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Comparison of Nicotinic Acetylcholine Receptor Subunit Alpha 9 between Rat and Guinea Pig

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Based on its high level of expression in hair cells, the recently-cloned nicotinic receptor subunit, α9, is likely to be the postsynaptic receptor for acetylcholine in hair cells either as a homomeric complex or with other subunits yet to be identified. To study this receptor further, we cloned and sequenced α9 cDNA from the guinea pig organ of Corti library. The sequence of the guinea pig α9 cDNA is similar to that of the rat, with homologies of 85% and 89% at the nucleotide and amino acid levels, respectively. Most differences are in the cytoplasmic loop domain between the transmembrane segments 3 and 4. We also observed minor differences in the putative ligand binding regions. These changes could account for the different responses to the agonist 1, 1-dimethyl-4-phenylpiperazinium (DMPP) reported for rat and guinea pig outer hair cells. (Ajou Med J 1998; 3(1): 20~24)

Key Words: *Hair cell, Efferent receptor, Guinea pig, Alpha 9 cDNA*

INTRODUCTION

Efferent innervation of the organ of Corti (OC) arises from neurons in the superior olivary complex nuclei.[19] Lateral olivocochlear neurons project to ipsilateral cochlea and terminate on spiral ganglion (SG) cell dendrites under the inner hair cells (IHCs), and medial olivocochlear neurons project to the contralateral cochlea and terminate on the bases of outer hair cells (OHCs).[8,21] Several neurotransmitters have been found to be associated with efferent terminals in the cochlea including acetylcholine, γ-aminobutyric acid (GABA), enkephalin, dopamine, calcitonin gene-related peptide (CGRP), and dynorphin.[8,15] Acetylcholine appears to be the predominant neurotransmitter for both lateral and medial efferents. While the evidence supporting acetylcholine as an efferent neurotransmitter is very strong, the nature of the postsynaptic acetylcholine receptor for either lateral or medial efferents remained unknown until recently; cholinergic agonists and antagonists, useful for characterizing other acetylcholine receptors, provided little information on the efferent receptor.[1,3,9,10] Several studies sought to determine which acetylcholine receptors are expressed in the OC and a number of nicotinic subunits, including α2, 4, 5, and 6 and β2, 3, and 4 were detected in the cochlea with PCR amplification.[5,13] The muscarinic acetylcholine receptor, M3, also was detected in the rat and guinea pig OC and in SG cells.[20] However, these receptors did not fit the unusual pharmacological properties of the efferent receptor of the hair cells, suggesting that they are not involved in efferent transmission or that they are associated with additional subunits that contribute to their unique properties.[4,7,12,16]

In 1994, Elgoyhen et al. reported the identification of a new member of the nAChR gene family, α9. They found that expression of α9 mRNA is restricted, with cochlear hair cells being among the tissues expressing the most receptor. When the α9 subunit was expressed in *Xenopus laevis* oocytes, the receptors responded to ACh but did not elicit any response to nicotine or muscarine and little response to 1, 1-dimethyl-4-phenylpiperazinium (DMPP), a nicotinic agonist. These functional characteristics are similar to those of the efferent cholinergic

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receptor on OHCs, leading to the suggestion that α9 is the efferent receptor on OHCs, either functioning alone or in combination with other subunits, yet to be identified. To further study this important cochlear receptor, we have characterized the guinea pig α9 receptor by sequencing its complementary DNA(cDNA) sequence from an organ of Corti cDNA library and compare it with rat α9 cDNA.

