Primary structure of porcine cardiac muscarinic acetylcholine receptor deduced from the cDNA sequence

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The complete amino acid sequence of the porcine cardiac muscarinic acetylcholine receptor has been deduced by cloning and sequencing the cDNA. The tissue location of the RNA hybridizing with the cDNA suggests that this muscarinic receptor species represents the M_2 subtype.

Muscarinic acetylcholine receptor (Porcine heart) cDNA cloning Nucleotide sequence RNA blot hybridization analysis M_2 subtype

1. INTRODUCTION

The muscarinic acetylcholine receptor (mAChR) mediates various cellular responses through the action of guanine nucleotide-binding regulatory proteins (G-proteins) [1-3]. Pharmacologically distinguishable subtypes of the mAChR occur in different tissues and have provisionally been classified into M_1 and M_2 subtypes on the basis of their difference in apparent affinity for the antagonist pirenzepine [4-6]. We have previously isolated DNA complementary to porcine cerebral mRNA encoding mAChR and have deduced the primary structure of the receptor protein from the nucleotide sequence of the cDNA [7]. The tissue location of the mRNA, in conjunction with the high apparent affinity for pirenzepine of the mAChR expressed from the cDNA, has led to the proposition that this protein is the mAChR of the M_1 subtype [7]. In the present investigation, we have cloned and sequenced cDNA encoding another species of porcine mAChR and have thus deduced the complete amino acid sequence of the protein. The preferential location of the RNA hybridizing with the cDNA in the heart and medulla-pons suggests that this mAChR species represents the M_2 subtype.

Abbreviation: mAChR, muscarinic acetylcholine receptor

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2. MATERIALS AND METHODS

Total RNA was extracted from porcine tissues [8], and poly(A)$^+$ RNA isolated as in [9]. Oligodeoxyribonucleotides were synthesized by the triester method [10], except that the primer 5′-AGAGGATGAAGGAGAGG-3′ was prepared with an automatic DNA synthesizer (Applied Biosystems). The procedures used to clone transcripts formed by extension of a synthetic primer into the *Pst*I site of the plasmid pBR322 have been described previously [11]. *Escherichia coli* HB101 or MC1061 was used for transforma-

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tion [12]. Transformants were screened [13], using a hybridization probe labelled with ^{32}P at the 5′-end. For constructing the Okayama-Berg cDNA library [14], 15.6 μg of porcine atrial poly(A)$^+$ RNA and 4.2 μg of the vector-primer DNA were used; the hybridization probe used to screen this library was labelled by nick-translation [15] with [α-^{32}P]dCTP. DNA sequencing was carried out according to [16]. RNA blot hybridization analysis was performed as in [7].

3. RESULTS AND DISCUSSION

In our previous study [7], the mAChR, purified from porcine cerebrum [17], was digested with trypsin, and the resulting peptides were fractionated by reverse-phase high-performance liquid chromatography. The amino acid sequences determined for peptide fractions corresponding to relatively large absorbance peaks (fractions I, II and III) were found to be encoded by the porcine cerebral mAChR cDNA isolated. However, analysis of peptide fractions corresponding to minor absorbance peaks disclosed that their amino acid sequences were not included in the sequence predicted from the cDNA. These were the sequences EPVANQEPVSPXLVQG and DDEITQDENTVXXSL (in one-letter code) determined from fraction IV (eluted at 31.8 min) and fraction V (eluted at 34.7 min), respectively, which were collected in the experiment shown in fig.1A in [7]. This finding raised the question of whether the tryptic peptides IV and V are derived from a different mAChR protein. An attempt was therefore made to clone cDNA encoding a second mAChR species.

