Cloning and expression of an isoform of the rat μ opioid receptor (rMOR1B) which differs in agonist induced desensitization from rMOR1

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Abstract A novel rat μ opioid receptor (rMOR1B) has been isolated. It shows identity to the recently published sequence of rMOR1 [Chen, et al., *Mol. Pharmacol.*, 44 (1993) 8–12] up to amino acid 386 and differs only in length and amino acid composition at the very carboxy-terminal tail. Both μ opioid receptor isoforms, when stably expressed in CHO-K1 cells, show similar affinities to opioid compounds and are equally effective in the inhibition of forskolin-induced cAMP formation. Reverse transcription polymerase chain reaction (RT-PCR) revealed that rMOR1B displays a similar distribution as rMOR1 in various rat brain areas. Studies measuring the inhibition of adenylate cyclase in cells that had been pre-exposed to the μ opioid agonist DAMGO indicated that rMOR1B is much more resistant to agonist-induced desensitization than rMOR1.

Key words: Opioid receptor; Alternative splicing; Desensitization; Cloning; G protein-coupled receptor

1. Introduction

Opioids exert their effects by specifically interacting with receptors which are widely distributed throughout the central and peripheral nervous system [1]. Until now, three subtypes of opioid receptors, termed μ [2–4], δ [4–6] and κ [7–12], have been cloned. They belong to the families of membrane receptors that transduce their intracellular signals via G protein-coupled pathways [13–15], and mediate the inhibition of adenylate cyclase, activation of potassium channels and/or inhibition of calcium channel activity through a pertussis-toxin-sensitive mechanism [13,16]. Most interesting among them is the μ receptor, because this receptor is thought to play an important role in mediating the opioid phenomena classically associated with morphine, including analgesia, opioid dependence and tolerance, and respiratory functions. In the present study, we report the cloning of a rat cDNA (rMOR1B) which shares sequence identity to the rat μ opioid receptor up to amino acid 386. This cDNA, which is very likely produced by alternative splicing, varies from rMOR1 in length and amino acid composition only at the C-terminal tail. Further, it is reported that both isoforms have similar binding properties and are equally effective in inhibiting forskolin-stimulated cAMP production. However, we demonstrate that rMOR1B is much more resistant to agonist-induced desensitization than rMOR1.

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2. Materials and methods

2.1. Cloning of cDNA

A mouse opioid receptor cDNA fragment was isolated by polymerase chain reaction (PCR) from reverse transcribed (RT) mRNA isolated from (NG108-15) cells using the following primers: AGACCGCCACCAACATCTACAT (sense), and CCCACCAC-CACCAGCACCAT (antisense), corresponding to transmembrane domains I and VI, respectively. Cycle conditions were: 1 min at 96°C, 1 min at 60°C and 1 min at 72°C for 40 cycles. The ends of the resulting product (562 bp) were filled in with Klenow enzyme and cloned into the *Eco*V site of pBluescript SK(−) (Stratagene). This fragment was then used to screen 1×10^6 plaques from a rat brain cDNA library (l ZAP II; Stratagene) under low stringency conditions (20% formamide, 5× SSC (1× SSC = 0.15 M sodium chloride; 0.015 M sodium citrate), 5× Denhardt solution, 100 μg/ml salmon sperm DNA, at 37°C overnight). Filters were washed in 2× SSC at 37°C for 2 h. Plasmids were autoexised from positive phage DNA. One 2.4 kb clone (rMOR1B) was sequenced and further characterized.

2.2. DNA transfection of CHO-K1 cells and binding analysis

A *Stu*I–*Xba*I fragment of rMOR1B, containing the entire protein-coding sequence, was cloned into pcDNAI.neo (Invitrogen). rMOR1 cloned into pRc/CMV was kindly provided by Dr. Lei Yu (Indianapolis, USA). For stable expression, CHO-K1 cells were grown to 50% confluency in a 35 mm dish and subsequently transfected with the receptor expression plasmids using lipofectin (Gibco/BRL) according to the instructions of the manufacturers. Stable transfectants were selected in NUT-F12 medium containing 10% FCS (fetal calf serum) and 600 μg G418 (Gibco/BRL) per ml, and checked for expression by Northern blot analyses. For binding studies, cells were grown to confluency and the membranes prepared as described [2]. For competitive binding studies, cell membranes (15–50 μg protein; measured by a protein assay kit (Bio-Rad)) were incubated with 2 nM [^{3}H]DAMGO (D-ala^2, *N*-methyl-phe^4, glyol enkephalin) in the presence of a competing ligand for 30 min at 25°C in a final volume of 500 μl. All assays were performed in duplicate using 50 mM Tris-HCl, pH 7.4, and 0.2% BSA (bovine serum albumin). The reactions were terminated by vacuum filtration through Whatman GF/B filters that had been pretreated with 1% polyethylenimine. Non-specific binding was determined using 10 μM DAMGO.

