Opiate Receptor Function May Be Modulated Through an Oxidation-Reduction Mechanism

Abstract. *Cupric ion, a thiol oxidant, caused naloxone-reversible analgesia when injected intracerebroventricularly in mice; its potency was close to that of morphine. Dithiothreitol, a thiol reductant, reversed the analgesia induced by cupric ion and antagonized analgesia induced by morphine. Oxidized dithiothreitol had no effect. These findings, together with evidence for redox modification of opiate receptor binding in vitro, suggest that a mechanism of oxidation-reduction of thiols may modulate opiate receptor function.*

Since the discovery of endogenous opioid peptides, numerous studies have demonstrated the importance of the levels of these peptides in the control of nociperception (*1*). Unlike the case for the more classical putative neurotransmitters (such as the catecholamines), there has been little evidence that changes in the sensitivity of opiate receptors also contribute to the regulation of nociperception. This report presents evidence indicating that the responsiveness of opiate receptors may be modified in vitro and in vivo through changes in oxidation-reduction state. We suggest that this may represent a physiological mechanism for modulating nociperception.

A nonendorphic, heat-stable, peptide-containing inhibitor of opiate receptor binding was extracted from rat brain in our laboratory (*2*) and identified as a complex of oxidized glutathione and cupric ion (*3*). The Cu^{2+} accounted entirely for the inhibition of opiate binding; the peptide was simply a carrier, binding this metal with high affinity. Since other transition cations also inhibit opiate binding with a potency rank order that is related to their affinity for protein SH groups, we suggested that inhibition by Cu^{2+} occurs through the oxidation of essential thiol groups, which are known to be present at opiate receptor sites (*4*).

In this report we describe further experiments demonstrating the reversibility by reducing agents of the inhibitory effects of Cu^{2+} in vitro and showing the close dependence of opiate receptor binding on the oxidation-reduction potential prevailing in the incubation medium. We also describe results showing that Cu^{2+}, when administered intracerebroventricularly (ICV) in mice, induces analgesia that is reversible with naloxone and dithiothreitol (DTT), and that administration of reduced DTT, but not oxidized DTT, antagonizes analgesia induced by both morphine and Cu^{2+}.

Figure 1 shows the results of an experiment in which binding of opiates to rat brain membranes was first totally inactivated by incubation of the membranes with 90 μM Cu^{2+}. After extensive washing of the metal, the membranes were incubated with various reducing agents and then assayed for opiate binding with [^{3}H]etorphine. Whereas Cu^{2+}-treated membranes that had first been incubated without reducing agents were totally inactive, prior incubation with DTT, 2-mercaptoethanol, reduced glutathione, or cysteine restored the binding activity of the membranes to various degrees. The ability of thiol-containing reagents to reactivate Cu^{2+}-inhibited receptors is clearly related to their negative redox potential (E_0') (*5*). Dithiothreitol ($E_0' = -0.33$) was most effective, and cysteine ($E_0' = -0.22$) was weakest. Penicillamine, a strong chelator of copper, has a poor E_0' (-0.18) and failed to reactivate the receptors. Interestingly, DTT caused stimulation of binding significantly above control levels in both normal and Cu^{2+}-treated membranes.

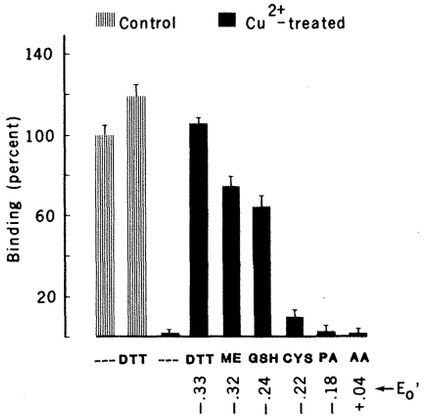

Fig. 1. Reversal of Cu^{2+} inactivation of opiate receptor binding by thiol reductants. Abbreviations: *DTT*, dithiothreitol; *GSH*, reduced glutathione; *PA*, penicillamine; *ME*, 2-mercaptoethanol; *CYS*, cysteine; *AA*, ascorbic acid. Brains (less cerebellums) were homogenized in 100 volumes of 0.05*M* tris (*p*H 7.4) and centrifuged at 13,000*g* for 15 minutes. After being resuspended in 10 volumes of tris, membrane samples were incubated with or without 90 μM $CuCl_2$ for 10 minutes at 36°C, diluted to 100 volumes with cold tris, and centrifuged at 13,000*g* for 10 minutes. After washing again with 100 volumes of tris, the membranes were suspended in 10 volumes of tris and incubated for 20 minutes in the absence or presence of one of the indicated SH reagents (all at 3 m*M*). Fifty-microliter samples of these membranes were assayed for [^{3}H]etorphine binding with 0.1 μM dextrorphan or levorphanol and 1 n*M* [^{3}H]etorphine (specific activity, 35 Ci/mmole). Data are mean percentages of control values ± standard errors. Control incubations gave 4704 ± 197 count/min with dextrorphan and 1160 ± 52 count/min with levorphanol, resulting in 38.1 fmole of etorphine bound per milligram of brain tissue.

