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IDENTIFICATION OF NOVEL MEMBERS OF G-PROTEIN COUPLED RECEPTOR SUPERFAMILY EXPRESSED IN BOVINE TASTE TISSUE

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SUMMARY : Using reverse transcription-polymerase chain reaction (RT-PCR) and degenerate oligonucleotide primers, we amplified novel members of two different subfamily of G-protein coupled receptor (GCR) superfamily from bovine taste tissue. Type A receptor clones composed of multiple cDNA clones had significant similarity with putative olfactory receptor subfamily, while a single type B clone had significant similarity with peptide receptor subfamily. Physiological functions of these receptors in taste cells are discussed.

In vertebrate taste systems, varieties of taste stimuli are received by taste receptor cells in taste buds located on the tongue epithelium. Several observations have suggested the involvement of G-protein mediated signaling system in taste transduction, especially for sweet and bitter stimuli (1,2). Therefore, it is highly plausible that some of the receptors for taste stimuli belong to the superfamily of G-protein coupled receptor (GCR). Furthermore, it is plausible that GCRs are involved in modulating taste sensitivity or regeneration of taste cells. GCRs are characterized by the presence of seven transmembrane (TM) domains which share conserved amino acid residues, especially in TM2, TM3, TM6 and TM7 (3,4). Recent development of polymerase chain reaction (PCR) using degenerate oligonucleotide primers deduced from these domains enabled the identification of new members of GCR superfamily (5,6). Herein, making use of the PCR technology, we identified novel members of two different subfamilies of GCR superfamily expressed in bovine taste tissues which show significant similarities with the putative odorant receptor subfamily and neuropeptide receptor subfamily.

MATERIALS AND METHODS

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from the bovine tongue taste papillae (circumvallate and fungiform papillae) according to the method of Chomczynski and Sacchi (7). To avoid false amplification from contaminating genomic DNA in the following PCR

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experiments, the taste RNA was treated with DNase I. Single-stranded cDNA was synthesized from the taste RNA with random hexamer and MMLV reverse transcriptase. Then, the cDNA was subjected to series of PCR experiments using degenerate oligonucleotide primers corresponding to the transmembrane (TM) domains of the G-protein coupled receptors as shown below: C1 (olfactory receptor TM3), 5'-TT(T/C)(T/C)TI(T/C)TIGTIGCIATIGCIT(A/T)(T/C)GA(T/C)(A/C)GITA-3'; D1 (olfactory receptor TM6), 5'-ACIACI(G/C)(A/T)IA(A/G)(A/G)TGI(G/C)(A/T)ICC-(A/G)CAIGTIGA(A/G)AA-3'; A1 (rhodopsin TM2), 5'-AA(T/C)T(G/A)(G/C)ATI(C/A)-TI(G/C)TIAA(T/C)(C/T)TIGCIGTIGCIGA-3'; B4 (thyrotropin receptor TM7), 5'-GC-(C/T)TTIGT(A/G)AAIATIGC(A/G)TAIAG(G/A)AAIGG(G/A)TT-3'. PCR was performed according to the following scheme: denaturation at 94 °C for 2 min, then, 5 cycles of 94 °C (1 min), 45 °C (1.5 min) and 72 °C (3 min) were followed by 40 cycles of 94 °C (1 min), 55 °C (1.5 min) and 72 °C (3 min). Amplified products of each PCR reaction was analyzed by agarose gel electrophoresis, and bands of interest were extracted and cloned into plasmid vector (pCR1000, INVITROGEN). Individual cDNA clones were sequenced by the dideoxy method (8).

Northern blot analysis

Poly $(A)^+$ RNAs (mRNAs) from various tissues were prepared by using oligo(dT)-latex beads (Oligotex-dT30, Japan Roche). Poly$(A)^+$ RNAs (10 μg per tissue) were electrophoresed on agarose gels and transferred to nylon membranes (8). The membranes were fixed by UV irradiation, prehybridized in the presence of 50 % formamide and 1 % SDS at 65 °C for 1 hr. Then, hybridization was carried out under the same condition with ^{32}P-labeled cRNA probe transcribed from pCR1000 plasmid clones containing PCR amplified cDNA fragments. The membrane was washed at 64 °C in 0.2 x SSC and 0.1 % SDS.

