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Short communication

Effect of opioid receptor ligands on the μ-S196A knock-in and μ knockout mouse vas deferens

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Abstract

We have determined the effect of naltrexone, naloxone, [D-Ala^2,D-Leu^5]enkephalin (DADLE), and morphine on the μ-S196A opioid receptor knock-in and μ-opioid receptor knockout mouse vas deferens preparations. The antagonists, naltrexone and naloxone, exhibited agonist activity and possessed IC_{50} values that were 14- and 37-fold greater than morphine on the S196A preparation. Morphine was found to be threefold more potent at S196A relative to wild-type μ-opioid receptor. The mouse vas deferens data suggest that S196 in transmembrane helix 4 of the μ-opioid receptor modulates efficacy. It is proposed that this may be due to decreased dimerization of the receptor. Identical IC_{50} values of DADLE obtained on the wild-type, S196A knock-in, and μ-opioid receptor knockout preparations support the absence of μ–δ heterodimers in the mouse vas deferens.

Keywords: μ-Ser196Ala; Vas deferens, mouse; Opioid receptor antagonist; μ-Opioid receptor knockout

1. Introduction

Opioid receptors are members of the rhodopsin subfamily in the superfamily of G protein-coupled receptors (Dhawan et al., 1996). The overall identity of amino acid residues within the opioid receptor family is approximately 60%. The different types of opioid receptors are characterized by a highly homologous heptahelical transmembrane domain and intracellular loops. The extracellular loops and both the N- and C-terminus sequences possess low homology.

In an effort to investigate the relationship between structure and function of opioid receptors, a substantial number of chimera and point mutations have been constructed (Law et al., 1999). A majority of the mutational analysis studies have focused on the seven-transmembrane helical domain because it is generally believed that the cavity formed from the bundle of helices contains an array of residues that comprises the recognition site for opioid ligands.

Of considerable interest are reports that the conserved serine residue at position 196 (S196) in transmembrane helix 4 of the μ-opioid receptor is required for the distinction between opioid receptor agonist and antagonist function (Claude et al., 1996; Yang et al., 2003). This effect, which is characterized by the transformation of an antagonist (e.g., naloxone) to an agonist, has been investigated in cultured cells and, most recently, in homozygous knock-in mice that contain the S196A mutant μ-opioid receptor. The binding of agonist and antagonist ligands to the S196A mutant μ-opioid receptor has been reported to be indistinguishable from that of the wild-type receptor.

In order to determine if this phenomenon can also be observed in a native system that is less complex than the central nervous system (CNS), we have evaluated two agonists, morphine and [D-Ala^2,D-Leu^5]enkephalin (DADLE), and two antagonists, naloxone and naltrexone, on the isolated mouse vas deferens preparation that contains the S196A mutant μ-opioid receptor. Additionally, the μ- and δ-opioid receptor agonists have been tested on the μ knockout mouse vas deferens preparation in order to determine whether or not there is interaction between these receptors, as there are reports implicating the existence of μ–δ heterodimeric opioid receptors in the CNS and in cultured cells (George et al., 2000; Gomes et al., 2000; Rothman et al., 1993; Vaught et al., 1981). While studies with selective opioid ligands support the presence of non-

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interacting μ- and δ-opioid receptors in the mouse vas deferens (Elliott and Traynor, 1995), the use of a μ-opioid receptor knockout preparation would offer a more conclusive answer to this question.

2. Materials and methods

Vasa deferentia derived from wild-type, homozygous S196A μ-opioid receptor mutant knock-in, and mu-opioid receptor knockout mice (Yang, et al.) were prepared using the method of Henderson et al. (1972). Mice (25–30 g) were sacrificed by cervical dislocation. Both vasa deferentia were removed and mounted between platinum ring electrodes and placed in a 10-ml organ bath containing a modified Kreb's solution (NaCl, 118 mM; KCl, 4.70 mM; $CaCl_2$, 2.52 mM; KH_2PO_4, 1.19 mM; $NaHCO_3$, 25 mM; glucose, 11.48 mM; pH = 7.4) at 37 °C. The bath was continuously bubbled with a 95% O_2, 5% CO_2, gas mixture. One end of the vas deferens was attached to the electrode assembly, the other is attached to a Statham-Gould UC-3 isometric force transducer using 6.0 surgical silk. The vasa deferentia were stimulated transmurally with a Grass S44 stimulator (square waves of supramaximal voltage (70 V) for 1 ms and a frequency of 0.1 Hz). Resting tension was 200 mg. Vasa deferentia were stimulated continuously for 20 min before each experiment to allow equilibration to occur. The tissues were washed every 10 min during this period.

