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Differential Blockade of Opioid Analgesia by Antisense Oligodeoxynucleotides Directed against Various G Protein α Subunits

KELLY M. STANDIFER,[1] GRACE C. ROSSI, and GAVRIL W. PASTERNAK

The Cotzias Laboratory of Neuro-Oncology, Memorial Sloan-Kettering Cancer Center (K.M.S., G.C.R., G.W.P.), New York, New York 10021, and Departments of Pharmacology and of Neurology and Neuroscience, Cornell University Medical College, New York, New York 10021 (G.W.P.)

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SUMMARY

Antisense oligodeoxynucleotides directed against various G protein α subunits differentially block the analgesic actions of μ-, δ-, and κ-opioid agonists in mice. Intracerebroventricular administration of oligodeoxynucleotides targeting $G_{i\alpha2}$, $G_{o\alpha}$, and $G_{s\alpha}$ block supraspinal μ-opioid analgesia, whereas $G_{i\alpha2}$ and $G_{x/z\alpha}$ antisense probes block spinal μ analgesia. Although supraspinal and spinal morphine-6β-glucuronide (M6G) analgesia also is sensitive to these antisense treatments, its sensitivity profile differs from that of morphine, implying the existence of a different analgesic system. $G_{i\alpha1}$ and $G_{x/z\alpha}$ antisense probes block supraspinal M6G analgesia, whereas $G_{i\alpha1}$, $G_{i\alpha3}$, $G_{o\alpha}$, and $G_{x/z\alpha}$ antisense probes block spinal M6G analgesia. Spinal δ-opioid analgesia is blocked by antisense probes to all of the G protein α subunits tested, whereas κ_1-opioid analgesia is sensitive to only $G_{q\alpha}$. The κ_3 agonist naloxone benzoylhydrazone produces its analgesia through supraspinal mechanisms and is blocked by $G_{i\alpha1}$, $G_{i\alpha3}$, $G_{s\alpha}$, $G_{q\alpha}$, and $G_{x/z\alpha}$ antisense oligodeoxynucleotides. Together, these results support the presence of seven different analgesic systems for these various opioid agonists.

Opioid receptors modulate pain through a a number of pharmacologically distinct receptor systems acting both spinally and supraspinally (1). Recently, four members of the opioid receptor family were cloned and found to belong to the G protein-coupled seven transmembrane spanning receptor family (for reviews, see Refs. 2–4). When expressed, these clones exhibit the binding selectivities and functional activities anticipated from studies of the receptors normally present in brain. Determining the role of these cloned receptors in the established pharmacology of opioid receptors has proved to be more difficult. Antisense approaches have been used to explore the role of these cloned receptors in the central nervous system, demonstrating their importance in eliciting opioid analgesia (5–13). Detailed studies have raised the possibility that subtypes within the cloned receptor families may result from alternative splicing (2, 6–8). Together, these studies have demonstrated the presence of distinct opioid analgesic systems within the brain.

Although all of the opioid receptors inhibit adenylate cyclase, different populations of G proteins mediate their responses in cell culture models (14–19). Extension of these studies to the *in vivo* pharmacology of opioids might offer insights into the mechanisms of action of this complex family of receptors. Recently, several approaches were used to explore the G protein α subunits responsible for transducing supraspinal μ and δ analgesia using either antisera or antisense approaches (20–26), but these studies did not address the full complexity of the various opioid receptor classes capable of mediating analgesia, especially the receptor subtypes. We used an antisense approach capable of selectively down-regulating individual G protein α subunits and defined seven distinct opioid receptor analgesic systems in the mouse.

Experimental Procedures

Materials. Morphine, M6G, DPDPE, and U50,488H were generously provided by the Research Technologies Branch, National Institute on Drug Abuse, National Institutes of Health, Bethesda, MD. NalBzoH was synthesized as described previously (27). All ODNs were synthesized by the Midland Certified Reagent Co. (Midland, TX) and were specific for the G protein α subunits for which they were targeted. Protogel was obtained from National Diagnostics

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[1] Current affiliation: Department of Pharmacological and Pharmaceutical Sciences, University of Houston, Houston, TX 77204-5515.

