The Journal of Neuroscience, January 15, 1997, 17(2):735–744

Multiple Receptors Involved in Peripheral α_2, μ, and A_1 Antinociception, Tolerance, and Withdrawal

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We examined the interactions among three classes of peripherally-acting antinociceptive agents (μ-opioid, α_2-adrenergic, and A_1-adenosine) in the development of tolerance and dependence to their antinociceptive effects. Antinociception was determined by assessing the degree of inhibition of prostaglandin E_2 (PGE_2)-induced mechanical hyperalgesia, using the Randall-Selitto paw-withdrawal test.

Tolerance developed within 4 hr to the antinociceptive effect of the α_2-adrenergic agonist clonidine; dependence also occurred at that time, demonstrated as a withdrawal hyperalgesia that was precipitated by the α_2-receptor antagonist yohimbine. These findings are similar to those reported previously for tolerance and dependence to μ and A_1 peripheral antinociception (Aley et al., 1995).

Furthermore, cross-tolerance and cross-withdrawal between μ, A_1, and α_2 agonists occurred. The observations of cross-tolerance and cross-withdrawal suggest that all three receptors are located on the same primary afferent nociceptors. In addition, the observations suggest that the mechanisms of tolerance and dependence to the antinociceptive effects of μ, A_1, and α_2 are mediated by a common mechanism.

Although any of the agonists administered alone produce antinociception, we found that μ, A_1, and α_2 receptors may not act independently to produce antinociception, but rather may require the physical presence of the other receptors to produce antinociception by any one agonist. This was suggested by the finding that clonidine (α_2-agonist) antinociception was blocked not only by yohimbine (α_2-antagonist) but also by PACPX (A_1-antagonist) and by naloxone (μ-antagonist), and that DAMGO (μ-agonist) antinociception and CPA (A_1-agonist) antinociception were blocked not only by naloxone (μ-antagonist) and PACPX (A_1-antagonist), respectively, but also by yohimbine (α_2-antagonist). This cross-antagonism of antinociception occurred at the ID_{80} dose for each antagonist at its homologous receptor. To test the hypothesis that the physical presence of μ-opioid receptor is required not only for μ antinociception but also for α_2 antinociception, antisense oligodeoxynucleotides (ODNs) for the μ-opioid and α_{2C}-adrenergic receptors were administered intrathecally to reduce the expression of these receptors on primary afferent neurons. These studies demonstrated that μ-opioid ODN administration decreased not only μ-opioid but also α_2-adrenergic antinociception; A_1 antinociception was unaffected. In contrast, α_{2C}-adrenergic ODN decreased antinociception induced by all three classes of antinociceptive agents.

In conclusion, these data suggest that peripheral antinociception induced by μ, α_2, and A_1 agonists requires the physical presence of multiple receptors. We propose that there is a μ, A_1, α_2 receptor complex mediating antinociception in the periphery. In addition, there is cross-tolerance and cross-dependence between μ, A_1, and α_2 antinociception, suggesting that their underlying mechanisms are related.

Key words: pain; analgesia; dorsal root ganglion; opioid; antisense oligodeoxynucleotide; receptor cross-talk

Both μ-opioid and A_1-adenosine agonists have been shown to produce a potent antinociception when administered in the periphery (Taiwo and Levine, 1990; Aley et al., 1995). This antinociception is detected in the presence of hyperalgesia produced by numerous inflammatory mediators including PGE_2 (Taiwo and Levine, 1990). Many of the effects produced by μ and A_1 agonists are mediated through a common second messenger, specifically, activation of an inhibitory guanine nucleotide binding (G_i) protein (Sharma et al., 1975; Law et al., 1981; Childers and LaRiviere, 1984; Mankman et al., 1988).

We have found that tolerance and dependence develop to both μ and A_1 peripheral antinociception. In addition, a symmetric cross-tolerance and cross-dependence exists between the μ and A_1 antinociceptive mechanisms (Aley et al., 1995).

Received Sept. 3, 1996; revised Oct. 22, 1996; accepted Oct. 25, 1996.

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α_2-adrenergic agonists have also been shown to produce antinociception when administered peripherally (Khasar et al., 1995). Because many of the effects of α_2 agonists, like those produced by μ and A_1 agonists, involve G_i protein signaling (Sharma et al., 1975; Law et al., 1981; Childers and LaRiviere, 1984; Mankman et al., 1988), we hypothesized that they all produce antinociception in the periphery through common cellular mechanisms. In addition, we hypothesized that there would be symmetric cross-tolerance and cross-dependence between the peripheral antinociceptive actions of these three agonists. Because α_2-adrenergic, μ-opioid, and A_1-adenosine agonists are widely used clinically, interactions between their peripheral antinociceptive effects is of significant interest.

MATERIALS AND METHODS

Animals

Experiments were performed on male Sprague Dawley rats (250–300 gm, Bantin and Kingman, Fremont, CA). Animals were housed in groups of two under a 12 hr light/dark cycle (light on 6.0 hr). Food and water were available *ad libitum*. All testing was done between 10.0 and 16.0 hr.

Table 1. Abbreviations of agents used

Abbreviation(s)	Agent	Action
PGE_2 or E_2	Prostaglandin E_2	Hyperalgesic inflammatory mediator
DAMGO or D	[D-Ala^2,N-Me-Phe^4,gly^5-ol] enkephalin	μ-opioid receptor agonist
N	Naloxone	Opioid receptor antagonist
Cl	Clonidine	α_2-adrenergic agonist
Yo	Yohimbine	α_2-adrenergic antagonist
CPA	N^6-cyclopentyl-adenosine	A_1-adenosine receptor agonist
PACPX	1,3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine	A_1-adenosine receptor antagonist
μ-antisense	μ-opioid receptor oligodeoxynucleotide (ODN)	Downregulation of μ-opioid receptors
μ-sense		No effect
α_2-antisense	α_{2C}-adrenergic receptor oligodeoxynucleotide (ODN)	Downregulation of α_{2C}-adrenergic receptors
α_2-sense		No effect

Experiments were carried out under approval of the Institutional Animal Care Committee of the University of California, San Francisco.

Behavioral testing

The nociceptive flexion reflex was quantified with a Basile Analgesymeter (Stoelting, Chicago, IL), which applies a linearly increasing mechanical force to the dorsum of the hindpaw of the rat. Before the experiments, rats were exposed to the procedure for 3 d (1 hr daily at 5 min intervals, i.e., 12 exposures), a procedure that produces a stable baseline threshold measurement and enhances the ability to detect the action of hyperalgesic agents (Taiwo et al., 1989; Aley et al., 1995). On the day of the experiment, rats were exposed to the same procedure, and the mean of the last 6 of the 12 readings was considered to be the baseline mechanical nociceptive threshold. The mean baseline threshold in these experiments was 110.9 ± 0.4 gm (n = 466; mean ± SEM). Mechanical threshold was again determined at different time points (15, 20, and 25 min) after various treatments. The mean of these three readings was defined as the paw-withdrawal threshold post-treatment for that paw, and this value was used to calculate the percentage change from the baseline threshold [% change in threshold = (pretreatment threshold - post-treatment threshold)/(pretreatment threshold)] × 100.

