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Antagonism of Ethanol Intoxication in Rats by Inhibitors of Phenylethanolamine N-Methyltransferase

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The probable involvement of brain epinephrine in the expression of the acute sedative and intoxicating effects of ethanol and pentobarbital is demonstrated. Two selective inhibitors of phenylethanolamine N-methyltransferase (PNMT), LY134046 and LY78335, proved to be potent and long-lasting antagonists of ethanol intoxication in rats. Acute antagonism of pentobarbital-induced intoxication was observed with LY134046. The present results are compatible with a role for central epinephrine synthesis in ethanol and pentobarbital-induced sedation and intoxication in rats.

THE BIOCHEMICAL MECHANISMS by which the intoxicating and addictive effects of ethanol are mediated are not understood. Consumed in small quantities, ethanol can produce anxiolytic and euphoric effects.[1] In larger quantities, ethanol consumption is associated progressively with sedation, coma, and death.[2] Chronic intoxication can lead to physical or psychological dependence on ethanol so that cessation of consumption precipitates withdrawal symptoms.[3] These symptoms of withdrawal are not unique to ethanol dependence. Similar physiological responses are seen in withdrawal following chronic use of a variety of central nervous system (CNS) depressants, such as barbiturates, ethyl ether, chloral hydrate, and nitrous oxide.[1,4,5] Several of these symptoms are effectively treated with alpha-2 adrenoreceptor agonists such as clonidine.[6,7] Interestingly, abrupt discontinuation of chronically administered clonidine[8] or of chronic intraventricularly administered epinephrine,[9] both of which are sedative, can also precipitate a withdrawal syndrome in rats. This suggests the involvement of central alpha-2 adrenoreceptors in the development of at least one type of dependence and withdrawal syndrome.

Factors controlling alpha-2 receptor number and affinity have been studied extensively. It was recently demonstrated that phenylethanolamine N-methyltransferase (PNMT, E.C.2.1.1.28), the enzyme that converts norepinephrine to epinephrine in brain, is an important regulatory factor in determining alpha-2 receptor number in rat brain.[10-12] This is particularly true in the brainstem and hypothalamus where there is both a high density of alpha-2 receptors and relatively high PNMT activity.[13-15] As a result, it seemed plausible that the alpha-2 receptor changes associated with withdrawal symptoms might be a consequence of some effect of ethanol intoxication on brain epinephrine formation. Finally, the possible involvement of epinephrine synthesis in reward has been suggested by Katz and coworkers,[16,17] who showed that PNMT inhibition caused a dose dependent decrease in intracranial self-stimulation in rats.

The ability of the brain to synthesize epinephrine has been known for many years[18] although the presence of PNMT-containing neurons was not demonstrated until 1973.[19,20] Since that time it has been generally assumed that epinephrine acts primarily as a neurotransmitter in the brain. A nontransmitter role for epinephrine, as a brain neuromodulator or hormone in the hypothalamus and active intraneuronal metabolite in the brainstem has been proposed.[21,22]

In initial experiments we examined the effects of acute and chronic exposure to ethanol as well as withdrawal from exposure to ethanol on brain contents of biogenic amines and metabolites.[23] Epinephrine content of both hypothalamus and brainstem C1-C3 areas was significantly reduced 90 min following acute intraperitoneal administration of ethanol while only hypothalamic epinephrine was reduced following 24-hr and chronic (14-day) exposure and during withdrawal. Norepinephrine was significantly reduced in hypothalamus following acute, intraperitoneal administration as well as during withdrawal following 14 days of ethanol exposure. Reduction of both norepinephrine and epinephrine in the hypothalamus was highly correlated with blood ethanol concentration.[23] Biogenic amines and metabolites in areas other than the hypothalamus were unaffected. These data indicated a selective effect of ethanol on epinephrine synthesis and/or release in the hypothalamus.

In order to test the hypothesis that newly synthesized epinephrine plays a role in the sedative and intoxicating effects of ethanol, two selective, competitive inhibitors of brain PNMT, LY78335, 2,3-dichloro-α-methylbenzylamine and the rigid conformer, LY134046, 8,9-dichloro-2,3,4,5-tetrahydro-1H-2-benzazepine, were employed.[24] LY134046 was used in subsequent experiments because of its greater selectivity for PNMT compared to alpha-2 receptors.[24] Furthermore, in order to assess whether the effects of PNMT inhibitors were specific to the measured

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effects of ethanol, two other classes of sedative hypnotic compounds, barbiturates and benzodiazepines, were tested as well.