ABBREVIATIONS: M6G, morphine-6β-glucuronide; NalBzoH, naloxone benzoylhydrazone; ODN, oligodeoxynucleotide; PAG, periaqueductal gray; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; DPDPE, [D-Pen^2,D-Pen^5]enkephalin.

(Atlanta, GA). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Western blot analysis. Western blot analysis was performed as described previously (7). In brief, membrane homogenates (10 μg) made from the PAG region of control and antisense-treated mice were solubilized in SDS buffer and separated on 10% polyacrylamide gels (1.5 mm). Proteins were transferred onto nitrocellulose (1.5 hr at 190 mA), and the membrane was blocked in PBS containing 10% nonfat dried milk for 1 hr before the addition of the primary antisera. AS/7 ($G_{i\alpha1/2}$), EC/2 ($G_{i\alpha3}$), GC/2 ($G_{o\alpha}$), and RM/1 ($G_{s\alpha}$) (DuPont-New England Nuclear Research Products, Boston, MA) were incubated overnight at 4° (1:1000 dilution). After being washed with PBS containing 0.1% Tween, the nitrocellulose was incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antisera (1:10,000) in PBS/0.1% Tween containing 0.5% bovine serum albumin for 2 hr at ambient temperature. The blot was visualized after a chemiluminescent reaction (Renaissance kit; New England Nuclear, Boston, MA) and exposure to Kodak XAR-5 film. Protein was determined using bovine serum albumen, as described previously (28).

Antinociception assays. Male CD-1 mice (25–35 g; Charles River Breeding Laboratories, Portage, Canada) were maintained on a 12-hr light/dark cycle with food and water available *ad libitum*. Mice were housed in groups of five until testing. Both intracerebroventricular and intrathecal injections were made with the animals under light Halothane anesthesia (Halocarbon Laboratories, Hackensack, NJ) using a Hamilton 10-μl syringe fitted to a 30-gauge needle with V1 tubing. Under these conditions, anesthesia wore off within several minutes and did not interfere with subsequent tailflick testing. The intracerebroventricular injections were administered ~2 mm caudal and ~2 mm lateral to bregma at a depth of 3 mm (29), whereas intrathecal injections were by lumbar puncture (30). Analgesia was determined in the radiant heat tailflick assay (31), as described previously (8, 32–34). Baseline tailflick latencies were determined individually for each mouse as the mean value of two trials (ranging from 2 to 3 sec) and were not affected by ODN treatment. Animals received a single injection of ODN (5 μg/2 μl saline intracerebroventricularly) 48 hr before testing. Analgesia was assessed 15 min after administration of an opioid. The maximal latency was 10 sec to minimize tissue damage. Analgesia was defined quantally as doubling or greater of baseline tailflick latencies. Statistical analysis was determined using the Fisher exact test.

Results

Down-regulation of G protein α subunits by antisense ODNs. The PAG is important in the production of opioid analgesia. Located within the brainstem, the PAG should be particularly susceptible to antisense ODNs administered within the cerebrospinal fluid because of its close proximity to the ventricular system. To validate the antisense model, we examined the effects of various antisense ODNs on the levels of the individual G protein α subunits in the PAG by Western blotting. Mice were treated with various antisense probes (20 μg intracerebroventricularly), and 48 hr later the PAG was dissected and examined for levels of the individual G protein α subunits. Although activity could be seen as soon as 24 hr after injection, optimal results were observed at 48 hr. To ensure the selectivity of the actions of the antisense probes for an individual α subunit, we treated mice with ODNs targeting $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, or $G_{o\alpha}$ (Table 1) and probed with the antisera GC/2, looking for down-regulation of $G_{o\alpha}$ on Western blots of PAG membranes (Fig. 1A). On the Western blot, only the ODN selectively targeting $G_{o\alpha}$ down-regulated its levels (Table 1). The other three antisense ODNs directed at other G protein α subunits were ineffective, although they did lower the levels of their targeted G protein