Screening of cDNA library

A cDNA library which consists of 6×10^5 independent clones was constructed in the λZAP cloning vector (Stratagene) using 5 μg poly$(A)^+$ RNA prepared from the bovine taste papillae total RNA. The cDNA library was screened with a ^{32}P-labelled cDNA probe (random prime kit, Boehringer) which was a PCR product obtained using A1 and B4 primers. A single positive phage clone was obtained, and converted to pBluscriptII SK(-) plasmid according to the instructions of the manufacturer (Stratagene).

RESULTS AND DISCUSSION

To identify G-protein coupled receptors expressed in mammalian taste tissue, RT-PCR was performed with total RNA prepared from bovine circumvallate and fungiform papillae. PCR was performed under low stringent conditions with various degenerate primer pairs corresponding to the transmembrane domains (TM2, 3, 6, 7) of known subfamilies of G-protein coupled receptors including the putative olfactory receptors. Consequently, two types of cDNA fragments (type A, 408 bp; type B, 690 bp) were amplified and, then cloned into plasmid (pCR1000) and analyzed further.

Type A clones which are homologous to putative olfactory receptors

Sequencing of type A cDNA clones amplified with a pair of C1 (TM3) and D1 (TM6) primers deduced from putative rat olfactory receptors identified more than ten independent clones which had high homology among each other (40 - 90 %). Deduced amino acid sequences of five representative clones (TAS3, 4, 7, 8 and 38)

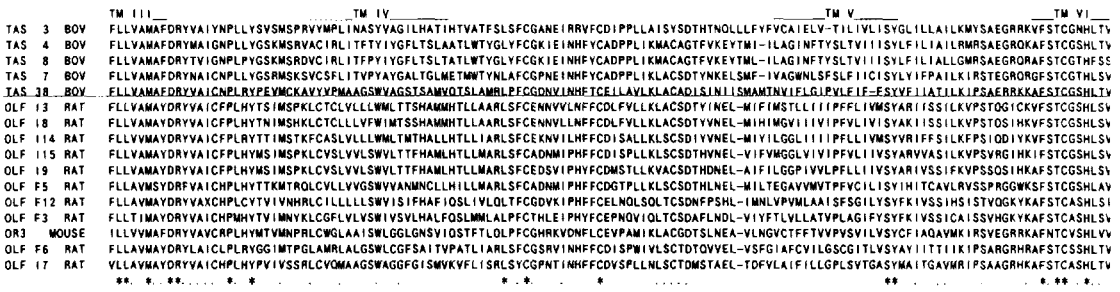

Fig. 1. Alignment of amino acid sequences of 5 clones of type A GCR cDNA fragments amplified from bovine taste tissue with rat and mouse olfactory receptor clones (6,9). Amino acid residues conserved in all of the bovine, rat and mouse clones are indicated by asterisks (*). Amino acid residues conserved in most of clones and varied within amino acid residues of similar physicochemical properties are indicated by period (.). Putative transmembrane domains are overlined.

are shown in Fig. 1. The amino acid sequences of Type A clones had several features common to GCR superfamily including the presence of potential transmembrane domains (TM4 and TM5) deduced from hydropathy profiles (data not shown). Not surprisingly, sequence homology search in SWISS-PROT database using FASTA software indicated that type A receptors are most homologous to the family of putative rat olfactory receptors (31 - 47 %)(6,9). But, the sequence alignment analysis using CLUSTAL software indicated the type A cDNA clones isolated from bovine taste tissue and putative olfactory receptors isolated from rat and mouse olfactory epithelium form distinct branches in the resulting dendrogram and do not mix each other (data not shown).

Northern blot analysis was performed to examine the tissue distribution of type A receptor expression (Fig. 4AB). With cRNA probes synthesized from TAS3,4 cDNA and TAS38 cDNA, hybridization band at 2.4 kb was detected in mRNA from circumvallate and fungiform papillae. The hybridization band of the same size was also detected in tongue epithelium devoid of taste papillae with strength weaker than in taste papillae. Interestingly, mRNA from olfactory epithelium hybridized with TAS3,4 and 38 probes at 3.5-4 and 1.5 kb in addition to 2.5 kb. While mRNAs from most of other tissues including brain, liver and lung did not hybridize with TAS3,4 and 38 probes, kidney mRNA showed a hybridization band at 1.5 kb with both TAS3,4 and TAS38 probes. There is a possibility that the same species of type A receptors are expressed in these tissues. But there is also a possibility that similar but different species of type A receptors are expressed in these tissues because members of a particular GCR subfamily in general have strong similarity each other. In this context, it is interesting to note that the putative olfactory receptors exist in sperm cells (10). These observations suggest that members of olfactory subfamily of GCR play yet unidentified roles in various tissues and cell types including kidney and germ cells.