METHODS

Male Sprague-Dawley rats, 250 to 400 g, were used throughout. Animals were provided food and water ad libitum and were handled regularly for 2 weeks prior to testing. On the day of the test, animals were removed from their home cages but kept with cage mates and transported to the test room where they were allowed to acclimate for approximately 2 hr. The acclimation period and prior handling were found to be necessary to achieve reproducible effects. Doses of pentobarbital and benzodiazepines were chosen empirically to give intoxication ratings similar to that observed with the dose of ethanol used. LY134046 and LY78335 were administered (40 or 50 mg/kg intraperitoneal in normal saline) 5 min prior to administration of ethanol (2.4 g/kg, 30% v:v in normal saline, intraperitoneal, pentobarbital (21 mg/kg intraperitoneal in normal saline), or diazepam (40 mg/kg intraperitoneal). This short time interval was chosen to assure that the effects observed were due to acute inhibition of epinephrine synthesis rather than depletion of tissue stores.[24,25] Animals were then rated blindly for intoxication.[26] Animals were placed individually in an open field, $\approx 24 \times 24$ inches and observed for 1 to 2 min. Differences between intermediate intoxication scores were discerned by manipulating the animal and observing the ability of the animal to regain balance or position. Intoxication was scored on a continuous scale with unimpaired animals rating = 0, slight ataxia = 1, moderate ataxia = 2, severe ataxia with difficulty maintaining balance and locomotion = 3, loss of locomotor capacity with near complete loss of righting reflex = 4, and 5 = sedation with complete loss of righting reflex.

Antagonism of ethanol induced intoxication has been produced by drugs that act at the benzodiazepine/GABA receptor complex.[27] In order to exclude the direct involvement of this receptor system in the observed effects, three PNMT inhibitors, LY78335, LY134046, and SKF64139 (7,8-dichloro, tetrahydroisoquinoline) were tested in vitro for specific binding at the benzodiazepine receptor (^{3}[H]flunitrazepam displacement) and the chloride channel (*t*-butylbicyclophosphoro[^{35}S]thionate (TBSP) displacement).[28]

RESULTS

The ability of PNMT inhibitors to reduce the intoxicating effects of ethanol in this paradigm is shown in Fig. 1. Median scores show that most animals receiving the PNMT inhibitors showed no signs of intoxication following the subsequent administration of ethanol. The antagonism of ethanol intoxication was long-lasting, providing significant prophylaxis throughout the 60- and 90-min test periods.

The effects observed following ethanol consumption, including sedation, euphoria, CNS depression, physical dependence, and resultant withdrawal symptoms, are not unique to ethanol but are observed with a variety of sedative hypnotics and general anesthetics.[1,4,5] Consequently, the ability of LY134046 to antagonize intoxication by barbiturates and benzodiazepines was tested. Pretreatment with LY134046 significantly decreased pentobarbital-induced intoxication measured by loss of righting reflex, ataxia and sedation[26] as seen in Fig. 2. This antagonism was not as long-lasting nor as marked as with alcohol. In preliminary experiments, antagonism of pentobarbital-induced intoxication was also observed using a third PNMT inhibitor. SKF64139 (Mefford IN, Ota M, unpublished observations). This antagonism was observed only during the early portion of the experiment. As these animals recover more quickly from pentobarbital intoxication than ethanol intoxication, differences between the groups are not seen at later test points. LY134046 (50 mg/kg intraperitoneal) provided no prophylaxis against intoxication after a higher dose of pentobarbital which produced anesthesia (40 mg/kg intraperitoneal, data not shown).

The effects of PNMT inhibition on benzodiazepine-induced intoxication are seen in Fig. 3. Against diazepam-induced intoxication, LY134046 was completely ineffective. The failure to observe significant reversal of benzodiazepine intoxication may in part be due to the low degree of ataxia and the ability of animals to maintain righting reflex following drug administration; however, no indication of antagonism by PNMT inhibition was observed.

The three PNMT inhibitors tested, SKF64139, LY78335, and LY134046, were found to have no specific binding at the benzodiazepine receptor (3[H]flunitrazepam displacement) nor at the chloride channel (TBSP displacement)

DISCUSSION

Two PNMT inhibitors, LY134046 and LY78335, are demonstrated to provide potent and long lasting prophylaxis against the intoxicating effects of ethanol in the rat. LY134046 is also demonstrated to provide partial antagonism of the intoxicating effects of pentobarbital as measured by loss of righting reflex, ataxia and sedation. These compounds failed to antagonize the sedating effects of diazepam at the doses employed in the present study. Because the antagonism of intoxication was observed within 5 to 7 min of administration of the PNMT inhibitors, we reasoned that newly synthesized epinephrine might play a role in the induction of ataxia, sedation and loss of righting reflex observed after administration of ethanol or barbiturates to the rat.

