kv3.3 (204 bp), and Kv3.4 (287 bp), respectively. The GenBank accession numbers are indicated in the parentheses following the gene names.

RT-PCR analysis was performed for mRNAs derived from the tongue tip portion containing fungiform papillae, the region containing vallate papillae (center rear portion of the tongue), the both side portions of the tongue containing foliate papillae, and the epithelium not containing taste buds, and mRNA derived from the kidney. As controls, the same reaction was performed without adding a reverse transcriptase for mRNAs derived from those tissues.

As a result, in the tongue tip portion containing fungiform papillae, the region containing vallate papillae, and the both side portions of the tongue containing foliate papillae, which were epithelial tissues including the taste buds, DNA fragments of expected sizes were amplified for all the proteins, Kv3.1, Kv3.2, Kv3.3 and Kv3.4 (Fig. 1). These results revealed that expression of mRNAs of the ion channel proteins of the Kv3 family was observed in portions containing the taste buds in the tongue epithelium. In particular, it was clarified that the Kv3.2 gene localized only in portions containing taste buds, i.e., the tongue tip portion containing fungiform papillae, the region containing vallate papillae, and the both side portions of the tongue containing foliate papillae, in the tongue epithelium. At the same time, DNA fragments of expected sizes were also amplified for Kv3.1, Kv3.2 and Kv3.3 for the kidney. As for Kv3.4, a DNA fragment of a size different from the expected size was amplified, and there was suggested a possibility of the existence of a splice variant.

Example 2: Isolation of polynucleotide encoding human Kv3.2 protein and construction of expression vector therefor

Although the full length cDNA encoding a human salty taste receptor protein can be cloned from, for example, human mRNA, it can also be purchased as a corresponding full length cDNA among those included in The Mammalian Gene Collection (<http://mgc.nci.nih.gov/>) (MGC: 120670, IMAGE: 7939480, catalog number: MHS1010-98052225, Open Biosystems). By using this commercially available plasmid as a template, an oligonucleotide consisting of the nucleotide sequence shown in SEQ ID NO: 19 (*Kpn*I recognition sequence was added to the 5' end) as a forward primer, and an oligonucleotide consisting of the nucleotide sequence shown in SEQ ID NO: 20 (*Xho*I recognition sequence was added to the 5' end) as a reverse primer, PCR was performed. The obtained DNA fragment was digested with the restriction enzymes *Kpn*I and *Xho*I, and then cloned into the plasmid pcDNA3.1(+). The obtained clone was designated pcDNA3.1-KCNC2. The plasmid pcDNA3.1(+) has a promoter sequence derived from cytomegalovirus, and can be used for expression of a polypeptide encoded by a cloning fragment in an animal cell.

The nucleotide sequence of the obtained clone pcDNA3.1-KCNC2 was analyzed using a DNA sequencer (3130xl Genetic Analyzer, Applied Biosystems) according to the dideoxy terminator method, and the nucleotide sequence shown in SEQ ID NO: 5 was obtained. The nucleotide sequence shown in SEQ ID NO: 5 has an open reading frame consisting of 1677 base pairs. The amino acid sequence predicted from the open reading frame consists of 558 amino acid residues (SEQ ID NO: 6).

Example 3: Expression of Kv3.2 protein in *Xenopus laevis* oocyte

In order to detect the cation channel activity of a protein having the amino acid sequence shown in SEQ ID NO: 5, which activity changes according to the change of extracellular sodium ion concentration, cRNA was synthesized by using the expression vector pcDNA3.1-KCNC2 obtained in Example 2 described above as a template, and injected into a *Xenopus laevis* oocyte, so that the protein was expressed. The aforementioned expression vector pcDNA3.1-KCNC2 was digested with the restriction enzyme *Xho*I at one site and thereby made linear, and then cRNA encoding the Kv3.2 protein was synthesized by using a commercially available cRNA synthesis kit, Megascript High Yield Transcription Kit (Ambion). The synthesized cRNA was injected into a *Xenopus laevis* oocyte prepared in a conventional manner using a glass capillary and a microinjector (World Precision Instruments), so that the Kv3.2 protein was expressed. Furthermore, as a control cell, an oocyte into which water was injected was also prepared in the same manner. The obtained these oocytes were stored in a standard oocyte culture medium such as the ND96+ solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, 2 mM sodium pyruvate, 1/100 penicillin/streptomycin mixture (15140-122, Invitrogen), adjusted to pH 7.4 with NaOH) at 18°C for two to four days, and then used in Examples 4 described below.

