K.-P.L. and W.W. The excellent technical assistance of A. Stillbauer is gratefully acknowledged.

Note Added in Proof: After this paper had been submitted, the recent work of Cain et al.[25] also describing the synthesis and benzodiazepine receptor interaction of

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(25) M. Cain, R. W. Weber, F. Guzmann, J. M. Cook, S. A. Barker, K. C. Rice, J. N. Crawley, S. M. Paul, and P. Skolnick, *J. Med. Chem.*, **25**, 1081 (1982).

several β-carboline derivatives came to our attention.

Alkylation of Opioid Receptor Subtypes by α-Chlornaltrexamine Produces Concurrent Irreversible Agonistic and Irreversible Antagonistic Activities[1]

L. M. Sayre,[†,§] A. E. Takemori,[‡] and P. S. Portoghese*[,†]

Department of Medicinal Chemistry, College of Pharmacy, and Department of Pharmacology, Medical School, University of Minnesota, Minneapolis, Minnesota 55455. Received September 22, 1982

α-Chlornaltrexamine (1a, α-CNA), the C-6 epimer of the opioid receptor affinity label β-CNA (1b), has been synthesized and tested in vitro and in vivo. In vitro, α-CNA appears to alkylate opioid receptor subtypes (μ, κ, and δ) and is similar to β-CNA in its ability to produce irreversible antagonism at all three subtypes. However, 1a differs from 1b in that it exhibits additionally an irreversible agonist activity in the guinea pig ileum preparation but not in the mouse vas deferens preparation. This latter activity is discussed in terms of an irreversible mixed agonism–antagonism at κ receptors, or, alternatively, it may reflect differences between μ receptors in the two in vitro preparations.

The affinity label β-chlornaltrexamine (1b, β-CNA) has
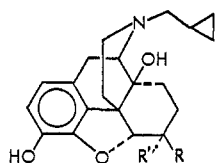
1a, R = H; R¹ = N(CH₂CH₂Cl)₂
1b, R = N(CH₂CH₂Cl)₂; R¹ = H
2a, R = H; R¹ = N(CH₂CH₂OH)₂
3a, R = H; R¹ = NH₂

4b, R = NHCOC=CCOOMe; R¹ = H
        | |
        H   H

found considerable use in the pharmacological characterization of opioid receptors.[2-9] This ligand irreversibly inhibits the stereospecific binding of [³H]naloxone in the opiate receptor binding assay[3] and behaves as an irreversible narcotic antagonist in the guinea pig ileum (GPI)[4,5] and mouse vas deferens (MVD)[6] preparations. Moreover, β-CNA produces ultralong-lasting antagonism (≥3 days) of morphine in vivo.[7-9] At least three opioid receptor subtypes (μ, κ, and δ) are apparently covalently bound by interaction with β-CNA.

In view of the properties of β-CNA (1b), it was therefore of interest to investigate the pharmacological profile of its C-6 epimer, α-CNA (1a), because its nitrogen mustard electrophile is in a different orientation and, hence, might alkylate different receptor nucleophiles. In this report we describe the synthesis and remarkable biological properties of α-CNA. A key feature distinguishing α-CNA from β-CNA is its behavior as an irreversible agonist in addition to its irreversible antagonist activity.

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† Department of Medicinal Chemistry.
‡ Department of Pharmacology.
§ Present address: Department of Chemistry, Case Western Reserve University, Cleveland, OH 44106.

Table I. Agonist Effect of α-CNA on the Untreated and β-FNA-Treated GPI

GPI preparation	IC₅₀ ± SE,[a] nM	
	EKC	α-CNA
untreated	0.72 ± 0.21	5.5 ± 2.0
β-FNA treated[b]	0.69 ± 0.22	2.4 ± 0.8

[a] All IC₅₀ values represent means plus or minus standard errors determined from four GPI preparations. [b] GPI was incubated with 2 × 10⁻⁷ M β-FNA for 60 min and washed thoroughly 20 times. Such preparations have been shown to be μ-less, since all available μ sites are irreversibly bound by β-FNA and become essentially preparations with κ sites. The IC₅₀ values of the κ agonist, EKC (ethylketazocine), indicate that the κ sites are intact in the β-FNA-treated GPI.