Drug administration

The drugs used in this study were PGE_2 [100 ng; direct-acting hyperalgesic inflammatory mediator (Pitchford and Levine, 1991, Gold et al., 1994)], DAMGO (μ-opioid receptor agonist), clonidine (α_2-adrenergic receptor agonist), CPA (A_1-adenosine receptor agonist), naloxone methyl iodide (opioid receptor antagonist), yohimbine HCl (α_2-adrenergic receptor antagonist), and PACPX (A_1-adenosine receptor antagonist), all from Research Biochemicals International (Natick, MA). The selection of the drug doses used in this study was based on the dose–response curves determined during this study or from previous work done in this laboratory (Aley et al., 1995). The stock solution of PGE_2 (1 μg/2.5 μl) was prepared in 10% ethanol and further dilutions were made in saline; the final concentration of ethanol was ≤1%. DAMGO, clonidine, CPA, naloxone, yohimbine, and PACPX were dissolved in saline. When drug combinations were used, they were administered from the same syringe so that the drug mentioned first reached the intradermal site first; the two drugs were separated in the syringe by a small air bubble to avoid the problem of diffusion. When an antagonist was included to antagonize the effect of an agonist, it was always injected first. The ID_{80} dose of each antagonist (i.e., naloxone 200 ng, yohimbine 100 ng, and PACPX 100 ng), calculated from its dose–response curve for reversal of the effect of its homologous agonist, was used throughout the study. All the drugs except the oligodeoxynucleotides were administered intradermally in a volume of 2.5 μl/paw.

Intrathecal cannulation

To administer drugs intrathecally, a catheter (PE-10 polyethylene tubing) was inserted caudally 8.5 cm into the subdural space through a midline incision made in the atlanto-occipital membrane of rats anesthetized with pentobarbital (50 mg/kg, i.p.); the external end of the catheter was secured to the skull with screws and dental acrylic (Yaksh and Rudy, 1976). The skin incision was sutured closed, and the animals were allowed to recover. Two days after surgery, rats that showed no motor deficits were used for experimental studies involving intrathecal administration of ODNs.

Antisense oligodeoxynucleotides

μ-Opioid receptor oligodeoxynucleotides. The μ-opioid receptor antisense and sense ODNs used in this study were synthesized using a Nucleic Acid Synthesizer model 391 (PCR Mate; Applied Biosystems, Foster City, CA). The μ-opioid receptor antisense ODN, 5′-CGCCCCAGCC-TCTTCCTCT-3′, is directed against the 5′-untranslated region of μ-opioid receptor-1 (MOR-1) clone, located between bases 87 and 69 upstream from the initiating ATG. The sense ODN, 5′-AGAGG-AAGAGGCTGGGGCG-3′ (Rossi et al., 1994), is complementary to the antisense sequence. Concentrations of ODN stocks were determined by spectrophotometry. Before their use, ODNs were lyophilized and resuspended in 0.9% NaCl to a concentration of 1 μg/10 μl.

Rats were divided into three groups: one group was untreated (without cannulae), a second group was treated with sense ODN (1 μg), and the third group was treated with antisense ODN (1 μg/rat). Using a microsyringe (Hamilton, Nevada City, UT), a dose of 1 μg ODN was administered to each rat intrathecally, in a volume of 10 μl, followed by 10 μl of saline (the dead space of the intrathecal catheter), on alternate days (days 1, 3, and 5). Behavioral tests were done 24 hr after the last dose of ODN. We have found that antisense ODN against MOR-1 attenuates μ-opioid receptor-like immunoreactivity in the dorsal horn of the spinal cord and peripheral nerve, DAMGO-induced inhibition of calcium current in cultured dorsal root ganglion neurons and DAMGO-induced inhibition of PGE_2-induced hyperalgesia (Khasar et al., 1996).

α_{2C}-opioid receptor oligodeoxynucleotides. We have demonstrated previously that the α_2-adrenergic receptor mediating peripheral antinociception has the pharmacological characteristics of the α_{2C} subtype (Khasar et al., 1995). Therefore, we also synthesized the antisense and sense ODNs for the α_{2C} receptor subtype, using Nucleic Acid Synthesizer model 391 PCR Mate. The α_{2C} receptor antisense ODN, 5′-ACCTGC-GGAGTACTG-3′, was developed by Lingen and Ordway (1995). The sense ODN sequence 5′-CAGTACTCCGCAGGT-3′ is complementary to the antisense sequence.

Rats were divided into three groups: one group was untreated (without cannulae), a second group was treated with sense ODN (1 μg/rat), and the third group was treated with antisense ODN (1 μg). Treatment and behavioral testing were as described for μ-opioid receptor ODN experiments.

Abbreviations for the drugs used in this study and their actions are shown in Table 1; experimental protocols are shown in Table 2.

Statistical analysis

Data are presented as mean ± SEM of six or more observations in each of the experimental groups. Statistical significance was determined by ANOVA, followed by Scheffe's *post hoc* test; $p < 0.05$ was considered statistically significant. Some data are repeated for comparison (see figure legends).

RESULTS

Tolerance to clonidine antinociception

In the present study, we found that after repeated administration, clonidine (100 ng) produces tolerance for its inhibition of PGE_2 (100 ng)-induced hyperalgesia (Fig. 1*A*), similar to that seen for μ and A_1 agonists (Aley et al., 1995).

Bidirectional cross-tolerance for μ, A_1, and α_2 peripheral antinociception

In paws made tolerant to DAMGO or CPA, clonidine failed to produce a significant antinociceptive effect when injected at the

Table 2. Experimental protocols

Group	*N*	Treatment	Dose(s)
I–A			
1	24	PGE_2	100 ng
2	16	Clonidine + PGE_2	100 ng + 100 ng
3	6	Clonidine hourly × 3	100 ng × 3
4	12	Clonidine hourly × 3, fourth hour clonidine + PGE_2	100 ng × 3, 100 ng + 100 ng
I–B			
1	12	DAMGO hourly × 3, fourth hour clonidine + PGE_2	1 μg × 3, 100 ng + 100 ng
2	12	CPA hourly × 3, fourth hour clonidine + PGE_2	1 μg × 3, 100 ng + 100 ng
3	16	Clonidine + PGE_2	100 ng + 100 ng
4	12	Clonidine hourly × 3, fourth hour DAMGO + PGE_2	100 ng × 3, 1 μg + 100 ng
5	6	DAMGO + PGE_2	1 μg + 100 ng
6	6	DAMGO hourly × 3	1 μg × 3
7	6	CPA hourly × 3	1 μg × 3
8	12	Clonidine hourly × 3, fourth hour CPA + PGE_2	100 ng × 3, 1 μg + 100 ng
9	6	CPA + PGE_2	1 μg + 100 ng
II-A			
1	24	PGE_2	100 ng
2	16	Clonidine + PGE_2	100 ng + 100 ng
3	6	Clonidine × 3	100 ng × 3
4	8	Clonidine hourly × 3, fourth hour yohimbine	100 ng × 3, 100 ng
II-B			
1	10	Clonidine hourly × 3, fourth hour naloxone	100 ng × 3, 200 ng
2	10	Clonidine hourly × 3, fourth hour PACPX	100 ng × 3, 100 ng
3	6	Clonidine × 3	100 ng × 3
4	10	DAMGO hourly × 3, fourth hour yohimbine	1 μg × 3, 100 ng
5	6	DAMGO × 3	1 μg × 3
6	10	CPA hourly × 3, fourth hour yohimbine	1 μg × 3, 100 ng
7	6	CPA hourly × 3	1 μg × 3
III-A			
1	24	PGE_2	100 ng
2	6	DAMGO + PGE_2	1 μg + 100 ng
3	6	Naloxone + DAMGO + PGE_2	1 ng + 1 μg + 100 ng
4	6	Naloxone + DAMGO + PGE_2	10 ng + 1 μg + 100 ng
5	6	Naloxone + DAMGO + PGE_2	100 ng + 1 μg + 100 ng
6	6	Naloxone + DAMGO + PGE_2	1 μg + 1 μg + 100 ng
III-B			
1	24	PGE_2	100 ng
2	16	Clonidine + PGE_2	100 ng + 100 ng
3	6	Yohimbine + clonidine + PGE_2	1 ng + 100 ng + 100 ng
4	6	Yohimbine + clonidine + PGE_2	10 ng + 100 ng + 100 ng
5	6	Yohimbine + clonidine + PGE_2	100 ng + 100 ng + 100 ng
6	6	Yohimbine + clonidine + PGE_2	1 μg + 100 ng + 100 ng
III-C			
1	24	PGE_2	100 ng
2	6	CPA + PGE_2	1 μg + 100 ng
3	6	PACPX + CPA + PGE_2	1 ng + 1 μg + 100 ng
4	6	PACPX + CPA + PGE_2	10 ng + 1 μg + 100 ng
5	6	PACPX + CPA + PGE_2	100 ng + 1 μg + 100 ng
6	6	PACPX + CPA + PGE_2	1 μg + 1 μg + 100 ng
IV-A			
1	24	PGE_2	100 ng
2	16	Clonidine + PGE_2	100 ng + 100 ng
3	6	Yohimbine + clonidine + PGE_2	100 ng + 100 ng + 100 ng
4	10	Naloxone + clonidine + PGE_2	200 ng + 100 ng + 100 ng
5	10	PACPX + clonidine + PGE_2	100 ng + 100 ng + 100 ng
IV-B			
1	24	PGE_2	100 ng
2	6	DAMGO + PGE_2	1 μg + 100 ng
3	6	Naloxone + DAMGO + PGE_2	200 ng + 1 μg + 100 ng
4	8	Yohimbine + DAMGO + PGE_2	100 ng + 1 μg + 100 ng
5	6	PACPX + DAMGO + PGE_2	100 ng + 1 μg + 100 ng
IV-C			
1	24	PGE_2	100 ng
2	6	CPA + PGE_2	1 μg + 100 ng

