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Expression of two variants of the human μ opioid receptor mRNA in SK-N-SH cells and human brain

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Abstract A partial μ opioid receptor gene was isolated from a human genomic library using a mouse δ opioid receptor cDNA as a probe. Using information from this genomic clone and the published human μ receptor, MOR1, a cDNA was isolated from SK-N-SH mRNA that codes for a variant of the MOR1 mRNA, MOR1A. The presence of MOR1A is also shown in human brain using RT-PCR. MOR1A differs from MOR1 in that the 3′ terminal intron has not been removed. An in-frame termination codon is found four amino acids after the 5′ consensus splice site, making MOR1A eight amino acids shorter than MOR1. Both receptors show similar ligand binding and coupling to cAMP in CHO-K1 cells. The C-terminal differences between MOR1 and MOR1A could have effects on receptor coupling or receptor transport and localization.

Key words: G protein-coupled receptor; Cyclic AMP; SK-N-SH cell; Brain; Pain

1. Introduction

The opioid receptors are G-protein coupled receptors that play a major role in pain management. However, they are also associated with many side effects, such as constipation, respiratory depression, dependence and tolerance [1]. Pharmacologist have identified 3 basic types of opioid receptors, μ, κ and δ, as well as subtypes of these receptors [2–5]. It has been suggested that many of the opioid side-effects will be associated with a particular receptor subtype and that the development of specific agonists and antagonists would eliminate many of these side-effects [6]. The recent cloning of the mouse δ opioid receptor, DOR1 [7,8], which has lead to the cloning of other opioid receptors [9–17], may help bring this idea to fruition. However, as yet no receptor subtype cDNAs of μ, κ or δ have been cloned. While looking for receptor subtypes, we discovered a splice variant of the human μ opioid receptor. The human μ opioid receptor, MOR1, and this variant, MOR1A, have similar ligand affinity. The variant is also negatively coupled to cAMP in CHO-K1 cells, and mRNA coding for the variant can be found in human brain. It is possible that the changes in the variants C-terminus may modulate the receptor's G-protein coupling or affect its cellular distribution.

2. Materials and methods

2.1. Cloning of the human opioid receptor variants

DOR1 [7,8] was cloned and used to screen a human genomic library (Stratagene) as described in detail by Mansson [17]. Two clones, OR7 and OR4, were identified with homology to rat μ opioid receptor (rMOR1) [9,10].

The human μ opioid receptor cDNA variants were cloned from SK-N-SH mRNA using RT-PCR. Reverse transcription was performed on 0.5 μg of mRNA using 0.1 μM of either a specific primer (RTM1, GCCTCCTACACATTCTTGAAG) corresponding to the 3′ non-coding region of the published human MOR1 [15], or a specific primer to the new variant (RTM2, AGAACCAGAGCAAGACTGGC) corresponding to 3′ non-coding sequences in the genomic μ opioid receptor clone, OR7 (i.e. proposed intron 3 region of hMOR). The products from the RT reactions were purified using Glassmax spin columns (Gibco/BRL) and used as substrates for PCR reactions. A 1447 bp MOR1 clone was PCR amplified using the forward primer (MF1, CTAGGTACCGCAGAGGAGAATGTCAGAT) with an added *Kpn*1 site and a reverse primer (MR1, GATCTCGAGCTAAGCTTGGTGAAGGTCGG) with an added *Xho*1 site based on the published 5′ and 3′ non-coding sequence of the human MOR1 [15]. A 1485 bp μ receptor variant, MOR1A, was cloned using the same forward primer used to clone MOR1 and a reverse primer (MR2, CTAGGTACCAAGCTTCCCTCCATTCTCATCCTC) specific for the 3′ non-coding sequence in the genomic μ opioid receptor clone, OR7 (i.e. the proposed intron 3 region of hMOR1) plus an added *Kpn*1 site. All PCR reactions were performed using 1 μM primers, 1/10 of the purified cDNA, and 1 U *Taq* Polymerase (Perkin-Elmer) in the supplied buffer for 35 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C followed by a 10 min 72°C extension. The PCR products, representing the published human MOR1 and a variant of the human MOR1, were cloned into SK plasmids (Stratagene), sequenced and the inserts subcloned into the expression vector pcDNA3 (Invitrogen) to create pcMOR1 and pcMOR1A. The above PCR reactions were also done with mRNA from SK-N-SH and human brain in which the forward primers were replaced with the primer (MF2, CAATGTCTGCAACTGGATCC) which is specific to TM #4 of both MOR1 and MOR1A cDNAs.