Chemistry. α-CNA was prepared in two steps from α-naltrexamine (3a)[10] by exhaustive reductive alkylation of the latter with glycoaldehyde (NaBH₃CN, MeOH) and

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(1) Stereochemical Studies on Medicinal Agents. 27. For paper 26, see Portoghese, P. S.; Poupaert, J. H.; Larson, D. L.; Groutas, W. C.; Meitzner, G. D.; Swenson, D. C.; Smith, G. D.; Duax, W. L. *J. Med. Chem.* **1982**, *25*, 685.

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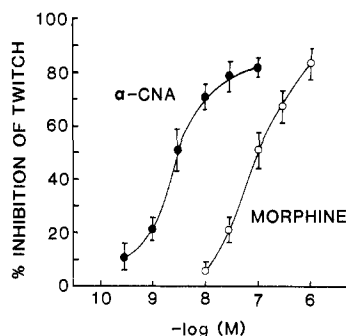(10) Sayre, L. M.; Portoghese, P. S. *J. Org. Chem.* **1980**, *45*, 3366.

Figure 1. The agonist activity of α-CNA and morphine on the GPI. The mean IC_{50} ± SE of morphine was 121.0 ± 0.36 nM and that of α-CNA was 5.5 ±2.0 nM in four preparations. The vertical lines represent standard errors.

conversion of the intermediate bis(2-hydroxyethyl)amine (**2a**) to the bis(2-chloroethyl)amine with Ph_3P/CCl_4 in CH_3CN. The crude product, isolated as its dihydrochloride salt, was triturated with benzene and then with EtOAc and was purified at 4 °C by silica gel chromatography (EtOAc-CH_3CN, 3:1) of the free base liberated upon neutralization with Et_3N on the column. The desired product was precipitated as the dihydrochloride salt by collection in HCl-acidified flasks.

Pharmacological Results

α-CNA (**1a**) was first examined in the guinea pig ileal longitudinal muscle preparation (GPI). The cumulative agonist concentration–response relationship revealed that the compound was approximately 22 times more potent than morphine (Figure 1). A component of this agonism could not be reversed by extensive washing or treatment with naloxone, though *prior* incubation with naloxone completely protected against the expression of any agonist activity of α-CNA. The level of agonism that could not be reversed increased with increasing concentration or time of incubation, and the ileal twitch was permanently eradicated by a 30-min treatment with 20 nM α-CNA.

In order to assess the characteristics of the agonist activity in terms of opioid receptor subtypes, some experiments were performed with a GPI preparation that had been depleted of functional μ receptors (a μ-less, essentially κ preparation)[11] by pretreatment with β-funaltrexamine (β-FNA, **4b**). The IC_{50} value of α-CNA on this preparation did not differ significantly from that on untreated preparations (Table I), suggesting that the agonist activity of α-CNA is wholly mediated by κ receptors. However, the *irreversible component* of the agonist activity was diminished in the μ-less preparation.