We next investigated the possible effects of Cu^{2+} in vivo. Cupric chloride was administered ICV to male Swiss-Webster mice (15 to 20 g); nociperception was assessed by measuring the latency of the tail-flick response to a heat stimulus (*6*). These measurements indicated that marked analgesia occurred. Analgesia was observed soon after the injection, and a dose as low as 3 nmole was effective. At low doses, analgesia disappeared rapidly; at 22 nmole, it lasted at least 1 hour (Fig. 2A). The median effective dose (ED_{50}) for Cu^{2+}-induced analgesia in control animals was 4.8 nmole (Fig. 2B), with 95 percent confidence limits of 2.7 to 9.0 (*6*). The analgesic effects were nearly the same as those of morphine sulfate, which had an ED_{50} of 3.5 nmole when administered ICV under the same conditions. However, it must be noted that the sensitivity of mice to Cu^{2+}-induced analgesia varied considerably with animal supplier and time of day (*7*). Since peak sensitivity occurred in the afternoon, all tests were carried out during that period with a carefully staggered order of control and treated animals.

Subcutaneous pretreatment of mice with naloxone reduced the degree and duration of the analgesia (Fig. 2A) and resulted in a parallel shift of the dose-response curve from the control ED_{50} of 4.8 nmole to one of 30.2 nmole (confidence limits, 18 to 48) (Fig. 2B). As Fig. 3A shows, naloxone not only antagonized the analgesic effects of Cu^{2+}, it reversed the effects when administered ICV after injection of the cation.

Figure 3A also shows that post-treatment with low intracerebroventricular doses of DTT resulted in a faster loss of Cu^{2+}-induced analgesia than post-treatment with saline. Dithiothreitol antagonized not only Cu^{2+}-induced analgesia, but also analgesia induced by morphine sulfate. Intracerebroventricular doses of DTT as low as 25 nmole caused a parallel shift of the morphine sulfate dose-response curve and raised the ED_{50} from 6.5 mg/kg (confidence limits, 5.3 to 8.1) in saline-pretreated animals to 11 mg/kg (confidence limits, 8.9 to 13.0) in animals first treated with DTT. This ef-

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fect is related to the reducing activity of DTT, since *trans*-4,5-dihydroxy-1,2-dithiane, the oxidized form of DTT, had no effect.

We also administered mice $ZnCl_2$ ICV, and found that 40 nmole was the ED_{50} for analgesic effects (*7*). The potency difference between Zn^{2+} and Cu^{2+} in vivo is consistent with previous results in vitro (*3*) showing that Zn^{2+} affects opiate binding at a level about one-tenth that of Cu^{2+}. On the other hand, it is of interest that La^{3+} and Ba^{2+}, which were reported to cause analgesia after central administration in mice (*8*), did not inhibit [^{3}H]etorphine binding in our test in vitro even at a concentration of 1 m*M* (*7*). Thus La^{3+} and Ba^{2+}, which have low oxidation potentials and little binding affinity for thiols, appear to act by a mechanism different from that of Cu^{2+}.

The ability of Cu^{2+} and other transition cations to inhibit opiate receptor binding in vitro paralleled the potencies with which these cations bind to protein SH groups (*3*). The affinity of this binding is related to the oxidation potential of the cations, and the reaction involves an oxidation-reduction (*9*). In agreement with such a mechanism, our experiments showed that the inhibitory effects of Cu^{2+} on opiate binding are reversed by thiol reagents at levels that are correlated with their reduction potentials, DTT being the most active. As indicated above (*9*) and as expected from other studies (*10*), the redox reaction between Cu^{2+} and receptor thiols may result in binding of the metal into stable (di)thiol-copper complexes rather than in the formation of disulfide and liberation of the reduced metal. However, our experiments did not distinguish between these possibilities. Since DTT enhances the binding of opiates to untreated membranes (Fig. 1), it is possible that opiate receptors are not fully reduced in their physiological state and thus are not optimally active. This possibility is also suggested by the fact that, while a highly reducing environment is maintained intracellularly by the glutathione redox system, a more oxidizing milieu (which favors disulfide formation) prevails extracellularly (*11*), where opiate receptors are believed to occur. A disulfide-sulfhydryl equilibrium condition would allow for a mechanism of modification of receptor binding tuned to the status of the redox buffers in the system.

