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Ro 15-4513 Alteration of Pentobarbital Dependence

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YUTRZENKA, G. J., T. STONE AND S. ANDERSON. *Ro 15-4513 alteration of pentobarbital dependence.* PHARMACOL BIOCHEM BEHAV **55**(3) 379–386, 1996.—This study investigated the ability of the benzodiazepine inverse agonist, Ro 15-4513, to alter the expression of physical dependence on pentobarbital. Male Sprague–Dawley rats were made physically dependent on pentobarbital by continuous, IP, infusion of escalating doses of pentobarbital for 12 days. In Experiment 1, pentobarbital dependent rats received either vehicle or Ro 15-4513, in doses of 5, 10, or 15 mg/kg, IP, periodically during the pentobarbital abstinence period. As expected, Ro 15-4513 produced a significant, dose-dependent, exacerbation of withdrawal signs in the pentobarbital dependent rats. In Experiment 2, either vehicle or Ro 15-4513, at a dose of 15 mg/kg, was administered, IP, once daily during the 12 days of continuous pentobarbital infusion. During the subsequent pentobarbital abstinence period it was noted that the withdrawal signs were significantly reduced in the rats receiving the daily administration of Ro 15-4513. It is hypothesized that the benzodiazepine inverse agonist, Ro 15-4513, may inhibit the development of physical dependence on pentobarbital through an opposing action on the GABA-A receptor. **Copyright © 1996 Elsevier Science Inc.**

Inverse agonists Dependence Pentobarbital Ro 15-4513 Rats

THE role of the GABA-A receptor in the action of central nervous system depressants has been well established (16,19, 38,45). Several studies have also established a link between the alteration of GABA-A receptor function and the chronic administration of CNS depressants (18,21,22,32). Chronic pentobarbital administration results in both a decreased number of GABA binding sites (21) and an inhibition of pentobarbital induced facilitation of binding to the GABA-A receptor (42). In addition, pentobarbital has been shown to enhance the flux of chloride ion through the chloride ion channel of the GABA-A receptor (1,2,19,43).

Similarly, ethanol interaction with the GABA-A receptor has been established. A variety of GABAergic agonists have been noted to enhance ethanol-induced motor incoordination and sedation while picrotoxin appears to reverse these effects (26,27,30,31). In addition, ethanol enhances both ^{36}Cl ion uptake (44) and muscimol stimulated ^{36}Cl flux in membrane preparations (3). Interestingly, ethanol appears to have greater effects on chloride ion flux in rodents that have been selectively bred for high sensitivity to ethanol (4,5).

A number of previous investigations have noted that the benzodiazepine partial inverse agonist, Ro 15-4513 (ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5-a][1,4] benzodiazepine-3-carboxylate) may alter the action of CNS depressants. Ro 15-4513 has been shown to inhibit pentobarbital-induced motor incoordination and hypothermia (9,23) and was able to partially antagonize pentobarbital enhancement of GABA-activated chloride ion flux (20). Ro 15-4513 has also been demonstrated to inhibit ethanol stimulated chloride ion uptake (44). Likewise, many of the acute behavioral effects of ethanol, including motor incoordination, sedation, amnesia, and mortality, can be inhibited by the administration of Ro 15-4513 (7,14,15,23,36). Importantly, the benzodiazepine antagonist, Ro 15-1788, was found to block the effects of Ro 15-4513, thus indicating that the action of Ro 15-4513 was directed at the benzodiazepine binding site on the GABA-A receptor (11,12).

The previous investigations support the hypothesis that Ro 15-4513 may play a role in inhibiting the behavioral effects of CNS depressants through an interaction with the GABA-A receptor. The current study was designed to investigate the ability of Ro 15-4513 to alter the development of physical dependence on pentobarbital.

METHOD

Animals

Male, Sprague–Dawley rats (Sasco, Omaha, NE), weighing 160–180 g, were individually housed and allowed ad lib access

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to food and water. All rats were maintained on a 12 L:12 D cycle (lights on at 0730 h).

Drugs

Pentobarbital sodium was dissolved in 0.9% saline as a 30 mg/ml stock solution. All solutions were filtered through 0.2 micron filters prior to infusion into rats. Ro 15-4513 was suspended in Tween 80/0.9% saline and was administered, IP, in a volume of 1 ml/kg.

