Research report

Improgan, a cimetidine analog, induces morphine-like antinociception in opioid receptor-knockout mice


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Abstract

Improgan is an analog of the H₂ antagonist cimetidine that does not act on known histamine receptors, but induces highly effective analgesia in rodents following intracerebroventricular (icv) administration. Since the mechanism of action of this compound remains unknown, improgan analgesia was characterized presently with the tail immersion nociceptive test in mutant mice lacking either the μ (exon 1 of MOR-1), δ (exon 2 of DOR-1) or κ (exon 3 of KOR-1) opioid receptor. Improgan (30 μg, icv) induced reversible, maximal analgesia in both sexes of all three genotypes (+/+ and −/−) of MOR-1 mutant mice 10 and 20 min after administration, whereas morphine analgesia was reduced (+/−) or abolished (−/−) in these subjects. In DOR-1 mutant mice, improgan was equally effective in all three genotypes, despite the reduction (+/−) or complete loss (−/−) of δ opioid receptor ([H-[D-Pen²,D-Pen⁵]enkephalin, DPDPE) binding. Similarly, improgan analgesia was equivalent in all three genotypes of KOR-1 mutant mice, whereas κ-mediated analgesia ([H-U69,593] binding were abolished in the homozygous (−/−) mice. These studies demonstrate that improgan analgesia does not require intact MOR-1, DOR-1, or KOR-1 genes, and support the hypothesis that improgan-like analgesics act in the CNS by non-opioid mechanisms.

Theme: Sensory systems
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1. Introduction

Pain continues to be a worldwide health problem, and there is an urgent need to discover new kinds of pain-relieving medications [6]. Despite progress, the opioid morphine remains the standard by which new medications are compared. Thus, an ideal analgesic would show morphine-like activity in pain tests, but lack opioid side-effects, both of which result from stimulation of opioid receptors. Improgan (N-cyano-N’-[3-(imidazole-4-yl)propyl]-N”’-methylguanidine, formerly known as SKF92374) is an analog of the histamine H₂ receptor antagonist cimetidine, that has been shown to inhibit thermal and mechanical nociception in rodents following icv administration [13,14]. Of particular note is the efficacy of this compound in attenuating high-temperature tail flick responses in rats, an activity shared with morphine, but with few other classes of analgesic agents [13,14]. At maximal antinociceptive doses in rats, improgan does not alter locomotor or rotorod performance, suggesting a selective analgesic activity [14]. Furthermore, daily injections of morphine and improgan showed tolerance to the analgesic activity of the former, but not to the latter, suggesting a favorable clinical profile [1]. Other com-
pounds structurally related to \( H_2 \) and \( H_3 \) antagonists also induce analgesia, and a novel structure–activity relationship for this effect has been established [7,9].

In vitro screens at more than 50 receptors [6] have not yet identified a site of improgan action, and little is known about the mechanism of this drug. Several studies have shown that improgan analgesia is not mediated by known \((H_1, H_2, \text{ or } H_3)\) histamine receptors [8,13,15]. Furthermore, improgan antinociception was unaffected by the opioid antagonist naltrexone [15], suggesting an activity that is independent of opioid receptors. Since multiple types of opioid receptors can contribute to pain-relieving mechanisms [11], and, because improgan analgesia is poorly understood, the activity of this compound was presently studied in three types of mutant mice: those lacking the \( \mu \) (MOR-1), \( \delta \) (DOR-1) or \( \kappa \) (KOR-1) opioid receptors.

2. Materials and methods

2.1. MOR-1 knockout mice

Mice deficient in exon 1 of the MOR-1 gene were generated by use of a targeting vector in which exon 1 was replaced by a neomycin resistance cassette, as characterized in detail [20]. Offspring derived from heterozygous matings showed a Mendelian inheritance pattern, producing the three expected genotypes studied presently. Radioligand binding assays with the \( \mu \) agonist \(^3\)H-DAMGO found approximately one-half and no specific binding in the brains of heterozygous \((+/−)\) and homozygous \((−/−)\) mice, respectively [20]. Hybridization studies found no changes in mRNA levels representative of \( \delta \) (DOR-1) receptors, \( \kappa \) (KOR-1) receptors, proenkephalin or prodynorphin in MOR-1 mutant mice as compared with wild-type controls [20].