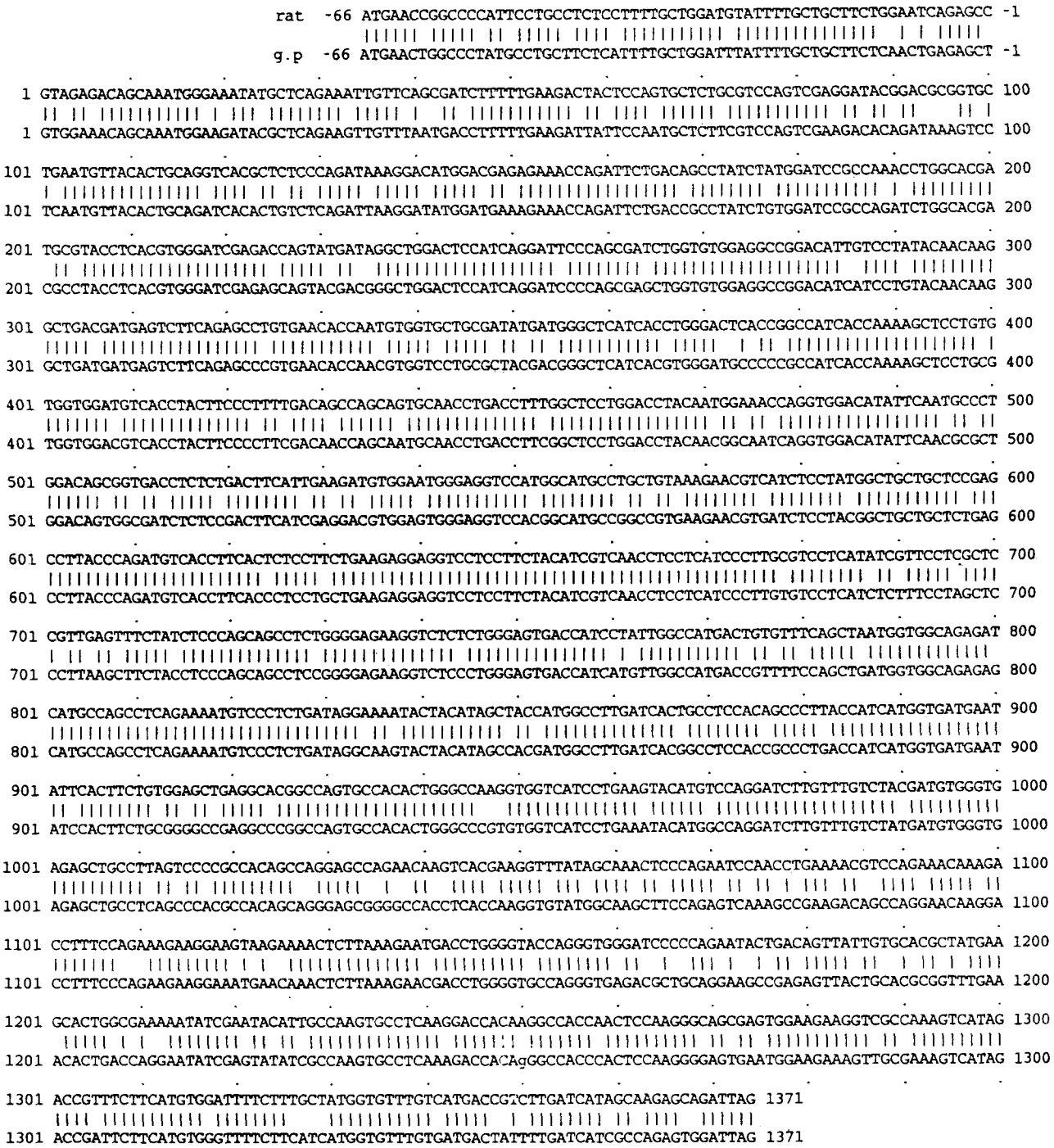

Fig. 1. Nucleotide sequences of rat and guinea pig α9 cDNAs.
Alignment of nucleotide sequences shows 85% homology between the rat and guinea pig sequences. Nucleotides are numbered in the 5′- to 3′ direction starting with the first nucleotide of the codon for the first amino terminal amino acid of the mature protein. Nucleotides −1 to −66 correspond to putative signal peptides and nucleotides 1~1371 are coding sequence regions.

MATERIALS AND METHODS

The primers were designed based on the rat α9 sequence[6] and were used to obtained PCR products from guinea pig OC library cDNA[22]: sense primer, nucleotides 784~808; antisense primer, nucleotides 1359~1331. The PCR product (expected size 576 bp) was cut and purified after agarose gel electrophoresis, ligated into the pCRII vector, and transformed with plasmid using TA Cloning kit (Invitrogen Corp., San Diego, CA). The conditions of cloning and transformation followed the manufacturer's instructions. The plasmid with insert was identified in ampicillin (100 mg/ml) and X-gal (50 mg/ml) agar plates. Selected clones were grown overnight in the LB broth with ampicillin (100 mg/ml) and plasmid DNA was purified using Plasmid Miniprep (Qiagen, Chats-