2.2. PCR analysis and Southern blots

20 μl of the 50 μl PCR reactions were analyzed by electrophoresis on a 1% agarose gel, and stained with ethidium bromide. The gels were blotted onto nylon filters and hybridized in 50% formamide at 42°C to either a [^{32}P]dCTP nick-translated pchMor1 insert or to the oligo (GTACGCAGTCTCTAGAATTAGGTATATCTACTGGGGATGACAT), which is specific to the 3′ non-coding region of MOR1A, and was [^{32}P]dATP poly(A) tailed with terminal transferase. The filters were washed twice in 2 × SSC, 0.1% SDS, and twice in 0.5 × SSC, 0.1% SDS, all at 42°C. In addition, the oligo-probed filter was washed once in 0.25 × SSC, 0.1% SDS at 42°C and the MOR1-probed filter was washed in 0.2 × SSC, 0.1% SDS at 55°C. The filters then were exposed to X-ray film with intensifying screens.

2.3. Expression and characterization of hMOR1 and hMOR1A

Stable clones expressing the MOR1 and the MOR1A receptors were made by transfecting 70% confluent CHO-K1 cells in a 35 mm dish with 8 μg of either pcMOR1 or pcMOR1A and 8 μl of lipofectAMINE (Gibco/BRL) for 5 h in 1 ml of serum free Ham's F-12 media. 1 ml of media with 20% fetal calf serum (FCS) was added, which was replaced with 10% serum after 24 h from the start of transfection. After 48 h the cells were split 1 to 30 into ten 100 cm dishes with media containing

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Abbreviations: DPDPE, cyclic [D-Pen^2,D-Pen^5]enkephalin; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly^5-ol]enkephalin; U69593, D(5a,7a,8b)-(+)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxa spiro[4,5]-dec-8yl]benzene acetamide.

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10% FCS and 1 mg/ml Geneticin (Gibco/BRL). After 14 days colonies were picked and grown to confluency in 24 well microtiter dishes. Receptor expression was confirmed using [^{3}H]diprenorphine in a whole cell binding assay [21], and a high expressing cell line was selected for further studies. For binding studies, cells were grown to confluency, and membranes were prepared according to Raynor et al. [18]. For competitive binding studies, cell membranes (40–60 μg) were incubated with [^{3}H]diprenorphine (0.4 nM) in the presence of a competing ligand for 60 min at 25°C in a final volume of 500 μl. Saturation binding studies were done as described using increasing concentrations of [^{3}H]diprenorphine or [^{3}H]DAMGO. Non-specific binding was defined as the amount of binding in the presence of 10 μM levorphanol (≈5–10% of the total binding). All assays were performed in triplicate using 50 mM Tris, pH 7.4, and analyzed using the program LIGAND [22].

For cAMP analysis, cells were grown to confluency in 12 well costar plates, treated with 1 mM 3-isobutyl-1-methylxanthine and 1 mM forskolin for 30 min with or without the agonist DAMGO (0.5 mM), dissolved in 0.5 ml 1 M HCl, lyophilized and analyzed for cAMP using an Amersham kit (TRK432).

3. Results and discussion

Screening of a human genomic library at low stringency with a 1 kb DOR1 cDNA fragment was used to isolate two clones, OR7 and OR4. Subsequent restriction mapping and Southern blotting revealed that OR7 and OR4 represented the same gene. DNA sequencing of subclones hybridizing to the DOR1 probe showed the greatest homology to rat μ opioid receptor (rMOR1) [9,10]. Six transmembrane regions (TM) corresponding to TM #2–7 of rMOR1 were identified. The 5′ end of the gene containing TM #1 either is not present in these clones or does not hybridize to the DOR1 probe under the conditions used. A termination codon was identified 52 amino acids following TM #7, which is similar to the termination codon found 60 amino acids following TM #7 in rMOR1 [9,10]. We demonstarted the presence of mRNA by RT-PCR, amplifying fragments of the receptor from SK-N-SH messenger RNA with primers RTM2, MR2 and MF2, which are based on the 3′ non-coding and TM #4 regions of the OR7 clone (Fig. 1, lane3). Recently, Wang et al. [15] published a human μ cDNA sequence which is identical to exon regions of OR7 except for the 3′ end. Part of this discrepancy was explained by an intron/exon boundary that Wang et al. [15] identified 12 amino acids from the 3′ end of hMOR1. However, because we could PCR-amplify fragments of MOR from SK-N-SH mRNA containing a different 3′ end than reported for MOR1, we speculated that we may have identified a splice variant of the human MOR1 receptor which we call MOR1A.