Table 2. continues.

Table 2. *continued*

Group	*N*	Treatment	Dose(s)
IV-C			
3	6	PACPX + CPA + PGE_2	100 ng + 1 μg + 100 ng
4	8	Naloxone + CPA + PGE_2	200 ng + 1 μg + 100 ng
5	6	Yohimbine + CPA + PGE_2	100 ng + 1 μg + 100 ng
V-A			
1	8	Clonidine hourly × 3, fourth hour yohimbine	100 ng × 3, 100 ng
2	6	Clonidine hourly × 3, fourth hour clonidine + yohimbine	100 ng × 3, 100 ng + 100 ng
3	8	Clonidine hourly × 3, fourth hour DAMGO + yohimbine	100 ng × 3, 1 μg + 100 ng
4	8	Clonidine hourly × 3, fourth hour CPA + yohimbine	100 ng × 3, 1 μg + 100 ng
V-B			
1	6	DAMGO hourly × 3, fourth hour naloxone	1 μg × 3, 200 ng
2	6	DAMGO hourly × 3, fourth hour DAMGO + naloxone	1 μg + 1 μg + 200 ng
3	8	DAMGO hourly × 3, fourth hour clonidine + naloxone	1 μg + 100 ng + 200 ng
4	8	DAMGO hourly × 3, fourth hour CPA + naloxone	1 μg + 1 μg + 200 ng
V-C			
1	8	CPA hourly × 3, fourth hour PACPX	1 μg × 3, 100 ng
2	6	CPA hourly × 3, fourth hour CPA + PACPX	1 μg + 1 μg + 100 ng
3	8	CPA hourly × 3, fourth hour clonidine + PACPX	1 μg + 100 ng + 100 ng
4	8	CPA hourly × 3, fourth hour DAMGO + PACPX	1 μg + 1 μg + 100 ng
VI-A			
1	24	PGE_2	100 ng
2	6	DAMGO + PGE_2	1 μg + 100 ng
3	6	μ-antisense intrathecally alternate days × 3, 24 hr after DAMGO + PGE_2	1 μg × 3, 1 μg + 100 ng
4	6	μ-sense intrathecally alternate days × 3, 24 hr after DAMGO + PGE_2	1 μg × 3, 1 μg + 100 ng
VI-B			
1	24	PGE_2	100 ng
2	16	Clonidine + PGE_2	100 ng + 100 ng
3	6	μ-antisense intrathecally alternate days × 3, 24 hr after clonidine + PGE_2	1 μg × 3, 100 ng + 100 ng
4	6	μ-sense intrathecally alternate days × 3, 24 hr after clonidine + PGE_2	1 μg × 3, 100 ng + 100 ng
VI-C			
1	24	PGE_2	100 ng
2	6	CPA + PGE_2	1 μg + 100 ng
3	6	μ-antisense intrathecally alternate days × 3, 24 hr after CPA + PGE_2	1 μg × 3, 1 μg + 100 ng
4	6	μ-sense intrathecally alternate days × 3, 24 hr after CPA + PGE_2	1 μg × 3, 1 μg + 100 ng
VII-A			
1	24	PGE_2	100 ng
2	6	DAMGO + PGE_2	1 μg + 100 ng
3	6	α_2-antisense intrathecally alternate days × 3, 24 hr after DAMGO + PGE_2	1 μg × 3, 1 μg + 100 ng
4	6	α_2-sense intrathecally alternate days × 3, 24 hr after DAMGO + PGE_2	1 μg × 3, 1 μg + 100 ng
VII-B			
1	24	PGE_2	100 ng
2	16	Clonidine + PGE_2	100 ng + 100 ng
3	6	α_2-antisense intrathecally alternate days × 3, 24 hr after clonidine + PGE_2	1 μg × 3, 100 ng + 100 ng
4	6	α_2-sense intrathecally alternate days × 3, 24 hr after clonidine + PGE_2	1 μg × 3, 100 ng + 100 ng
VII-C			
1	24	PGE_2	100 ng
2	6	CPA + PGE_2	1 μg + 100 ng
3	6	α_2-antisense intrathecally days × 3, 24 hr after CPA + PGE_2	1 μg × 3, 1 μg + 100 ng
4	6	α_2-sense intrathecally alternate days × 3, 24 hr after CPA + PGE_2	1 μg × 3, 1 μg + 100 ng

Abbreviations: PGE_2, Prostaglandin E_2 (EP receptor agonist); DAMGO, [D-Ala^2, N-Me-Phe^4, gly^5-ol] (μ-opioid receptor agonist); Cl, clonidine (α_2 agonist); Yo, yohimbine (α_2 antagonist); CPA, N^6-cyclopentyl adenosine (A_1-adenosine agonist); PACPX, 1,3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine (A_1-adenosine antagonist). There are repetitions for the sake of comparison.