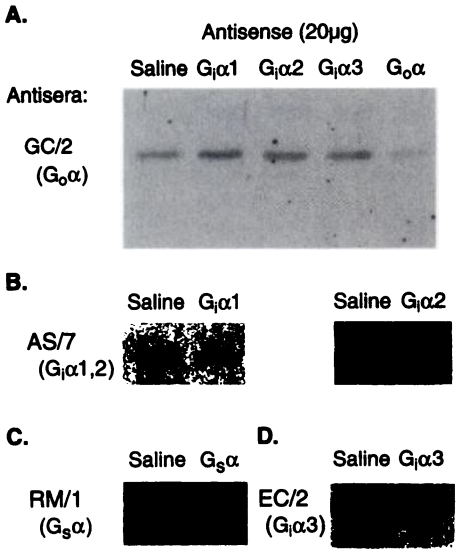

Fig. 1. Effect of antisense ODN treatment on G protein α subunit levels in membranes from mouse PAG region. Mice received single injections of antisense ODN (20 μg/2 μl intracerebroventricularly) or saline, and membranes from the PAG region were prepared 48 hr later. Membrane proteins (10 μg) were solubilized in SDS, separated on an SDS-polyacrylamide gel (10%), transferred to nitrocellulose, and probed with the indicated antibody, as described in Experimental Procedures. A, Samples obtained after treatment with either saline or antisense ODNs to $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, or $G_{o\alpha}$ were probed with antisera to $G_{o\alpha}$ (GC/2). B, Samples obtained after treatment with the antisense ODNs to $G_{i\alpha1}$ or $G_{i\alpha2}$ or saline were probed with antisera AS/7, which recognizes both $G_{i\alpha1}$ and $G_{i\alpha2}$. C, Samples obtained after treatment with the antisense ODN to $G_{s\alpha}$ or saline were probed with antisera RM/1, which recognizes $G_{s\alpha}$. D, Samples obtained after treatment with the antisense ODN to $G_{i\alpha3}$ or saline were probed with antisera EC/2, which recognizes $G_{i\alpha3}$.

TABLE 1

Base composition of ODNs

Antisense ODNs were designed that were selective for the indicated G protein α subunit. All sequences were searched through GenBank and found to be unique to their respective G protein α subunit and were based on mouse sequences, with the exception of $G_{i\alpha3}$ (rat) and $G_{x/z\alpha}$ (human).

Target protein	Sequence (5′–3′)
$G_{i\alpha1}$	AGA CCA CTG CTT TGT A
$G_{i\alpha2}$	CTT GTC GAT CAT CTT AGA
$G_{i\alpha3}$	AAG TTG CGG TCG ATC AT
$G_{o\alpha}$	CGC CTT GCT CCG CTC
$G_{s\alpha}$	TTG TTG GCC TCA CGC TG
$G_{q\alpha}$	GCT TGA GCT CCC GGC GGG CG
$G_{x/z\alpha}$	GGG CCA GTA GCC CAA TGG G
Nonsense	GGG GGA AGT AGG TCT TGG

α subunits (Table 2). The levels of $G_{i\alpha1}$ and $G_{i\alpha2}$ were decreased by treatment with their respective antisense probes, as demonstrated by the antisera AS/7, which recognizes both proteins (Fig. 1B). Similarly, the levels of $G_{s\alpha}$ (Fig. 1C) and $G_{i\alpha3}$ (Fig. 1D) were lowered by their selective antisense ODNs. Thus, these antisense treatments selectively lowered the levels of the expected G protein α subunits in brain regions important in opioid analgesia. However, other brain regions, such as the thalamus, were not as sensitive, presumably due to difficulties with diffusion of the ODN deep into tissue.

Role of various G protein subunits in opioid analgesia. Supraspinal and spinal opioid analgesic systems are pharmacologically distinct. Supraspinally, morphine acts

TABLE 2

Down-regulation of G protein α subunits by antisense ODNs

The Western blots described in Fig. 1 were digitized using Quantity One (PDI, Huntington Station, NY), and the signal intensity of various bands was determined after correction for background intensity. The intensity of each treatment was compared with its saline control and expressed as percentage of the saline control. The results represent the mean ± standard error. All of the bands were significantly different, with the exception of the lower band in the $G_{s\alpha}$ experiments.