In the mechanism of taste transduction, roles of GCR and second messenger systems have been implicated in the reception of sweet and bitter substances (1,2). In the reception of sweet substances (sugars), it was suggested that activation of adenylate cyclase in an G-protein dependent manner is involved (1). Although different sugar compounds induce similar sweet taste, it was suggested that there are multiple types of receptors for sugar compounds (11,12). On the other hand, transduction of bitter substances was suggested to be coupled to phospholipase C to increase inositol triphosphate (IP3) (2). The presence of multiple types of receptors for bitter substances also has been suggested (2). It is interesting to note that olfactory responses are evoked also by bitter substances which are usually hydrophobic compounds like odorants (Shoji and Kurihara, unpublished observation). Alternatively, gustatory responses are evoked by odorants (13). This notion suggests that some members of type A receptors function as receptors for bitter substances.

During the preparation of this manuscript, we noticed a report by Abe *et al.* (14) describing the identification of novel members of GCR subfamily expressed in rat tongue epithelium which are similar, but not identical to the type A receptors described in the present study.

Type B clone which is homologous to peptide receptors

A single cDNA clone (AL11) was identified by sequencing type B cDNA band amplified with a pair of A1 and B4 primers and had similarity with the member of peptide receptor branch of the GCR superfamily. ^{32}P-labelled PCR product of the Type B cDNA fragment (AL11) was used as probe to screen the 6×10^5 recombinants of a λZAP cDNA library prepared from bovine circumvallate and fungiform papillae. A single positive clone (PPR1) containing 2 kb insert was isolated. Sequence analysis of PPR1 identified a translational open reading frame encoding a protein of 350 amino acids (Fig. 2). There are two potential in-frame ATG initiation codons (underlined in Fig. 2). The region around the first ATG is closer to the consensus sequence for translation initiation (15). Hydropathy profile of PPR1 is consistent with presence of seven stretches of hydrophobic transmembrane domains. As shown in Fig. 2, there are a number of conserved motifs typical for GCR superfamily including GN**V (TM1), LA*AD (TM2), I**DRY (TM3) and NP**Y (TM7). Furthermore, there are two potential N-glycosylation sites (N-X-S/T) in extracellular N-terminal end typical for the GCR superfamily. There are two conserved cysteine residues in the second and third extracellular domains necessary to form a disulfide bridge typical for some members of GCR superfamily. Several consensus acceptor sites for phosphorylation by ser/thr-protein kinases are found in the carboxy terminus region.

Search of the SWISS-PROT protein databases revealed that PPR1 protein has not been previously identified, but has significant similarity with other known