Example 4: Detection of cation channel activity of Kv3.2 protein, which activity changes according to change of extracellular sodium ion concentration

The total cell current was measured for each of the oocytes obtained in Example 3, of which membrane potential was clamped by the two electrode voltage clamp method. For this measurement, the ND96 solution (96 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L MgCl₂, 1.8 mmol/L CaCl₂, and 5 mmol/L HEPES (pH 7.5)) was used.

In the *Xenopus laevis* oocyte injected with cRNA prepared using the plasmid pcDNA3.1-KCNC2 as the template,

an inward current higher than 100 nA was measured at a holding potential of -80 mV, when the NaCl concentration in the extracellular fluid was set to be 96 mM. Increase of this current with increase of the extracellular sodium ion concentration was observed, when the NaCl concentration in the extracellular fluid was changed between 0 to 96 mM by replacing NaCl in the aforementioned ND96 solution with N-methyl-D-glucamine hydrochloride (Fig. 2). On the other hand, in the control oocyte injected with water, such a significant inward current was not observed at a holding potential of -80 mV, when the NaCl concentration in the extracellular fluid was set to be 96 mM, and change of the current was not also observed, even when the sodium ion concentration in the extracellular fluid was changed.

Example 5: Confirmation of presence or absence of cation channel activity that changes according to change of extracellular sodium ion concentration for Kv3 family proteins

Expression vectors were constructed for the channels Kv3.1, Kv3.3 and Kv3.4 belonging to the Kv3 family, like Kv3.2, in the same manner as that of Example 2. Full length cDNAs encoding the corresponding proteins were purchased (Kv3.1: catalog number ORK11519, Promega, Kv3.3: catalog number OHS4559-99848374, Open Biosystems, Kv3.4: catalog number MHS1010-98052650, Open Biosystems). By using each of these full length cDNAs as a template and a combination of the primers shown in Table 2, PCR was performed to prepare gene DNA fragments encoding the proteins of Kv3.1, Kv3.3, and Kv3.4, respectively. Both the 5' and 3' ends of each obtained DNA fragment was digested with restriction enzymes (Kv3.1: *Xba*I and *Xho*I, Kv3.3: *Nhe*I and *Eco*RI, Kv3.4: *Nhe*I and *Xho*I), then the digestion product was cloned into the plasmid pcDNA3.1(+). The obtained clones were designated pcDNA3.1-KCNC1 (Kv3.1), pcDNA3.1-KCNC3 (Kv3.3) and pcDNA3.1-KCNC4 (Kv3.4),

respectively.

Table 2

Gene	Forward primer SEQ ID NO:	Reverse primer SEQ ID NO:
Kv3.1	29	30
Kv3.3	31	32
Kv3.4	33	34

cRNAs were synthesized using these plasmids as templates, and injected into *Xenopus laevis* oocytes in the same manner as that of Example 2, so that the proteins were expressed. The aforementioned expression vectors pcDNA3.1-KCNC1, pcDNA3.1-KCNC3 and pcDNA3.1-KCNC4 were digested with a restriction enzyme (Kv3.1: *Xho*I, Kv3.3: *Eco*RI, Kv3.4: *Xho*I) at one site and thereby made linear, and then cRNAs encoding the Kv3.1, Kv3.3 and Kv3.4 proteins were synthesized respectively using a commercially available cRNA synthesis kit, Megascript High Yield Transcription Kit (Ambion). The synthesized cRNAs were each injected into *Xenopus laevis* oocytes prepared in a conventional manner by using a glass capillary and a microinjector (World Precision Instruments), so that the proteins encoded by the cRNAs were expressed. As control cells, an oocyte injected with Kv3.2 in the same manner as that of Example 2 so that the protein was expressed, and an oocyte into which water was injected were also prepared. The obtained oocytes were stored in a standard oocyte culture medium such as the ND96+ solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, 2 mM sodium pyruvate, 1/100 penicillin/streptomycin mixture (15140-122, Invitrogen), adjusted to pH 7.4 with NaOH) at 18°C for two to four days, and then the total cell current was measured for each of the oocytes, of which membrane potential was clamped by the dual electrode voltage clamp method. For this measurement, the ND96 solution (96 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L MgCl₂, 1.8 mmol/L CaCl₂, and 5 mmol/L

HEPES (pH 7.5)) was used.

