acetylcholine concentration of approximately 0.3 m*M* (*22*). Simultaneous or successive release of multiple quanta is likely to result in even larger concentrations of the hydrolyzed product choline, although neuronal choline uptake and diffusion away from the neuromuscular junction cause uncertainty as to the maximum choline concentrations actually attained. Similar local concentrations of choline may occur at other nicotinic cholinergic synapses. Thus, choline concentrations comparable to those used in the present experiments may occur physiologically and exert effects because of choline's activity as a partial nicotinic agonist.

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13. Mecamylamine can inhibit processes other than nicotinic receptor activation [G. M. Lees and S. Nishi, *Br. J. Pharmacol.* **46**, 78 (1972)]. In chromaffin cell cultures 1 μ*M* mecamylamine inhibited carbachol- or acetylcholine-induced secretion but had no effect on secretion caused by solutions containing 56 m*M* K^+ (Na^+ concentration was reduced to 92 m*M* to maintain tonicity). Hence it is likely that the inhibitory effect of mecamylamine on the action of cholinergic agonists was caused by blockade of nicotinic receptors and not by blockade of other processes involved in secretion.
14. Choline acetyltransferase was measured by the method of F. Fonnum [*J. Neurochem.* **24**, 407 (1975)]. Based on the sensitivity of the assay, the maximal amount of acetylcholine that could have been synthesized would have resulted in a maximal acetylcholine concentration of 0.5 μ*M* in the cultures. This would not account for the secretion observed with choline.
15. Maximal catecholamine secretion is induced by 0.1 m*M* acetylcholine. Concentrations of acetylcholine as high as 1 m*M* do not inhibit this secretion. Hence, acetylcholine synthesized in the cultures would not be expected to inhibit the response to 30 μ*M* acetylcholine.
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Multiple Opiate Receptors: Alcohol Selectively Inhibits Binding to Delta Receptors

Abstract. *The addition of ethanol or other aliphatic alcohols to rat brain membranes strongly inhibits binding of enkephalins at concentrations at which little inhibition of opiate alkaloids is seen. Inhibition is reversible, and potency increases with chain length of the alcohol. The results suggest that* δ *receptors are considerably more sensitive to alcohols than* μ *receptors. This is the first demonstration of selective inhibition of one of the postulated classes of opiate receptors by a reagent that is not a ligand for the receptor.*

In recent years numerous studies have suggested the existence of several classes of opiate receptors. Martin and coworkers (*1*), on the basis of pharmacological studies on spinal dogs, concluded that there are three such classes (μ, κ, and σ). Lord *et al.* (*2*) discovered a difference between the receptors that predominate in guinea pig ileum and those in mouse vas deferens. They called them μ, or morphine-preferring, and δ, or enkephalin-preferring, respectively. Receptor binding studies (*2–4*) produced evidence for the same two types of receptors.

The existence of separate classes of receptors would be supported by evidence for the selective inhibition or inactivation of one of the receptor types. Attempts to inactivate or inhibit μ or δ opiate receptors by reagents other than receptor ligands and their derivatives have been unsuccessful. Irreversible inhibitors such as *N*-ethylmaleimide (*5, 6*) and phenoxybenzamine (*7*) and enzymes such as phospholipase A (*8*) and trypsin (*9*) were found to produce equal inactivation of enkephalin and opiate binding. This has necessitated the use of indirect approaches, such as studies of the protection of enkephalin and opiate binding against inhibitors by various receptor ligands (*6, 7*). We now report that ethanol and other aliphatic alcohols selectively inhibit the binding of enkephalin and its stable analogs at concentrations that have little or no effect on the binding of opiate alkaloids. Our results indicate that δ receptors are selectively inhibited.

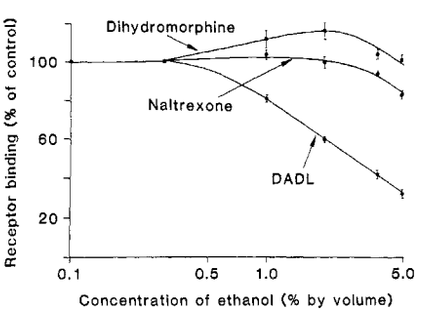

Fig. 1. Effects of ethanol on the binding of ^{3}H-labeled opioids to opiate receptors in rat brain membrane preparations. Duplicate 2-ml samples (0.9 to 1.1 mg of protein per milliliter) in 0.05*M* tris HCl (*p*H 7.4) containing 1 m*M* dipotassium EDTA were incubated with 1 n*M* [^{3}H]dihydromorphine (specific activity, 73.2 Ci/mmole), [^{3}H]naltrexone (8.5 Ci/mmole), and [^{3}H]DADL (31.0 Ci/mmole). To assess specific binding, samples were incubated in the presence or absence of 1 μ*M* unlabeled ligand. Incubations were followed by cooling in an ice water bath for 10 minutes before filtration. The values represent the means ± standard errors for at least three experiments.

Membrane preparations were made from rat brain, toad brain, and neuroblastoma cells (N4TG1) (*10*). Binding studies were carried out with [^{3}H]naltrexone, [^{3}H]dihydromorphine, and tritiated D-Ala^2-D-Leu^5-enkephalin (DADL). The alcohol was added just before addition of the labeled ligand and incubation was carried out at 37°C for 15 minutes in the presence or absence of a 1000-fold excess of unlabeled ligand. Filtration and scintillation counting were done as previously described (*10*).