The synthetic oligodeoxyribonucleotide primer 5′-GT$^{A}_{G}$TT$^{T}_{C}$TC$^{A}_{G}$TC$^{T}_{C}$TG-3′, corresponding to the partial sequence QDENT of peptide V, was extended by reverse transcriptase using porcine cerebral poly(A)$^+$ RNA as template. The resulting single-stranded cDNA was converted to double-stranded cDNA, which was cloned in the plasmid pBR322. These clones were screened by hybridization at 36°C with the synthetic oligodeoxyribonucleotide probe

$$\text{nucleotide probe } 5'\text{-GTTAT}^{T}_{C}\text{TC}^{A}_{G}\text{TC}^{A}_{G}\text{TC-3}' \text{ cor-}$$

responding to the amino-terminal sequence DDEIT of peptide V. Thus, two clones were isolated from $\sim 6.7 \times 10^5$ transformants. Nucleotide sequence analysis of one of these clones (pmACR423) revealed that it carried a 596-base-pair cDNA insert including the sequences that encode the amino acid sequences determined for peptides IV and V in the same reading frame.

Because RNA blot hybridization analysis indicated that porcine atrium contained a relatively large amount of RNA hybridizable with a cDNA probe derived from pmACR423, a library of cDNA clones was constructed using poly(A)$^+$ RNA from this tissue and the Okayama-Berg vector [14]. Screening of this library ($\sim 2.4 \times 10^5$ transformants) by hybridization at 60°C with the *Hin*fI(337)-*Dde*I(813) fragment (numbers indicating the 5′-terminal nucleotide generated by cleavage; for nucleotide numbers, see fig.1) derived from pmACR423 yielded one positive clone (phmACR15), which harboured a ~3-kilobase-pair cDNA insert.

Because the cDNA sequence carried by pmACR423 did not extend to the translational initiation site, the synthetic oligodeoxyribonucleotide primer 5′-AGAGGATGAAGGAGAGG-3′, which is complementary to the sequence of nucleotide residues 447–463 in pmACR423, was extended using porcine atrial poly(A)$^+$ RNA as template and then cloned in the plasmid pBR322 as described above. Screening of these clones ($\sim 1.2 \times 10^5$ transformants) by hybridization at 50°C with the *Hin*fI(337)-*Ava*II(405) fragment from pmACR423 yielded five positive clones. Nucleotide sequence analysis of one of them (phmACR905) showed that it carried a 589-base-pair cDNA sequence, which extended beyond the translational initiation site.

Fig.1 shows the 1665-nucleotide cDNA sequence determined with clones phmACR905, pmACR423 and phmACR15. The reading frame corresponding to the amino acid sequences determined for the tryptic peptides IV and V (amino acid residues 223–238 and 298–312) was used to deduce the primary structure of the protein encoded by this cDNA (referred to hereafter as the cardiac mAChR). The translational initiation site was assigned to the methionine codon composed of nucleotide residues 1–3 because this is the first ATG triplet that appears downstream of a nonsense codon, TAG (nucleotide residues −108 to −106), found in frame. A translational ter-