Antisense target	n[a]	Loss of immunoreactivity	P
		%	
$G_{i\alpha1}$	3	74.2 ± 18	<0.05
$G_{i\alpha}$	5	65.1 ± 6.5	<0.001
$G_{i\alpha}$	5	86.5 ± 7.0	<0.001
$G_{o\alpha}$	5	72.9 ± 13.6	<0.01
$G_{s\alpha}$ (top band)	4	74 ± 11.7	<0.01
$G_{s\alpha}$ (bottom band)	3	21.7 ± 23.4	N.S.

[a] Total number of replications from three animals for each treatment.

through the μ_1 receptor subtype, whereas NalBzoH acts through κ_3 receptors. Prior studies indicated that M6G analgesia is mediated through another independent analgesic system (2, 8, 34). Having established the ability of antisense treatments to selectively down-regulate selective G protein α subunits, we next examined the effects of these treatments on opioid analgesia. Preliminary studies revealed effects at doses as low as 5 μg. We used the lowest effective doses in these behavioral assays to ensure the selectivity of the responses. Groups of mice were treated with antisense ODNs targeting $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, $G_{o\alpha}$, $G_{s\alpha}$, $G_{q\alpha}$, or $G_{x/z\alpha}$ (5 μg intracerebroventricularly; Table 1) and were tested with morphine (0.7 μg intracerebroventricularly), M6G (20 ng intracerebroventricularly), or NalBzoH (15 μg intracerebroventricular) 48 hr later. The sensitivity profile of each opioid toward the various G protein α subunits was quite distinct (Fig. 2 and Table 3). Supraspinal morphine analgesia was most potently blocked by the antisense ODN against $G_{i\alpha2}$ ($p < 0.0005$) and only slightly less by ODNs against $G_{o\alpha}$ ($p < 0.005$) and $G_{s\alpha}$ ($p < 0.03$) (Fig. 2A). The other antisense probes did not significantly lower the analgesic response to morphine.

In contrast, M6G analgesia was antagonized only by the probes targeting $G_{i\alpha1}$ ($p < 0.003$), $G_{s\alpha}$ ($p < 0.01$), and $G_{x/z\alpha}$ ($p < 0.03$) (Fig. 2B). The sensitivity profile of NalBzoH analgesia differed from those of both of the others (Fig. 2C). Like M6G, NalBzoH analgesia was decreased by the $G_{i\alpha1}$ ODN ($p < 0.01$), but its sensitivity toward $G_{i\alpha3}$ ($p < 0.005$)