The possibility that α-CNA possesses irreversible *antagonist* activity was assessed by using incubation conditions that did not lead to full irreversible agonism. Thus, although treatment with 20 nM β-CNA produced a >90% inhibition of the ileal twitch, limiting the incubation time to 10 min allowed recovery of approximately one-half the original twitch response upon thorough washing. The ability of morphine and ethylketocine (EKC) to inhibit the remaining twitch was determined and compared to the control responses to the two agonists obtained prior to incubation with α-CNA. The post-α-CNA concentration–response curve of morphine was shifted to the right, and the maximal response was reduced (Figure 2). EKC agonism was also inhibited but to a lesser degree. However, when the incubation time was lengthened to 30 min by (using the β-FNA-treated GPI), α-CNA showed substantial antagonism of EKC (Table II). These results suggest that, in addition to its irreversible agonist activity,
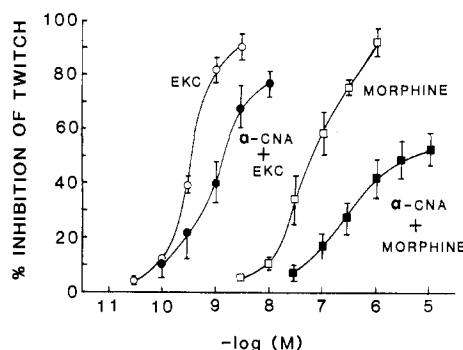
Figure 2. The antagonist activity of α-CNA on ethylketocine (EKC) and morphine in the GPI. After the control agonist concentration–response curves were determined, the preparations were incubated with 20 nM α-CNA for 10 min. The concentration–response curves were redetermined after thorough washing (20X) of the preparations. Values on the curve represent means plus or minus standard errors from three experiments.
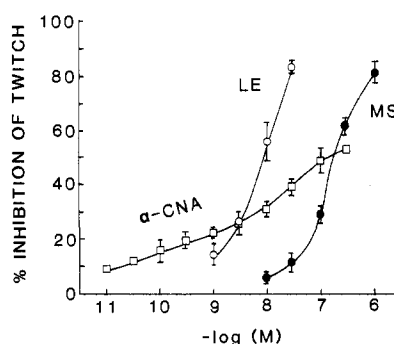

Figure 3. The agonist activity of Leu-enkephalin (LE), α-CNA, and morphine sulfate (MS) on the MVD. The mean IC_{50} ± SE of LE was 7.6 ± 1.2 nM and that of morphine sulfate was 139.5 ± 20.6 nM in four preparations. The vertical lines represent standard errors.

Table II. κ Antagonist Effect of α-CNA on the β-FNA-Treated GPI

treatment[a]	EKC IC_{50} ratio[b]
β-FNA	1.21 ± 0.21
β-FNA + α-CNA	10.14 ± 2.47

[a] GPI was incubated with 2×10^{-7} M β-FNA for 60 min, followed by thorough washing (see legend of Table I for the consequences of this treatment). α-CNA was employed at 2×10^{-8} M and incubated with GPI for 30 min, followed by thorough washing. Approximately 60% of the twitch returned after washing. [b] IC_{50} ratio = IC_{50} after treatment/IC_{50} before treatment.

α-CNA possesses a simultaneous irreversible antagonist activity that affects morphine (μ agonist) much more so than EKC (κ agonist).

In the mouse vas deferens preparation (MVD), α-CNA was found to be a potent but partial agonist, as compared to either morphine or Leu-enkephalin (Figure 3). This agonist activity was completely reversible under all conditions, in contrast to the behavior observed in the GPI. That α-CNA acts as a reversible agonist at δ receptors was confirmed by the finding that the agonism displayed in a special MVD preparation devoid of functional μ and κ receptors was not significantly altered. This "pure δ" MVD preparation[11] was obtained by alkylating opioid receptors with β-CNA in the presence of a protecting concentration of the highly selective δ-agonist DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr).[12]

(11) Ward, S. J.; Portoghese, P. S.; Takemori, A. E. *Eur. J. Pharmacol.* **1982**, *85*, 163.
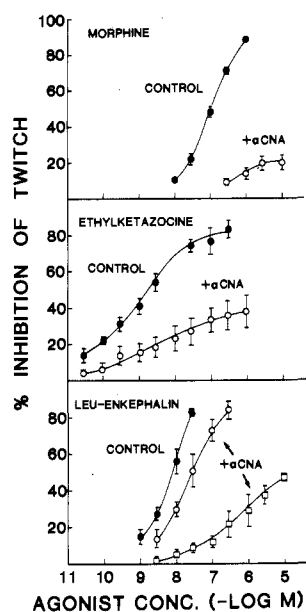
Figure 4. The antagonist activity of α-CNA on morphine, ethylketazocine, and Leu-enkephalin in the MVD. After the control agonist concentration–response curves were determined, the preparations were incubated with 20 nM α-CNA for 30 min. The concentration–response curves were redetermined after thorough washings (20X) of the preparations. The open squares in the lowest panel represent experiments in which 200 nM α-CNA was used. Values on the curves represent means plus or minus standard errors from three to five experiments.