Intraperitoneal Infusion Procedure

Rats were surgically prepared with an indwelling, IP, polyethylene cannula (PE90) while under halothane/nitrous oxide anesthesia (52). All rats received 0.25 ml of a broad spectrum antibiotic (Combiotic) following the surgical procedure and were allowed 3 days for postsurgical recovery. Rats were placed into an infusion harness attached to a constant infusion pump and were continuously infused with 0.9% saline for 2 days prior to the start of the pentobarbital infusion period.

At the start of the pentobarbital infusion period all rats were randomly assigned to treatment groups and pentobarbital-infused rats received pentobarbital at an initial dose of 100 mg/kg/24 h (52). During the next 12 days rats were continuously infused with escalating doses of pentobarbital sodium reaching a final dose of 1000 mg/kg/24 h. Following the initial dose, pentobarbital doses were increased by 100 mg/kg/24 h for the first 8 infusion days with subsequent pentobarbital doses being increased by 50 mg/kg/24 h until day 12 of the infusion period. A CNS depression rating scale was employed each day to aid in the determination of the degree of CNS depression exhibited by the rat (52). Slight alteration of the dosing schedule was made for individual rats as a result of the degree of CNS depression exhibited. In our experience, nearly all rats tolerate the established pentobarbital dosing schedule without alteration. Control rats were continuously infused with 0.9% saline. Pentobarbital and 0.9% saline were delivered at a rate of 10 ml/24 h.

On day 13 there began a drug abstinence period, during which saline was substituted for pentobarbital. Body weight was obtained daily and behavioral signs indicative of pentobarbital withdrawal were determined hourly for the first 12 h and then at 14, 16, 20, 24, 28, 32, 36, and 48 h. The withdrawal rating scale (Table 1) has been previously described (52). The withdrawal signs were rated by two trained observers who were blind to the treatment protocol and an average withdrawal score was determined at each time point.

In Experiment 1, rats were continuously infused with either saline (n = 24) or with escalating doses of pentobarbital (n = 24) during the 12-day infusion period. On day 13, the control rats were placed into treatment groups 1–4 (n = 6/group) and the pentobarbital infused rats were placed into treatment groups 5–8 (n = 6/group). During the withdrawal period rats were treated with either vehicle (GP 1 and GP 5) or with Ro 15-4513 in doses of 5 mg/kg (GP 2 and GP 6), 10 mg/kg (GP 3 and GP 7), or 15 mg/kg (GP 4 and GP 8). Both the Ro 15-4513 and vehicle were administered, IP, at 0, 4, 8, 12, 24, 28, 32, and 36 h of the pentobarbital abstinence period. Either Ro 15-4513 or vehicle were administered 30 min prior to the scheduled withdrawal observation time point. This pretreatment time was utilized in an attempt to limit the possible contaminating effect induced by the handling of the rats for injection on the subsequent withdrawal score.

In Experiment 2, rats were continuously infused with either saline (control) or pentobarbital sodium for 12 consecutive days as was previously described. At the beginning of the infusion period control rats were placed into treatment groups 1–3 (n = 8/group) and pentobarbital-infused rats were placed into treatment groups 4–6 (n = 8/group). Rats in GP 1 and GP 4 received once daily administration of Tween 80/0.9% saline vehicle. Rats in GP 2 and GP 5 were treated with Ro 15-4513, 15 mg/kg, IP, once daily during the infusion period while rats in GP 3 and GP 6 were treated with Ro 15-4513, 15 mg/kg, IP, once daily during both the infusion period and the subsequent abstinence period. Rats received either vehicle or Ro 15-4513 at 0900 h each day. The 15 mg/kg dose of Ro 15-4513 was used in Experiment 2 because this dose appeared to produce the greatest effect on withdrawal scores in Experiment 1.

TABLE 1
WITHDRAWAL SYNDROME RATING SCALE

A) Response to air puff
0 = No response
1 = Jumps
2 = Jumps and vocalizes

B) Response to prodding of flank
0 = No response
1 = Jumps away and /or vocalizes

C) Presence of "High "Posture
0 = Not present
1 = Present

D) Response to being picked up and held
0 = No response
1 = Struggles or vocalizes
2 = Struggles and vocalizes
3 = Struggles, vocalizes, scratches, bites

Statistical Analysis

Differences in withdrawal scores between control and pentobarbital-treated rats were analyzed for significance using the Mann–Whitney U-test. Changes in body weight were analyzed for significant difference using ANOVA followed by post hoc t-test analysis where indicated.