For the case where the NaCl concentration in the extracellular fluid was set to be 96 mM using ND960, and the case where the NaCl concentration in the extracellular fluid was set to be 0 mM by substitution of N-methyl-D-glucamine hydrochloride for NaCl in the ND96 solution, the difference of current amounts at a holding potential of -80 mV was measured for each oocyte. As a result, only when the cRNA encoding the Kv3.2 protein was injected, a current amount difference of about 80 nA or larger in average was measured (Fig. 3). On the other hand, such current amount difference could not be observed for the control oocyte injected with water. For the oocytes injected with each of cRNAs encoding Kv3.1, Kv3.3 and Kv3.4 proteins, which are the other channels of the Kv3 family, only a current smaller than 15 nA was measured, and significant difference was not observed with respect to the control oocyte injected with water. On the basis of these measurement results, it was confirmed that, among the quite similar Kv3 family channels, only the Kv3.2 showed increase in the current amount in connection with the increase of the extracellular sodium ion concentration.

The above results revealed that the Kv3.2 protein could constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration.

Example 6: Comparison of expression levels of Kv3.2 gene in strains showing difference in salty taste perception sensitivity

A method for measuring mouse salty threshold value by using behavioral procedures has been reported (Ishiwatari, Y. and Bachmanov, A.A., Chemical Senses, 34:277-293 (2009)). Moreover, it has also been reported that there is difference in salty taste perception sensitivity among mouse strains (Ishiwatari, Y., and Bachmanov, A.A., Chemical Senses, 32:A26 (2007)). By

using the aforementioned behavioral method, difference of salty threshold values was measured for two strains showing difference in salty taste perception sensitivity, C57BL/6J (strain showing high salty taste perception sensitivity), and A/J (strain showing low salty taste perception sensitivity). As a result, it was confirmed that there was difference in the threshold value of about 5 times between the both (Table 3). Then, expression amount of the gene encoding the Kv3.2 protein in the tongue epithelium of both the strains were quantified and compared.

Table 3

Strain	Salty threshold value (mM)
C57BL/6J	8.6
A/J	43.3

The epithelia were isolated from the tongues of mice of the two strains (C57BL/6J, A/J), and a tongue tip portion containing the fungiform papillae and epithelium not containing taste buds were excised, respectively, from each of them. RNA was extracted from each tissue using an RNA extraction kit (Absolutely RNA Microprep Kit, Stratagene). The extracted RNA was reverse-transcribed using SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) to synthesize a first strand cDNA. By using the obtained first strand cDNA as a template, quantitative RT-PCR analysis was conducted with TaqMan Gene Expression Assays (Life Technologies) and StepOnePlus Real-time PCR System (Life Technologies). The quantification results were corrected by expression amount of β -actin gene (actb) in each site, and then the expression levels of the strains were compared. Two kinds of assays (Probe 1: Mm01234233_m1, Probe 2: Mm01234232_m1) were performed for the Kv3.2 gene, and as a result, difference was observed between expression levels in the tongue tip portions of both the strains, A/J and C57BL/6J.

The expression level of the strain C57BL/6J was 3 to 5 times higher than the expression level of the strain A/J, and it is considered that this difference in the expression levels affects the difference of the salty taste perception sensitivity (Table 4). On the other hand, no expression was detected in the epithelia not containing the taste buds of both the strains.

Table 4

Site	B6/A (actb normalized)*	
	Probe 1	Probe 2
Tongue tip portion (containing fungiform papillae)	3.43	5.21

* Ratio of expression amounts of B6 (C57BL/6J) and A (A/J) standardized with actb gene expression amount

On the basis of the above results, it was confirmed that the Kv3.2 gene coded for a protein contributing to salty taste perception.