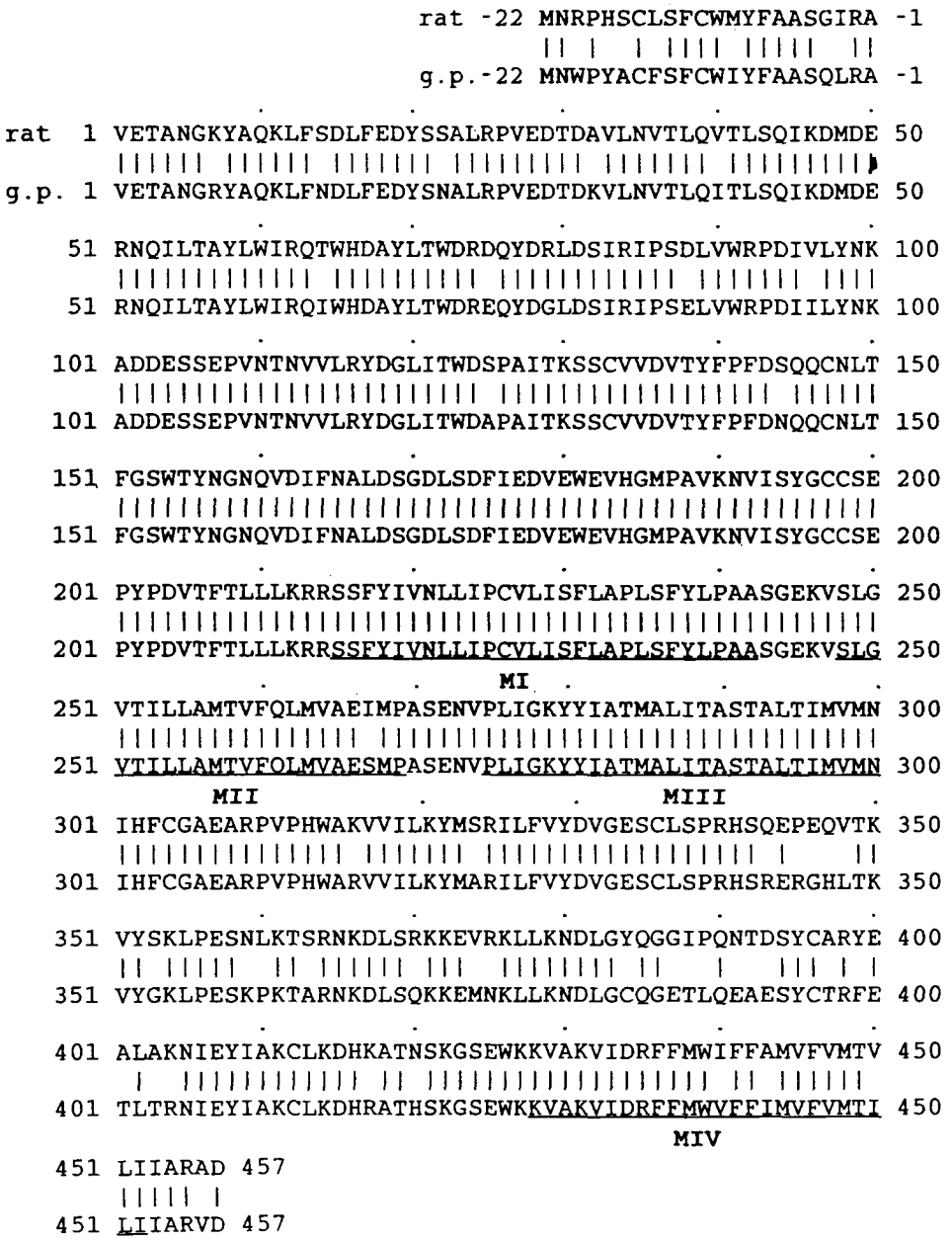

Fig. 2. Amino acid sequences of rat and guinea pig α9 cDNAs.
Alignment of deduced amino acid sequences showed 89% homology between the rat and guinea pig sequences. Amino acids −1 to −22 are a putative signal peptide. Sequences are presented in single letter code from amino to carboxy terminus. M: transmembrane segment.

	Loop A	Loop B	Loop C
Torpedo D	VWLPDLVLYNNAD	CEIIVTHFPFDQQNCTMKLGIWTYDGT	KHWVYTCCPDTPYLDITYHFIMQR
	88 96	144	
rat	DLVWRPDIVLYNKAD	CVVDVTYFPFDSQQCNLTFGSWTYNG	KNVISYGCCSEPYPDVTFTLLLKR
	|||||||||||||	||||||||||*||||||||||||	||||||||||||||||||||
g.p.	ELVWRPDIILYNKAD	CVVDVTYFPFDNQQCNLTFGSWTYNG	KNVISYGCCSEPYPDVTFTLLLKR

Fig. 3. Alignment of amino acid sequences in loops A, B, C of the proposed ligand binding sites of the *Torpedo* a subunit with rat and guinea pig corresponding regions of α9 subunits. The conserved amino acid residues are presented in bold letters. Asterisks denote amino acid residues that are different in the rat and guinea pig α9 subunits. Corresponding amino acid sequences of rat and guinea pig for each loop are: loop A (88~102), loop B (133~158) and loop C (190~213).