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                                                                    60
GAGGAGACTTTGTTTTATAAAAGCCAACAACTAAAATTAAGGACAATTCTGAGATAATTT
                                                                   120
ATTTAACTTAATCTAAAATAAATTCATGAATAAATATAAGTAAAATATTTATTTTAAATA
                                                                   180
ATTCATTTTAATTTATTGTTGATATACTGCATAAGAATCACAAAGTTGTGTATGTACAAG
                                                                   240
TTTTAGGGCTTAATGATTAAGAACATTTCTCAGGGTATTATTTTTTTTATCTTCCCTCTG
                                                                   300
TAAATCCCGTTTCCTGTACAGACTTGTAAAAACAAGCAATGAAAAACTACTCTTGAGGAT
                                                                   360
ATACTCTAATGTGACTTCCATTAAAGAGTTTCAAAGAGTGGCAGACTCAGGCTCACATAT
                                                                   420
GGTATAACAACAAACTATTCTCTAATAAAAGCATAAAACATATGCTAGAGCAAAAGTATT
                                                                   480
TCCATTTAAGCAATATTAAAATAAATATGCTGTATTCTTTTTGCCATTTAGATTGGAACC
                                                                   540
ATGGCTGTGGAATACAACCAGTCAACAGATTACTATTATGAGGAAAATGAAATGAATGAC
  1 M  A  V  E  Y  N  Q  S  T  D  Y  Y  Y  E  E  N  E  M  N  D
                    *                                   *
                                                                   600
ACTCATGACTATAGTCAGTATGAAGTGATCTGTATAAAAGAAGAGGTCAGAAAATTTGCA
 21 T  H  D  Y  S  Q  Y  E  V  I  C  I  K  E  E  V  R  K  F  A
                                                                   660
AAAGTTTTCCTACCTGCCTTCTTCACAATAGCTTTCATCATTGGACTTGCAGGCAATTCC
 41 K  V  F  L  P  A  F  F  T  I  A  F  I  I  G  L  A  G  N  S
                                                                   720
ACTGTAGTGGCGATTTATGCCTATTACAAAAAGCGGAGAACCAAAACAGATGTGTACATC
 61 T  V  V  A  I  Y  A  Y  Y  K  K  R  R  T  K  T  D  V  Y  I
                                                                   780
TTGAATTTGGCAGTGGCGGATTTATTCCTTCTATTCACTTTGCCTTTTTGGGCAGTTAAT
 81 L  N  L  A  V  A  D  L  F  L  L  F  T  L  P  F  W  A  V  N
                                                                   840
GCAGTTCATGGGTGGGTTTTAGGGAAAATCATGTGCAAAGTCACTTCAGCCTTGTACACA
101 A  V  H  G  W  V  L  G  K  I  M  C  K  V  T  S  A  L  Y  T
                                                                   900
GTCAATTTTGTGTCTGGAATGCAGTTTCTGGCTTGTATCAGCACAGACAGATACTGGGCA
121 V  N  F  V  S  G  M  Q  F  L  A  C  I  S  T  D  R  Y  W  A
                                                                   960
GTAACTAAAGCTCCCAGTCAATCGGGAGTGGGAAAACCATGCTGGGTCATCTGTTTCTGC
141 V  T  K  A  P  S  Q  S  G  V  G  K  P  C  W  V  I  C  F  C
                                                                  1020
