other mRNA sequences in these tissues.

In conclusion, we have used pure molecular hybridization probes for ovalbumin mRNA and intervening sequence RNA to measure their concentration in the nucleus and cytoplasm of the hormone-stimulated oviduct cell. We found that, unlike mRNA transcripts of the structural sequences of the avian ovalbumin gene, nuclear intervening sequence RNA was preferentially turned over and not transported efficiently to the polysomes.

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### References and Notes

1. Intervening sequences in eukaryotic DNA interrupt the structural DNA sequences coding for mRNA. Intervening sequences were first observed by Leder, Tonegawa, and other workers [see (*8, 9*)] in globin and immunoglobulin systems and were subsequently observed in several other systems (*8–11*).
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## Chloroxymorphamine, an Opioid Receptor Site–Directed Alkylating Agent Having Narcotic Agonist Activity

Abstract. *Chloroxymorphamine, the 6β-N,N-bis(2-chloroethyl) derivative of oxymorphone, is a potent nonequilibrium narcotic agonist in the longitudinal muscle preparation of guinea pig ileum. The corresponding naltrexone analog, chlornaltrexamine, is a potent nonequilibrium antagonist of morphine. These receptor site–directed alkylating agents possess considerable potential as pharmacologic and biochemical probes of opioid receptors.*

The development of highly selective opioid receptor site–directed alkylating agents that act both in vivo and in vitro should contribute significantly to the armamentarium of compounds that are employed as investigational tools. We reported (*1*) on an alkylating analog of naltrexone, chlornaltrexamine (CNA) (Fig. 1), which affords long-lasting narcotic antagonism in mice and exhibits avid stereospecific binding in vitro. We now report on a second compound, chloroxymorphamine (COA) (Fig. 1), whose interaction with opioid receptors leads to sustained agonism. We also report on experiments that further demonstrate the nonequilibrium nature and specificity of CNA.

Since CNA is a potent antagonist, we postulated that an identical modification of oxymorphone (Fig. 1) (*2*), a potent analgesic, might afford an affinity labeling agent, COA, with agonist properties. The synthesis of COA, which is similar to that for CNA (*1*), involves $NaCNBH_3$, reduction of oxymorphone in the presence of diethanolamine, followed by treatment of the bis(hydroxyethyl)amino intermediate with $CCl_4$-$Ph_3P$. After washing with hot ethyl acetate, the crude COA was purified on a dry silica-gel column and eluted with ether-methanol-am-

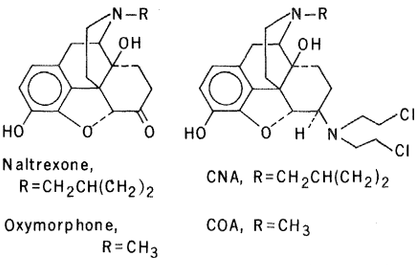


Fig. 1. The structures of oxymorphone, chloroxymorphamine (COA), naltrexone, and chlornaltrexamine (CNA).