2.3. RT-PCR

Total RNA was isolated from tissues of adult Wistar rats as described [17], digested with RQ1 RNase-free DNase (Promega) and converted to single-stranded cDNA by priming of 2 μg total RNA with hexanucleotide primers and reverse trancriptase (Superscript; BRL). cDNAs were amplified using *Taq* polymerase (Pharmacia) and up- and downstream primers PMOR-1, PMOR-2 and PMOR-3, as indicated in Fig. 1 for 35 cycles (96°C for 1 min, 55°C for 1 min, and 72°C for 2 min). To test the conditions for the PCR reaction, cDNAs were subjected to 30, 35, 38 or 40 cycles of amplification. Saturation was observed after 38 and 40 rounds of amplification. The resulting products for rMOR1 (404 bp) and rMOR1B (364 bp) were electrophoresed and stained with ethidium bromide.

2.4. Adenylate cyclase experiments

Accumulation of cAMP was measured as follows: 1.5×10^5 stable transfected CHO-K1 cells were seeded in 22 mm twelve-well dishes with NUT-F12 medium containing 10% FCS. cAMP assays were performed

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24 h later. Cells were washed twice with serum-free medium (SFM) and pretreated with SFM containing 200 μM isobutyl methyl xanthine for 20 min at 37°C. Thereafter, forskolin (25 μM final concentration) or a combination of forskolin and DAMGO were added at the indicated concentration and the incubation continued for 15 min at 37°C. The reaction was terminated by removing the medium, scraping and sonicating the cells in 1 ml of ice-cold HCl/EtOH (1 vol. of 1 N HCl/100 vols. EtOH). After evaporation of the solution, the residue was dissolved in 10 mM Tris, 1 mM EDTA (TE) buffer (pH 7.4), and cAMP content measured using a commercially available kit (Amersham; TRK 432). For time–course experiments, cells were seeded as described above and DAMGO (1 μM) was added into the growth media at 37°C at the indicated times before assay. For some studies cells were incubated with pertussis toxin (PTX, 100 ng/ml) (List Biological Laboratories Inc.) for 24 h before assay.

3. Results

3.1. Cloning of the cDNA

A rat brain cDNA library was screened under low hybridization conditions with a fragment of the mouse δ opioid receptor as a probe. One positive plaque contained a 2.4 kb cDNA fragment. Sequencing revealed that it encodes a protein of 391 residues, which shares 100% homology with rMOR1 up to amino acid 386. The encoded protein is seven residues shorter than that predicted from the rMOR1 sequence and differs in five amino acids at the C-terminus. The clone was therefore termed rMOR1B (Fig. 1). Recently the genomic organization of the mouse μ opioid receptor was elucidated and an intron–exon boundary has been found between amino acids 386 and 387 [18]. As the cDNAs of both receptors start to differ from each other at exactly the same position it is very likely that rMOR1B, in addition to rMOR1, derives from a common precursor mRNA by alternative splicing.

3.2. Expression of receptor isoforms in CHO-K1 cells

To test whether rMOR1B differs in its binding properties from rMOR1, expression studies were performed. CHO-K1 cells were stable transfected with rMOR1 and rMOR1B and competition binding experiments were performed using the μ-specific ligand [^{3}H]DAMGO as specific radioligand. Representative competition curves are shown in Fig. 2. In general, the relative affinities of both receptor variants for opioid ligands are similar. Non-labeled DAMGO displaced [^{3}H]DAMGO binding with high affinity (IC_{50} = 5 nM), whereas the δ-selective agonist DPDPE [D-Pen^{2,5}-enkephalin] and the κ-selective agonist U-50488 showed low affinities, with IC_{50} values in the micromolar range. DADLE [D-ala^2, D-Leu^5-enkephalin], a predominantly δ agonist, displayed nanomolar affinities (IC_{50} = 70 nM and 100 nM, respectively). The μ_1-selective antagonist naloxonazine also shows high affinities (IC_{50} = 5 nM and 6 nM, respectively). Taken together, these results suggests that rMOR1B encodes μ receptors and that the divergence in the C-terminal tail does not essentially change the ligand specifities.