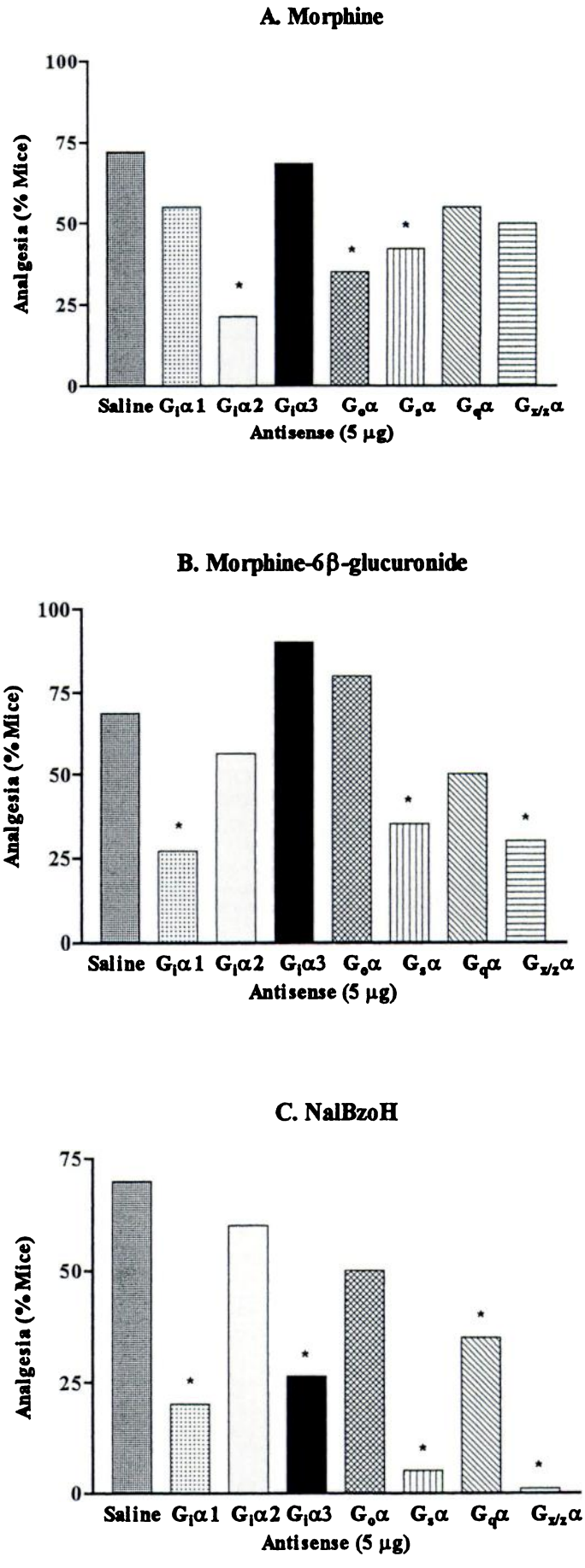

Fig. 2. Effects of antisense ODN administration on supraspinal opioid analgesia. A, Morphine (700 ng) produced analgesia in 72% of saline-treated mice (50 mice). Morphine analgesia was significantly blocked by a single intracerebroventricular injection of antisense to $G_{i\alpha2}$ ($p < 0.0005$, 19 mice), $G_{o\alpha}$ ($p < 0.005$, 20 mice), and $G_{s\alpha}$ ($p < 0.03$, 19 mice). Although $G_{i\alpha3}$ treatment had no effect, a slight (20%) decrease in morphine analgesia was noted with $G_{i\alpha1}$ (20 mice), $G_{q\alpha}$ (20 mice), and $G_{x/z\alpha}$ (10 mice) antisense administration but was not significant. B, M6G (20 ng) produced analgesia in 69% of saline-treated mice (48 mice). M6G analgesia was significantly blocked by a single intracerebroventricular injection of antisense to $G_{i\alpha1}$ ($p < 0.003$, 18 mice), $G_{s\alpha}$ ($p < 0.01$, 20 mice), and $G_{x/z\alpha}$ ($p < 0.03$, 10 mice). $G_{i\alpha2}$ (18 mice) and $G_{q\alpha}$ (20 mice) treatments had slight effects, but they did not approach significance. C, NalBzoH (15 μg) produced analgesia in 70% of saline-treated mice (40 mice). NalBzoH analgesia was significantly blocked by a single intracerebroventricular injection of antisense to $G_{i\alpha1}$ ($p < 0.01$, 10 mice), $G_{i\alpha3}$ ($p < 0.005$, 19 mice), $G_{s\alpha}$ ($p < 0.0001$, 20 mice), $G_{q\alpha}$ ($p < 0.01$, 20 mice), and $G_{x/z\alpha}$ ($p < 0.0001$, 10 mice). Although it produced a small decrease in NalBzoH analgesia, $G_{o\alpha}$ antisense treatment (20 mice) was not significantly different from saline controls.