RESULTS

In Experiment 1 administration of Ro 15-4513 to pentobarbital-dependent rats during the abstinence period resulted in a dose-dependent increase in withdrawal scores (Fig. 1). While Ro 15-4513 at 5 mg/kg had no significant effect on withdrawal scores (Fig. 1A), there was a significant increase in scores noted following administration of Ro 15-4513 at doses of either 10 mg/kg (Fig. 1B) or 15 mg/kg (Fig. 1C). Increased withdrawal scores were noted as early as 8 to 12 h into the abstinence period and withdrawal scores tended to remain significantly elevated throughout the withdrawal period. Administration of Ro 15-4513 to control rats had no significant effect.

Ro 15-4513 did appear to exacerbate the weight loss of dependent rats during the abstinence period (Fig. 2). Pentobarbital-dependent rats treated with vehicle exhibited about 9% loss of body weight at 24 h of abstinence with a partial return to initial body weight evidenced by 48 h. Ro 15-4513, on the other hand, induced a greater degree of body weight loss at both 24 and 48 h of abstinence, with the greatest loss

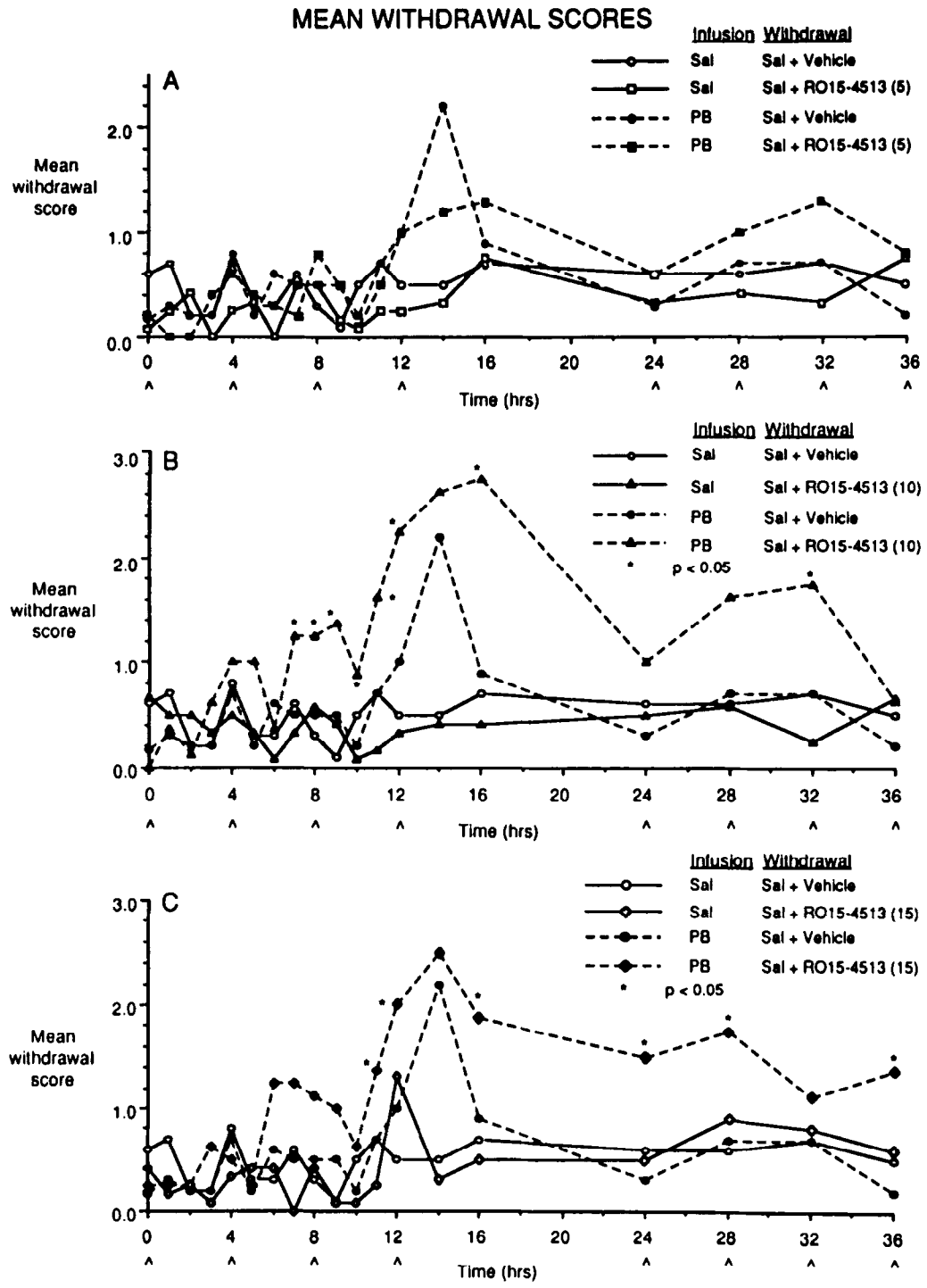