3.3. RT-PCR

RT-PCR was carried out to investigate the mRNA expression pattern of both isoforms in various brain regions. PCR primers were chosen in such a way that either rMOR1 or rMOR1B was specifically amplified (Fig. 1). PMOR-1 and PMOR-2 primers were used to obtain a 404 bp fragment consistent with rMOR1 mRNA, and PMOR-1 and PMOR-3 to obtain a 364 bp fragment consistent with rMOR1B mRNA. After 35 PCR cycles similar concentrations of amplified receptor mRNA levels for both rMOR1 and rMOR1B could be detected in various brain areas (Fig. 3). In general, the expression pattern of rMOR1 and rMOR1B mRNA in rat brain is similar, with highest mRNA concentrations in the frontal cortex (lane 2) and the striatum (lane 4) and lowest concentrationes in the bulbus olfactorius (lane 1) and the cerebellum (lane 10). On the other hand, the tegmentum (lane 9) and the pons (lane 11) appear to contain smaller concentrations of rMOR1B than rMOR1 mRNA, indicating a tissue-specific splicing.

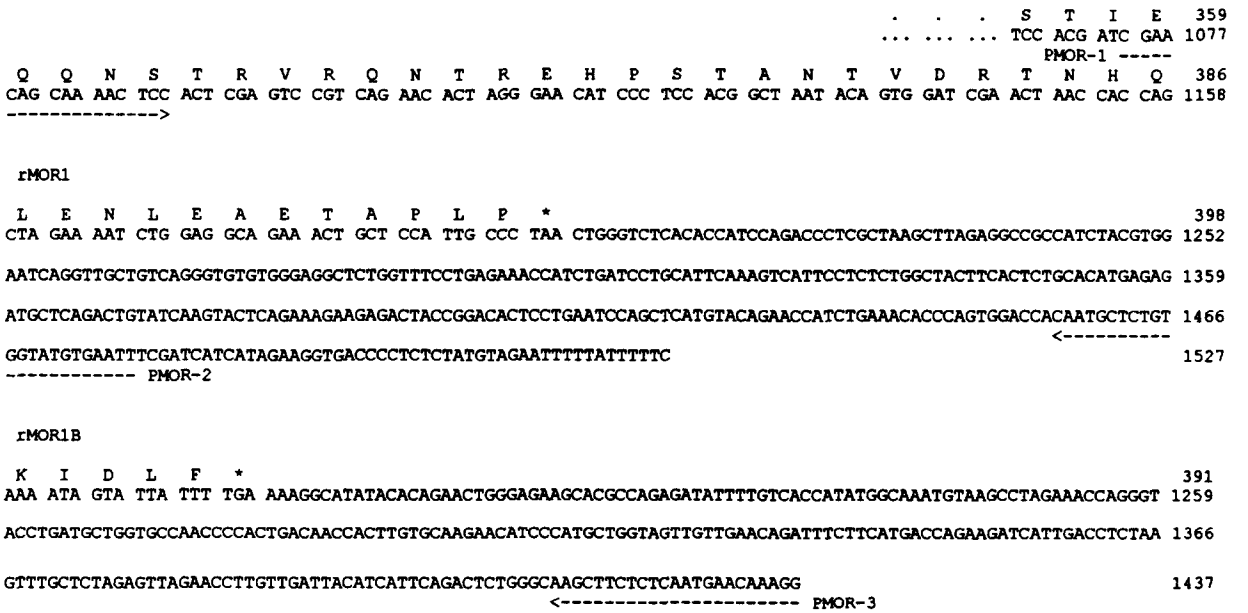

Fig. 1. The carboxy-termini of rMOR1 and rMOR1B. The deduced amino acid sequence is shown above the nucleotide sequence. Primers used for PCR (PMOR-1, PMOR-2 and PMOR-3) are marked by dashed lines with arrows. Note that both receptor variants share identical nucleotide sequences up to amino acid 386.