The presence of full-length mRNAs for human MOR1 [15] and for the μ variant, MOR1A, derived from sequence information of OR7, MOR1A, was demonstrated in SK-N-SH mRNA using RT/PCR. Both the RT and 3′ PCR primers were specific to either MOR1 or MOR1A. MOR1's RT primer (RTM1), 5′ PCR primer (MF1), and 3′ PCR primer (MR1) were designed from the published sequence [15]. The MOR1A variant's RT primer (RTM2), and 3′ PCR primer (MR2), were designed using information obtained from sequencing of the OR7 clone. The 5′ PCR primer (MF1) was the same one used to clone MOR1. A shorter PCR product could also be amplified in the reactions by substituting MF1 with a new 5′ PCR primer (MF2), the sequence of which is common to TM #4 of OR7 and the published MOR1 sequence [15]. The results of the RT-PCR on the SK-N-SH message is shown in Fig. 1. Lane 1 shows the presence of a 679 PCR product containing the 3′-half

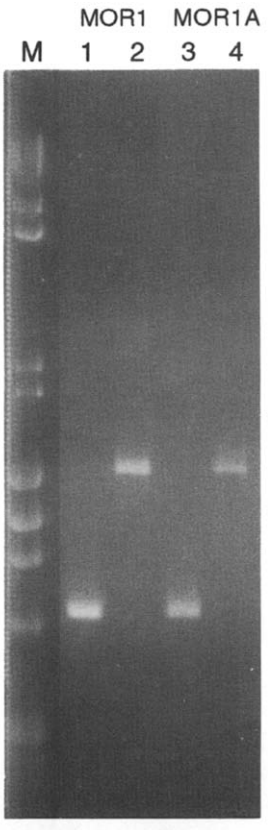

Fig. 1. Agarose gel demonstrating the presence of MOR1 and MOR1A mRNA in SK-N-SH cells. Ethidium bromide-stained agarose gel of the RT-PCR products from SK-N-SH mRNA. Lanes 1 and 2 used RT and 3′ PCR primers specific to MOR1 and lanes 3 and 4 used RT and 3′ PCR primers specific to MOR1A. Lanes 1 and 3 used a 5′ PCR primer common to TM #4 of MOR1 and MOR1A. Lanes 2 and 4 used a 5′ PCR primer to the 5′ non-coding region of MOR1 to yield complete cDNA coding regions. Lane M contains φX174/*Hae*III and λ/*Hin*dIII markers (Gibco/BRL).

of the MOR1 cDNA, and lane 2 shows the presence of a 1447 bp PCR product containing a full-length MOR1 cDNA. The presence of MOR1A mRNA in SK-N-SH cells is confirmed in lanes 3 and 4 of Fig. 1. Lane 3 shows the presence of a 717 PCR product containing the 3′-half of the MOR1A cDNA, and lane 2 shows the presence of a 1485 bp PCR product containing a full-length MOR1A cDNA. The difference in staining intensity suggests that MOR1A mRNA is about 5-fold less abundant than MOR1 mRNA in SK-N-SH cells. The full-length cDNAs of MOR1 and MOR1A were gel-purified, cloned, and sequenced. The MOR1 clone contained a reading frame coding for the complete 400 amino acid MOR1 receptor [15], and the MOR1A clone contained a reading frame coding for a 392 amino acid protein that differs from MOR1 only at its 3′ end.

It is unlikely that the MOR1A product is PCR amplification of unspliced heterogenous nuclear RNA, since PCR amplification of the 1485 bp MOR1A cDNA requires that intron 1 and 2 be removed. Larger PCR products, or no products at all, would have been the result if these introns were present. The PCR primers for the short product span intron 2 (0.8 kb). If intron 2 were not removed, some 1.5 kb product would have been observed during the PCR. Instead only the 717 base pair product is observed.