2.2. DOR-1 knockout mice

Exon 2 of the murine DOR-1 gene was replaced with a neomycin resistance cassette, as described in detail [28]. Heterozygote matings of the mutants showed Mendelian inheritance of the three genotypes studied presently. Immunostaining [28] and radioligand binding studies ([28] and present) showed no detectable DOR-1 protein product and either trace amounts or zero levels of pharmacologically-defined \( \delta \), or \( \delta \), receptors [28] in the homozygote mutant mouse brain. Additional characterization of these mice found normal levels of \( \mu \) (\(^4\)H-DAMGO) and \( \kappa \) (\(^3\)H-U69593) receptor binding; in situ hybridization found similar levels of mRNA for proenkephalin, prodynorphin, and pro-opiomelanocortin in mutant and wild-type brains [28].

2.3. KOR-1 knockout mice

Exon 3 of the murine KOR-1 gene was replaced with the neomycin resistance cassette [27]. Homozygous knockout mice were born viable, grossly normal and fertile, and contained no detectable \(^3\)H-U69,593 binding in brain homogenates.

2.4. Animals

Mice were labeled with coded genotypes and shipped to Albany Medical College, where antinociceptive testing and radioligand binding were performed. They were housed in groups of 4 to 6, separated by sex and genotype, and maintained on a normal 12 h light/dark cycle with food and water freely available. All procedures were approved by the Albany Medical College Institutional Animal Care and Use Committee.

2.5. Drugs and solutions

DPDPE (Sigma, St Louis, MO), morphine sulfate (Mallinckrodt, Paris, KY) and U50,488 (\( \pm \)-trans-U-50,488 methanesulfonate, RBI) were dissolved in saline. Improgan mice deficient in exon 1 of the MOR-1 gene were base was ... which was prepared according to [25]. The compound (mp=152–153\(^\circ\)C) was dissolved in 1.0 M HCl at a concentration of 150 mg/ml, titrated to pH 7.0 and diluted with saline. Doses of all drugs refer to the chemical form administered.

2.6. Tail-immersion nociceptive testing

Animals were loosely restrained in a conical polypropylene tube. The tail (2–3 cm) was immersed into a 55\(^\circ\)C water bath and the latency to sudden movement (flick) or removal of the tail was recorded with an 8-s cutoff [14].

2.7. Microinjection and testing procedure in mice

Following base-line testing, animals were lightly anesthetized with ether. A microliter syringe was connected to a 26-gauge needle with PE20 tubing. The needle was inserted into the lateral ventricle through a stereotaxically drilled Plexiglas plate as described previously in detail [4]. A total of 2 \( \mu\)l of drug solution was injected over a 1-min period. The animals were conscious within 3 min after the injection and were tested several times up to 2 h later. One to two weeks later, mice received systemic or icv opioid treatments, and were re-tested. Following all testing, animals received pentobarbital, and an icv injection of India Ink (1 \( \mu\)l) to verify the accuracy of injections into the lateral ventricle.
2.8. Data analysis

Antinociceptive responses for each animal were calculated as percent of maximum possible effect (% MPE), where:

\[ \text{\% MPE} = \frac{\text{drug latency} - \text{baseline latency}}{\text{cutoff latency} - \text{baseline latency}} \times 100 \]

Results are expressed as \% MPE (mean±S.E.M.), which were subjected to multi-factor analysis of variance (ANOVA) with repeated-measures (time). Newman–Keuls comparisons were used for post-hoc testing (Statistica, CSS, Inc., Tulsa, OK).

2.9. Radioligand binding

In some experiments, whole brains were harvested and homogenized following nociceptive testing. Membrane fractions were assayed for \( \delta \) or \( \kappa \) opioid receptor binding by radioligand methods [19]. The former was studied with \(^{3}H\)-DPDPE (2 nM, 33 Ci/mmol) incubated for 4 h at 25°C with naloxone (10 \( \mu \)M) for non-specific binding; the latter was performed with \(^{3}H\)-U69,593 (2 nM, 44 Ci/mmol) incubated for 30 min at 37°C with naloxone (10 \( \mu \)M) for non-specific binding.

3. Results

3.1. MOR-1-deficient mice

Improgan induced maximal antinociception in male and female mice of all three genotypes 10 and 20 min after administration (Fig. 1). This activity was reversible, with responses declining over the subsequent 120 min period. Saline-treated mice (not shown) exhibited antinociceptive scores between 0 and 5%. A two-factor repeated-measures ANOVA found significant (\( P<0.05 \)) interaction terms (genotype–time, gender–time, genotype–gender–time). In females, improgan analgesia was enhanced in heterozygous (+/−) and homozygous (−/−) mutants, as compared with control (+/+ ) subjects; no such differences were found in males. As expected, morphine analgesia was attenuated (+/−) or abolished (−/−) in these mice as compared with +/+ controls (Fig. 1).