The observation that centrally administered epinephrine can induce sedation and analgesia was made many years ago.[29,30] Although the relative concentration of epinephrine in brain is quite low with respect to norepinephrine, the higher affinity of epinephrine versus norepinephrine for clonidine binding sites suggests that outside the synaptic cleft, epinephrine might be the primary agonist for these sites.[10-12,31] Several effective sedative hypnotics and anesthetics including clonidine are potent agonists at alpha-2 receptors.[32,33] Further, alpha-2 antagonists have been reported to be effective in reversing xylazine- and thiopental-induced anesthesia.[34,35] These observations suggest a role for epinephrine or another endogenous alpha-2 agonist in sedation.

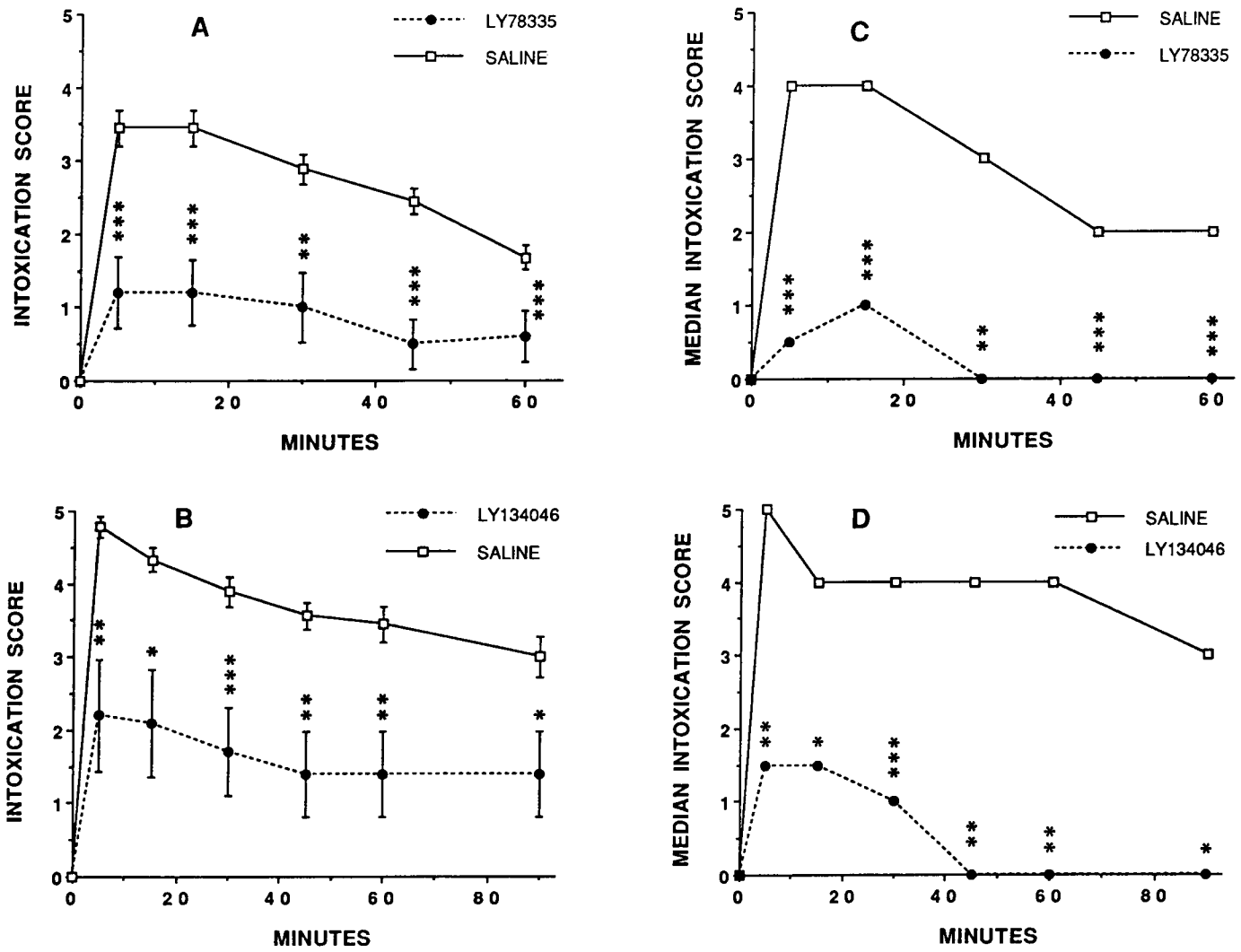

Fig. 1. Effects of PNMT inhibition on acute ethanol intoxication. Male Sprague-Dawley rats, 250 to 350 g, were pretreated with either LY134046 or LY78335, 40 mg/kg intraperitoneal or saline. After 5 min, each group was administered 2.4 g/kg of ethanol, (30% v:v in normal saline). Animals were rated blindly using a standard intoxication scale.[26] Most animals receiving pretreatment with PNMT inhibitors were unaffected by the administration of ethanol. As a result, the data is presented as both the mean intoxication score (A,B) and median intoxication score (C,D). $n = 9$ or 10 of each time point. Both LY134046 and LY78335 produced significant antagonism of intoxication at each time point by the Mann-Whitney rank order U test. $^{***}p < 0.01$, $^{**}p < 0.025$, $^{*}p < 0.05$.

Our observation of antagonism of ethanol and barbiturate intoxication by PNMT inhibitors is not likely due to direct effects of the drugs at alpha-2 receptors, although SKF64139 has been shown to be a potent alpha-2 blocker and LY78335 a less potent alpha-2 blocker. LY134046 does not exhibit potent alpha-2 blocking activity relative to its potency as a PNMT inhibitor.