Example 7: Confirmation of expression of mouse Kv3.2 gene in taste buds by in situ hybridization method

A cDNA encoding the full length of the mouse salty taste receptor protein can be cloned by designing a primer set with reference to SEQ ID NO: 9 and a similar sequence registered at a gene database (GenBank accession number: BY281762), and performing PCR with it. By using an oligonucleotide consisting of the nucleotide sequence shown in SEQ ID NO: 35 as a forward primer, an oligonucleotide consisting of the nucleotide sequence shown in SEQ ID NO: 36 as a reverse primer, and the first strand cDNA obtained in Example 6, which was prepared from the tongue tip portion containing the fungiform papillae, as a template, PCR was performed. The obtained DNA fragment was cloned in the plasmid pGEM-T Easy (Promega). The obtained clone was designated pGEM-T-mKcnc2. The nucleotide sequence of the obtained clone was analyzed

using a DNA sequencer (3130xl Genetic Analyzer, Applied Biosystems) according to the dideoxy terminator method, and it was confirmed that the same nucleotide sequence as that of SEQ ID NO: 9 was obtained. Furthermore, the plasmid pGEM-T-mKcnc2 was digested with the restriction enzyme *SphI* at one site and thereby made linear, and an antisense RNA of the mouse Kv3.2 gene labeled with digoxigenin was synthesized by using DIG RNA Labeling Kit (Roche Diagnostics).

From a portion of mouse tongue containing taste buds immersed in a frozen tissue embedding agent and cryopreserved at -80°C , sections were prepared using a cryostat. After a fixation treatment (immersion in 4% paraformaldehyde solution for 10 minutes), the sections were subjected to an acylation treatment for 10 minutes, washed 3 times with PBS for 10 minutes, and then immersed in a hybridization buffer (5 x SSC, 50% formamide, 1 x Denhardt's solution, 1 mg/ml of salmon sperm DNA, 1 mg/ml of tRNA). A cRNA probe was prepared using T7 RNA polymerase in the presence of UTP labeled with digoxigenin. Hybridization was performed at 72°C in a 5 x SSC, 50% formamide solution. For signal detection, a color reaction was performed using an alkaline phosphatase-bound anti-digoxigenin antibody (Roche Diagnostics) and NBT/BCIP as the substrate, and then the sections were observed under a light microscope (BX61, Olympus). As a result of hybridization using the antisense RNA of the mouse Kv3.2 gene labeled with digoxigenin as a probe, signals were detected in the taste buds in a region containing the vallate papillae of the mouse tongue epithelium (Fig. 4). Therefore, it became clear that the Kv3.2 gene was expressed in the taste buds.

From the above results, it was confirmed that the Kv3.2 protein is present in the taste buds. Taking into consideration that the Kv3.2 protein contributes to the salty taste perception sensitivity, and constitutes a cation channel of which activity changes according to

change of extracellular sodium ion concentration, it is concluded that the Kv3.2 protein constitutes a salty taste receptor ion channel. The Kv3.2 protein can be used for screening for a novel salty taste modulating substance.

Example 8: Screening for channel activity modulating compound using Kv3.2

cRNA of the human Kv3.2 channel was injected into a matured oocyte taken out from *Xenopus laevis* (female), and the oocyte was stored in a standard oocyte culture medium such as the ND96+ solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, 2 mM sodium pyruvate, 1/100 penicillin/streptomycin mixture (15140-122, Invitrogen), adjusted to pH 7.4 with NaOH) at 18°C. Two to four days after the injection, the oocyte was set on a parallel clamp system for *Xenopus laevis* oocytes, OpusXpress 6000A (Molecular Devices Japan). The oocyte was perfused with an assay buffer (66 mM N-methyl-D-glucamine hydrochloride, 30 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, adjusted to pH 7.4 with KOH), and the membrane potential was maintained at -80 mV. The cell membrane current was measured in this state, and screening was performed on the basis of detection of change of the current at the time of adding a test substance solution. It is considered that when an inward current is observed for the electric current, the Kv3.2 channel is activated with a compound, and when an outward current is observed, the Kv3.2 channel activity is suppressed. Moreover, in order to distinguish from nonspecific change of electric current due to influence of a test substance on the oocyte itself, the test substance solution was also added to the oocyte injected with water instead of cRNA to confirm that there was no change in the current. For example, a low molecular organic compound A retrieved by the screening increased the current in a concentration dependent manner in the oocyte expressing the human Kv3.2 when the compound was added, but such

change of the current was not confirmed in the oocyte injected with water (Fig. 5).

Example 9: Salty taste enhancing effect of obtained activity modulating compound

Influence on salty taste of the compound for which Kv3.2 channel activity modulating action was found was confirmed by performing a quantitative sensory evaluation test for the compound.