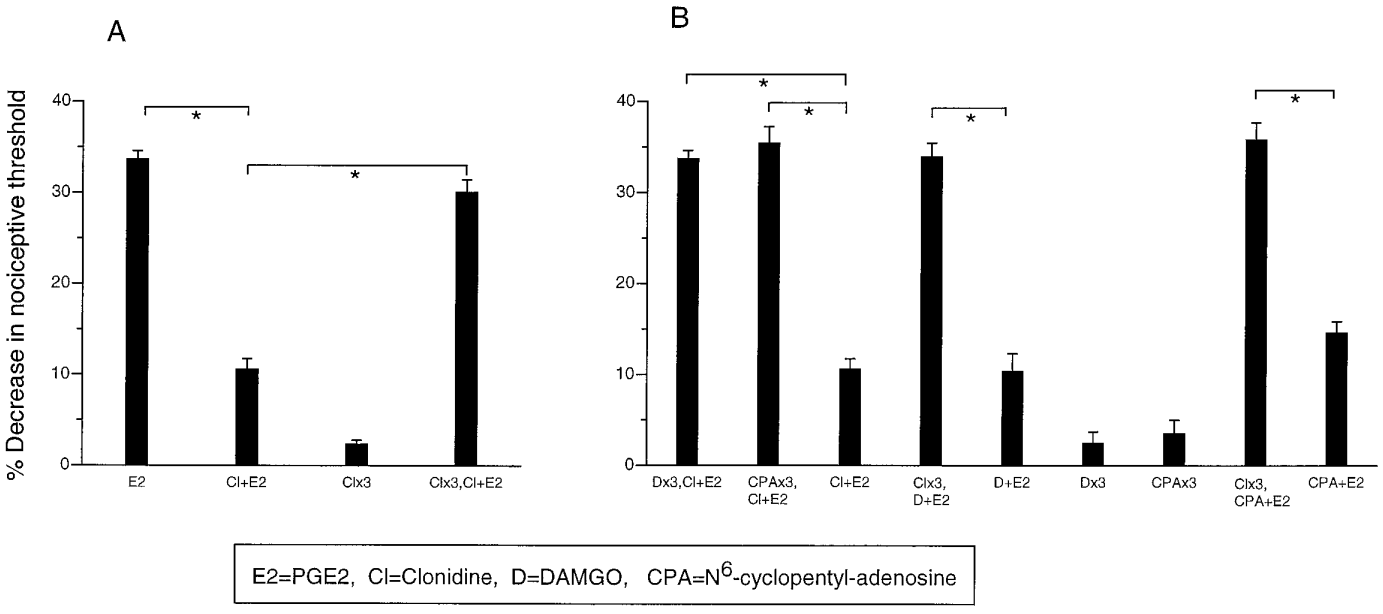

Figure 1. *A*, Repeated administration of clonidine produces tolerance to antinociception. Effect of PGE_2 (*E2*), clonidine plus PGE_2 (*Cl+E2*), clonidine once hourly for 3 hr (*Clx3*), clonidine once hourly for 3 hr, and at the fourth hour clonidine plus PGE_2 (*Clx3, Cl+E2*) on mechanical paw withdrawal threshold in the rat. *B*, Bidirectional cross-tolerance develops among A_1, α_2, and μ antinociception. Effect of clonidine plus PGE_2 (*Cl+E2*), DAMGO once hourly for 3 hr (*Dx3*), DAMGO once hourly for 3 hr and at the fourth hour clonidine plus PGE_2 (*Dx3,Cl+E2*), CPA once hourly for 3 hr and at the fourth hour clonidine plus PGE_2 (*CPAx3, Cl+E2*), CPA once hourly for 3 hr (*CPAx3*), clonidine once hourly for 3 hr and at the fourth hour DAMGO plus PGE_2 (*Clx3, D+E2*), and clonidine once hourly for 3 hr and at the fourth hour CPA plus PGE_2 (*Clx3, CPA+E2*) on mechanical paw withdrawal threshold in the rat.

fourth hour (Fig. 1B). Also, in paws made tolerant to clonidine, DAMGO and CPA were no longer antinociceptive (Fig. 1*B*). These observations suggest that there is bidirectional cross-tolerance between α_2, μ, and A_1 to their peripheral antinociceptive effects.

Yohimbine precipitated withdrawal hyperalgesia in clonidine-tolerant paws

In the present study, we found that after induction of tolerance with three hourly injections of clonidine (100 ng), administration of its receptor antagonist yohimbine (100 ng) precipitated a withdrawal hyperalgesia, revealing the development of dependence (Fig. 2*A*).

Bidirectional cross-withdrawal for μ, A_1, and α_2 tolerance/antinociception

In paws made tolerant to clonidine, administration of the μ- and A_1-antagonists, naloxone and PACPX, respectively, precipitated withdrawal hyperalgesia. In paws made tolerant to DAMGO and CPA, yohimbine precipitated withdrawal hyperalgesia (Fig. 2*B*). These observations suggest that there is bidirectional cross-withdrawal between μ, A_1, and α_2 after the development of peripheral tolerance to their peripheral antinociceptive effects.

Multiple receptors involved in μ, A_1, and α_2 antinociception

Naloxone, yohimbine, and PACPX dose-dependently blocked the antinociceptive effects of their homologous agonists DAMGO, clonidine, and CPA, respectively (Fig. 3*A–C*). However, in addition, naloxone (200 ng) also blocked clonidine antinociception but not CPA antinociception (Fig. 4*A,C*), and PACPX (100 ng) blocked clonidine antinociception but not DAMGO antinociception (Fig. 4*A,B*). Yohimbine (100 ng) blocked clonidine, DAMGO, and CPA antinociception (Fig. 4*A–C*). These data suggest that the α_2 receptor is involved not only in α_2 antinociception but also in μ and A_1 antinociception. In addition, the data suggest that the μ and A_1 receptors are involved in α_2 antinociception.

Multiple receptors involved in μ, A_1, and α_2 tolerance and withdrawal

A similar profile of receptor interactions was seen for tolerance and withdrawal to μ, A_1, and α_2 antinociception. This was demonstrated by examining which receptor agonists (μ, A_1, and α_2) could block antagonist-induced withdrawal hyperalgesia. Yohimbine-induced withdrawal, in clonidine-tolerant paws, was blocked by coadministration of clonidine with yohimbine, as well as by coadministration of DAMGO and CPA with yohimbine (Fig. 5*A*). Naloxone-induced withdrawal, in DAMGO-tolerant paws, was blocked by coinjection of DAMGO with naloxone or clonidine with naloxone, but was not blocked by coadministration of CPA with naloxone (Fig. 5*B*). Similarly, PACPX-induced withdrawal in CPA-tolerant paws was blocked by coinjection of CPA with PACPX or clonidine with PACPX, but was not blocked by coadministration of DAMGO with PACPX (Fig. 5*C*). These data suggest, as in previous experiments, that μ, α_2, and A_1 receptors are involved in α_2 antinociception, tolerance, and withdrawal; that μ and α_2 receptors are involved in μ antinociception, tolerance, and withdrawal; and that A_1 and α_2 are involved in A_1 antinociception, tolerance, and withdrawal.

Antisense ODN treatment supports the hypothesis that multiple receptors are involved in μ, α_2, and A_1 antinociception

Antisense μ-opioid receptor ODN significantly attenuated not only the μ antinociception but also α_2 antinociception 24 hr after