3.2. DOR-1-deficient mice

Improgan also induced antinociception in both sexes of all DOR-1 genotypes 10 and 20 min after icv administration (Fig. 2). A time-related reversal of the analgesia was observed 90 and 120 min later. ANOVA found significant effects (\( P<0.01 \)) across time, but not across genotype or gender. Thus, improgan induced equivalent antinociception in all three DOR-1 genotypes. Subsequent challenge of these mice with DPDPE (8 \( \mu \)g, 20 min, icv) unexpectedly showed equivalent analgesia in all three genotypes (mean \( \% \) MPE±S.E.M., were 87.1±6.2, 66.9±8.5 and 86.4±7.2 for +/+ [\( n=12 \)], +/− [\( n=11 \)] and −/− [\( n=10 \)] groups, respectively). As expected however, brain \(^{3}H\)-DPDPE binding was either reduced (+/−) or abolished (−/−) in the DOR-1 mutants, as compared with +/+ controls (Fig. 2).

3.3. KOR-1-deficient mice

As found in the other mutant mice (above), improgan caused analgesia in all KOR-1 genotypes (Fig. 3). The time-course of action was similar to that seen in other mutant mice (Fig. 3). A 20 \( \mu \)g dose of improgan was used in the KOR-1 mice because pilot studies in +/+ mice found that a higher dose (30 \( \mu \)g) caused maximal responses that did not return to baseline within 2 h (data not shown). ANOVA found significant effects (\( P<0.01 \)) across time, but not across genotype or gender. Subsequent analgesic studies with U50,488, a selective \( \kappa \) opioid agonist (Fig. 3), found mild antinociceptive responses in +/+ control mice, but not in −/− mutant mice. ANOVA showed this effect to be significantly (\( P<0.05 \)) different across genotype, although the lack of a significant time by genotype interaction term prevented post-hoc comparisons. However, a gender by genotype interaction term was significant from the ANOVA (\( P<0.05 \)), with males showing significantly more U50,488 analgesia (not shown). As expected, \(^{3}H\)-U69,593 binding to \( \kappa \) opioid sites was virtually abolished in the −/− KOR-1 mice (Fig. 3).

4. Discussion

Improgan is a cimetidine analog, which shows characteristics of a selective, highly effective analgesic agent after CNS administration [13,14]. However, the mechanism of improgan analgesia has not yet been discovered. Bioassays [13], agonist and antagonist treatments [15] and in vivo structure–activity studies [9] have excluded roles for known histamine (\( H_1 \), \( H_2 \) and \( H_3 \)) receptors. Radioligand receptor screening for improgan is not yet complete, but more than 40 G-protein-coupled receptors and ion channels have been excluded [6], suggesting the possibility that the improgan target is novel.

Because improgan attenuates high-temperature nociceptive responses in rats (an effect shared with morphine), opioid receptors have been considered as potential sites of improgan action. However, binding studies with brain membranes and cloned cells found improgan (10 \( \mu \)M) to have no affinity at \( \mu \) opioid receptors [6]. The compound also showed very low affinity at \( \delta \) opioid receptors by radioligand binding techniques and bioassay [6]. Improgan
Fig. 1. Effects of improgan (left) and morphine (right) on antinociceptive scores (% MPE, mean±S.E.M., ordinate) in three genotypes of MOR-1 knockout mice. (Left) Mice (sexes shown separately) were tested for baseline responses, received improgan (30 μg, icv) and were re-tested at the times indicated. (Right) One to two weeks later, the same mice were tested for baseline responses, received morphine sulfate (10 mg/kg, s.c.) and were re-tested at the times shown. Following the last test, the animals were re-anesthetized, and injected with India Ink to verify icv injection sites. Baseline latencies (s, mean±S.E.M., females and males, respectively) were improgan: 1.05±0.07, 1.28±0.06; morphine: 1.01±0.08, 1.11±1.10. Data from one improgan-treated female (−/−) were omitted because the icv injection was not verified to be in the lateral ventricle. Morphine results from this subject were included. Another subject (+/+ male) died several days after improgan treatment, and thus was not included in the morphine experiment. For all panels, significant differences (P<0.05, 0.01, respectively) were: *,** time differences vs. first time period in the same group; genotype differences vs. control (+/+) mice in the same gender at the same time; #,#,#,# gender differences in the same groups.

also had low affinity at κ1 and ORL1 sites by radioligand binding [6]. Thus, improgan does not appear to act directly on μ, δ, κ, or ORL1 opioid receptors.

Although direct opioid actions of improgan have been excluded, the drug could still be activating endogenous opioid circuitry. For example, supraspinally-administered GABA_A antagonists [2], excitatory amino acids [5] and β-endorphin [24] are all thought to work in part by activation of spinal opioid mechanisms. Thus, further analysis of the significance of opioid receptors as mediators of improgan analgesia was needed.