The ability of α-CNA to effect irreversible antagonism of morphine, EKC, and Leu-enkephalin in the MVD was determined by obtaining concentration–response curves for these agents before and after treatment with α-CNA. The antagonist potency of α-CNA was highest against morphine, slightly less so against EKC, and weakest against Leu-enkephalin (Figure 4). In each case, flattening of the concentration–response curve was observed at the levels of α-CNA that were effective in producing a significant rightward shift of the curve. This indicates that the maximal responses of all three agonists are reduced by the irreversible actions of α-CNA. A nearly identical profile of irreversible antagonist activity in MVD was observed for β-CNA.[6] The irreversible antagonist activity of α-CNA in MVD is probably responsible for the flattening of its own agonist concentration–response curve in this tissue (Figure 3).

The pharmacological profile of α-CNA was also assessed in vivo. α-CNA was administered intracerebroventricularly (icv) to male Swiss–Webster mice (16–23 g) at doses of between 0.625 and 5.0 nmol/mouse. After 10 min, analgesia was measured according to the tail-flick assay. The observed dose–response relationship indicated an ED_{50} of 1.3 (1.0–1.6) nmol/mouse with toxic effects being observed at higher doses. The duration of analgesia was relatively short, lasting about 30 min. However, 24 h after the administration of α-CNA icv, substantial antagonism of morphine was observed when the mice were challenged with 10 mg/kg of morphine sulfate (only about 40% of the animals displayed analgesia after 1.25 nmol of α-CNA/mouse). Complete antagonism of the analgesic effect of the challenge dose of morphine was seen at the higher doses of α-CNA where toxic effects were observed (2.5 and 5.0 nmol/mouse).

Discussion

The covalent bonding of selective affinity labels to opioid receptors depends on two recognition processes.[13] The first is a manifestation of affinity and has been termed primary recognition. This is followed by a secondary recognition step that involves the proper juxtaposition of the electrophilic center of the ligand and a receptor nucleophile so that covalent bonding can occur. Primary recognition without secondary recognition affords a reversibly acting ligand, whereas secondary without primary recognition gives an indiscriminate alkylating agent.

Using this concept, we chose to study α-CNA (1a) and compare its irreversible activity with that of its C-6 epimer, β-CNA (1b). Since the aziridinium ion derived from the $N(CH_2CH_2Cl)_2$ group is very reactive and differs in its orientation in these molecules, we considered the possibility that α-CNA might alkylate receptor nucleophiles that are different from those that react with β-CNA.

Evidence consistent with this idea was obtained from studies on the GPI and MVD. The interaction of α-CNA with different opioid receptor subtypes (μ, κ, and δ) suggested that covalent bonding occurs in each case, as was observed previously for β-CNA. However, for β-CNA, covalent bonding was manifested entirely in irreversible antagonism of all agonists tested, and only a feeble or transient agonist activity could be detected (in both muscle preparations).[3–6] On the other hand, for α-CNA, in addition to the irreversible antagonism, there was displayed a potent (partial) agonist activity in MVD and an even more pronounced, as well as irreversible, agonist activity in GPI.