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                                                                                                                5'-----CGAAGG  -121
GCTCCCCACTCCTAGCCAGCCCACACCAAGCTTCTTGCAGCCCGGGGAGCAAGTGGAACTAAACCTGCGGCAGGTTTAAATGTGTATTTGGCTACTTGGCTACTGAGTAGAGAACACAAA    -1
  1                                10                                  20                                  30
Met Asn Asn Ser Thr Asn Ser Ser Asn Ser Gly Leu Ala Leu Thr Ser Pro Tyr Lys Thr Phe Glu Val Val Phe Ile Val Leu Val Ala
ATG AAT AAC TCC ACC AAC TCC TCT AAC AGT GGC CTG GCT CTG ACC AGT CCT TAT AAG ACA TTT GAA GTG GTT TTT ATT GTC CTT GTC GCC     90
                                 40                                  50                                  60
Gly Ser Leu Ser Leu Val Thr Ile Ile Gly Asn Ile Leu Val Met Val Ser Ile Lys Val Asn Arg His Leu Gln Thr Val Asn Asn Tyr
GGA TCC CTC AGT TTG GTG ACC ATT ATT GGG AAC ATC CTG GTC ATG GTC TCC ATC AAA GTC AAC CGA CAC CTC CAG ACA GTC AAC AAT TAC    180
                                 70                                  80                                  90
Phe Leu Phe Ser Leu Ala Cys Ala Asp Leu Ile Ile Gly Val Phe Ser Met Asn Leu Tyr Thr Leu Tyr Thr Val Ile Gly Tyr Trp Pro
TTT TTG TTC AGC TTG GCC TGT GCT GAC CTC ATC ATT GGT GTT TTC TCC ATG AAC CTG TAC ACT CTT TAC ACT GTG ATT GGC TAC TGG CCT    270
                                100                                 110                                 120
Leu Gly Pro Val Val Cys Asp Leu Trp Leu Ala Leu Asp Tyr Val Val Ser Asn Ala Ser Val Met Asn Leu Leu Ile Ile Ser Phe Asp
TTG GGC CCC GTG GTG TGT GAC CTT TGG CTA GCT CTG GAC TAC GTG GTC AGT AAT GCC TCA GTA ATG AAT CTG CTC ATC ATC AGC TTT GAC    360
                                130                                 140                                 150
Arg Tyr Phe Cys Val Thr Lys Pro Leu Thr Tyr Pro Val Lys Arg Thr Thr Lys Met Ala Gly Met Met Ile Ala Ala Ala Trp Val Leu
AGG TAC TTC TGT GTC ACG AAG CCG CTC ACC TAC CCC GTC AAG CGG ACC ACA AAA ATG GCA GGT ATG ATG ATT GCT GCT GCG TGG GTC CTC    450
                                160                                 170                                 180
Ser Phe Ile Leu Trp Ala Pro Ala Ile Leu Phe Trp Gln Phe Ile Val Gly Val Arg Thr Val Glu Asp Gly Glu Cys Tyr Ile Gln Phe
TCC TTC ATC CTC TGG GCT CCG GCC ATT CTC TTC TGG CAG TTC ATT GTA GGG GTG AGA ACT GTG GAG GAT GGT GAA TGC TAT ATA CAG TTT    540
                                190                                 200                                 210
Phe Ser Asn Ala Ala Val Thr Phe Gly Thr Ala Ile Ala Ala Phe Tyr Leu Pro Val Ile Ile Met Thr Val Leu Tyr Trp His Ile Ser
TTT TCC AAC GCT GCT GTC ACC TTT GGC ACT GCC ATT GCA GCC TTC TAT TTG CCT GTG ATC ATC ATG ACT GTA TTA TAC TGG CAC ATA TCC    630
                                220                                 230                                 240
Arg Ala Ser Lys Ser Arg Ile Lys Lys Asp Lys Lys Glu Pro Val Ala Asn Gln Glu Pro Val Ser Pro Ser Leu Val Gln Gly Arg Ile
CGA GCC AGT AAG AGC AGG ATT AAG AAG GAC AAG AAG GAG CCT GTG GCC AAC CAA GAA CCA GTT TCT CCA AGT TTG GTA CAA GGA AGA ATA    720
                                250                                 260         Ser                     270
Val Lys Pro Asn Asn Asn Asn Met Pro Gly Ser Asp Glu Ala Leu Glu His Asn Lys Ile Gln Asn Gly Lys Ala Pro Arg Asp Ala Val
GTG AAG CCG AAC AAC AAT AAT ATG CCT GGC AGT GAT GAA GCC CTG GAG CAC AAC AAA ATC CAG AAT GGC AAA GCT CCC AGG GAT GCT GTG    810