Our investigation of possible effects of cupric ion on nociperception was further stimulated by the realization that the concentration of copper in rat brain (17 μmole/kg) (*12*) is of the same order as the median effective concentration for inhibition of opiate binding by Cu^{2+} in vitro (12 μ*M*) (*13*).

Our finding that Cu^{2+} has potent analgesic effects that are reversed by DTT supports the oxidation-reduction hypothesis for modulation of opiate receptor function. The evidence that Cu^{2+}-induced analgesia is antagonized and reversed by naloxone and that low doses of reduced DTT, but not oxidized DTT, can antagonize the analgesic effects of morphine indicates that these oxidation and reduction effects on nociperception reflect changes at opiate receptor sites. The competitive effects of naloxone on Cu^{2+}-induced analgesia and of DTT on morphine analgesia are not surprising, since a competitive interaction between opiates and thiol reagents at receptor binding sites were reported (*14*).

Since Cu^{2+} has agonistlike pharmacologic effects in vivo, but inhibits rather than facilitates the binding of opiate agonists and opioid peptides in vitro, it appears that Cu^{2+} itself may act as an agonist at the receptor site (*15*). Since details of the reaction between Cu^{2+} and receptor thiols have not been studied, the mechanism of this action is unknown. However, we can suggest an explanation by using an analogy from studies of the effects of sulfhydryl agents on the acetylcholine receptor (which contains disulfhydryl groups). It had formerly been thought that modifications by DTT of the redox state of disulfides in the electroplax and in motor end plates causes a simple inactivation of the binding sites. However, such modifications were later found to produce complex changes in the conformation of the receptor, resulting in an antagonist becoming an agonist or in an agonist becoming a more potent agonist (*16*). Similar com-

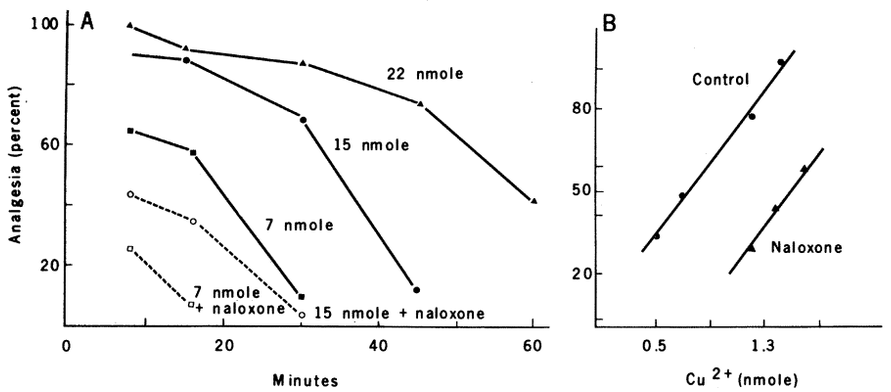

Fig. 2. Analgesia induced by Cu^{2+} and antagonism of this effect by naloxone. (A) Duration of analgesia after intracerebroventricular administration of various doses of $CuCl_2$ to mice previously injected with saline (solid line) or naloxone (dashed line) (*6*). (B) Log-probit plot of dose-response curves for Cu^{2+} analgesia in control and naloxone-treated mice (*6*).

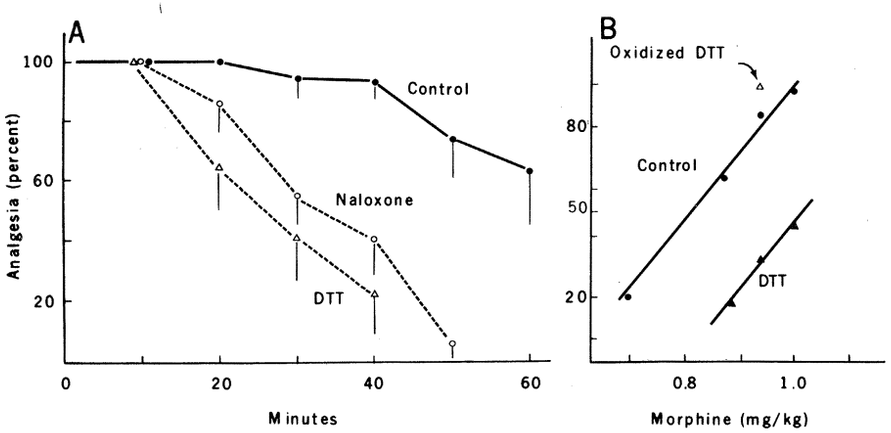

Fig. 3. Reversal of Cu^{2+} analgesia by DTT and by naloxone, and antagonism of morphine analgesia by DTT. (A) Ten minutes after injection of 37 nmole of $CuCl_2$, mice received a second injection of saline (5 μl), DTT (10 nmole), or naloxone (5 nmole). Both injections were made into the same brain site. Intervals indicate elapsed time between the second treatment and tail-flicking testing. (B) Soon after receiving an intracerebroventricular injection of saline (5 μl), DTT (25 nmole), or oxidized DTT (25 nmole; Sigma), mice were injected subcutaneously with various doses of morphine sulfate. Analgesia was tested after 30 minutes, and the results were plotted on log-probit paper (*6*). Oxidized DTT, tested with one dose of morphine sulfate, did not significantly alter the degree of analgesia (control, $N = 10$; oxidized DDT, $N = 8$), although there was a tendency toward a lowered ED_{50} for morphine.