GTCTGGGTGGCTGCCATCTTGCTGAGTATCCCTCAGTTGGTTTTTTATACAGTAAATCAT
161 V  W  V  A  A  I  L  L  S  I  P  Q  L  V  F  Y  T  V  N  H
                                                                  1080
AAAGCTAGGTGTGTTCCCATCTTTCCATACCACCTAGGAACATCAATGAAAGCATCAATT
181 K  A  R  C  V  P  I  F  P  Y  H  L  G  T  S  M  K  A  S  I
                                                                  1140
CAAATCCTGGAAATCTGCATTGGATTTATAATACCCTTTCTTATCATGGCAGTGTGCTAC
201 Q  I  L  E  I  C  I  G  F  I  I  P  F  L  I  M  A  V  C  Y
                                                                  1200
TTCATCACGGCAAAGACACTCATCAAGATGCCTAACATTAAAAAATCTCAGCCCCTCAAA
221 F  I  T  A  K  T  L  I  K  M  P  N  I  K  K  S  Q  P  L  K
                                                                  1260
GTTCTGTTCACAGTGGTCATAGTTTTCATTGTCACTCAATTACCTTATAATATTGTCAAG
241 V  L  F  T  V  V  I  V  F  I  V  T  Q  L  P  Y  N  I  V  K
                                                                  1320
TTCTGCCAAGCCATAGACATCATCTACTCCCTGATCACTGACTGTGACATGAGCAAACGC
261 F  C  Q  A  I  D  I  I  Y  S  L  I  T  D  C  D  M  S  K  R
                                                                  1380
ATGGACGTTGCCATCCAAATCACAGAGAGTATTGCACTCTTTCACAGCTGCCTCAACCCC
281 M  D  V  A  I  Q  I  T  E  S  I  A  L  F  H  S  C  L  N  P
                                                                  1440
GTCCTATATGTTTTCATGGGAACCTCTTTTAAAAACTACATCATGAAAGTTGCCAAGAAA
301 V  L  Y  V  F  M  G  T  S  F  K  N  Y  I  M  K  V  A  K  K
                                                                  1500
TACGGATCCTGGAGAAGACAAAGACAAAATGTGGAGGAGATTCCTTTCGAATCTGAAGAT
321 Y  G  S  W  R  R  Q  R  Q  N  V  E  E  I  P  F  E  S  E  D
                                                                  1560
GCTACAGAGCCAACCAGTACTTTCAGCATTTAATATAAAGACCCATCTGTCTCTTGCTTC
341 A  T  E  P  T  S  T  F  S  I  #
                                                                  1620
AATACACATGAATGATGCTTCCTGCTGAGAACATCTGCATCTGCATCAAACATCTGCATT
                                                                  1680
TTTCAGAAATTCAAATTTCAAACACTGTGGTGGCAACTTGTAGCAAAGAAGAGGTTGGGG
                                                                  1740
TGGGAAAGGGGAGTGGGGTAGAAGCCAAGAAAAAGAAGAAACAAAATAATAAATTCATAA
                                                                  1800
AACACAGAAATTAAAGCTAACAATATAAGAAAATAGTATTAACAGTCATACATAAGAAAA
                                                                  1860
ACACTACAAGTTAGGTCATCTAAAACAGATTATTAAAGAGGTTTAATTTAAGGGTCATTT
                                                                  1920
ACAGTTATTTTAAATTATCTAAATTTTAACATAACAATTTTCCTGCATAATTTTAATACT
                                                                  1980
TGAATAATCATGCAGCAACATTTAATGACTTTTTCTTTTTCCTGTTCCTAATTTGTAAGT
                                                                  2040