The receptor subtype in the GPI responsible for both the reversible and irreversible agonist activity of α-CNA is probably κ because the agonist response of α-CNA was preserved in the β-FNA-treated GPI. This conclusion is consistent with the observed lack of an irreversible agonist effect in MVD, since this tissue possesses much fewer (and perhaps dissimilar) κ receptors relative to the GPI.[14] In this case, since other experiments indicated the ability of α-CNA to block the effect of the κ-agonist EKC in the GPI, the irreversible activity of α-CNA at κ receptors appear to be of the mixed agonist–antagonist type. If this assignment is correct, it suggests that possibly two distinctly different interactions of α-CNA with κ opioid receptors lead to a mixture of covalently bound receptors that are either in an agonist or antagonist state. We postulate that the ratio of these two states determines the balance of agonism and antagonism exerted by α-CNA. This can result as a consequence of binding of α-CNA to two different (conformational) states of the κ receptor or two different modes of binding of α-CNA to a receptor in a single state.[15] The fact that the α and β epimers of CNA have their electrophilic moiety in a different orientation is a factor that is thought to influence the relative rates of alkylation of different nucleophiles in the agonist and antagonist states.

An alternative explanation for the irreversible agonist

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activity of α-CNA in the GPI can be given in light of recent studies[16] in our laboratories that suggest possible differences between μ receptors in GPI and those in MVD. The fact that the irreversible component of α-CNA agonism in GPI is diminished by β-FNA pretreatment suggests that this activity is μ related. The lack of observed irreversible activity in MVD would then require different types of μ receptors in the two tissues. If this assignment is correct, the activity of α-CNA at κ receptors would simply be reversible agonism and irreversible antagonism.

It might have been expected that a long-term agonism would have been seen in vivo to match the irreversible agonist effect observed in the GPI. This was not the case. Since α-CNA was administered icv, it is unlikely that the observed lack of long-term tail-flick antinociception is due to the inability of α-CNA to reach the central receptors. There are undoubtedly other reasons why irreversible agonism in vitro may not necessarily translate into long-term agonism in vivo. For example, β-COA (5), an agent

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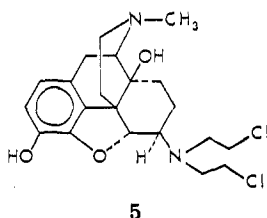
that exhibits irreversible (presumably μ) agonist activity in vitro,[4] does not display an unusually long-term agonism in vivo, and a much longer-lasting antagonist activity is observed after the agonism disappears.[7,8] Events that occur due to persistent drug–receptor interaction (e.g., tolerance) could be responsible for extinguishing agonist but not antagonist effects produced by virtue of receptor alkylation.

In summary, α-CNA, like β-CNA, appears to alkylate all opioid receptor subtypes. The two epimers are similar insofar as they both produce an irreversible antagonism at μ, κ, and δ receptors. However, α-CNA additionally exhibits a *reversible* agonist activity in GPI and MVD and an *irreversible* agonist activity in GPI. The unique, irreversible, mixed agonist–antagonist activity of α-CNA is consistent with covalent bonding to different opioid receptor subtypes in either agonist or antagonist modes.

Experimental Section

Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by MHW Laboratories, Phoenix, AZ. IR spectra were obtained on a Perkin-Elmer 281 infrared spectrometer. NMR spectra were taken at ambient temperature with tetramethylsilane as internal standard on either a Varian T-60 or A-60D instrument. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. All evaporations were performed at reduced pressure between 25 and 40 °C.

$N,N^{6\alpha}$-**Bis(2-hydroxyethyl)-6α-naltrexamine Dihydrochloride (2a).** α-Naltrexamine diacetate (0.925 g, 2 mmol), HOCH$_2$CHO (0.72 g, 12 mmol), and NaBH$_3$CN (0.314 g, 5 mmol) were stirred in 60 mL of MeOH over 3Å sieves under N$_2$ for 12 h. Complete reaction was indicated by total conversion of the amine to product 2a [R_f 0.5 (EtOAc–MeOH–NH$_4$OH, 80:20:2)]. The mixture was filtered, the solids were washed with MeOH, and the filtrate was concentrated and partitioned between dilute aqueous NH$_4$OH and CHCl$_3$. The combined organic extracts were evaporated, and the residue was diluted with MeOH and acidified to pH 1.5 with concentrated HCl. Evaporating, drying (EtOH–

benzene), and recrystallizing from MeOH–i-PrOH yielded 0.97 g of 2a (96%) in two crops: mp 195 °C; $[\alpha]^{25}_D$ −148° (c 1.1, MeOH); EIMS (20 eV), m/e 430 (M$^+$, 1.4), 399 (M$^+$ − CH$_2$OH, 51); NMR (deuterium exchanged free base in CDCl$_3$) δ 6.56–6.13 (2 d, 2 H, J = 7.5 Hz, Ar H), 4.57 (br s, 1 H, C-5 H). Anal. (C$_{24}$H$_{36}$N$_2$O$_5$Cl$_2$·CH$_3$OH) C, H, N.