Fig. 2 shows the differences in the 3′ coding regions of MOR1

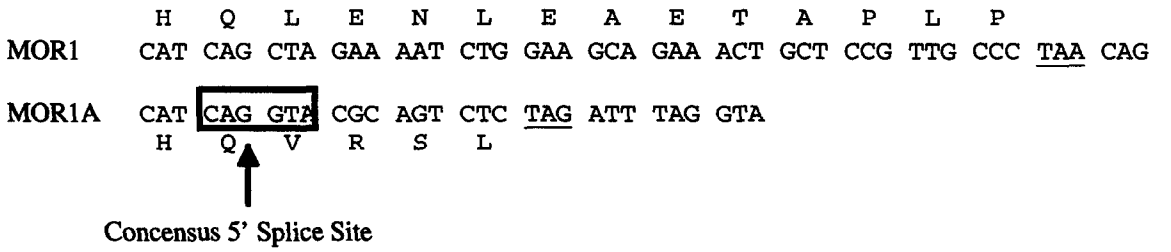

Fig. 2. Comparison of the DNA and amino acid sequences at the 3′ end of MOR1 and MOR1A. Stop codons are underlined, and a consensus 5′ splice site is boxed in MOR1A. All the 5′ coding sequence not shown is identical between receptors. The nucleotide sequence has been submitted to Genbank (#U12569).

and MOR1A. The nucleotides coding for the first 388 amino acids of MOR1A are identical to MOR1*. MOR1 continues for an additional 12 amino acids before terminating, and MOR1A continues for four amino acids before terminating. A consensus 5′ splice site can be seen in MOR1A's DNA sequence (and in the OR7 clone) at the position that MOR1 and MOR1A's sequences differ, suggesting that only in MOR1 has this splice site been used. A similar event in the somatostatin gene (i.e. alternative use of a 3′ splice site) leads to two functional somatostatin receptors, SSTR2A and SSTR2B [18,19].

Both MOR1 and MOR1A mRNAs are present in human brain, although MOR1A mRNA appears to be less abundant in human brain than in SK-N-SH cells. Fig. 3A shows the ethidium bromide-stained RT-PCR products from human brain mRNA using RT and 3′ PCR primers specific to either MOR1 or MOR1A and a common 5′ PCR primer to TM #4. The MOR1A PCR product is 717 bp and the MOR1 PCR product is 679 bp. MOR1A appears to be at least 10-fold less abundant than MOR1 in human brain mRNA as compared to about 5-fold in SK-N-SH mRNA. To further demonstrate the specificity of the PCR reactions, the gel was Southern-blotted and probed using a 43 nucleotide probe specific to MOR1A's 3′ end. Fig. 3B shows that only the MOR1A PCR products hybridize to the MOR1A specific probe. Furthermore, probing the blot with a probe containing the common regions of MOR1 and MOR1A demonstrates that the PCR products are of the μ opioid gene (Fig. 3C). The PCR product of MOR1 and MOR1A containing the complete coding region could also be PCR amplified from human brain mRNA, and showed the same hybridization specificity as the shorter product shown in Fig. 3 (data not shown).

The results of receptor binding experiments performed using membranes isolated from CHO-K1 cells stably expressing MOR1 or MOR1A are shown in Table 1. Data for the saturation binding of diprenorphine to MOR1 and MOR1A are shown in Fig. 4. MOR1 and MOR1A have similar affinities to diprenorphine (both K_d = 0.13 nM) and to DAMGO (K_d = 0.26 and 0.15 nM, respectively). Neither κ or δ selective ligands at high concentrations (>1 μM) displace diprenorphine from the MOR1 or MOR1A, but the μ specific ligand DAMGO does effectively displace diprenorphine.

MOR1 and MOR1A were analyzed for their ability to couple to cAMP (Table 2). Treatment of CHO-K1 cells stably expressing MOR1 or MOR1A with 0.5 mM DAMGO and forskolin caused a 32% (MOR1) and 39% (MOR1A) reduction in cAMP compared to cells treated with only forskolin, indicating that, like other opioid receptors, both MOR1 and MOR1A are functionally coupled to cAMP in CHO-K1 cells.

We re-examined the genomic clone OR7 for the presence of the 3′ terminus of MOR1 using a probe specific to MOR1's 3′ non-coding region (data not shown), but could not identify a hybridizing fragment. Since OR7 is approximately 15 kb, the human MOR gene is large. This also is true of the human κ receptor (>15 kb) which contains a large first intron, and a 3.5 kb second intron [16]. Our data indicates that the MOR1 gene's first intron between TM #1 and TM #2 is at least 8 kb, that the second intron between TM #4 and TM #5 is 0.8 kb and that

*Nucleotide differences between MOR1 [15] and MOR1A are noted in Genbank U12569.