Graded concentrations of morphine sulfate, naloxone.HCl, naltrexone.HCl (Mallinckrodt Chemical, St. Louis, MO), and [D-Ala^2,D-Leu^5]enkephalin (Multiple Peptide Systems, San Diego, CA) dissolved in 100 μl water were added to the organ bath to obtain a cumulative concentration–response relationship. Testing of naloxone.HCl and naltrexone.HCl on the wild-type preparation was conducted at three concentrations (100, 300, 1000 nM), whereas all other testing was carried out at four to five dose levels. IC_{50} values were estimated by the parallel line assay of Finney (1964).

The animal protocols used in these experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC).

3. Results

The δ-opioid receptor-selective peptide, DADLE, exhibited no significant difference in the inhibition of electrically stimulated contractions of the wild-type, μ-S196A knock-in, and μ-opioid receptor knockout mouse vas deferens preparations, with IC_{50} values in the 0.32–0.37 nM range (Table 1). Morphine, on the other hand, displayed greatest potency (IC_{50} = 18 nM) on the knock-in mouse vas deferens, with ~ 3-fold greater potency over the wild-type. On the μ-opioid receptor knockout preparation, morphine was a partial agonist (38%) with a 60-fold decrease in potency relative to wild-type. Naltrexone and naloxone exhibited full agonist activity when tested on the knock-in preparation, with ED_{50} values of 270 and 400 nM, respectively. Both antagonists displayed little, if any, agonism at 1-μM on the wild-type mouse vas deferens. In this regard, the naloxone and naltrexone exhibited 5% and 21% inhibition of the preparation at 1 μM.

Table 1
Potencies of opioid ligands on the wild-type, μ-S196A knock-in and μ-opioid receptor knockout vas deferens preparations

Agonist	IC_{50} ± S.E.M. (*n*)		
	Wild-type	μ-S196A knock-in	μ Knockout
DADLE	0.37 ± 0.11 (4)	0.32 ± 0.11 (4)	0.35 ± 0.06 (6)
Morphine	54 ± 19 (5)	18 ± 2.9 (11)	3300[a] (2)
Naloxone	[b]	400 ± 120 (3)	–
Naltrexone	[c]	270 ± 24 (4)	–

[a] 38% maximal effect at 10 μM.
[b] 5% maximal effect at 1 μM.
[c] 21% maximal effect at 1 μM.

4. Discussion

The present study using morphine in the knock-in mouse vas deferens containing S196A mutant μ-opioid receptors has provided data that are generally in agreement with the reported (Yang et al., 2003) agonist activity of naloxone and naltrexone in knock-in mice. The IC_{50} values of naloxone and naltrexone were 22- and 15-fold greater than that of morphine in the μ knock-in mouse vas deferens (Fig. 1). In the wild-type preparation, these opioid receptor antagonists were very feebly active. The finding that the maximal agonist effect at 1 μM on the wild-type preparation was substantially greater for naltrexone (21%) than for naloxone (5%) may reflect the greater efficacy of naltrexone over naloxone. When compared to the partial agonist activity reported for naltrexone and naloxone on knock-in mice, these ligands appeared to possess substantially greater efficacy in the μ-S196A preparation.

The three greater agonist potency of morphine in the knock-in relative to the wild-type preparation (Table 1) differs from that in mice where no significant difference in antinociception was reported. However, since there was an 85% decrease in the number of S196A mutant μ-opioid receptors in the brains of knock-in mice and no significant difference between K_d values of wild-type and mutant receptors, it appears likely that the greater potency of morphine on the knock-in mouse vas deferens is due to enhanced efficiency of activation by the S196A mutant μ-opioid receptor.