                                280                                 290          A                      300
Thr Glu Asn Cys Val Gln Gly Glu Glu Lys Glu Ser Ser Asn Asp Ser Thr Ser Val Ser Ala Val Ala Ser Asn Met Arg Asp Asp Glu
ACT GAG AAC TGT GTC CAG GGA GAG GAG AAA GAA AGC TCC AAC GAT TCC ACC TCA GTC AGT GCT GTT GCC TCT AAT ATG AGA GAT GAT GAA    900
                                310                                 320                                 330
Ile Thr Gln Asp Glu Asn Thr Val Ser Thr Ser Leu Gly His Ser Lys Asp Glu Asn Ser Lys Gln Thr Cys Ile Lys Ile Val Thr Lys
ATA ACC CAG GAT GAA AAC ACA GTT TCC ACT TCC CTG GGC CAT TCC AAA GAT GAG AAC TCA AAG CAA ACA TGC ATC AAA ATT GTC ACC AAG    990
                                340                                 350                                 360
Thr Gln Lys Ser Asp Ser Cys Thr Pro Ala Asn Thr Thr Val Glu Leu Val Gly Ser Ser Gly Gln Asn Gly Asp Glu Lys Gln Asn Ile
ACC CAA AAA AGT GAC TCA TGC ACC CCA GCT AAT ACC ACT GTG GAG CTT GTT GGT TCT TCA GGT CAG AAT GGA GAT GAA AAA CAG AAC ATT   1080
                                370                                 380                                 390
Val Ala Arg Lys Ile Val Lys Met Thr Lys Gln Pro Ala Lys Lys Lys Pro Pro Pro Ser Arg Glu Lys Lys Val Thr Arg Thr Ile Leu
GTC GCT CGC AAG ATT GTG AAG ATG ACC AAG CAG CCT GCA AAA AAG AAG CCG CCT CCT TCC CGG GAA AAG AAA GTG ACC AGG ACG ATC TTG   1170
                                400                                 410                                 420
Ala Ile Leu Leu Ala Phe Ile Ile Thr Trp Ala Pro Tyr Asn Val Met Val Leu Ile Asn Thr Phe Cys Ala Pro Cys Ile Pro Asn Thr
GCT ATT CTG TTG GCT TTC ATC ATC ACT TGG GCC CCG TAC AAC GTC ATG GTG CTC ATT AAT ACC TTC TGT GCA CCC TGC ATC CCC AAC ACA   1260
                                430                                 440                                 450
Val Trp Thr Ile Gly Tyr Trp Leu Cys Tyr Ile Asn Ser Thr Ile Asn Pro Ala Cys Tyr Ala Leu Cys Asn Ala Thr Phe Lys Lys Thr
GTG TGG ACA ATT GGT TAT TGG CTC TGT TAC ATC AAC AGC ACT ATC AAC CCT GCC TGC TAT GCA CTT TGT AAT GCC ACC TTC AAG AAG ACC   1350
                                460
Phe Lys His Leu Leu Met Cys His Tyr Lys Asn Ile Gly Ala Thr Arg
TTT AAA CAC CTT CTT ATG TGT CAT TAT AAG AAC ATA GGC GCT ACA AGG TAA AACATCTTTGTAAAGAAGGAAGGTAGTCAAGAGGAGCTTGAGGAACAGAAAA   1453
AGAATGAAAGAGCTCCTAGTTTTAAAATCTCTGCCATTGCACTTTATAGTCTTATTAATGGAATGTGCAATTAAGGAGCCCTACAG-----3'
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Fig.1. Nucleotide sequence of the cDNA encoding the porcine cardiac mAChR. The nucleotide sequence was determined using clones phmACR905 (nucleotides −126 to 463), pmACR423 (nucleotides 324–919) and phmACR15 (nucleotides 756–1539); the 3′-terminal ~2-kilobase-pair cDNA sequence of phmACR15 was not determined. Nucleotide residues are numbered in the 5′- to 3′-direction, beginning with the first residue of the ATG triplet encoding the initiating methionine, and the nucleotides on the 5′-side of residue 1 are indicated by negative numbers; the number of the nucleotide residue at the right end of each line is given. The deduced amino acid sequence of the porcine cardiac mAChR is shown above the nucleotide sequence, and amino acid residues are numbered beginning with the initiating methionine. Neither the 5′- nor the 3′-noncoding sequence presented is complete. A difference in nucleotide residue 787 is observed between clones phmACR15 (G) and pmACR423 (A), resulting in replacement of Gly by Ser (residue 263).