The quantitative sensory evaluation test was performed as follows. The compound A as a sample was mixed in an amount of 0.0001 to 0.002 g/dl with distilled water containing sodium chloride (0.5 g/dl), and intensity of salty taste enhancing activity of the compound was measured. Distilled water containing 0.55 g/dl, 0.60 g/dl or 0.65 g/dl of sodium chloride was used as objects of comparison. A method according to the linear scale method was used, wherein the panelists marked positions corresponding to their grading on a straight line on which positions of sodium chloride concentrations corresponding to 1.0 time (0.50 g/dl), 1.1 times (0.55 g/dl), 1.2 times (0.60 g/dl), and 1.3 times (0.65 g/dl) the concentration of the mixture were indicated. Test scores were obtained by averaging the positions marked by the panelists and indicated as a salty taste enhancing ratio (times). The test was performed with $n = 6$. The panelists consisted of persons who experienced development of flavoring of foodstuffs for one year or more in total, and could distinguish sodium chloride solutions having different concentrations of 0.50 g/dl, 0.55 g/dl and 0.60 g/dl (this ability was periodically confirmed). As the "initial taste", intensity of salty taste within 2 seconds after holding in the mouth was evaluated, and the "middle and after tastes" means the total of middle taste and after taste, and evaluated as intensity of salty taste after 2 seconds from holding in the mouth. The results for typical concentrations are shown in Table 5. As shown by

the results, it was found that addition of the compound A enhanced salty taste intensity in a concentration dependent manner.

Table 5

Sample	Concentration (g/dl)	Salty taste enhancing ratio (times)		Evaluation comment
		Initial taste	Middle and after tastes	
No additive	-	1	1	-
Compound A	0.0001	1.01	1.01	Almost no effect of addition
	0.001	1.04	1.04	Salty taste was slightly enhanced, after taste included bitter taste
	0.002	1.05	1.07	Salty taste was enhanced in middle and after tastes, after taste included bitter taste

Example 10: Cloning of *Xenopus laevis* Kv3.2 gene

There has been no information on cDNA encoding the Kv3.2 channel protein of *Xenopus laevis*. However, in the gene database of the closely related species, *Xenopus tropicalis* (<http://genome.jgi-psf.org/Xentr4/Xentr4.home.html>), a nucleotide sequence predicted to code for Kv3.2 (C_scaffold_541000003) is registered. With reference to this sequence as well as the gene sequence information on the human, mouse and rat Kv3.2 genes, PCR was performed using an oligonucleotide consisting of the nucleotide sequence shown in SEQ ID NO:

37 as a forward primer, an oligonucleotide consisting of the nucleotide sequence shown in SEQ ID NO: 38 as a reverse primer, and cDNA of *Xenopus laevis* brain as a template. The obtained DNA fragment was cloned into the plasmid pGEM-T Easy. The obtained clone was designated pGEM-T-xlKcnc2. The nucleotide sequence of the obtained clone was analyzed by using a DNA sequencer (3130xl Genetic Analyzer, Applied Biosystems) according to the dideoxy terminator method, and it was confirmed that the nucleotide sequence as shown in SEQ ID NO: 31 was obtained. The nucleotide sequence shown in SEQ ID NO: 39 has an open reading frame consisting of 1716 base pairs. The amino acid sequence predicted from the open reading frame consists of 571 amino acid residues (SEQ ID NO: 40).

Example 11: Expression of *Xenopus laevis* Kv3.2 protein in *Xenopus laevis* oocyte

In order to detect the cation channel activity of a protein having the amino acid sequence shown in SEQ ID NO: 40, which activity changes according to the change of extracellular sodium ion concentration, cRNA was synthesized by using the clone obtained in Example 10 described above as a template, and injected into a *Xenopus laevis* oocyte, so that the protein was expressed. PCR was performed using the aforementioned plasmid pGEM-T-xlKcnc2 as a template, an oligonucleotide consisting of the nucleotide sequence shown in SEQ ID NO: 41 as a forward primer, and an oligonucleotide consisting of the nucleotide sequence shown in SEQ ID NO: 42 as a reverse primer. The obtained DNA fragment was digested with the restriction enzymes *NheI* and *XhoI*, and then cloned into the plasmid pcDNA3.1(+) (Invitrogen). The obtained clone was designated pcDNA3.1-xlKcnc2. The obtained plasmid was digested with the restriction enzyme *XhoI* at one site and thereby made linear, and then cRNA encoding the Kv3.2 protein was synthesized using a commercially available cRNA synthesis kit, Megascript High Yield Transcription