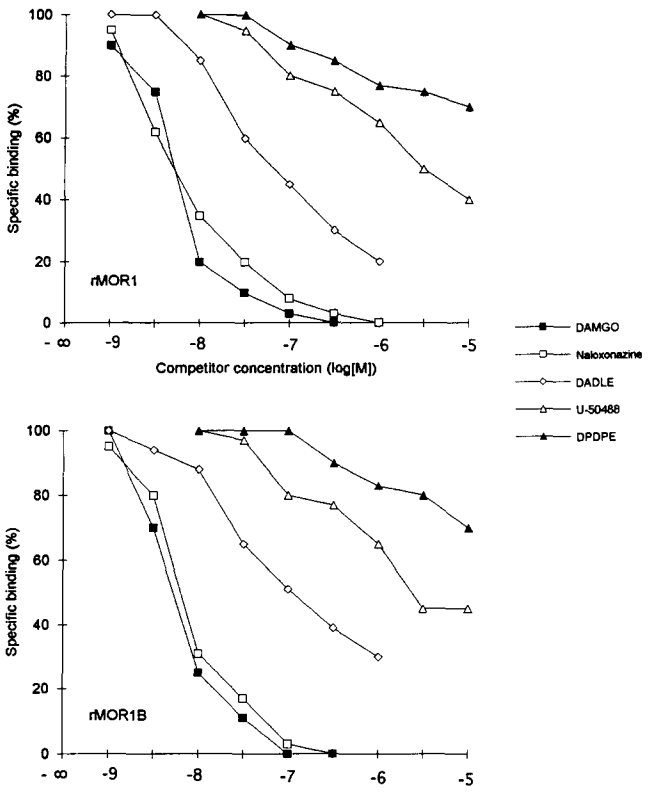

Fig. 2. Displacement of [^{3}H]DAMGO binding from rMOR1- and rMOR1B-expressing CHO-K1 cells by unlabeled ligands. Data from a representative experiment are presented for each ligand. IC_{50} values were calculated by log probit analysis of two independent displacement curves. IC_{50} (nM; rMOR1B/MOR1): DAMGO (5/5); naloxonazine (6/5); DADLE (100/70); U-50488 (2000/3000); DPDPE (>10,000/>10,000).

3.4. *Second messenger coupling of receptor isoforms*

To test the ability of both variants to inhibit forskolin-stimulated cAMP accumulation, cells were incubated with increasing concentrations of DAMGO (1 nM to 1 μM) followed by the determination of the levels of cAMP (Fig. 4). Both rMOR1 and rMOR1B inhibit stimulated adenylate cyclase to the same extent (69 ± 3% and 73 ± 2%, respectively) and with a similar potency (EC_{50} = 150 nM). Pretreatment of the cells with pertussis toxin (100 ng/ml) abolished the ability of the activated receptors to inhibit cAMP formation, indicating that both variants inhibit adenylate cyclase activity via pertussis toxin-sensitive G proteins (data not shown). However, a significant difference between the two variants in their desensitization behavior upon agonist treatment could be observed. Pretreatment of the cells with DAMGO (1 μM) attenuates the ability of the agonist to inhibit forskolin-stimulated adenylate cyclase faster in MOR1- than in MOR1B-expressing cells (Fig. 5). Thus, as early as 30 min following preincubation with 1 μM DAMGO, a significant reduction by the agonist of the inhibition of forskolin-induced cAMP levels (from 69 ± 3% to 59 ± 2%) was observed in cells expressing rMOR1. In contrast, in rMOR1B-expressing cells, pretreatment with DAMGO for 30 min, 1 h and 2 h had no influence on agonist-induced inhibition of stimulated adenylate cyclase; pretreatment with DAMGO for 5 h was required to obtain a significant effect (from 73 ± 3% to 57 ± 2%), indicating that rMOR1B is less prone than rMOR1 to undergoing agonist-promoted desensitization (Fig. 5). After 24 h of preincubation with DAMGO, however, both receptors entirely lost their ability to inhibit adenylate cyclase.

4. Discussion

Our results provide evidence for the existence of another μ opioid receptor. Two subtypes of μ opioid receptors (μ_1 and μ_2), which differ in their binding of morphine and enkephalin, have already been proposed [19]. However, the observation that rMOR1B binds to the μ_1-selective antagonist naloxonazine with a similar high affinity to rMOR1, excludes the possibility that rMOR1B encodes a μ_2 receptor.