Fig. 3. Identification of MOR1 and MOR1A mRNA in human brain. (A) Ethidium bromide-stained agarose gels of the RT-PCR products from human brain. The RT and 3′ PCR primers were specific to either MOR1 or MOR1A. The same 5′ PCR primer to TM #4 was used for both PCR reactions. The gel was Southern-blotted and probed with an oligomer 43 nucleotide long that is specific to MOR1A (B) or the pchMOR1 insert that is common to both MOR1 and MOR1A receptors (C).

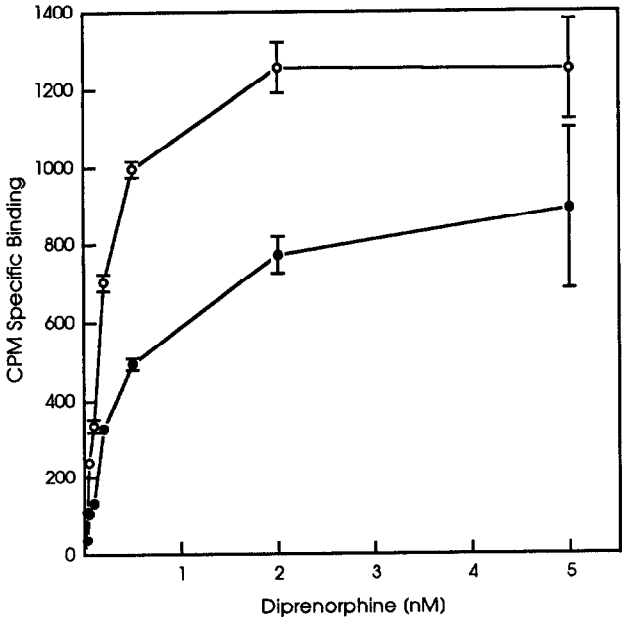

Fig. 4. Saturation binding of [^{3}H]diprenorphine using membranes from CHO-K1 cell stably expressing the cDNA for MOR1 (B_{max} = 760 fmol/mg) or MOR1A (B_{max} = 520 fmol/mg). Data are expressed as ± S.E.M.

the third intron is greater that 3 kb. It is this third intron that is alternatively spliced to produce MOR1 and MOR1A variants.

There appears to be at least two possible explanations of the MOR1A clone. First, it may be a true variant with ligand affinities similar or identical to MOR1 [15] and similar cAMP coupling. We have identified this transcripts in the mRNA of both SK-N-SH cells and in human brain mRNA via RT-PCR. Precedence for alternative splicing of a 3′ intron comes from the alternative splicing of the somatostatin receptor gene to form the SSTR2A and SSTR2B variants [18,19]. These somatostatin receptor variants have similar ligand affinities and cAMP coupling, but were shown to have tissue specific regulation [18].

The second possibility is that MOR1A is heterogeneous nuclear RNA that has been incompletely spliced. This seems unlikely, because if heterogeneous nuclear RNA were present additional PCR products containing the 0.8 kb intron 2 would have been detected in Fig. 1.

Further studies to detect the MOR1A receptor protein and to determine the effects of its unique C-terminus will help to establish and clarify the role of the MOR1A variant. MOR1A's unique C-terminus could have effects on G-protein coupling or on tissue distribution. It could also be involved in determining whether the receptor is localized to specific regions of the neuron. In addition, the high homology between human and rat μ opioid receptors suggests that MOR1A may be found in rat brain, where regional brain distributions can be more easily determined.

Table 1
Pharmacological characterization of the cloned μ opioid receptors

Ligand	MOR1		MOR1A	
	K_d (nM)	K_i (nM)	K_d (nM)	K_i (nM)
Non-selective diprenorphine	0.13 ± 0.08		0.13 ± 0.07	
μ-selective DAMGO	0.26 ± 0.06	0.5 ± 0.1	0.15 ± 0.04	1.0 ± 0.1
κ-selective U69,593		> 1000		> 1000
δ-selective DPDPE		> 1000		> 1000

The binding data were collected in triplicate and analyzed with the LIGAND program. Data are expressed as ± S.E.M.

Table 2
Receptor coupling to cAMP activity (inhibition)

Receptor	MOR1	MOR1A
Control	1.5 ± 0.5	2.0 ± 0.5
+ Forskolin	24 ± 2.5	25 ± 1.5
+ Forskolin + 0.5 mM DAMGO	16 ± 1.6	15 ± 1.0
% inhibition	32	39

Synthesis of cAMP (pmol/5 × 10^5) in stable cell lines expressing the receptor cDNAs. Data are expressed as ± S.E.M.

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