mination codon (TAA) occurs in frame after the 466th codon specifying arginine. Thus, the cardiac mAChR consists of 466 amino acid residues (including the initiating methionine) and has a calculated M_r of 51 670, which agrees with the reported M_r of the protein moiety of the porcine cardiac mAChR [18].

Fig.2 shows the alignment of the amino acid sequences of the cardiac and cerebral mAChRs. The two sequences are highly homologous, except that amino acid residues 219–370 of the cardiac mAChR and residues 221–347 of the cerebral mAChR showed no apparent sequence homology. Of the positions aligned, 60% are occupied by identical residues and 22% by conservative residues. In general, the putative transmembrane segments (see below) are better conserved. It is noteworthy that the non-homologous segments correspond to the region that represents an insertion in the mAChR as compared with the β-adrenergic receptor [20,21] and rhodopsin [22]; the structural homology among the three proteins has been discussed [7].

The cardiac mAChR shares characteristic structural features with the cerebral mAChR [7]. The hydropathy profiles [23] of the two mAChRs are very similar (see [7]), suggesting the presence of seven hydrophobic segments (I–VII), which represent putative transmembrane α-helices; segment VII is less hydrophobic than the others. The only charged amino acid residues present in these segments are two aspartic acid residues (residue 69 in segment II and residue 103 in segment III), which are conserved in the two mAChRs; the amino acid numbers refer to those of the cardiac mAChR. The cardiac mAChR, like the cerebral mAChR, lacks an amino-terminal signal sequence [24], and the amino-terminal region preceding segment I contains potential *N*-glycosylation sites [25] (asparagine residues 2, 3 and 6); *N*-glycosylation of residues 2 and 3 may be mutually exclusive. The carboxy-terminal region of the cardiac mAChR contains several potential sites of phosphorylation (threonine residues 446, 450 and 465), as is the case for the cerebral mAChR. These structural features are generally shared by rhodopsin [22,26] and the β-adrenergic receptor [20,21,27]. Thus it is suggested that the cardiac mAChR, like the cerebral