There are an increasing number of reports demonstrating that splicing is a common mechanism to produce different C-termini of G protein-coupled receptors (GPC-Rs) [20–22]. Furthermore, the carboxy-terminal tail of GPC-Rs was shown to influence G protein selectivity [22] and/or the desenzitization characteristics of GPC-Rs [23]. Our group recently reported that the somatostatin receptor subtype 2 (SSTR 2) occurs in two isoforms. The spliced receptor SSTR 2B differed from the unspliced receptor (SSTR 2A) only at the C-terminus. The shorter form (SSTR 2B) was more resistant to agonist-induced desensitization than the longer form (SSTR 2A) [23]. In addition, recent data provide evidence that the C-terminus also plays a crucial role in the desensitization process of other GPC-Rs. Thus, progressive truncation of the C-terminus of the rat substance P receptor attenuates agonist-induced desensitization [24]. Similarly, the platelet-activating factor (PAF) recep-

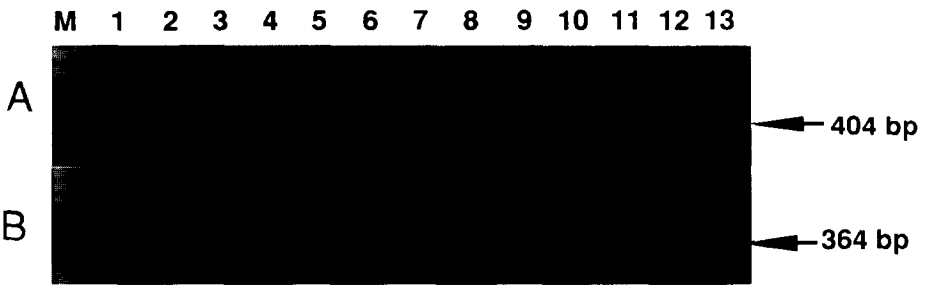

Fig. 3. PCR analyses of rMOR1 (A) and rMOR1B (B) receptor transcripts from various rat brain tissues. Lanes: 1, bulbus olfactorius; 2, frontal cortex; 3, motor cortex; 4, striatum; 5, thalamus; 6, hypothalamus: 7, hippocampus; 8, tegmentum; 9, colliculi; 10, cerebellum; 11, pons; 12, medulla oblongata; 13, negative control. For amplification of rMOR1 the primer pair PMOR-1 (sense) and PMOR-2 (antisense) were used, and for amplification of rMOR1B, PMOR-1 (sense), PMOR-3 (antisense) were used; see Fig. 1. M (marker), *Hae*III-digested fragments of Fx 174 phage (in bp 1353, 1078, 872, 603, 310).

tor is rapidly desensitized in response to prolonged exposure to agonists, whereas a C-terminally truncated form of this receptor shows almost no desensitization characteristics [25]. Similar findings were noted in another mutant of the PAF receptor in which the serine and threonine residues in the carboxy tail were substituted with alanines [25]. This indicates that phosphorylation of the C-terminal part of the receptor by protein kinases (e.g. protein kinase A (PKA) or beta-adrenergic receptor kinase (βARK)) plays a crucial role in agonist-induced desensitization. It is noteworthy that within that region of the C-terminus in which rMOR1 differs from rMOR1B, one potential phosphorylation site for βARK is found (threonine-394). It remains to be clarified by mutation studies whether phosphorylation of this threonine is responsible for the faster desensitization of rMOR1B as compared to that of rMOR1, or if other mechanisms are also involved.

While preparing this manuscript the isolation of another spliced variant of the human μ opioid receptor was reported [26]. This isoform, in which the 3′ terminal intron has not been removed, encodes a μ receptor, which is eight amino acids shorter than hMOR1 and differs by four amino acids at the C-terminus (which showed no homology to rMOR1B). This human μ receptor variant was shown to be expressed in SK-N-SH cells and in human brain. It would be interesting to know whether a similarly spliced isoform of MOR1 may also occur in rats. Moreover, it is not unreasonable to assume that in various species several alternatively spliced forms of the MOR1 receptor may exist which differ in amino acid composition at the very carboxy-terminus. It would be interesting to know whether they also differ in agonist-induced desensitization. Presently experiments are being carried out in our laboratory to search for additional C-terminally spliced isoforms of rMOR1.