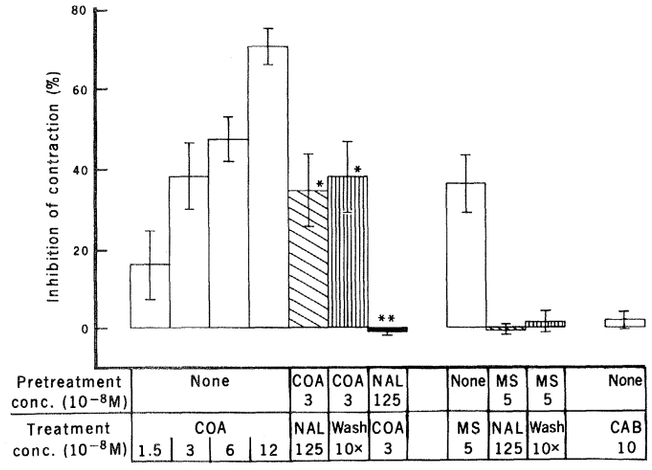


Fig. 2. The effect of chloroxymorphamine on the coaxially stimulated longitudinal muscle of guinea pig ileum. The muscle was prepared by the method of Rang (*4*). A strip of longitudinal muscle was removed with myenteric plexus intact from a segment of the guinea pig ileum. A 1- to 2-cm portion of this muscle was suspended between two platinum electrodes in a 10-ml bath containing Krebs-Ringer bicarbonate solution with 1.25 $\mu M$ chlorpheniramine maleate. The organ bath was bubbled with a mixture of 95 percent $O_2$ and 5 percent $CO_2$ while being maintained at 36° to 37°C. Contractions in the muscle strip were initiated by supramaximal rectangular pulses (80 V, 0.5-msec duration, 0.1 Hz) and recorded by an isometric transducer connected to one end of the tissue. After equilibration the ileal muscles were exposed to either chlorambucil (*CAB*), morphine sulfate (*MS*), or chloroxymorphamine (*COA*) for 15 minutes or naloxone (*NAL*) for 5 minutes. Open bars represent percentage of inhibition of muscle twitch after exposure to these drugs. A computer program was used to analyze the results (*8*). Diagonally hatched bars represent percentage of inhibition remaining after subsequent treatment with naloxone. Vertically hatched bars represent percentage of inhibition remaining after the treated preparations were washed ten times with Krebs-Ringer bicarbonate solution. The solid bar represents percentage of inhibition observed 15 minutes after COA treatment of muscle strips that had been treated with naloxone. Each value given is the mean ± standard error of three to seven experiments; *, significantly different ($P < .05$) from similarly treated morphine control; **, significantly different ($P < .05$) from nonpretreated control.


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monia (90:10:2). The COA was converted to its dihydrochloride salt with the following properties: melting point > 285°C; $R_F$ = 0.65 on silica gel in ethyl acetate–ammonium hydroxide (100:1); electron ionization mass spectrometry *m/e* 426 ($M^+ - 2HCl$), 428 ($M^+ + 2 - 2HCl$). The configuration is assigned to the C-6 center in COA on the basis of the nuclear magnetic resonance coupling constant (*1*, *3*) ($J_{56}$ = 7.5 Hz) of the C-6 proton. As we suspected, COA, as indicated by initial studies in vivo, is a long-acting narcotic agonist. Our work with the coaxially stimulated longitudinal muscle preparation of guinea pig ileum indicates that COA has characteristics of a narcotic agonist the effects of which are blocked but not reversed by naloxone or washing. We describe these experiments here as evidence that COA, like CNA, binds specifically and irreversibly to opioid receptors.

Chloroxymorphamine inhibited contractions in coaxially stimulated longitudinal muscle strip of guinea pig ileum (*4*) in a concentration-dependent fashion with concentrations between $1.5 \times 10^{-8}$ to $1.2 \times 10^{-7}M$ (Fig. 2). The $IC_{50}$ (concentration of drug which inhibits contractions by 50 percent) of COA was $5.4 \times 10^{-8}M$, which was not significantly different ($P < .05$) from the $IC_{50}$ of morphine ($6.2 \times 10^{-8}M$). The depressant effect of COA on contractions of electrically stimulated ileum could not be reversed by the addition of naloxone or by ten washes. The 38 percent inhibition of contraction produced by $3 \times 10^{-8}M$ COA was essentially unchanged by the addition of $1.25 \times 10^{-6}M$ naloxone, whereas complete reversal of the inhibition of an equally effective concentration of morphine was observed after addition of the same amount of naloxone. Washing the muscle strips ten times after treatment with this concentration of morphine also completely reversed the inhibition of contraction, but the effect of COA persisted after washing.

The agonistic effect of $3 \times 10^{-8}M$ COA could be completely blocked by treating the muscle strips with $1.25 \times 10^{-6}M$ naloxone before testing with COA. As further evidence of the specificity of the effect of COA on the muscle strip, the nitrogen mustard chlorambucil had no effect on the contractions even at a concentration as high as $1 \times 10^{-7}M$.

We also investigated the possibility of an antagonistic action of COA. Treatment of muscle strips with $5 \times 10^{-8}M$ morphine or with $1.5 \times 10^{-8}M$ COA for 15 minutes followed by ten washes had no effect on the subsequent responses to morphine. Higher concentrations of COA could not be used because of irreversible inhibition of contraction.

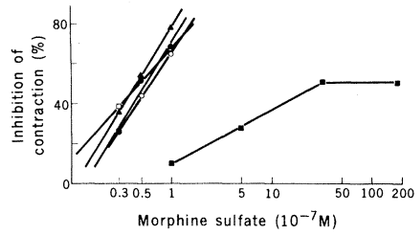


Fig. 3. Inhibition of morphine action on electrically stimulated longitudinal muscle of guinea pig ileum by chlornaltrexamine and the blockade of this effect by naloxone. By the methods described for Fig. 2, the response of the longitudinal muscle to morphine was determined before treatment (●, control). After a 15-minute incubation with either $5 \times 10^{-8}M$ naltrexone (▲), $2 \times 10^{-8}M$ CNA (■), $1 \times 10^{-7}M$ chlorambucil (○), or $2 \times 10^{-8}M$ CNA and $1.25 \times 10^{-6}M$ naloxone (□), the preparations were washed 20 times with Krebs-Ringer bicarbonate solution. Subsequent responses to morphine were recorded. Each point represents the average of values from at least three preparations.