Kit (Ambion). The synthesized cRNA was injected into a *Xenopus laevis* oocyte prepared in a conventional manner using a glass capillary and a microinjector (World Precision Instruments), so that the Kv3.2 protein was expressed. Furthermore, as a control cell, an oocyte into which water was injected was also prepared in the same manner. The obtained these oocytes were stored in a standard oocyte culture medium such as the ND96+ solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, 2 mM sodium pyruvate, 1/100 penicillin/streptomycin mixture (15140-122, Invitrogen), adjusted to pH 7.4 with NaOH) at 18°C for two to four days, and then used in Examples 12 described below.

Example 12: Detection of cation channel activity of *Xenopus laevis* Kv3.2 protein, which changes according to change of extracellular sodium ion concentration

The total cell current was measured for each of the oocytes obtained in Example 11, of which membrane potential was clamped by the dual electrode voltage clamp method. For this measurement, the ND96 solution (96 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L MgCl₂, 1.8 mmol/L CaCl₂, and 5 mmol/L HEPES (pH 7.5)) was used. In the *Xenopus laevis* oocyte injected with cRNA prepared using the plasmid pcDNA3.1-*xlKcnc2* as the template, an inward current higher than 100 nA was measured at a holding potential of -80 mV, when the NaCl concentration in the extracellular fluid was set to be 96 mM. When the NaCl concentration in the extracellular fluid was 0 mM, this current was not observed, and decrease in the current amount was observed with decrease of the extracellular sodium concentration (Fig. 6). On the other hand, in the control oocyte injected with water, such a significant inward current as described above was not observed at a holding potential of -80 mV, when the NaCl concentration in the extracellular fluid was set to be 96 mM, and change of the current amount was not also observed, even when the

sodium ion concentration in the extracellular fluid was changed to 0 mM.

The above results demonstrated that the *Xenopus laevis* Kv3.2 protein could constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration.

Example 13: Cloning of mouse Kv3.2 gene expressed in taste buds

One kind of mouse Kv3.2 gene has been registered (SEQ ID NO: 9). As for human, four kinds of genes considered to be splice variants are registered at a gene database (SEQ ID NOS: 1, 3, 5 and 7) as Kv3.2 genes, and a plurality of splice variants may similarly exist for mouse. Therefore, for the purpose of obtaining Kv3.2 genes that were expressed and functioned in the mouse taste buds, mouse Kv3.2 genes were cloned from the tongue epithelium containing the taste buds. First, the epithelium was isolated from the tongue of mouse, a portion containing the taste buds was excised, and RNA was extracted using an RNA extraction kit (Absolutely RNA Microprep Kit, Stratagene). The extracted RNA was reverse-transcribed by using SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) to synthesize a first strand cDNA. By using the obtained first strand cDNA as a template, cDNA was amplified by PCR. As a forward primer, an oligonucleotide consisting of the nucleotide sequence shown in SEQ ID NO: 35 was used, and as a reverse primer, an oligonucleotide consisting of the nucleotide sequence shown in SEQ ID NO: 36, and oligonucleotides consisting of the nucleotide sequences shown in SEQ ID NOS: 43, 44 and 45, respectively, were used. The oligonucleotides consisting of the nucleotide sequences shown in SEQ ID NOS: 43, 44 and 45 were designed as reverse primers corresponding to mouse Kv3.2 splice variants expected from comparison of human Kv3.2 gene sequences (SEQ ID NOS: 1, 3, 5 and 7) and the mouse genomic DNA sequence containing the

mouse Kv3.2 gene (GenBank accession number: NC_000076). For PCR, Phusion Hot Start High-Fidelity DNA Polymerase (New England Biolabs) was used.