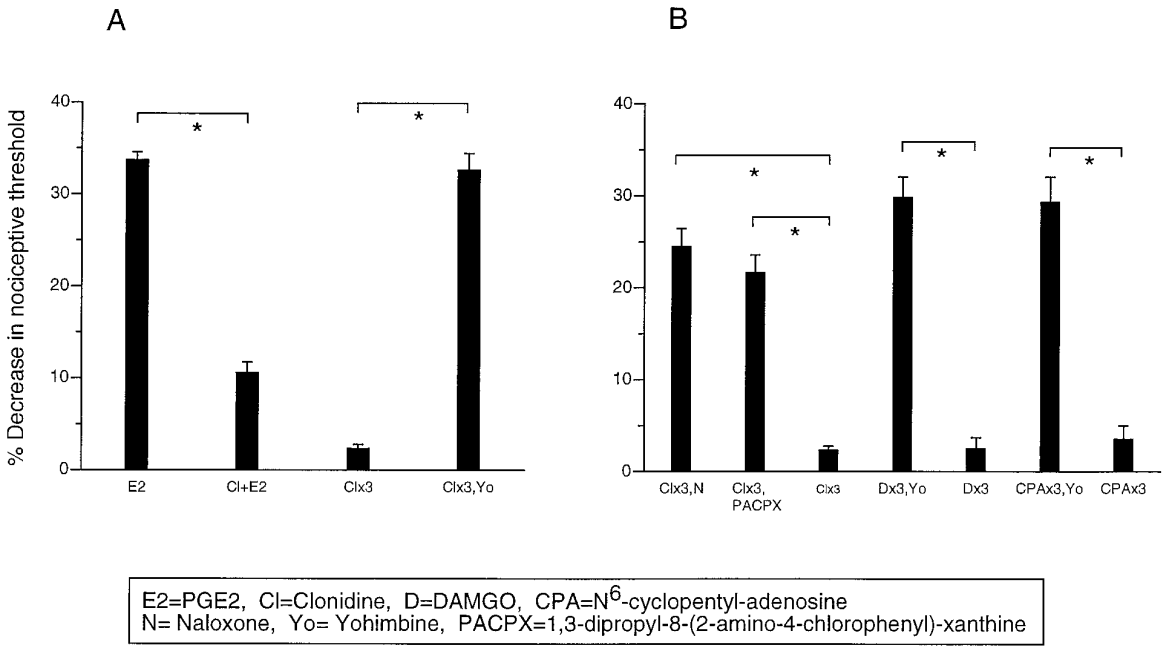

Figure 2. *A*, Yohimbine precipitates withdrawal hyperalgesia in clonidine tolerant paws. Effect of PGE_2 (*E2*), clonidine plus PGE_2 (*Cl+E2*), clonidine once hourly for 3 hr (*Clx3*), clonidine once hourly for 3 hr (*Clx3*), clonidine once hourly for 3 hr, and at the fourth hour yohimbine (*Clx3, Yo*) on mechanical paw-withdrawal threshold in the rat. *B*, Bidirectional cross-withdrawal develops among A_1, α_2, and μ antinociception. Effect of clonidine once hourly for 3 hr and at the fourth hour naloxone (*Clx3, N*), clonidine once hourly for 3 hr and at the fourth hour PACPX (*Clx3, PACPX*), clonidine once hourly for 3 hr (*Clx3*), DAMGO once hourly for 3 hr and at the fourth hour yohimbine (*Dx3, Yo*), DAMGO once hourly for 3 hr (*Dx3*), CPA once hourly for 3 hr (*CPAx3*), CPA once hourly for 3 hr and at the fourth hour yohimbine (*CPAx3, Yo*), and CPA once hourly for 3 hr (*CPAx3*) on mechanical paw withdrawal threshold in the rat.

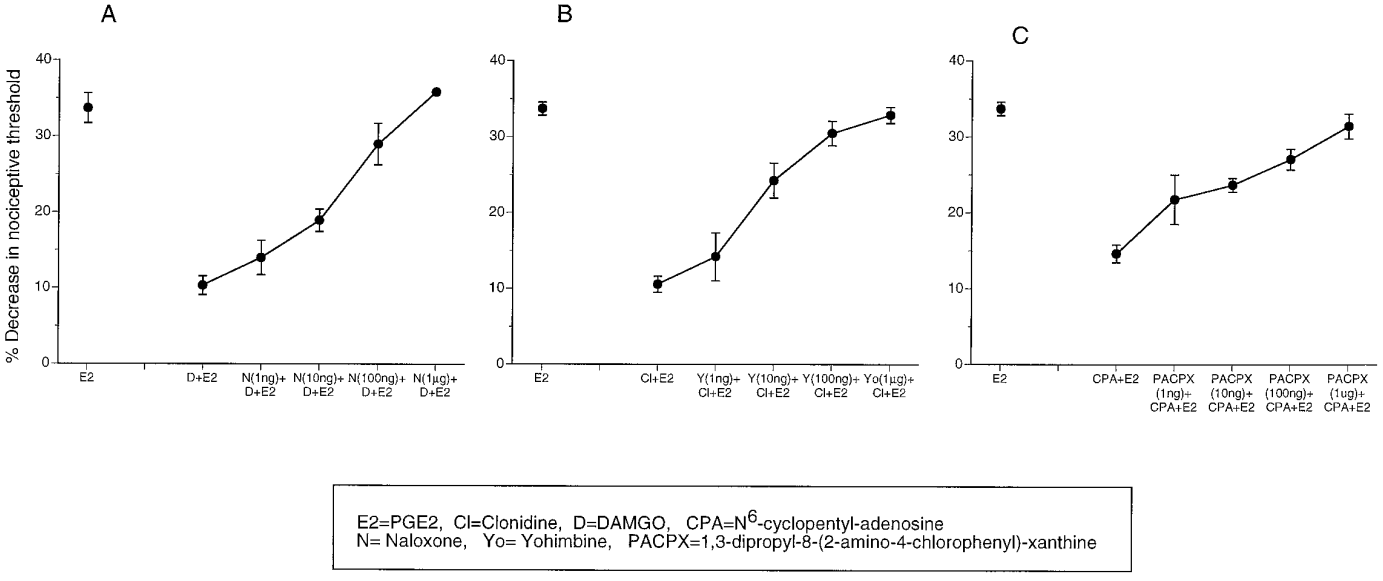

Figure 3. μ, α_2, and A_1 antagonists dose-dependently block μ, α_2, and A_1 antinociception, respectively. *A*, Naloxone dose-dependently blocks DAMGO antinociception. Effect of PGE_2 (*E2*), DAMGO plus PGE_2 (*D+E2*) and various doses of naloxone (1 ng to 1 μg), and DAMGO plus PGE_2 (*N+D+E2*), on mechanical paw withdrawal threshold in the rat. *B*, Yohimbine dose-dependently blocks clonidine antinociception. Effect of PGE_2 (*E2*), clonidine plus PGE_2 (*Cl+E2*), and various doses of yohimbine (1 ng to 1 μg) and clonidine plus PGE_2 (*Yo+Cl+E2*) on mechanical paw withdrawal threshold in the rat. *C*, PACPX dose-dependently blocks CPA antinociception. Effect of PGE_2 (*E2*), DAMGO plus PGE_2 (*CPA+E2*), and various doses of PACPX (1 ng to 1 μg) and CPA plus PGE_2 (*PACPX+CPA+E2*) on mechanical paw withdrawal threshold in the rat.