Initial studies show that naloxone can block the analgesic effects of COA in vivo in the tail-flick assay. Analgesia induced by COA apparently persists in mice four times longer than morphine-induced analgesia after an intracerebroventricular injection of equally effective doses. We had expected the duration of the COA effect to be even longer because of the long-lasting effects produced by CNA in vivo and the irreversible effects of COA seen on ileal muscle strips. This shorter duration of action relative to CNA may be caused by the development of acute tolerance in mice treated with COA which could mask the continued expression of the agonistic effects of COA.

Treatment of muscle strips with CNA produced a transient inhibition of contraction. This effect correlates with the initial analgesia in mice given an intracerebroventricular injection of CNA which could not be attributed to a nonspecific effect of the alkylating agent (*1*).

When muscle strips were washed 20 times after a 15-minute incubation with $2 \times 10^{-8}M$ CNA, their response to morphine was greatly attenuated (Fig. 3). In contrast, muscle strips treated similarly with $5 \times 10^{-8}M$ naltrexone were as responsive to morphine as were untreated control strips. In addition to decreased sensitivity to morphine, the maximum inhibition of contraction attainable (100 percent) was decreased to 50 percent by CNA treatment. Further, the morphine concentration-response curve after CNA treatment was significantly nonparallel to curves of naltrexone-treated and untreated controls. A similar pattern of effects has been demonstrated for other known receptor alkylating agents such as phenoxybenzamine and dibenamine (*5*). These results suggest that CNA antagonizes morphine in a nonequilibrium fashion characteristic of receptor-specific alkylating agents.

Like antagonism of morphine-induced analgesia by CNA, this antagonism of morphine action on the ileal muscle strips reflects a specific action of the alkylating agent. Treatment of the strip with $1.25 \times 10^{-6}M$ naloxone completely blocks the antagonistic action of CNA (Fig. 2). Also, treatment of strips with $1 \times 10^{-7}M$ chlorambucil, a nonspecific alkylator, has no effect on the morphine concentration-response curve. Phenoxybenzamine, an $\alpha$-adrenergic receptor alkylating agent, did not antagonize the morphine response at a concentration of $1 \times 10^{-7}M$ (Fig. 4).

The possibility that CNA might antagonize the effects of other ileal muscle depressants prompted us to study the effects of CNA and of phenoxybenzamine on the response of the muscle to norepinephrine (Fig. 4). The norepinephrine $IC_{50}$ of muscle strips after 20 washes was $9.2 \times 10^{-8}M$. When the strip was treated for 15 minutes with phenoxybenzamine

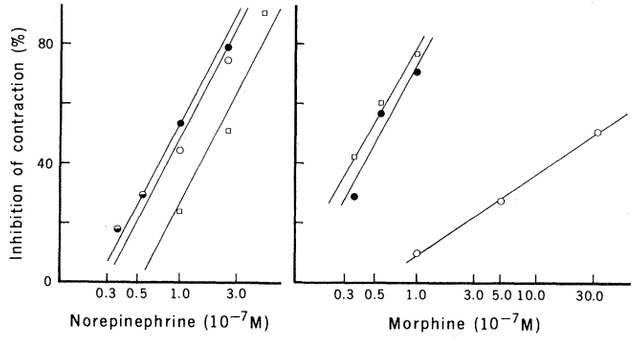


Fig. 4. The effect of chlornaltrexamine or phenoxybenzamine on the response of the coaxially stimulated longitudinal muscle of guinea pig ileum to either norepinephrine or morphine. Guinea pig muscle strips were either not treated (●) or treated with $2 \times 10^{-7}M$ chlornaltrexamine (○) or $1 \times 10^{-7}M$ phenoxybenzamine (□) for 15 minutes and then washed 20 times with Krebs-Ringer bicarbonate solution. Each muscle strip was then used to determine the $IC_{50}$ of either morphine or norepinephrine. Each point represents the average of at least three experiments.

before washing, the $IC_{50}$ increased significantly ($P < .05$) to $19.7 \times 10^{-8}M$, which is reflected in Fig. 4 as a shift of the concentration-response curve to the right. Gyang and Kosterlitz (*6*) have reported similar results after phenoxybenzamine treatment of guinea pig ileum. However, a 15-minute incubation with $2 \times 10^{-8}M$ CNA had no effect on the norepinephrine $IC_{50}$. After these same treatments, opposite results were seen in the responsiveness of muscle strips to morphine; phenoxybenzamine treatment had no effect but CNA treatment significantly inhibited the morphine response. These results indicate that CNA selectively inhibits the depressant effects of morphine on the ileal muscle strips but does not affect the norepinephrine depression, which is not mediated by opiate receptors.