FIG. 1. Mean withdrawal scores of saline (SAL)-infused control rats (open symbol) and pentobarbital (PB)-infused rats (filled symbol) treated with either Vehicle or with Ro 15-4513 in doses of 5 mg/kg (A), 10 mg/kg (B), or 15 mg/kg (C) during the pentobarbital withdrawal period. During the withdrawal period all rats receive continuous infusion of saline. Vehicle or Ro 15-4513 were administered, IP, 30 min prior to the withdrawal sign observation period. The arrowhead indicates time of administration of vehicle or Ro 15-4513. Each point represents the mean value for six rats. *Indicates significant difference ($p \leq 0.05$) between pentobarbital-dependent rats treated with vehicle and pentobarbital-dependent rats treated with Ro 15-4513.

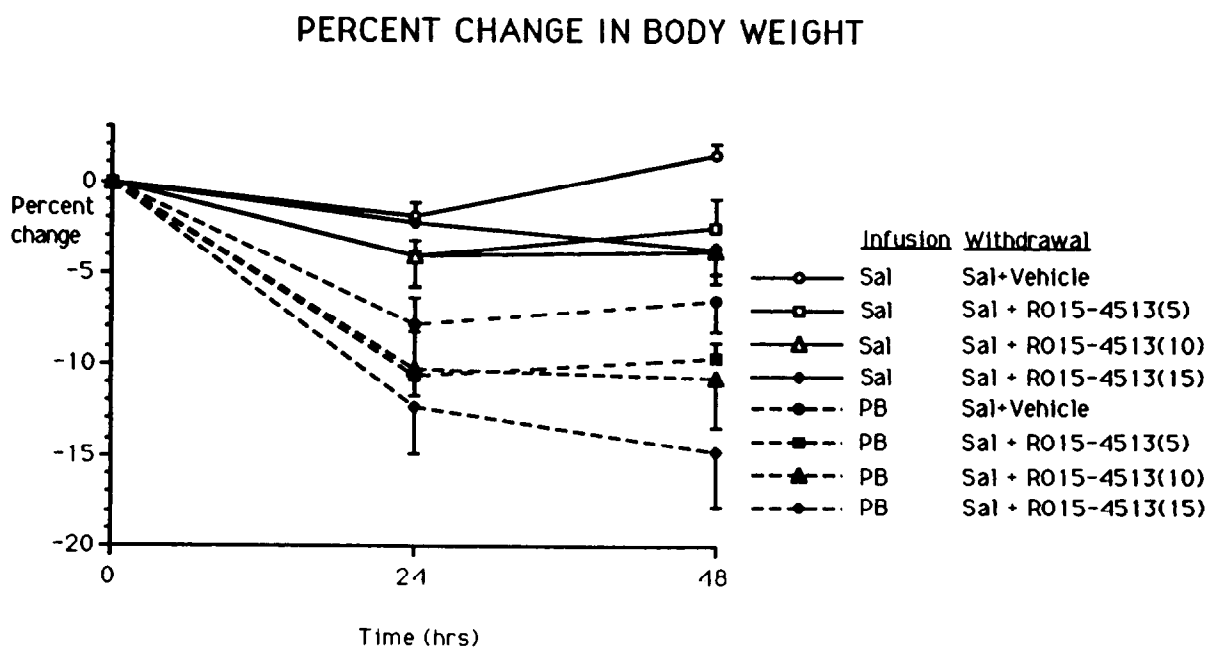

FIG. 2. Percent change in body weight of saline (SAL)-infused control rats (open symbol) and pentobarbital (PB)-infused rats (closed symbol) treated during the withdrawal period with either vehicle or Ro 15-4513 in doses of 5, 10, or 15 mg/kg. Each point represents the mean ± SEM of the percent change in body weight at 24 and 48 h of withdrawal as compared to the body weight at the 0 h time point. n = 6 rats per treatment group.

of body weight being observed with the highest dose of the inverse agonist. At the beginning of the abstinence period the average body weight for the control rats was 211 ± 3.53 g, while the pentobarbital-infused rats had an average body weight of 218 ± 3.56 g (not significantly different from control).