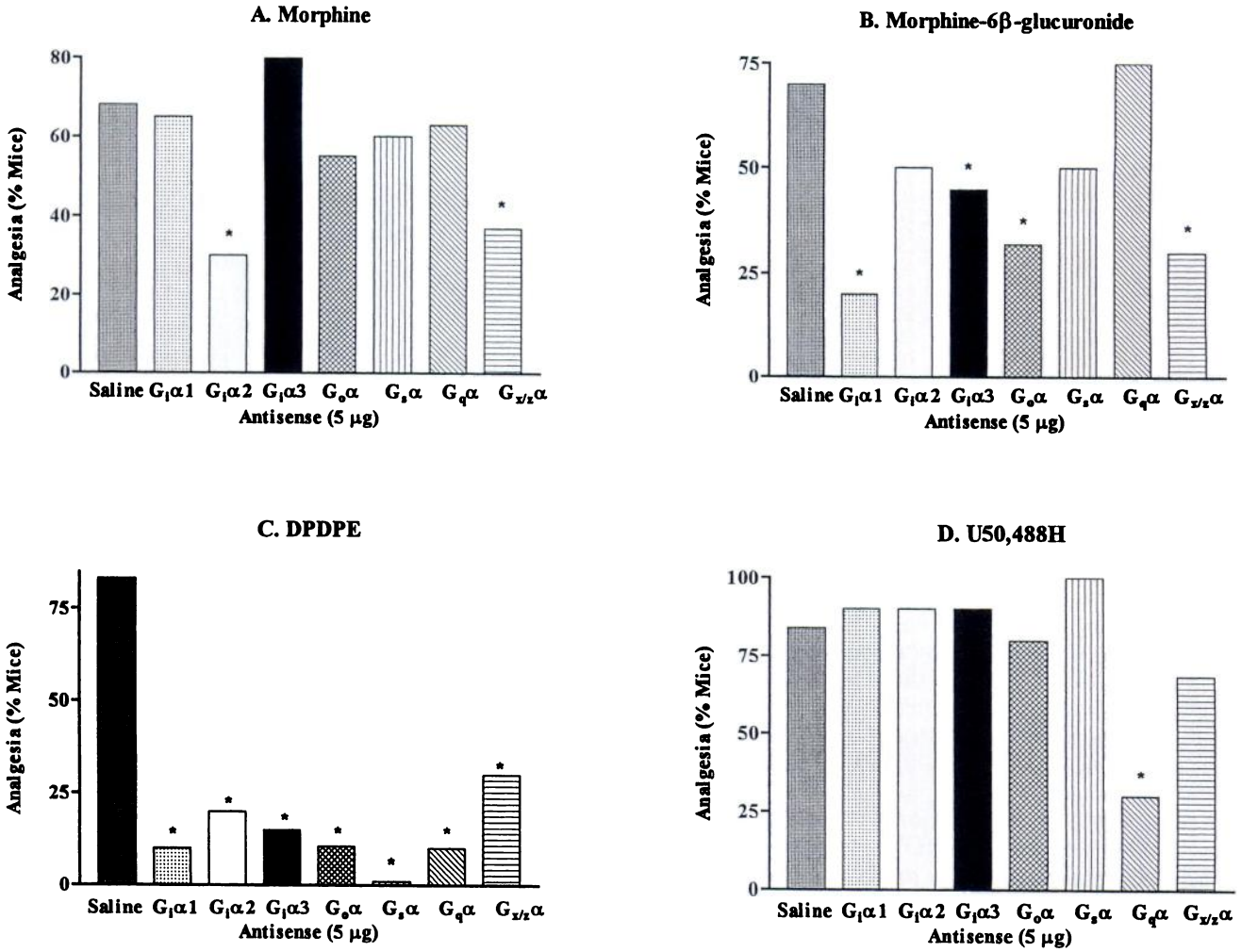

Fig. 3. Effects of intrathecal antisense ODN administration on spinal opioid analgesia. A, Morphine (600 ng) produced analgesia in 68% of saline-treated mice (50 mice). Morphine analgesia was significantly blocked by a single intrathecal injection of antisense to $G_{i\alpha 2}$ ($p < 0.001$, 30 mice) and $G_{x/z\alpha}$ ($p < 0.005$, 30 mice). All other treatments were without effect. B, M6G (7.5 ng) produced analgesia in 70% of saline-treated mice (50 mice). M6G analgesia was significantly blocked by a single intrathecal injection of antisense to $G_{i\alpha 1}$ ($p < 0.0001$, 40 mice), $G_{i\alpha 3}$ ($p < 0.05$, 20 mice), $G_{o\alpha}$ ($p < 0.005$, 28 mice), and $G_{x/z\alpha}$ ($p < 0.005$, 20 mice). Small reductions in M6G analgesia also were noted with $G_{i\alpha 2}$ (20 mice) and $G_{s\alpha}$ (20 mice) antisense treatment, but these changes were not significant. C, The δ agonist DPDPE (500 ng) produced analgesia in 83% of saline-treated mice (30 mice). DPDPE analgesia was significantly blocked by a single intrathecal injection of antisense to each antisense ODN tested except for nonsense: $G_{i\alpha 1}$ ($p < 0.0001$, 20 mice), $G_{i\alpha 2}$ ($p < 0.0001$, 20 mice), $G_{i\alpha 3}$ ($p < 0.0001$, 20 mice), $G_{o\alpha}$ ($p < 0.000001$, 19 mice), $G_{s\alpha}$ ($p < 0.0001$, 10 mice), $G_{q\alpha}$ ($p < 0.0001$, 10 mice), and $G_{x/z\alpha}$ ($p < 0.003$, 10 mice). D, The κ_1 agonist U50,488H (40 μg) produced analgesia in 84% of saline-treated mice (49 mice) and was blocked only by pretreatment with antisense to $G_{q\alpha}$ ($p < 0.003$, 10 mice).

clearly distinguished it from M6G. The $G_{x/z\alpha}$ ($p < 0.0001$) and $G_{s\alpha}$ ($p < 0.0001$) ODNs virtually abolished NalBzoH analgesia, whereas $G_{q\alpha}\alpha$ was somewhat less active ($p <$ 0.01). Treatment of mice with a nonsense antisense ODN had no effect on any of the analgesics tested (data not shown).

TABLE 3

Summary of analgesic sensitivities toward antisense ODNs against the G protein α subunits

	Blockade of analgesia by antisense ODN						
	$G_{i\alpha 1}$	$G_{i\alpha 2}$	$G_{i\alpha 3}$	$G_{o\alpha}$	$G_{q\alpha}$	$G_{x/z\alpha}$	$G_{s\alpha}$