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Figure 1 depicts the effect of increasing concentrations of ethanol on opiate receptor binding. The binding of DADL was strongly inhibited at concentrations at which naltrexone binding was inhibited only slightly and dihydromorphine binding not at all. In fact, the stimulation of dihydromorphine binding shown here has been consistently observed, although we cannot explain why. Binding of the putative κ receptor ligand ethylketocyclazocine (1 n*M*) and the σ ligand SKF 10,047 (1 n*M*) was essentially unaffected by ethanol up to 5 percent, a concentration at which the binding of DADL (1 n*M*) was inhibited 70 percent (Fig. 1).

As shown in Fig. 2, inhibitory potency increased exponentially with the chain length of straight-chain aliphatic alcohols. The median inhibitory concentration for inhibition of DADL binding ranged from 5 percent (by volume) for methanol to 0.2 percent for *n*-amyl alcohol, which has the longest chain of the alcohols tested. Median inhibitory concentrations for opiate alkaloids were three to five times higher.

When membrane preparations were exposed to alcohol for 15 minutes at 37°C, centrifuged to remove the alcohol, and washed, the inhibition of binding was completely reversed. Scatchard analysis of saturation curves representing DADL binding in the presence or absence of *n*-butanol yielded linear plots in the concentration range 0.5 to 10 n*M*. In two similar experiments with 0.5 percent *n*-butanol, inhibition resulted from a decrease in binding affinity (4.8 n*M* for control preparations, 11.9 n*M* for experimental preparations). There was no significant decrease in the maximum number of binding sites (0.11 pmole per milligram of protein for control preparations and 0.10 pmole/mg for experimental preparations).

It is possible, though unlikely, that alcohol affects the peptide rather than the receptor. The following evidence supports a selective effect on the δ opiate receptor. Binding of the enkephalin analog [^{3}H]FK33-824, reported to be a better ligand for μ than δ receptors (*11*), is significantly less inhibited by a given concentration of alcohol than binding of DADL. Thus, binding of FK33-824 (1 n*M*) is inhibited 24 ± 2.7 percent by *n*-butanol at a concentration (0.5 percent) that inhibits naltrexone binding 9 ± 1.9 percent and DADL binding 55 ± 1.3 percent. As shown in Table 1, in membrane preparations from toad brain, which we have found to contain predominantly μ receptors (*12*), the inhibition of DADL binding is much less pronounced than in rat brain. Perhaps most significantly, in a neuroblastoma cell line (N4TG1) reported to have receptors primarily of the δ type (*3*), inhibition of opiate binding was found to be even stronger than that of enkephalins.

Table 1. Inhibition of DADL and naltrexone binding by butanol in different tissues reported to have different ratios of μ and δ opiate receptors. The data are percentages of control binding. Where standard errors are given the value represents the mean for at least three experiments. Where no standard errors are shown the value represents the mean for two experiments.

Tissue	Concentration of *n*-butanol (%)	[^{3}H]DADL (5 n*M*)	[^{3}H]Naltrexone (5 n*M*)
Rat brain	0.5	53 ± 0.3	96 ± 4.6
	1.0	27 ± 2.5	65 ± 4.2
Toad brain	0.5	88	100
	1.0	56	107 ± 2.0
Neuroblastoma cells	0.5	72	52
	1.0	48 ± 2.4	29 ± 6.0

The mechanism by which alcohols inhibit opiate binding and, at low concentrations, selectively inhibit δ receptor binding is not known. Aliphatic alcohols increase the fluidity of cell membranes (*13, 14*), and the efficacy of this effect is greater the longer the chain length of the alcohol. This is thought to be related to the rise in lipid solubility of alcohols as their chain length increases. Changes in membrane fluidity affect the normal function of some membrane-bound proteins (*15, 16*). We suggest, as a working hypothesis, that δ receptors are more strongly influenced by changes in membrane fluidity than μ receptors.

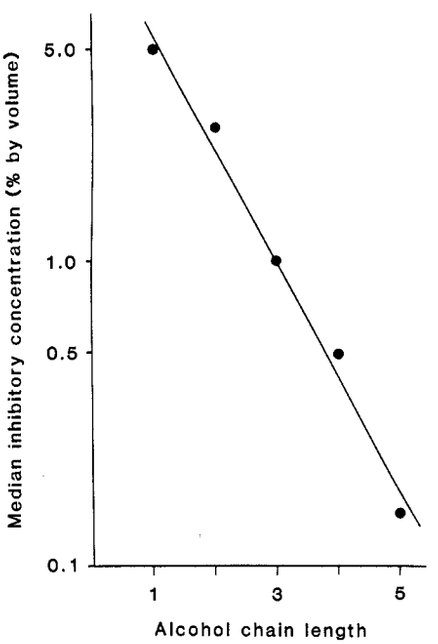

Fig. 2. Inhibitory potency of straight-chain aliphatic alcohols on [^{3}H]DADL binding as a function of chain length. Specific binding of DADL (1 n*M*) was measured in the presence of five concentrations of methyl, ethyl, *n*-propyl, *n*-butyl, and *n*-amyl alcohols under the conditions described in the legend to Fig. 1. The median inhibitory concentration of alcohol was obtained from a linear log-probit plot of the data. Each value represents the mean for at least two closely similar experiments.

These data strongly support the existence of separate μ and δ receptors. To our knowledge this is the first demonstration of selective inhibition of one of the postulated classes of opiate receptors by a reagent that is not a ligand for the receptor. These results should stimulate studies on the effects of ethanol ingestion on the endogenous opioid system.

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17. We dedicate this report to David Shemin in honor of his 70th birthday. We thank R. S. Zukin for supplying us with the neuroblastoma cells. This work was supported by grant DA-00017 from the National Institute on Drug Abuse. Gifts from Hoffmann–LaRoche, Inc., and E. I. du Pont de Nemours & Co. are gratefully acknowledged.

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