In Experiment 2, the once daily administration of Ro 15-4513 during the 12 day infusion period was noted to significantly reduce the withdrawal scores in the dependent rats as compared to the pentobarbital-dependent rats treated with vehicle (Fig. 3B). There was no significant difference in withdrawal scores between the groups of pentobarbital-infused rats that had been treated with Ro 15-4513 during the infusion period, only, and those rats that continued to receive the inverse agonist during the subsequent abstinence period. There was no significant effect of the Ro 15-4513 when administered to control rats (Fig. 3A).

At 24 h of abstinence Ro 15-4513 produced no significant effect on body weight loss in pentobarbital-dependent rats as compared to saline-infused control rats (Fig. 4). Rats that continued to receive Ro 15-4513 during the abstinence period did demonstrate a significantly greater loss of body weight at 48 h as compared to the other two groups of pentobarbital-infused rats. During this abstinence period there were no significant changes in body weight of control rats administered Ro 15-4513. In addition, daily administration of Ro 15-4513 to either control or pentobarbital-infused rats produced no significant effect on either mean body weight (control + vehicle = 213 ± 7 g; control + Ro 15-4513 = 211 ± 5 g; pentobarbital + vehicle = 223 ± 4 g; pentobarbital + Ro 15-4513 = 220 ± 5 g) or gross behavior during the 12-d infusion period.

DISCUSSION

The current study indicated that when Ro 15-4513 was administered periodically during the abstinence period the inverse agonist significantly increased the degree of expression of abstinence signs (Fig. 1). While Ro 15-4513 exacerbated withdrawal signs, beginning at about 8 h of abstinence, there was no indication that the inverse agonist directly precipitated withdrawal activity. This was evidenced, in part, by the lack of effect of Ro 15-4513 when administered to the pentobarbital-dependent rats at 0 h and 4 h of the abstinence period. These findings are in agreement with previous reports for ethanol-dependent mice (6).

Importantly, when Ro 15-4513 was administered once daily during the 12-day pentobarbital infusion period there was observed a significant attenuation of withdrawal signs exhibited during the abstinence period (Fig. 3). The reduction in withdrawal signs was also observed in those rats that continued to receive Ro 15-4513, once daily, during the abstinence period. It would appear that the daily treatment with Ro 15-4513 altered the development of physical dependence on pentobarbital.

The alterations in body weight observed in the dependent rats are characteristic of the typical changes that were previously observed during barbiturate abstinence (52). The reason for the loss of body weight is unclear, but may be due, in part, to the disruption of normal feeding patterns and a significant increase in general activity of the rats during the pentobarbital abstinence period (52). In Experiment 1, increasing doses of Ro 15-4513 administered during the abstinence period did appear to exacerbate the loss of body weight at 48 h (Fig. 2) in a manner that was consistent with the increased withdrawal scores.

Interestingly, in Experiment 2, even though there was observed a significant reduction of behavioral signs during withdrawal from pentobarbital in rats that had received once daily Ro 15-4513 administration, Ro 15-4513 did not appear to alter the degree of body weight loss at 24 and 48 h when compared