Supraspinal							
Morphine		++		+			+
M6G	++					+	+
NalBzoH	++		+		+	+++	+++
Spinal							
Morphine		+				+	
M6G	++		+	+		+	
U50,488					++		
DPDPE	+++	++	++	++	++	+	+++

At the spinal level, the various opioids also displayed different selectivity profiles. Groups of mice received the various antisense ODNs (5 μg intrathecally) and were tested 48 hr later with morphine (600 ng intrathecally), M6G (7.5 ng intrathecally), DPDPE (500 ng intrathecally), or U50,488H (40 μg). Morphine analgesia was blocked by both $G_{i\alpha 2}$ ($p < 0.001$) and $G_{x/z\alpha}$ ($p < 0.005$) antisense ODNs. This result was somewhat different from supraspinal morphine analgesia. Although $G_{i\alpha 2}$ seems to play a role at both levels, $G_{o\alpha}$ and $G_{s\alpha}$ were not active spinally. Spinal M6G analgesia also differed from supraspinal systems. $G_{i\alpha 1}$ ($p < 0.0001$) and $G_{x/z\alpha}$ ($p < 0.005$) are important at both levels, but $G_{o\alpha}$ ($p < 0.005$) worked spinally and $G_{s\alpha}$ worked only supraspinally. The $G_{i\alpha 3}$ antisense ODN also significantly lowers spinal M6G analgesia ($p < 0.05$), but the decrease is less than that of the others.

DPDPE analgesia, which is mediated through δ-opioid receptors, was potently blocked by all of the antisense ODNs tested, distinguishing it from all of the other agonists examined. In contrast, the analgesic actions of the κ_1 agonist U50,488H is blocked only by $G_{q\alpha}$ ($p < 0.003$). Treatment of mice with a nonsense ODN did not affect any of the analgesics tested.

Discussion

Examination of receptor/G protein interactions can help unravel the complex systems mediating the control of pain within the nervous system. Initial studies on supraspinal μ- and δ-opioid analgesia used antibodies targeting individual G proteins (21–24) or antisense ODNs against $G_{i\alpha 2}$, which blocked supraspinal morphine analgesia (20, 25, 26). A similar paradigm is effective in the rat (34). The current study provides a comprehensive examination of the role of a series of seven different G protein α subunits in supraspinal and spinal analgesia.