Example 14: Splice variants of mouse Kv3.2 gene isolated from taste buds

The obtained DNA fragments were cloned into the plasmid pGEM-T Easy. The nucleotide sequences of the obtained 30 clones were analyzed using a DNA sequencer (3130xl Genetic Analyzer, Applied Biosystems) according to the dideoxy terminator method, and they were roughly classified into 5 groups of similar kinds of nucleotide sequences. The five groups consisted of sequences having the same nucleotide sequence as that of SEQ ID NO: 9, which is uniquely registered at the gene database as the mouse Kv3.2 gene, and sequences having open reading frames shown in SEQ ID NOS: 46, 48, 50 and 52, respectively. The lengths of the nucleotide sequences and the lengths of the amino acid sequences predicted from the open reading frames (SEQ ID NOS: 47, 49, 51 and 53) are as shown in Table 6. These amino acid sequences have a common sequence except for the C terminal regions (part corresponding to the positions 1 to 597 of SEQ ID NO: 10), and they are considered as splice variants. These splice variants of the mouse Kv3.2 gene cloned from the taste buds may form a salty taste receptor channel by themselves or as a complex with another variant.

Table 6

	Nucleotide sequence		Amino acid sequence	
	SEQ ID NO:	Sequence length	SEQ ID NO:	Sequence length
Mouse Kv3.2	9	1920	10	639
Splice variant 1	46	1929	47	642
Splice variant 2	48	1854	49	617
Splice variant 3	50	1845	51	614
Splice variant 4	52	1968	53	655

Claims

1. A method for screening a salty taste modulating substance, which comprises the step of contacting a test substance with a cell that expresses a Kv3.2 protein, and comparing observed cation influx into the cell with cation influx into the cell observed when the test substance is not contacted with the cell.
2. The method according to claim 1, wherein the cation influx is measured by measuring cell membrane current of the cell in the presence of sodium ions.
3. The method according to claim 1, wherein the cation influx is measured by measuring cell membrane current of the cell in the absence of sodium ion.
4. The method according to claim 1 or 2, wherein the salty taste modulating substance is a salty taste enhancing substance or a salty taste inhibiting substance.
5. The method according to claim 1 or 3, wherein the salty taste modulating substance is a salt alternative substance.
6. The method according to any one of claims 1 to 5, wherein the Kv3.2 protein has the amino acid sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 40, 47, 49, 51 or 53.
7. The method according to any one of claims 1 to 5, wherein the Kv3.2 protein shows a sequence identity of at least 78% or more to the sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 40, 47, 49, 51 or 53, and is able to constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration.

8. The method according to any one of claims 1 to 5, wherein the Kv3.2 protein is has the amino acid sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 40, 47, 49, 51 or 53 including substitutions, deletions, insertions and/or additions of one or more amino acid residues, and is able to constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration.

9. The method according to any one of claims 1 to 5, wherein the Kv3.2 protein is encoded by a DNA shown in the following (a) or (b):

(a) a DNA having the nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 39, 46, 48, 50 or 52;

(b) a DNA which is able to hybridize with the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 39, 46, 48, 50 or 52, or a probe which can be prepared from the nucleotide sequence under stringent conditions, and codes for a protein that can constitute a cation channel of which activity changes according to change of extracellular sodium ion concentration.

10. The method according to any one of claims 1 to 5, wherein the Kv3.2 protein is selected from Kv3.2 protein homologues derived from human, mouse, rat, frog of the genus *Xenopus*, dog, horse, chimpanzee, rhesus monkey, fowl, opossum, swine or bovine, a mutant thereof, a fragment thereof, and a chimera protein thereof.

11. The method according to any one of claims 1 to 10, wherein the cell is an oocyte into which a polynucleotide encoding a Kv3.2 protein is introduced in an expressible form.

12. The method according to any one of claims 1 to 10, wherein the cell expresses a Kv3.2 gene isolated from

a tissue selected from taste cell, tongue epithelium, adrenal gland, pineal body, thyroid, melanocyte and kidney.

13. A method for searching a salty taste modulating substance, which comprises identifying a compound that acts on a Kv3.2 protein, or a cell expressing the protein.

14. A method for searching a taste substance or a flavor substance, which comprises identifying a compound that acts on a channel or complex which is constituted by a Kv3.2 protein and a protein specifically expressed in a taste cell.

ABSTRACT

By contacting a test substance with a cell that expresses a Kv3.2 protein, and comparing observed cation influx into the cell with cation influx into the cell observed when the test substance is not contacted with the cell, a salty taste modulating substance such as salt alternative compound, or salt perception enhancing or inhibiting compound is screened.