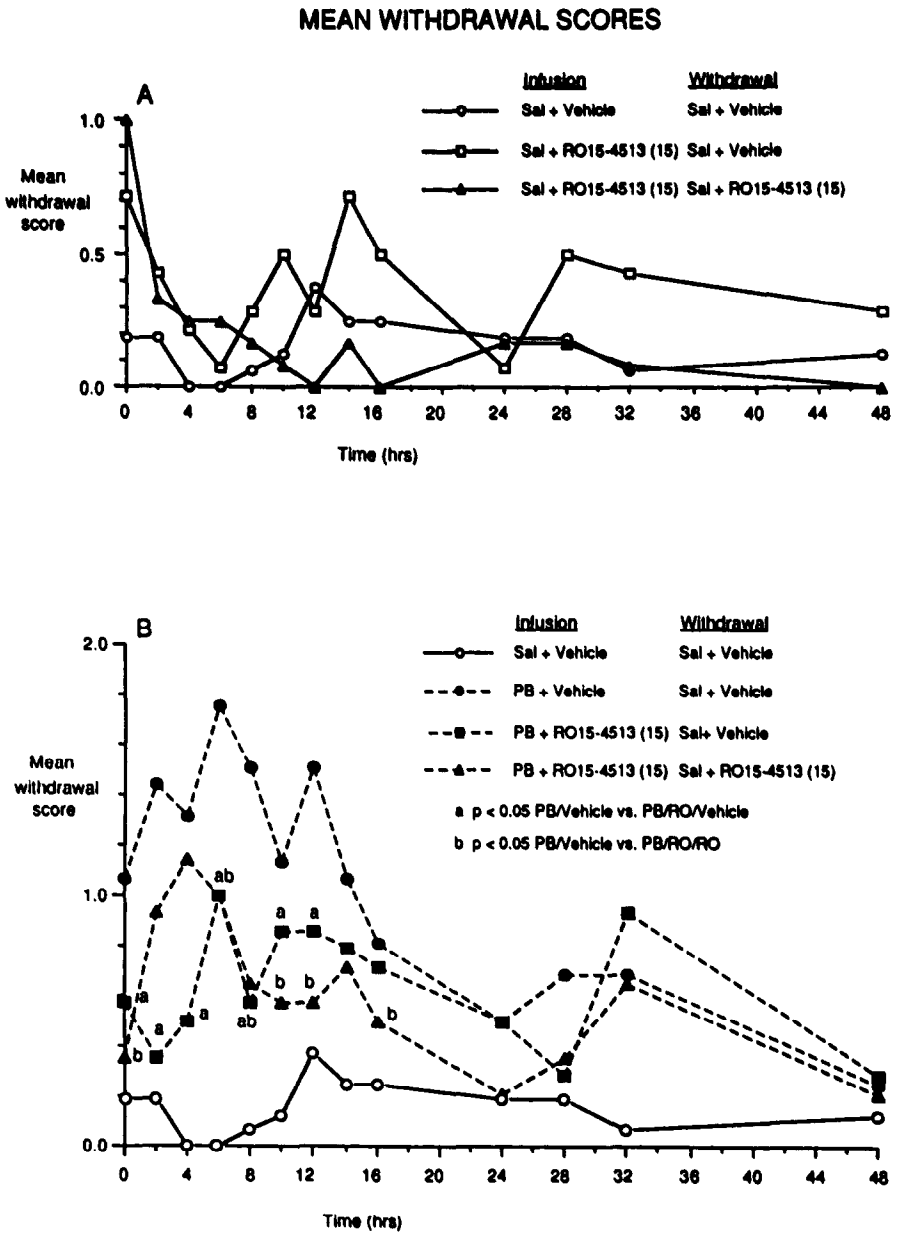

FIG. 3. Mean withdrawal scores of saline (SAL)-infused control rats (A) and of pentobarbital (PB)-dependent rats (B) administered either vehicle or Ro 15-4513, 15 mg/kg, IP, once daily for 12 consecutive days of pentobarbital infusion. All rats received saline infusion during the withdrawal period. Each point represents the mean withdrawal score of six to eight rats. (a) $p \leq 0.05$. Comparison between PB-infused rats receiving vehicle and PB-infused rats receiving Ro 15-4513 during the 12-day PB infusion period only. (b) $p \leq 0.05$. Comparison between PB-infused rats receiving vehicle and PB-infused rats receiving Ro 15-4513 during both the PB infusion period and the PB withdrawal period.

to pentobarbital-dependent rats that received once daily administration of vehicle (Fig. 4). Continued once daily administration of Ro 15-4513 during the abstinence period did produce a significant increase in body weight loss at 48 h. It is not readily apparent why Ro 15-4513 was unable to attenuate the characteristic weight loss during pentobarbital abstinence while it was able to reduce the other behavioral signs.

It is hypothesized that the observed effect of Ro 15-4513 on pentobarbital dependence is due to the opposing mechanisms of action at the GABA-A receptor. Pentobarbital binds to a barbiturate binding site on the GABA-A receptor and functions to increase chloride ion movement across the membrane, leading to hyperpolarization of the membrane and a reduction in membrane excitability (3,21,22). On the other hand, Ro 15-4513 binds to the benzodiazepine binding site of the GABA-A receptor and promotes the maintenance of the