$N,N^{6\alpha}$-**Bis(2-chloroethyl)-6α-naltrexamine Dihydrochloride (1a, α-CNA).** Compound 2a (0.60 g, 1.2 mmol) was stirred in 20 mL of CH$_3$CN over 3Å sieves for 20 min. Triphenylphosphine (1.58 g, 6 mmol) and 20 mL of CCl$_4$ were added, and the mixture was stirred for 15 h at 25 °C. Filtration, evaporation, resuspension in benzene (stirring for 10 h), and filtration afforded the crude product salt. The trituration/washing was repeated with EtOAc. The material at this point (0.60 g) was free of all but traces of contaminating Ph$_3$P and Ph$_3$P=O. Further purification was accomplished by loading the salt onto a silica gel column and *then* neutralizing with 2.5 equiv of Et$_3$N and eluting with EtOAc–CH$_3$CN (4:1) into collection tubes that contained HCl in THF. Yield after chromatography was 60%: mp 210 °C; $[\alpha]^{25}_D$ −141° (c 1.0, MeOH); R_f 0.49 (EtOAc–1% NH$_4$OH); EIMS (40–70 eV), m/e 466/468/470 (M$^+$), 417/419 (M$^+$ − CH$_2$Cl). Anal. (C$_{24}$H$_{34}$N$_2$O$_3$Cl$_4$) C, H, N.

Guinea Pig Ileal Longitudinal Muscle. Ilea from guinea pigs were taken approximately 10 cm from the ileocaecal junction, and a strip of longitudinal muscle with the myenteric plexus attached was prepared by the method of Rang.[17] A 1-cm portion of this strip was then mounted between two platinum electrodes, placed in a 10-mL organ bath, and connected to an isometric transducer, and contractions were recorded on a polygraph. Contractions of the ileal strip was initiated by supramaximal rectangular pulses in all preparations (80 V of 0.5-ms duration at a frequency of 0.1 Hz). Krebs bicarbonate solution containing 1.25 μM chlorpheniramine maleate was the bathing solution and was continuously bubbled with 95% O$_2$ and 5% CO$_2$. The organ bath was maintained at 36–37 °C. The longitudinal muscle strip was allowed to equilibrate with continuous stimulation for a minimum of 90 min. Drugs were added to the bath in 10- to 5-μL amounts and washed out usually with two 10-mL portions of buffer after noting their maximum effects. Log concentration-response curves were constructed, and IC$_{50}$ values were determined by the method of Finney.[18]

Mouse Vas Deferens. This assay was performed according to the description by Henderson et al.[19] Both vasa deferentia were dissected out of mice and mounted between two platinum electrodes in a 10-mL organ bath. The bath contained Krebs bicarbonate solution that was continuously bubbled with 95% O$_2$ and 5% CO$_2$. The organ bath was maintained at 37 °C. The tissue was attached to an isometric transducer and stimulated transmurally with rectangular pulses (0.1 Mz, 1-ms duration, supramaximal voltage). Drugs were added to the bath in 10- to 50-μL amounts and washed out after noting their maximum effect.

Analgesia. The tail-flick assay of D'Amour and Smith,[20] which was modified[21] for mice, was used to assess analgesic potency as well as antagonism of morphine analgesia. At least 24 animals were used to determine each dose–response curve and ED$_{50}$ values. α-CNA was injected icv (4 μL/mouse). The data were analyzed by the method of Litchfield and Wilcoxon.[22]

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