On the μ-opioid receptor knockout preparation, morphine behaved as a partial agonist (maximum effect, 38%) with greatly reduced potency. The partial agonist activity is most likely mediated through kappa receptors, given the reported change of pA_2 value to that characteristic of kappa receptors

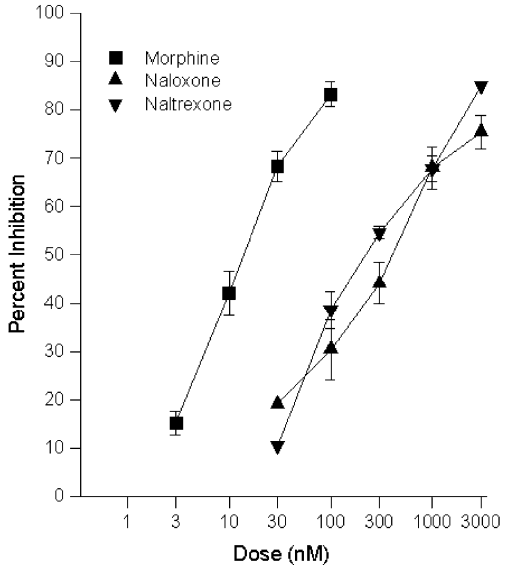

Fig. 1. Concentration–response curves of morphine, naloxone, and naltrexone from vasa deferentia derived from S196A knock-in mutant mice.

upon depletion of μ receptors by β-funaltrexamine treatment in the guinea pig ileum preparation (Ward et al., 1982).

The finding that the IC_{50} values of DADLE on S196A knock-in and μ-opioid receptor knockout preparations were not significantly different from that on the wild-type mouse vas deferens preparation has relevance in regard to the question concerning whether δ-opioid receptors function independently or in association with μ-receptors as heterodimers. The well-known in vivo synergism between μ- and δ-opioid receptor agonist ligands (Vaught et al., 1981), binding studies on brain membranes (Rothman et al., 1993), and recent studies on μ- and δ-opioid receptors co-expressed in cultured cells (George et al., 2000; Gomes et al., 2000) have led to the idea that these receptors may interact with one another. However, on the basis that co-administration of selective μ- and δ-opioid receptor agonists failed to give a synergistic effect, it was concluded that there was no interaction between these receptors in the mouse vas deferens preparation (Elliott and Traynor, 1995). The results of the present study provide definitive evidence in support of this conclusion.

The conserved S196 residue involved in modulating μ-opioid receptor activation is located in transmembrane helix 4 which is positioned more toward the exterior of the transmembrane domain that contributes to the recognition of opioid ligands. Among the receptors in the opioid receptor family, helix 4 has the lowest homology in the transmembrane domain and it is most exposed to the lipid bilayer. Due to the position of helix 4 with respect to the central cavity of the transmembrane bundle that contains the opioid recognition site, none of its residues appear to be direct participants in the binding of ligands to the μ-opioid receptor.

The relatively exposed nature of transmembrane helix 4 and the recently published direct demonstration that the prototypical G protein-coupled receptor, rhodopsin, is organized as a heterodimer with intradimer contacts between helices 4 and 5 for each subunit (Liang et al., 2003), may be of relevance in providing insight into the modulation of pharmacological efficacy by S196. If wild-type μ-opioid receptors are dimerized through the same interface, it is conceivable that mutations in helix 4 may inhibit dimerization.

But why would this effect efficacy? One possible explanation may be related to the report (Portoghese and Takemori, 1983) that naltrexone and naloxone are substantially more effective than μ-opioid receptor agonists in protecting μ-opioid receptors against alkylation by β-funaltrexamine in the guinea pig ileum preparation. Significantly, the corresponding N-methyl analogue, β-fuoxymorphamine, is a reversible μ-opioid receptor agonist and neither alkylates nor protects against alkylation by β-funaltrexamine. The lack of protection by agonists, and the inability of β-fuoxymorphamine to alkylate or protect was attributed to separate recognition sites for agonist and antagonist ligands. It was proposed that the μ-opioid receptor exists as a dimer in which one of the subunits modulates the efficacy of its neighbor as a regulatory mechanism for endogenous opioid peptides. Accordingly, signaling by an agonist at one of the sites would be dampened by the interaction of agonist at the neighboring site, and this was suggested to be a function of ligand concentration. It remains to be determined if this model represents a viable mechanism to account for the change in activity of naloxone and naltrexone from antagonist to agonist in the μ-S196A mutant receptor.

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