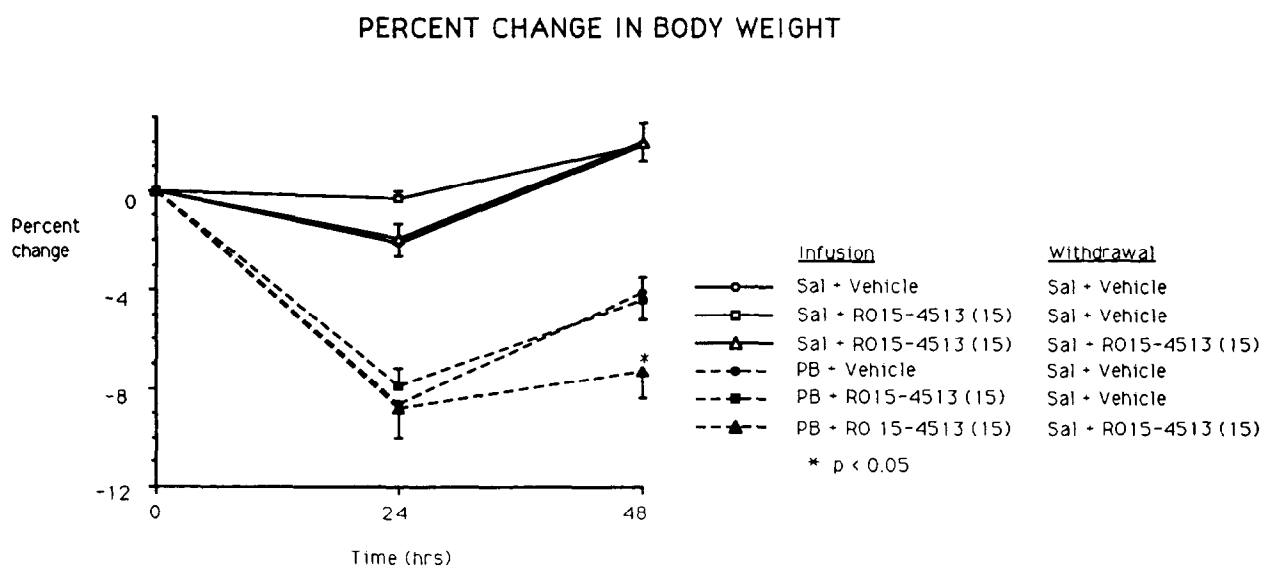

FIG. 4. Percent change in body weight of saline (SAL)-infused control rats (open symbol) and pentobarbital (PB)-infused rats (filled symbol) treated with either vehicle or Ro 15-4513, 15 mg/kg, IP, once daily during the PB infusion period only or during both the PB infusion period and the PB withdrawal period. Each point represents the mean ± SEM of six to eight rats. *$p < 0.05$ compared to PB-dependent rats receiving vehicle.

chloride ion channel in a closed configuration, thus inhibiting the action of GABA on the membrane (10,20,29,46).

The effect of barbiturates, benzodiazepines and inverse agonists on GABA-evoked chloride ion currents have been studied in cultured neurons (29,41,48). It has been determined that barbiturates act at specific binding sites on the GABA-A receptor to increase the duration of the open chloride ion channel and, thus, increase the duration of GABA-induced currents across the membrane. On the other hand, benzodiazepine agonists bind to the benzodiazepine site on the GABA-A receptor and function to increase the frequency of opening of the chloride ion channel. In an opposing action, benzodiazepine inverse agonists serve to reduce GABA-induced chloride ion currents by decreasing the frequency of opening of the chloride ion channel. Thus, it is hypothesized that the inverse agonists oppose the action of benzodiazepines by altering the ability of GABA to regulate chloride ion channel function (41,48).

Likewise, it is hypothesized that the benzodiazepine inverse agonists may be capable of opposing the action of barbiturates on GABA-induced chloride ion flux. While it is presumed that this interaction between barbiturates and inverse agonists occurs at differing binding sites on the GABA-A receptor, the consequence of this interaction is directed at modulation of the activity of the chloride ion channel.

The development of physical dependence is a dynamic process that includes the alteration of normal function at the site of drug action so as to allow the subject to function normally even in the chronic presence of the drug. It is suggested that chronic administration of pentobarbital would lead to a compensatory alteration in the normal function of the GABA-A receptor complex.

One means by which chronic pentobarbital administration may alter normal GABA-ergic activity is through pentobarbital-induced alteration in the molecular expression of GABA-A receptor subunits. The GABA-A receptor is comprised of at least five subunits coded for by specific mRNA (25,40,50,51). Each subunit consists as a family of related subtypes, which leads, in turn, to the possible expression of a variety of functionally different GABA-A receptors (39,49). The GABA recognition site is located on the β-subunit, while the benzodiazepine recognition site resides on the α-subunit of the GABA-A receptor (13).