The existence of isoforms of MOR1 in brain which differ in agonist-induced desensitization may explain the well-known

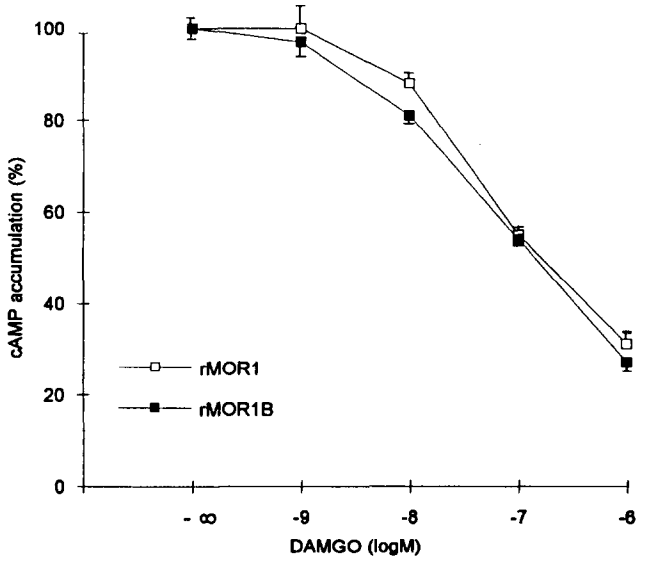

Fig. 4. Dose-dependent inhibition of DAMGO of forskolin-induced cAMP accumulation in stably transfected CHO-K1 cells. Cells were treated for 15 min with 25 μM forskolin in the presence of absence of various concentrations of DAMGO. For details see section 2. The results are shown as mean ± S.E.M. of three separate experiments performed in triplicate.

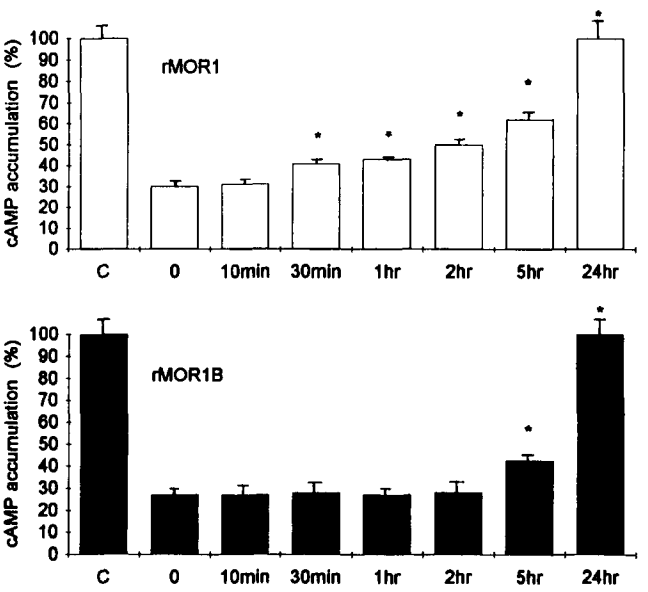

Fig. 5. Time-courses of agonist-induced desensitization of rMOR1 and rMOR1B. Transfected CHO-K1 cells were preincubated at 37°C with 1 μM DAMGO for the indicated time intervals. After washing twice with 1 × PBS (phosphate buffered saline) cells were treated with forskolin or forskolin plus DAMGO, and cAMP levels were determined as described in section 2. Values represent means ± S.E.M. from three separate measurements performed in triplicate. The asterisks (*) indicate significant difference ($p < 0.05$) from cells which were not preincubated with DAMGO (0). In addition, in the case of rMOR1, significant differences could be observed at 30 min vs. 10 min, 2 h vs. 1 h, 5 hr vs. 2 h, and 24 h vs. 5 h after exposure to DAMGO. In the case of rMOR1B, significant differences were observed at 5 h vs. 2 h, and 24 h vs. 5 h agonist exposure. C (control), forskolin treatment without DAMGO.

observation that the development of tolerance to the various effects of morphine is different. Thus, a rapid tolerance to the analgesic effects of morphine is generally observed, whereas tolerance is much less pronounced in the case of the respiratory depressant effects of the opioid agonist.

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