plex changes in the conformation of the opiate receptor may result from the redox actions of Cu^{2+} and DTT, and the effects of these agents on nociperception may be due to alterations in receptor efficacy resulting from such conformational changes.

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The Fetal Sound Environment of Sheep

Abstract. *Hydrophones implanted inside the intact amniotic sac recorded sounds available to fetal lambs. Unlike recordings made from outside the intact amnion in human subjects, sounds produced at levels similar to normal conversation from outside the ewe were picked up without masking by maternal cardiovascular sounds. Noises from inside the mother were intermittent and linked to her activity.*

In contrast to the rich and varied sound experience of birds before hatching (*1*), the predominating sounds available to the mammalian fetus are thought to come from the maternal cardiovascular system (*2-6*). These sounds are reported to be loud, 68 to 95 dB (*2, 3, 6, 7*); of low frequency, 20 to 700 Hz (*3, 4, 6*); in time with the maternal pulse (*2-4, 6*); and to provide a basis for prenatal conditioning (*5*). There is thought to be considerable attenuation of sounds arising from outside the mother, such attenuation varying from 19 to 90 dB (*2, 3, 6*). These results imply that fetal experience of sound is limited.

In one previous experiment (*7*), recordings were made from a hydrophone sutured to the outside of the uterine wall of a goat; most observations, however, have been made by inserting microphones into the uterine cavity (*3, 4, 6*) or against the cervix (*2*) of pregnant human subjects at term, before or after rupture of the fetal membranes, but never inside the intact amniotic sac. Therefore, sounds available to the fetus within the intact amnion have not, to our knowledge, been observed.

Through the use of hydrophones inside the amniotic sac of pregnant ewes, in the normal fluid environment of the fetus, we have found that the sounds of the mother's eating, drinking, rumination, breathing, and muscular movement were discernible, as also were sounds from outside the mother; external sounds were attenuated by 30 dB on average. Sounds from the maternal cardiovascular system were not perceptible, however.

Recordings were made within the amniotic sacs of two pregnant ewes. A hydrophone (Celesco LC-10) was sutured to the neck of the fetus on about day 120 of gestation. The ewes recovered rapidly from the implantation, behaved normally, and fed well. Neither animal had been shorn, although each had been shaved over the abdominal area before the operation.

For sounds generated from outside the ewe, attenuation was measured in two ways. First, a 100-mm loudspeaker was strapped to the shaven area of the ewe's flank, but separated from the skin by an annulus of foam rubber. The loudspeaker relayed patterned sound (bleats) from a tape recorder, and the sounds were then picked up by the implanted hydrophone and fed directly to a narrow band spectrum analyzer (Brüel and Kjaer No. 2031). The sound level outside the animal was measured with a probe microphone (Sennheiser), the tip of which was inserted between the loudspeaker and the skin. The output of this microphone was also fed directly to the spectrum analyzer. As the hydrophone and microphone systems were of equal sensitivity, attenuation could be measured by comparing the level of corresponding peaks from the record.

According to a second method, pure tones from a function generator were amplified and relayed by a loudspeaker about $1\frac{1}{2}$ m from and facing the sheep. The sound level outside the sheep's flank was measured by a sound level meter (Dawe) through the use of the "C" weighting. This level was then compared with that registered by the spectrum analyzer from the implanted hydrophone.

These two methods gave similar results (Fig. 1). Attenuation reached a maximum of 37 dB just below 1 kHz, but

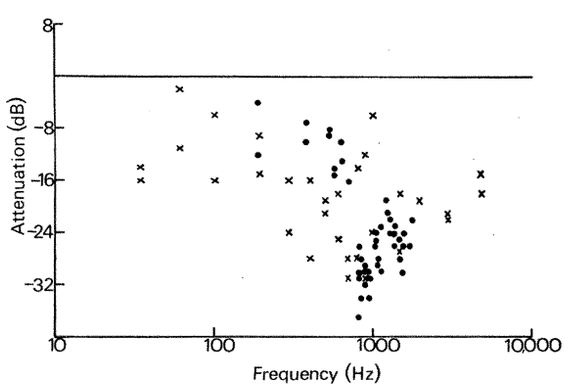

Fig. 1. Attentuation of sounds from outside the ewe. ×, Pure tones; ●, bleats.

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