Recent studies indicate that chronic administration of pentobarbital results in alteration in the expression of specific subtypes of the individual subunits that comprise the GABA-A receptor complex. Pentobarbital-tolerant rats showed decreased levels of α1-subunit mRNA in hippocampus with no change in β3-subunit mRNA (47). In addition, it was also demonstrated that at 24 h of pentobarbital abstinence there were increased levels of α1-subunit and β3-subunit mRNA in cerebral cortex and cerebellum. Similar changes in GABA-A subunit mRNA have been noted in response to chronic ethanol administration (35). These observations appear to be consistent with a downregulation of the GABA-A receptor in response to the chronic administration of ligands that bind to the α-subunit (benzodiazepines, barbiturates, ethanol) and a subsequent upregulation of GABA-A receptors during the abstinence period.

It is unclear, however, how the suggested downregulation of the α-subunit of the GABA-A receptor might ultimately enhance the ability of Ro 15-4513 to alter the development of physical dependence on pentobarbital. Presumably, the downregulation of the GABA-A receptor would also serve to limit the ability of the benzodiazepine inverse agonist to influence GABA-ergic function. Interestingly, studies have shown that chronic ethanol administration was observed to produce an upregulation of GABA receptors, which in turn, appeared to augment the action of Ro 15-4513 (33,34).

It has been shown that the expression of withdrawal signs during the pentobarbital abstinence period is temporally correlated with the elimination of pentobarbital (52). During the abstinence period, the elimination of pentobarbital allows whatever alterations that have already occurred at the GABA-A receptor to become manifested to a greater degree. Therefore,

if the GABA-A receptor has been downregulated as a consequence of chronic pentobarbital exposure, then elimination of pentobarbital could lead to a state in which the consequence of altered GABA-ergic function is expressed as the withdrawal syndrome. The coadministration of Ro 15-4513 during the abstinence period would be expected to exacerbate the withdrawal syndrome.

This was evidenced in Experiment 1 in which the administration of Ro 15-4513 to dependent rats during abstinence resulted in a significant increase in the intensity of the withdrawal syndrome. As would be expected, as the abstinence period progressed the intensity of the withdrawal signs also increased reflecting, in part, not only the elimination of pentobarbital from the rat but also the addition of Ro 15-4513.

The reduced expression of the withdrawal syndrome following the once daily administration of Ro 15-4513 during the chronic pentobarbital infusion period may be explained, in part, as a consequence of the opposing actions of pentobarbital and Ro 15-4513 on the GABA-A receptor complex. It is suggested that once daily administration of Ro 15-4513 serves to disrupt the continuity of pentobarbital-induced actions on the GABA-A receptor. It has been previously determined that the continuity of chronic drug exposure is an important factor in the establishment of physical dependence (8,17,24,37). The development of physical dependence on phenobarbital was inhibited if phenobarbital-treated mice were allowed one or two, 24-h long, drug "holidays" during a nine day chronic administration period (8). It was suggested that the drug holidays served to break the continuity of phenobarbital exposure, which in turn, allowed for the reversal of cellular adaptations that had occurred as a consequence of the chronic administration of phenobarbital.

It is uncertain how Ro 15-4513, which has a peak effect at about 15 min following administration and which has a relatively short duration of action (28) was capable of producing such a significant alteration of the development of physical dependence to pentobarbital. In line with this, it has been shown that a single dose of the benzodiazepine antagonist, flumazenil, delivered as long as 14 h prior to ethanol abstinence was able to reduce the severity of seizure activity during the ethanol withdrawal period (12). Additionally it has been reported that the periodic administration of the benzodiazepine antagonist, Ro 15-1788, disrupted the development of physical dependence on diazepam in primates (17,24). Thus, it is possible that even brief occupation of the GABA-A receptor by an antagonist or an inverse agonist may be sufficient to reset the cellular mechanisms that have been altered as a consequence of the chronic administration of ethanol or barbiturates.

The current study provides evidence that the benzodiazepine inverse agonists may attenuate the development of dependence on pentobarbital by an action at the GABA-A receptor. The chloride ion channel of the GABA-A receptor represents a final common pathway for the action of both pentobarbital and the benzodiazepine inverse agonists. Thus, the opposing actions of these two classes of compounds at the GABA-A receptor may represent the mechanism by which Ro 15-4513 alters the development of physical dependence on pentobarbital.

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