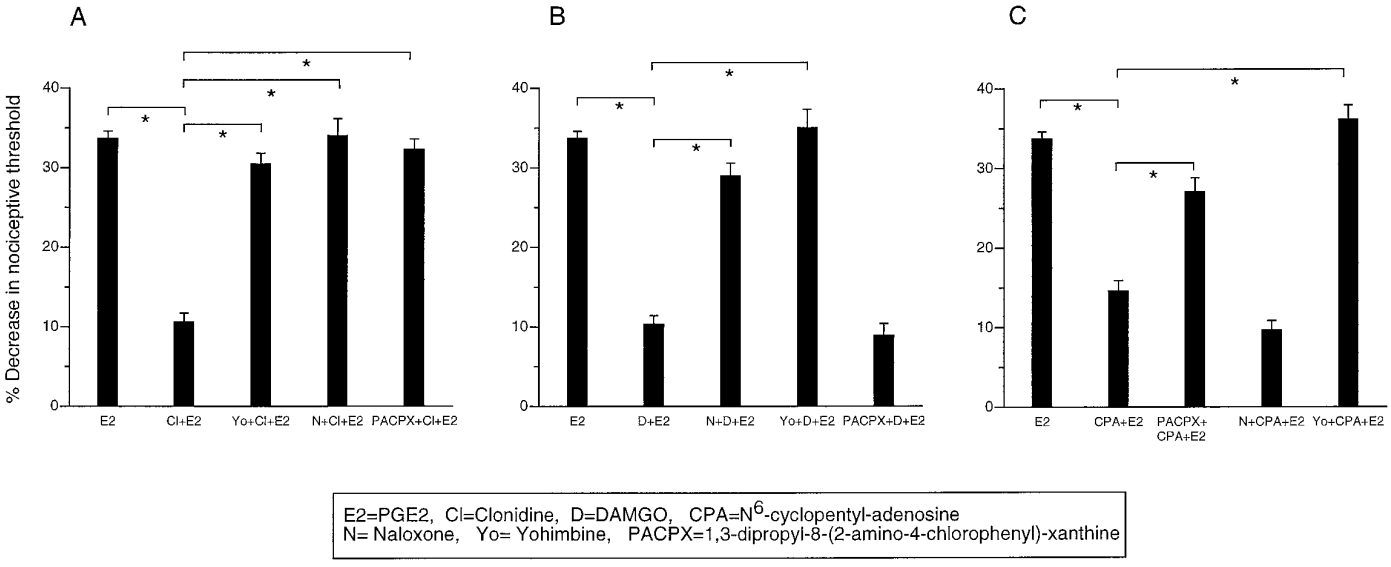

Figure 4. Multiple receptors are involved in μ, α_2, and A_1 antinociception. *A*, Clonidine α_2 antinociception is blocked not only by yohimbine but also by naloxone and PACPX. Effect of PGE_2 (*E2*), clonidine plus PGE_2 (*Cl+E2*), yohimbine plus clonidine plus PGE_2 (*Yo+Cl+E2*), naloxone plus clonidine plus PGE_2 (*N+Cl+E2*), and PACPX plus clonidine plus PGE_2 (*PACPX+Cl+E2*) on mechanical paw withdrawal threshold in the rat. *B*, DAMGO μ antinociception is blocked not only by naloxone but also by yohimbine. Effect of PGE_2 (*E2*), DAMGO plus PGE_2 (*D+E2*), naloxone plus DAMGO plus PGE_2 (*N+D+E2*), yohimbine plus DAMGO plus PGE_2 (*Yo+D+E2*), and PACPX plus DAMGO plus PGE_2 (*PACPX+D+E2*) on mechanical paw withdrawal threshold in the rat. *C*, CPA A_1 antinociception is blocked not only by PACPX but also by yohimbine. Effect of PGE_2 (*E2*), CPA plus PGE_2 (*CPA+E2*), PACPX plus CPA plus PGE_2 (*PACPX+CPA+E2*), naloxone plus CPA plus PGE_2 (*N+CPA+E2*), and yohimbine plus CPA plus PGE_2 (*Yo+CPA+E2*) on mechanical paw withdrawal threshold in the rat.

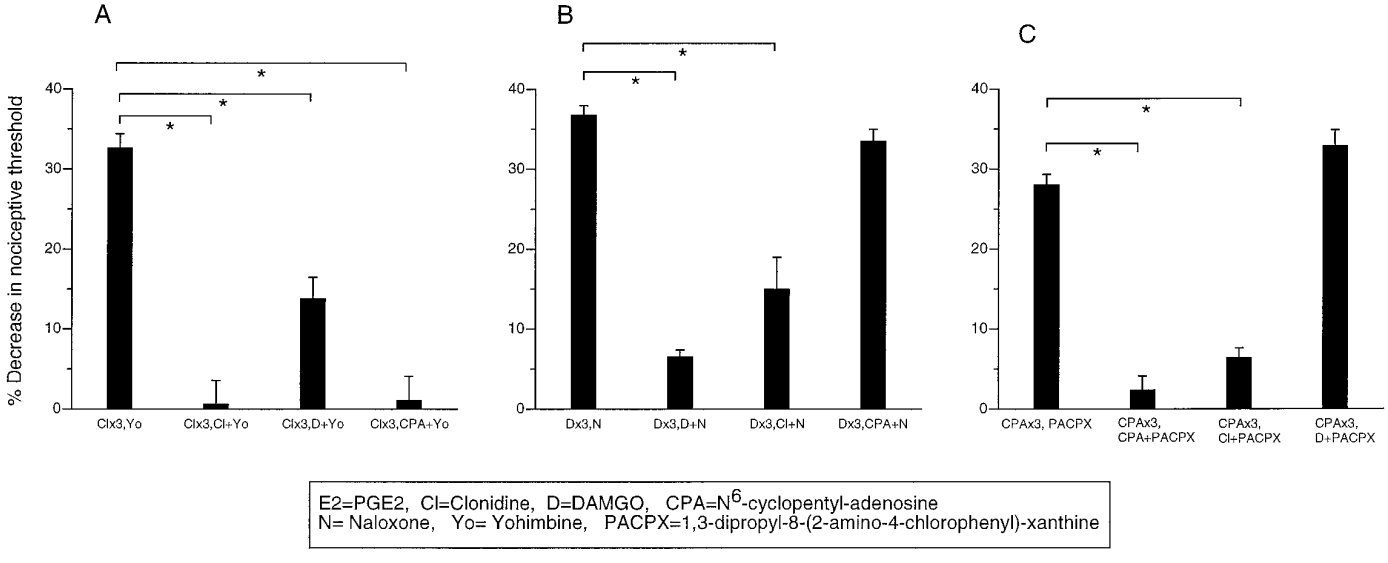

Figure 5. Multiple receptors are involved in α_2, μ, and A_1 tolerance and withdrawal. *A*, Yohimbine withdrawal is blocked not only by clonidine but also by DAMGO and CPA. Effect of clonidine once hourly for 3 hr and at the fourth hour yohimbine (*Clx3,Yo*), clonidine once hourly for 3 hr and at the fourth hour clonidine plus yohimbine (*Clx3,Cl+Yo*), clonidine once hourly for 3 hr and at the fourth hour DAMGO plus yohimbine (*Clx3,D+Yo*), and clonidine once hourly for 3 hr and at the fourth hour CPA plus yohimbine (*Clx3,CPA+Yo*) on mechanical paw withdrawal threshold in the rat. *B*, Naloxone withdrawal is blocked not only by DAMGO but also by clonidine. Effect of DAMGO once hourly for 3 hr and at the fourth hour naloxone (*Dx3,N*), DAMGO once hourly for 3 hr and at the fourth hour DAMGO plus naloxone (*Dx3,D+N*), DAMGO once hourly for 3 hr and at the fourth hour clonidine plus naloxone (*Dx3,Cl+N*), and DAMGO once hourly for 3 hr and at the fourth hour CPA plus naloxone (*Dx3,CPA+N*) on mechanical paw withdrawal threshold in the rat. *C*, PACPX withdrawal is blocked not only by CPA but also by clonidine. Effect of CPA once hourly for 3 hr and at the fourth hour PACPX (*CPAx3,PACPX*), CPA once hourly for 3 hr and at the fourth hour CPA plus PACPX (*CPAx3,CPA+PACPX*), CPA once hourly for 3 hr and at the fourth hour clonidine plus PACPX (*CPAx3,Cl+PACPX*), and CPA once hourly for 3 hr and at the fourth hour DAMGO plus naloxone (*CPAx3,D+PACPX*) on mechanical paw withdrawal threshold in the rat.