Our studies suggest that the sustained effects of COA and CNA are due to receptor alkylation. The ability of naloxone to block these effects and the absence of similar effects with phenoxybenzamine or chlorambucil demonstrate the specificity of action of COA and CNA. Moreover, the ability of both COA and CNA to act in a nonequilibrium fashion indicates that the aziridinium intermediates derived from these ligands are alkylating a nucleophilic group that is located in an identical or similar receptor environment during the equilibrium phase of binding. Thus we expect these compounds will be of value as pharmacologic and biochemical probes of opioid receptors.

Finally, the nonequilibrium behavior of COA is particularly relevant to the mode of interaction of narcotic agonists with receptors, since this indicates that receptor occupation rather than the rate of ligand-receptor association (*7*) is important for agonist activity.

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8. A computer program written for the parallel line assay [D. J. Finney, *Statistical Methods in Biological Assay* (Hafner, New York, 1964)] was used to analyze the data from at least three different preparations and to generate a concentration-response curve with three or four concentrations of drug. The program was also used to statistically compare two or more curves and to generate $IC_{50}$'s with a 95 percent confidence interval. These values were also used as a statistical comparison for concentration-response curves.
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## Evolutionary Relatedness of Viper and Primate Endogenous Retroviruses

Abstract. *A retrovirus previously isolated from a tumored Russell's viper is shown by molecular hybridization to be an endogenous virus of this reptilian species. Radioimmunologic techniques revealed that the viper retrovirus is immunologically and, hence, evolutionarily related to endogenous type D retroviruses of Old World primates. These findings extend the number of vertebrate classes possessing endogenous retroviruses and suggest that type D retroviruses may even be more widely distributed in nature than type C retroviruses.*

Retroviruses have been isolated from species representing several classes of vertebrates including mammals, birds, and reptiles. In many instances, it has been possible to show that retroviral genomes are endogenous to their species of origin. Moreover, the evidence indicates that at least some endogenous viruses have been genetically transmitted within a vertebrate family for many millions of years (*1, 2*). One major group of endogenous retroviruses of mammals includes type C virus isolates from species as diverse as rodents and primates. These viruses have been shown to share antigenic determinants among each of several of their respective translational products (*3*). More recently, morphologically distinguishable viruses, designated type D retroviruses, have been isolated from species of New and Old World primates (*4*). Evidence of the immunologic relatedness of their major structural proteins has implied that these viruses constitute a distinct group of evolutionarily related retroviruses (*5*).

The only known retrovirus of reptilian origin was isolated from spleen tissue of a tumor-bearing Russell's viper (*Vipera russellii*) (*6*). Initial evidence indicated that the viper retrovirus (VRV) morphologically resembled type C viruses. Yet, VRV was found to be biochemically and immunologically distinguishable from a number of mammalian and avian retroviruses (*7*). We have examined the origin of VRV and determined its relation to retroviruses of other vertebrate species. Viper retrovirus is shown to be genetically transmitted within the viper. Moreover, the demonstration of immunologic relatedness of the viper retrovirus to endogenous type D retroviruses of Old World primates establishes the widespread distribution of this virus group among vertebrates.

Retroviruses that are transmitted within the germ line of a species exhibit extensive nucleotide sequence homology with cellular DNA of their species of origin. To investigate whether or not VRV was endogenous to viper cells, a complementary DNA (cDNA) probe was synthesized in an endogenous reaction with the use of a sucrose gradient-purified VRV. In order to demonstrate that probe sequences were virus-specific, attempts were made to propagate the virus in cells unrelated to Russell's viper. Several mammalian and reptilian cell lines were tested, but VRV grew only in a cell line established from a timber rattlesnake (*8*). As is shown in Fig. 1A, VRV cDNA was hybridized to an extent of more than 70 percent by cellular DNA of rattlesnake cells chronically infected with VRV. In contrast, there was only about 15 percent hybridization with the DNA of uninfected rattlesnake cells. These results demonstrated that the VRV cDNA probe was composed substantially of VRV-specific nucleotide sequences.

In order to ascertain the representation of VRV sequences within viper cellular DNA, the VRV cDNA probe was tested for its ability to hybridize with normal tissue DNA of *V. russellii*. The maximum extent of annealing achieved was 70 percent with half-maximal hybridization of the cDNA probe occurring at a $C_0t$ of 85 (Fig. 1B). These findings demonstrated that the VRV was endogenous to *V. russellii* cells. By comparison with the $C_0t_{1/2}$ for unique sequence *V. russellii* DNA (1500), there were multiple


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