GATTTCATAAAACTTCAGAAAGAGTAAAAATTAGCAATCAAAAATAAAAAAAATAATCTTT
                                                                  2059
AAAAAAAAAAAAAAAAAAAA
```

Fig. 2. Nucleotide and deduced amino acid sequences of the type B GCR cDNA clone (PPR1). Nucleotide (right) and amino acid residues (left) are numbered. Potential initiation codons (ATG) and polyadenylation signals (AATAAA) are underlined. Putative transmembrane domains are underlined and N-linked glycosylation sites are denoted by stars.

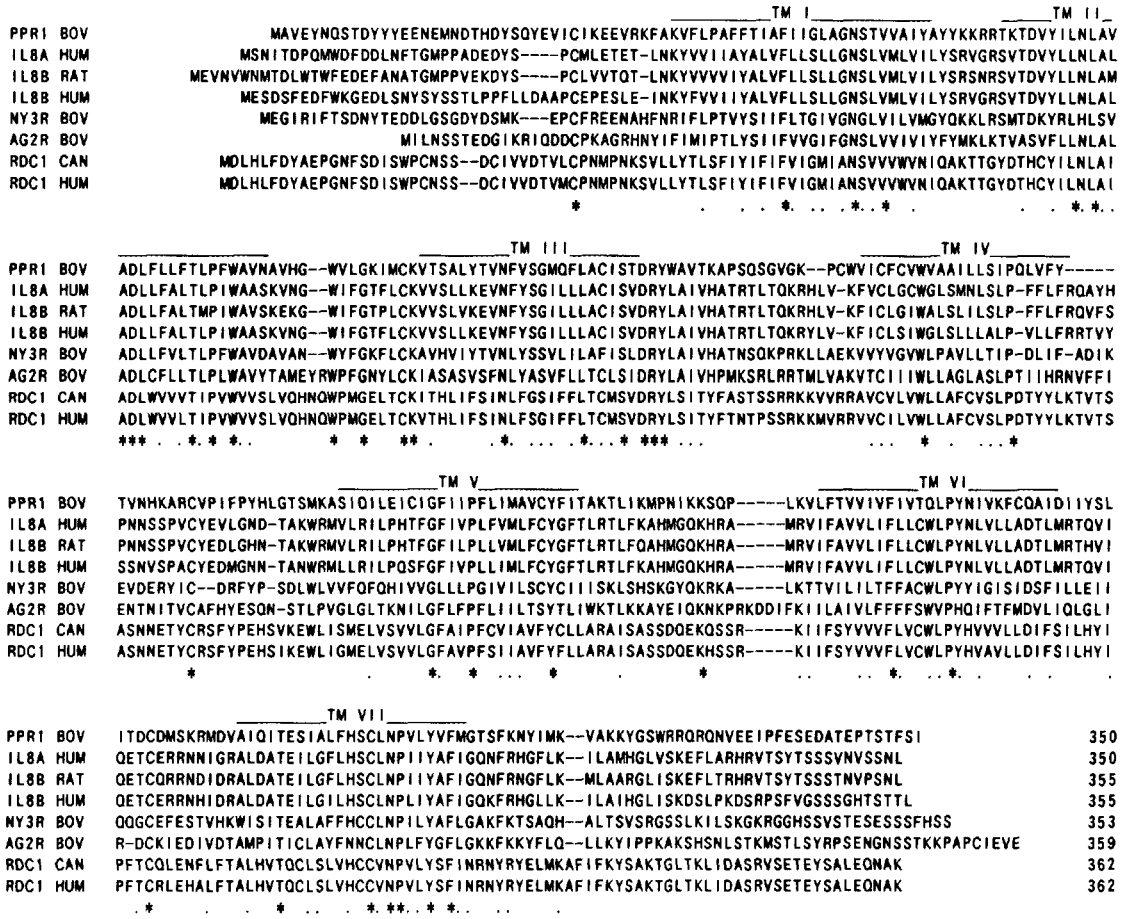

Fig. 3. Alignment of amino acid sequence of type B GCR cDNA clone (PPR1) with peptide receptors. Amino acid residues conserved in all the receptor clones are indicated by asterisks (*). Amino acid residues conserved in the most of clones and varied within amino acid residues of similar physicochemical properties are indicated by period (.). Putative transmembrane domains are overlined. Abbreviations are as follows: IL8A HUM, human interleukin 8 receptor; IL8B RAT, rat interleukin 8 receptor; IL8B HUM, human interleukin 8 receptor; NY3R BOV, bovine neuropeptide Y receptor; AG2R BOV, bovine type 1 angiotensin II receptor; RDC1 CAN, canine vasoactive intestinal peptide (VIP) receptor; RDC1 HUM, human VIP receptor. Total number of amino acid residues are indicated at C-terminus.

members of GCR especially in peptide receptor branch, e.g. the human interleukin-8 receptor (34.2 %), the bovine neuropeptide-Y receptor (35.2 %), the canine vasoactive intestinal peptide (VIP) receptor (RDC1, 30.0 %), and the bovine type 1 angiotensin II receptor (31.5 %). Most of the similarity between these receptors lies within the putative transmembrane domains (Fig. 3). The third cytoplasmic loop of most of these peptide receptors as well as PPR1 is also relatively short suggesting the same type of G-protein to be coupled as mentioned for type A receptors. From these observations, it is highly plausible that PPR1 protein is also a receptor for peptide ligands.

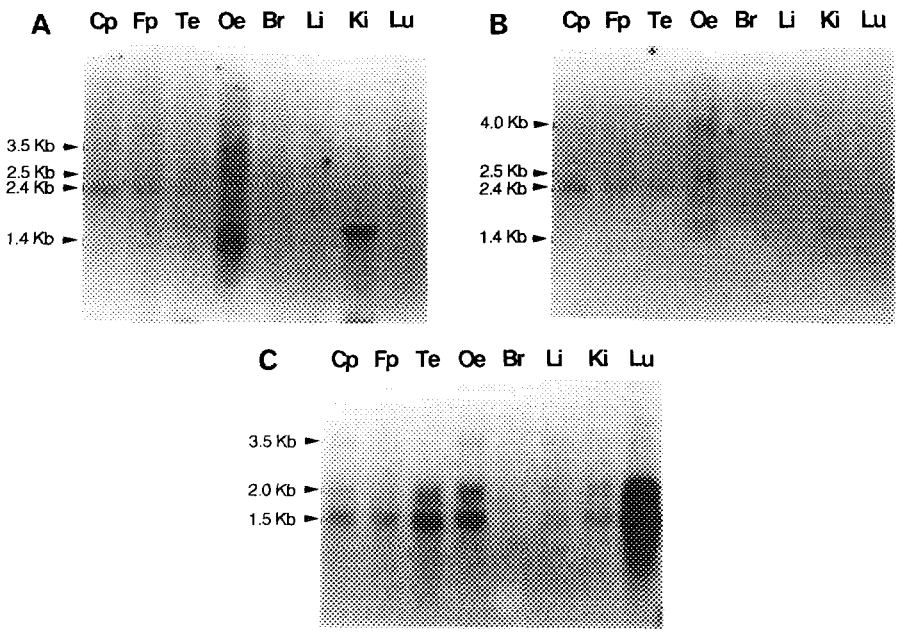

Fig. 4. Expression of type A and type B GCR mRNA in various bovine tissues. Northern blot analysis was performed with poly(A)$^+$-selected RNA from various bovine tissues (10 μg) as described in *Materials and Methods*. Blotted membrane was subjected to multiple hybridizations with mixture of TAS3 and TAS4 cRNA probes (A), TAS38 cRNA probe (B) and PPR1 cRNA probe (C). Tissues used are as follows: circumvallate papillae (Cp), fungiform papillae (Fp), tongue epithelium bearing no taste papillae (Te), olfactory epithelium (Oe), brain (Br), liver (Li), kidney (Ki), lung (Lu).