[24] Yet, all drugs prevented ethanol-induced intoxication. The doses used in the present experiments are comparable to those employed elsewhere in testing the behavioral and neuroendocrine effects of PNMT inhibition.[11,17,36] At 40 mg/kg, LY134046 has been demonstrated to cause an 84% decrease in brainstem PNMT activity within 1 hr, and to induce 70% decrease in brainstem PNMT activity at 20 mg/kg.[24] Maximal, and similar effects were observed within 1 hr using LY78335.[24] Others, using SKF64139, a PNMT inhibitor with less selectivity than LY134046 towards PNMT relative to alpha-2 receptor blockade,[13,24] have demonstrated that doses similar to those used in the present work do not block alpha-2 receptors in vivo. Clonidine stimulated growth hormone release, an index of central alpha-2 receptor integrity[37,38] occurs in the presence of 50 mg/kg SKF64139. However, inhibition of the formation of a potent endogenous alpha-2 adrenergic receptor agonist, epinephrine, should be analogous in some respects to administration of alpha-2 adrenergic receptor antagonists, yet providing site selectivity for these effects based on the distribution of PNMT.

The PNMT inhibitors used in this study have acute behavioral effects themselves,[36,39] inducing an acute reaction similar to ethanol withdrawal, including tremor. These effects, which are observed within 5 min and generally diminish within 30 min, may reflect the loss of tonic inhibition due to the acute depletion of extraneuronal or cytoplasmic pools of epinephrine.

It is not clear how our observations fit with previous

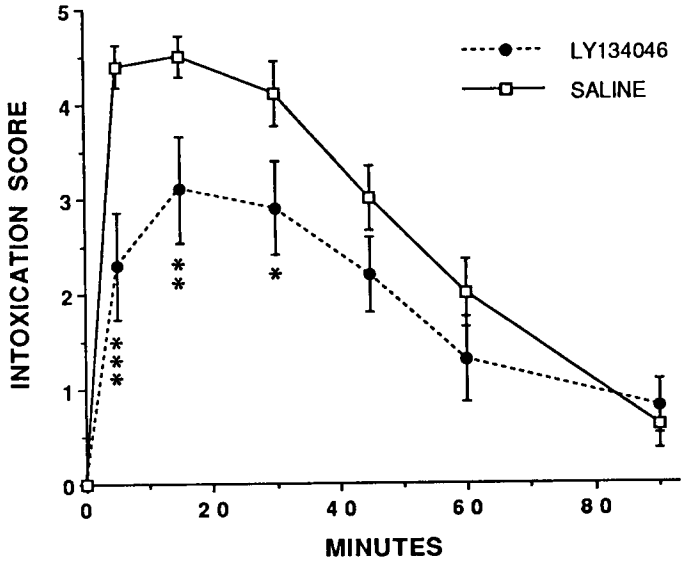

Fig. 2. Effect of PNMT inhibition on sodium pentobarbital-induced intoxication. Male Sprague-Dawley rats, 260 to 280 g, were pretreated with either saline or LY134046 (50 mg/kg intraperitoneal). After 5 min all animals were administered sodium pentobarbital, 21 mg/kg intraperitoneal. After 5 min and at regular intervals thereafter, animals were rated blindly for intoxication.[26] $n = 10$ in each case. ***$p < 0.005$, **$p < 0.025$, *$p < 0.05$, by Mann-Whitney rank order U test.