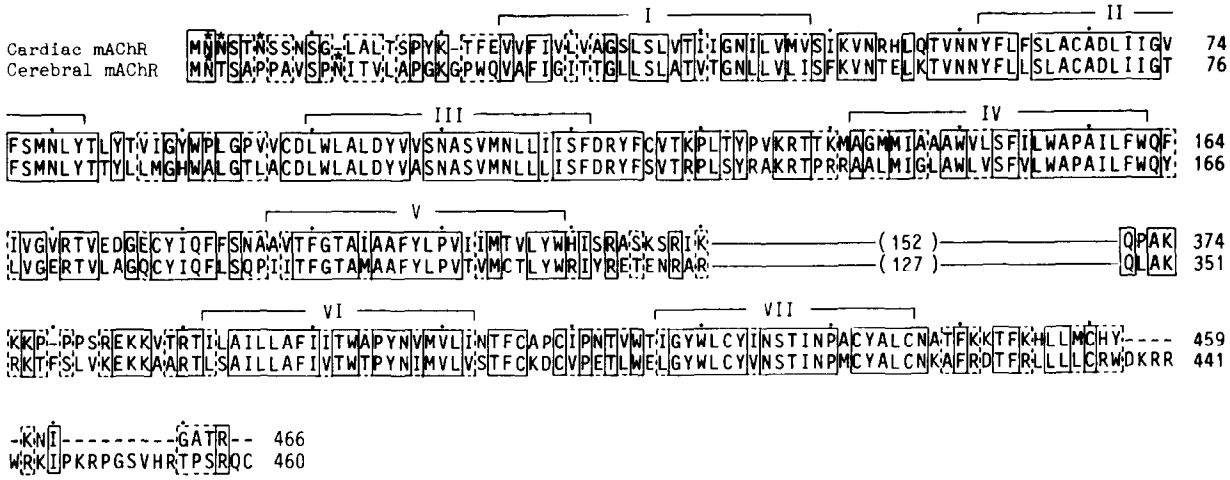

Fig.2. Alignment of the amino acid sequences of the porcine cardiac (top) and cerebral (bottom) mAChRs. The one-letter amino acid notation is used. The sequence data for the cerebral mAChR have been taken from [7]. Sets of identical residues are enclosed with solid lines, and sets of conservative residues with dashed lines; conservative substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A and G; N, D, E and Q; H, R and K; M, I, L and V; F, Y and W [19]. Gaps (–) have been inserted to achieve maximum homology. The non-homologous segments are shown by lines together with the sum of residues present in each segment given in parentheses. The number of the residue at the right end of each line is given. The positions of the putative transmembrane segments I–VII are indicated; the termini of each segment are tentatively assigned [7]. The asparagine residues as potential *N*-glycosylation sites in the amino-terminal region are marked with asterisks.

mAChR, has a transmembrane topography similar to that of bacteriorhodopsin [28–30] and rhodopsin [22,31,32].

In view of the different tissue locations of the mAChR subtypes [4–6], we examined poly(A)$^+$ RNA from several porcine tissues for the species hybridizing with a cardiac mAChR cDNA probe (fig.3). The heart (lane 4) and medulla-pons (lane 3), known to be representative sites for the M_2 subtype, contained a hybridizable RNA species of ~6100 nucleotides. On the other hand, the cerebral cortex (lane 1) and corpus striatum (lane 2), known to contain preferentially the M_1 subtype, contained only a small or hardly detectable amount of this RNA species. We have previously shown that the RNA species hybridizing with the cerebral mAChR cDNA is present selectively in the cerebral cortex and corpus striatum [7]. These results, taken together, suggest that the cerebral and the cardiac mAChR cDNA we have cloned encode the mAChRs of the M_1 and the M_2 subtype, respectively, implying that the mAChR subtypes represent distinct gene products.

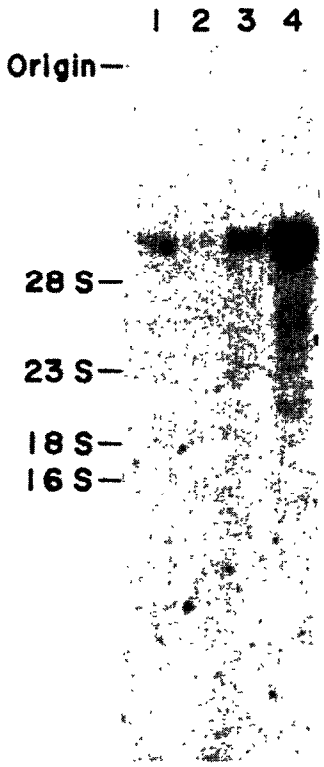

Fig.3. Autoradiogram of blot hybridization analysis of poly(A)$^+$ RNA from porcine cerebral cortex (lane 1), corpus striatum (lane 2), medulla-pons (lane 3) and atrium (lane 4) using a cardiac mAChR cDNA probe. The procedures used were as described previously [7], except that the hybridization probe (spec. act. 2.4×10^8 cpm/μg) was the *Hin*fI(337)-*Dde*I(813) fragment from clone pmACR423, labelled by nick-translation [15] with [α-^{32}P]dCTP; 15 μg of each poly(A)$^+$ RNA sample was used. Autoradiography was performed at −70°C for 90 h with an intensifying screen. The size markers were porcine and *E. coli* rRNAs [33].

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