worth, CA). Both strands of the α9 inserts were sequenced using the Sequenase 2.0 kit (United States Biochemical, Cleveland, Ohio) combined with an automated DNA sequencer (Model 373A, Applied Biosystems, Foster City, CA). New pairs of α9 primers were designed based on the guinea pig cDNA sequence: sense primer, CTCAGAAAATGTCCCTC and antisense primer, TGATGAAGAAAACCCAC (expected size of PCR products : 521 bp). To determine the N- and C-terminus sequences of guinea pig α9 cDNA, one of each primer set was designed in the vector (pSPORT 1, BRL) used in making the guinea pig OC library.[22] Primers for N-terminus were: sense (from vector), ATTCCCGGGTCGAC and antisense (from guinea pig cDNA), GTGGGTTTTCTTCATCA. Primers for C-terminus were: sense (from vector), CCTCTAGAGCGGCCGC and antisense (from guinea pig cDNA), GAGGGACATTTTCTGAG. PCR products were cloned and sequenced as described above. All PCR was performed with the *Pfu* polymerase (Stratagene, La Jolla, CA) with the final 10 min incubation with *Taq* polymerase (Perkin-Elmer, Foster City, CA) to add 3' A-overhangs (94°C, 1 min; 55°C, 1 min; 72°C, 1 min; for 30 cycles followed by a 10 min final extension at 72°C).

RESULTS

The sequence of the guinea pig α9 cDNA is similar to that of the rat, with similarities of 85% and 89% at the nucleotide and amino acid levels, respectively (Fig. 1, 2). Most differences are found in the cytoplasmic loop domain between the transmembrane segments III and IV. The possible phosphorylation sites in the rat α9 receptor which are not present in the guinea pig include Ser 324 and Ser 353 that are replaced by Ala 324 and Gly 353, respectively(Fig. 3) while the N-terminus and the transmembrane sequences themselves are highly conserved (Fig. 4).

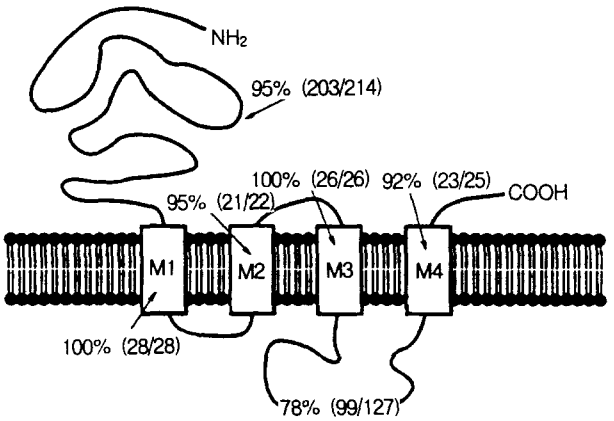

Fig. 4. Identities of amino acid sequences between rat and guinea pig. Both N-terminal and transmembrane segments showed high similarities compared to intracellular cytoplasmic loop area. M: transmembrane segment.

DISCUSSION

Analysis of the guinea pig α9 cDNA sequence shows that it is similar to that of the rat receptor with 85% and 89% homologies at the nucleotide and amino acid level, respectively. Sequence differences were the greatest in the cytoplasmic loop between transmembrane segments 3 and 4. Functionally, the acetylcholine receptors on the outer hair cells of the rat and the guinea pig are similar, except for one notable difference.[2] The guinea pig receptor responds to the agonist DMPP while the rat receptor responds to it only very weakly. While we cannot

rule out the possibility that this difference is due to the amino acid changes in the cytoplasmic loop, the ligand binding domain would be the most likely area where amino acid substitutions would affect ligand binding properties. A three loop model, made up of the N-terminal extracellular segment, has been proposed for the ligand binding site of the nicotinic a subunit of the *Torpedo*.[11] In comparing the corresponding regions of the rat and guinea pig α9 receptors, three amino acids are different between the two species. While all three changes are conservative, subtle changes such as these would be consistent with minor differences in the pharmacology of the receptors from rat and guinea pig. The amino acid differences in the intracellular loop between transmembrane segments 3 and 4 include consensus phosphorylation sites for several protein kinases. Protein kinase C (PKC) phosphorylates the α and δ AChR subunits.[14] According to the consensus sequences for PKC,[17] there are several possible phosphorylation sites in the rat α9 receptor which are not present in the guinea pig including Ser 324 and Ser 353 that are replaced by Ala 324 and Gly 353, respectively. Since protein phosphorylation is involved in regulating the function of ion channels and receptors such as the AChR,[18] the rat and guinea pig receptors may be modulated differently by phosphorylation.

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