Antisense ODNs can selectively down-regulate individual G protein α subunits *in vivo* within 48 hr of a single injection, as demonstrated by Western blot analysis. Other paradigms also can selectively lower individual G protein α subunits (26). In our study, not all brain regions were affected to a similar extent after intracerebroventricular treatment. The loss of G protein α subunits within a region depends in part on the levels of the ODN reaching it, which are the result of a number of variables, including cerebrospinal fluid flow and the proximity of the region to the ventricular system. Analgesia is mediated through periventricular brainstem regions, such as the PAG, which should be particularly susceptible to these treatments. The demonstration of selective decreases in the levels of the various G protein α subunits by the antisense ODNs in the brainstem confirms these predictions. In our paradigm, the sensitivity of each analgesic system to at least one ODN confirms that diffusion is not a problem because the distributions of all of the ODNs are likely to be the same. However, other behaviors mediated by different brain regions may not be equally sensitive to this antisense paradigm and may require direct administration of the ODN into the area involved.

The selective blockade of analgesia by the various antisense ODNs provides important evidence for a series of discrete analgesic systems involving the various opioid receptor subtypes. Pharmacological studies based on a variety of highly selective agonists and antagonists have suggested a number of distinct supraspinal mechanisms (1, 2), with morphine acting through the μ_1 receptor, NalBzoH acting through the κ_3 receptor, and M6G acting through a novel receptor. Results of antisense mapping of the cloned opioid receptors support these conclusions (2, 6–8, 34). At the spinal level, pharmacological evidence indicates the existence of distinct δ, μ_2, and κ_1 analgesic systems. In the current study, the different selectivity profiles against the antisense ODNs targeting the various G protein α subunits (Table 3) supports the existence of multiple, distinct analgesic opioid systems. Each of the various ligands demonstrates a different sensitivity profile to the panel of G protein α subunit antisense probes. The profile even differs for a single drug given spinally or supraspinally. $G_{o\alpha}$ has been implicated in μ receptor activation *in vitro* by several groups (14, 35–37). Likewise, we find that $G_{o\alpha}$ is involved with supraspinal morphine analgesia in the mouse, but earlier studies looking at the PAG in the rat revealed no effect on morphine analgesia when administered into the PAG of the rat (34). Thus, there may be species differences.

In vitro studies indicate that many, if not all, opioid receptors can activate multiple effectors through different G protein α subunits (19, 38–42). Thus, it is not surprising that multiple G protein α subunits are able to influence the analgesic actions of a single opioid agonist. However, it is interesting that the receptors mediating analgesia *in vivo* seem to be coupled to fewer types of G protein α subunits than do receptors studied in cell lines, with the exception of the δ receptor (14–19). This is more impressive in view of the differences between the two systems. Cell culture studies typically examine a homogeneous collection of noninteracting cells, whereas the situation *in vivo* is far more complex. Not only are receptors expressed on more than one cell type but also loss of function could result from an interruption anywhere along the pathway mediating the response. Thus, the loss of a response does not necessarily mean that the targeted G protein α subunit is physically associated with the receptor. The loss of function could be due to the down-regulation of a G protein located in cell far downstream in the analgesic circuit. Indeed, this might explain why down-regulation of $G_{s\alpha}$ blocks supraspinal morphine, M6G and NalBzoH analgesia, and spinal DPDPE analgesia so effectively, even though all of these receptors have been reported to inhibit adenylyl cyclase *in vitro*. Our *in vivo* observations regarding $G_{s\alpha}$ are consistent with previous studies in rats in which supraspinal morphine analgesia was blocked by both pertussis and cholera toxins (43). However, others have reported no blockade of analgesia (20, 23). It is not clear whether these differences reflect technical differences among the paradigms.

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Send reprint requests to: Gavril W. Pasternak, M.D., Ph.D., Department of Neurology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. E-mail: pasterng@mskmail.mskcc.org