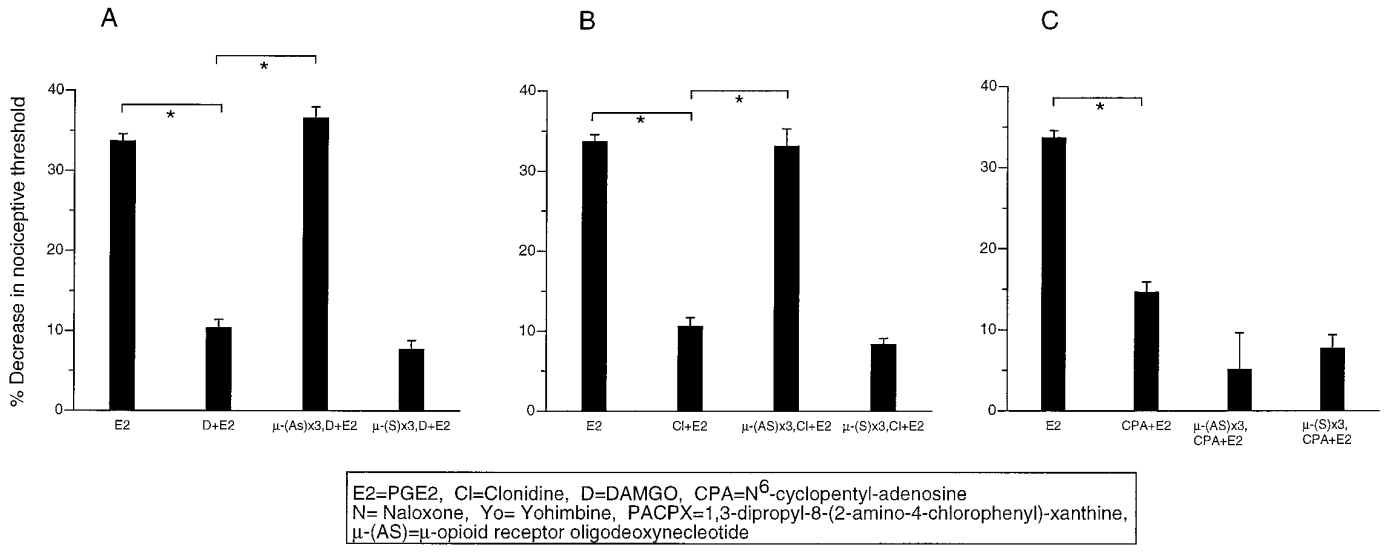

Figure 6. Antisense μ ODN treatment blocks not only μ antinociception but also α_2 antinociception. A_1 antinociception is unaffected. *A*, Effect of PGE_2 (*E2*), DAMGO plus PGE_2 (*D+E2*), μ-antisense (*AS*) ODN 1 μg intrathecally on alternate days × 3 and DAMGO plus PGE_2 [*μ-(AS)x3,D+E2*], μ-sense (*S*) ODN 1 μg intrathecally on alternate days × 3, and DAMGO plus PGE_2 [*μ-(S)x3,D+E2*] on mechanical paw-withdrawal threshold. *B*, Effect of PGE_2 (*E2*), clonidine plus PGE_2 (*Cl+E2*), μ-(AS) ODN 1 μg intrathecally on alternate days × 3, and clonidine plus PGE_2 [*μ-(AS)x3,Cl+E2*], μ-(S) ODN 1 μg intrathecally on alternate days × 3, and clonidine plus PGE_2 [*μ-(S)x3,Cl+E2*] on mechanical paw-withdrawal threshold. *C*, Effect of PGE_2 (*E2*), CPA plus PGE_2 (*CPA+E2*), μ-(AS) ODN 1 μg intrathecally on alternate days × 3, CPA plus PGE_2 [*μ-(AS)x3,CPA+E2*], μ-(S) ODN 1 μg intrathecally on alternate days × 3, and CPA plus PGE_2 [*μ-(S)x3,CPA+E2*] on mechanical paw withdrawal threshold in the rat.

the last injection of the antisense ODN. In contrast, A_1 antinociception was unaffected by μ ODN treatment (Fig. 6*A–C*). Sense μ-opioid receptor ODN was without effect on PGE_2 hyperalgesia, μ antinociception, or α_2 antinociception (Fig. 6*A–C*).

Antisense ODN for the α_{2C}-adrenergic receptor significantly attenuated not only α_2 antinociception but also μ and A_1 antinociception (Fig. 7*A–C*). Sense α_{2C}-adrenergic receptor ODN was without effect on PGE_2 hyperalgesia, μ antinociception, or α_2 antinociception.

The data from these antisense ODN experiments suggest that α_2-adrenergic receptors are required for μ and A_1 antinociception. In addition, they suggest that the μ-opioid receptor is required for α_2 antinociception. Thus, multiple receptors are involved in the production of antinociception by μ, α_2, and A_1 agonists.

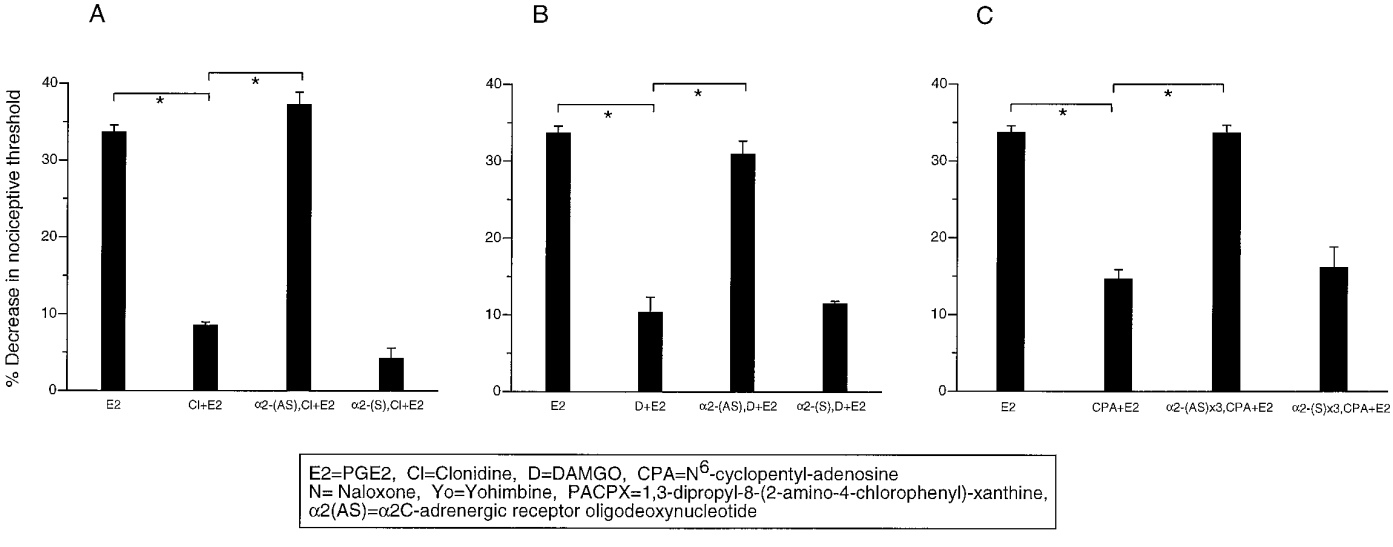

Figure 7. Antisense α_{2C} ODN treatment blocks not only α_2 antinociception but also μ and A_1 antinociception. *A*, Effect of PGE_2 (*E2*), clonidine plus PGE_2 (*Cl+E2*), α_2-(AS) ODN 1 μg intrathecally on alternate days × 3, and clonidine plus PGE_2 [*α_2-(AS)x3,CCl+E2*], α_2-(S) ODN 1 μg intrathecally on alternate days × 3, and clonidine plus PGE_2 [*α_2-(S)x3,Cl+E2*] on mechanical paw withdrawal threshold in the rat. *B*, Effect of PGE_2 (*E2*), DAMGO plus PGE_2 (*D+E2*), α_2-(AS) ODN 1 μg intrathecally on alternate days × 3, and DAMGO plus PGE_2 [*α_2-(AS)x3,D+E2*], α_2-(S) ODN 1 μg intrathecally on alternate days × 3, and DAMGO plus PGE_2 [*α_2-(S)x3,D+E2*] on mechanical paw withdrawal threshold in the rat. *C*, Effect of PGE_2 (E2), CPA plus PGE_2 (CPA + E2), α_2-(AS) ODN 1 μg intrathecally on alternate days × 3, and CPA plus PGE_2 [*α_2-(AS)x3,CPA+E2*], α_2-(S) ODN 1 μg intrathecally on alternate days × 3, and CPA plus PGE_2 [*α_2-(S)x3,CPA+E2*] on mechanical paw withdrawal threshold in the rat.