Northern blot analysis was performed to determine the tissue distribution of PPR1 mRNA expression (Fig. 4C). Multiple species of PPR1 mRNA (major, 1.5 kb; minor, 2.0 and 3.5 kb) were widely, but unevenly detected in various adult bovine tissues. Circumvallate and fungiform papillae from where original cDNA fragment (AL11) was amplified and olfactory epithelium expressed relatively high levels of PPR1 mRNA. Interestingly, lung expressed the most prominent level of PPR1 mRNA. Certain levels of PPR1 mRNA expression was also observed in liver, kidney and tongue epithelium bearing no taste papillae, while the expression in brain (mostly cerebral cortex) was very low.

Recently, using immunohistochemical technique, a variety of neuropeptides have been detected in vertebrate taste buds (16). Much of the attention has focused on the roles of neuropeptides in taste tissue including modulation of the sensitivity of taste cells and trophic action in maintenance and regeneration of taste cells (16). Identification of the authentic ligand for the PPR1 receptor protein would certainly facilitate the understanding the functions of neuropeptides in taste tissue. Works are currently underway in such direction using cells with artificially expressed PPR1 protein.

ACKNOWLEDGMENT

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REFERENCES

1. Striem, B., Pace, U., Zehavi, U., Naim, M., and Lancet, D. (1989) Biochem. J. 260, 121-126.
2. Hwang, P.M., Verma, A., Bredt, D.S., and Snyder, S.H. (1990) Proc. Natl. Acad. Sci. USA 87, 7395-7399.
3. Dohlman, H.G., Thorner, J., Caron, M.G., and Lefkowitz, R.J. (1991) Annu. Rev. Biochem. 60, 653-688.
4. Probst, W.C., Snyder, L.A., Schuster, D.I., Brosius, J., and Sealfon, S.C. (1992) DNA Cell Biol. 11, 1-20.
5. Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Jacqueline, V.S., Maehaut, C., Simons, M.J., Dumont, J.E., and Vassart, G. (1989) Science 244, 569-572.
6. Buck, L. and Axel, R. (1991) Cell 65, 175-187.
7. Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
8. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.
9. Nef, P., Hermans-Borgmeyer, I., Artires-Pin, H., Beasley, L., Dionne, V.E., and Heinemann, S.F. (1992) Proc. Natl. Acad. Sci. USA 89, 8948-8952.
10. Parmentier, M., Libert, F., Schurmans, S., Schiffmann, S., Lefort, A., Eggerickx, D., Ledent, C., Mollereau, C., Gerard, C., Perret, J., Grootegoed, A., and Vassart, G. (1992) Nature 355, 453-455.
11. Beidler, L.M. and Tonosaki, K. (1985) *in* Taste, Olfaction and the Central Nervous System (D.W. Pfaff, *Ed.*), pp. 47-64. Rockfeller University Press, New York.
12. Shimada, I., Shiraishi, A., Kijima, H., and Morita, H. (1974) J. Insect. Physiol. 20, 605-621.
13. Kashiwagura, T., Kamo, N., Kurihara, K., and Kobatake, Y. (1977) Comp. Biochem. Physiol. C. 56, 105-108.
14. Abe, K., Kusakabe, Y., Tanemura, K., Emori, Y., and Arai, S. (1993) FEBS Lett. 316, 253-256.
15. Kozak, M. (1989) J. Cell Biol. 108, 229-241.
16. Roper, S.D. (1992) J. Neurosci. 12, 1127-1134.