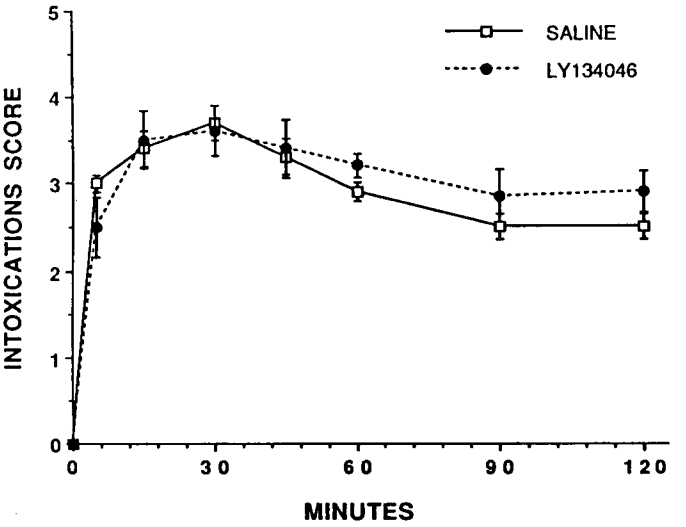

Fig. 3. Effect of PNMT inhibition on diazepam induced intoxication. Male Sprague-Dawley rats were pretreated with either saline or LY134046 (50 mg/kg intraperitoneal) 5 min prior to administration of diazepam, 40 mg/kg intraperitoneal. After 5 min and at regular intervals thereafter, animals were rated blindly for intoxication at regular intervals.[26] $n = 10$.

observations relating the benzodiazepine receptor complex to the mechanism of action of ethanol and barbiturates.[27] Both PNMT-containing neurons and GABA neurons acting via the GABA A receptor, provide inhibitory input to the locus coeruleus,[40] (Aston-Jones G, personal communication). Thus, our data may complement those of others who have observed antagonism of ethanol by a benzodiazepine inverse agonist[27] and represent blockade of a separate inhibitory mechanism stimulated by ethanol in vivo. Clonidine has been demonstrated to share many sedative properties with benzodiazepines and it has been suggested that the sedative effects of benzodiazepines may involve alpha-2 adrenergic receptors.[41] Our data imply that intoxication may be mediated in the phylogenetically older portions of the brain, notably the hypothalamus and brainstem, since PNMT is localized to these areas. Most of the work on description of the neuronal benzodiazepine receptor complex has been accomplished with cortically derived preparations[27] and thus is not necessarily comparable. It is likely that any inhibitory effects observed at the brainstem level would modify the transmission of information to the cortex and as a result these data might represent two different aspects of the same or related inhibitory phenomena. Comparisons between ethanol, barbiturate and benzodiazepine tolerance, intoxication and withdrawal do suggest that the actions of ethanol and barbiturates are more closely related to each other than to the benzodiazepines, consistent with the present observations.[42]

The rapid effect of PNMT inhibitors suggests that the active pool of epinephrine is very labile, and not protected by storage, consistent with cytoplasmic localization of PNMT and the high density of mitochondria in PNMT-containing neurons in the medulla.[43] Fuller and coworkers[24] have demonstrated that maximal PNMT inhibition with the two compounds used in the present study occurs within the 1st hr after administration. Previous data suggest that both epinephrine and PNMT-containing cells are associated closely with the projections of the A1/C1 and A2/C2 noradrenergic and PNMT-containing cell groups in the forebrain.[21] The locus coeruleus noradrenergic projections, implicated in withdrawal reactions, may also be regulated by inhibitory inputs from the C1 cell body group, capable of synthesizing epinephrine.[40,44]

It is not possible to exclude direct action at alpha-2 adrenergic receptors as providing at least partial antagonism of ethanol and barbiturate intoxication observed with the PNMT inhibitors used in the present study. However, the consistency of these observations with different inhibitors of epinephrine synthesis is suggestive of a role for central epinephrine in intoxication, sedation, and dependence following ethanol or barbiturate abuse. PNMT inhibitors provide partial prophylaxis against the acute effects of these agents and may be useful as antagonists of ethanol or barbiturate intoxication.

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