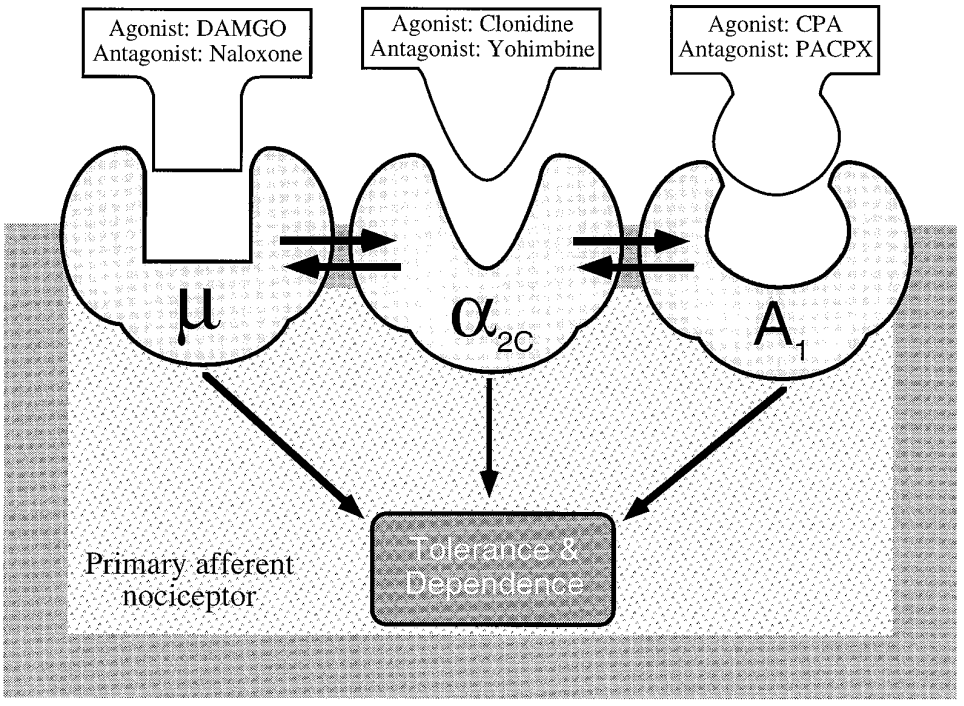

Figure 8. Schematic diagram of hypothesized topological/physical arrangement of the three receptors for peripheral antinociception in the cell membrane. μ (*DAMGO*), α_{2C} (*Clonidine*), and A_1 (*CPA*) agonism all result in peripheral antinociception mediated through a common second messenger pathway, leading to complete symmetrical cross-tolerance and cross-dependence. However, the asymmetrical interactions are proposed to be a result of the central position of the α_{2C} receptor leading to bidirectional interactions between this receptor and the two other receptors but no interaction between the μ and A_1 receptors.

DISCUSSION

In this study, we tested the hypothesis that peripheral antinociception produced by μ, α_2, and A_1 agonists exhibit cross-tolerance after repeated exposure to these agents. This hypothesis arose from previous data demonstrating that μ, α_2, and A_1 receptors can signal via a common second messenger, namely activation of an inhibitory G-protein (Sharma et al., 1975; Law et al., 1981; Childers and Rivere, 1989; Mankmann et al., 1988). In all experiments evaluating cross-tolerance and cross-dependence, complete symmetry was found for μ, α_2, and A_1. These findings support the hypothesis that there is a common signaling pathway for these three receptors. Although the mechanisms of tolerance and dependence in primary afferent nociceptors is unknown, in other systems the protein kinase C second messenger system has been implicated (Mao et al., 1995; Mayer et al., 1995). The role of this second messenger system in the tolerance and dependence to peripheral antinociception is currently being investigated. These data also suggest that clinically these pharmacologies may not be cross-substituted in dependent individuals.

As a control for the cross-withdrawal experiments, we tested whether there was blockade of antinociception by heterologous antagonists in naive animals. Unexpectedly, we found that the α_2 antagonist blocked not only α_2 antinociception but also A_1 and μ antinociception, that both μ and A_1 antagonists blocked α_2 antinociception, and that there was no such heterologous antagonism between μ and A_1 ligands in antinociception. The absence of an interaction between μ and A_1 antinociception is unlikely to be a result of an inadequate dose of antagonist because even at a very high dose (1 μg) no cross-antagonism was observed (unpublished observations).

There are known mechanisms by which antagonists can heterologously antagonize the actions of an agonist at another receptor class. First, when a receptor ligand is not highly selective, at sufficiently high doses it will bind to another receptor to displace a heterologous ligand (Cicero et al., 1974; Spiehler et al., 1978; Blank et al., 1983). However, clonidine binding is not displaced from neuronal membranes by morphine or naloxone (Golombiowska-Nikitkin et al., 1980). Second, there may be physical interactions between the receptors in the cell membrane. Such interactions have been suggested to explain effects of agonist combinations that are greater than additive (synergistic) or less than additive (antagonistic) than the effects seen at the different receptors. For example, an α_2 agonist attenuates both A_1 and μ mediated inhibition of norepinephrine release from sympathetic nerve endings; this interaction has been suggested to occur at the level of the receptor (Bucher et al., 1992). Furthermore, Bentley et al. (1983) have hypothesized that α-adrenoceptor and opioid receptors may be linked, either via second messenger systems or physically in the membrane.

We hypothesize from the data in our experiments that the α_2 receptor is arranged topologically between the μ and A_1 receptors to form a receptor complex (Fig. 8). This hypothesis is supported by the observation that antisense oligodeoxynucleotides against μ-opioid receptor reduced not only μ antinociception, but also α_2 antinociception, while preserving A_1 antinociception, whereas the α_{2C} antisense oligodeoxynucleotide reduced the antinociceptive effects of all three receptor systems, α_2, A_1, and μ. The preservation of A_1 antinociception after μ antisense treatment but not after α_{2C} antisense treatment suggests that the effect of receptor attenuation in the terminal is of short-range topologically. The same profile of interactions between α_2, μ, and A_1 were found in assessing the ability of a heterologous agonist to block withdrawal induced by the homologous antagonist in a tolerant paw.

In summary, we have demonstrated that there is a symmetry for the three agonists for production of tolerance and dependence. There was also an unexpected interaction, which seems to occur at the level of the receptor, between α_2 and μ receptors and α_2 and A_1 receptors, but not between μ and A_1 receptors. The results suggest the hypothesis that these three receptors may be coupled physically in the plasma membrane.

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