The present findings demonstrate that none of the intact genes studied presently (MOR-1, DOR-1 or KOR-1) are needed for production of improgan analgesia, and imply that these receptors (μ, δ and κ) do not participate in the action of this drug. Results showing that the analgesia evoked by morphine, but not by improgan (Fig. 1), is abolished in MOR-1−/− mutant mice are in agreement with several previous studies which have established the essential role of MOR-1 in morphine action [12,16,17,20,22]. The slight, but statistically significant gender difference in improgan analgesia in the MOR-1 knockout mice (Fig. 1) is interesting, but the explanation for this is unknown. Previous work has demonstrated sexual dimorphisms in both opioid [10] and non-opioid [23] analgesic responses.

Morphine analgesia is abolished in mice with MOR-1 mutations of exon 1 [12,20], exon 2 [17] and exons 2 and 3 [22], demonstrating that this μ opioid prototype requires an intact MOR-1 gene. However, some findings suggest
that other $\mu$-preferring opioids (e.g., fentanyl, morphine-6-glucuronide [M6G]) may act at another receptor, which is an alternate product of MOR-1. For example, M6G analgesia was found to be abolished in exon 2 mutants of MOR-1, but not in exon 1 knockouts [20]. However, another group reported that M6G analgesia was abolished in mice having a different mutation of exon 1 [12], and further studies are in progress to understand this discrepancy. If some $\mu$ opioids can indeed induce analgesia in exon 1 mutations of MOR-1, then it might be argued from the present data that improgan could also act in this manner. This is not likely, however, since there is a consensus that the analgesia elicited by all $\mu$ opioids (including M6G) is completely blocked by the opioid antagonist naltrexone. Since improgan analgesia is not blocked by naltrexone [15], or by other $\mu$ antagonists (unpublished studies), it seems clear that the MOR-1 gene does not participate in improgan analgesia.

Our present results, showing that improgan analgesia is unchanged in any of the DOR-1 genotypes (Fig. 2), suggest that the $\delta$ opioid receptor also does not play a role in the action of this drug. Although there is considerable pharmacological evidence for the existence of sub-types of the $\delta$ opioid receptor [26], both $\delta_1$ and $\delta_2$ binding sites were abolished in brains of homozygous DOR-1 mutant mice, suggesting that both sites are encoded by this gene [28]. The present studies, performed with the same DOR-1 mutants, independently confirm the loss of $\delta_1$ ($^3$H-DPDPE) binding (Fig. 2). Taken together, the two studies suggest that neither the $\delta_1$ nor the $\delta_2$ receptor contributes to improgan analgesia.

Some of experiments with the DOR-1 mutant mice showed surprising results. As expected, homozygous DOR-1 knockout mice showed a loss of analgesia following intrathecal administration of the $\delta$ agonist DPDPE [28], implying a functional role for this gene in this response. However, analgesia resulting from supraspinally-administered DPDPE was unexpectedly retained, suggesting the existence of more than one $\delta$-opioid receptor [28]. The present experiments, showing supraspinal DPDPE analgesia in homozygous DOR-1 mutants (see Results), are an independent confirmation of the earlier findings. The findings that both icv improgan and icv DPDPE induced analgesia in DOR-1 mutant mice could imply that improgan acts on this second $\delta$-like receptor. This is unlikely, however, because the analgesia resulting from icv DPDPE [28], but not that from icv improgan [15], is completely antagonized by naltrexone.

Our finding that improgan analgesia is unaffected by mutations in KOR-1 (Fig. 3A) suggest that $\kappa$ opioid receptors also do not contribute to improgan responses. The loss of $\kappa$ (U50,488) analgesia (Fig. 3B) and $\kappa$ opioid binding sites (Fig. 3C) in the homozygous KOR-1 mutants confirms the absence of functional $\kappa$ opioid receptors. Previous work with a KOR-1 mutant different from that studied presently also showed deficits in $\kappa$ binding and $\kappa$-mediated analgesic responses [21]. The moderate antinociceptive scores observed presently after U50,488 in control mice (25% MPE, Fig. 3B) are lower than found in the earlier study [21] because different water temperatures were used in the tail immersion tests. The apparent analgesic efficacy of kappa opioids is known to be related to the intensity of the thermal nociceptive stimuli used in the assay [18].

The present findings, showing that improgan analgesia is not diminished in mice lacking functional $\mu$, $\delta$ or $\kappa$ opioid receptors, add strong support for the contention that this class of agents reduces nociceptive responses in animals by a non-opioid mechanism. Although this mechanism presently remains unknown, the pain-relieving properties of
improgan-like compounds may lead to the development of brain-penetrating, clinically useful analgesics.

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References


