

Effects of "Nitrendipine" on Nitrous Oxide Anesthesia, Tolerance, and Physical Dependence

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Studies with ethanol have indicated that dihydropyridine-sensitive calcium (Ca^{++}) channels may be involved in the adaptation to prolonged exposure to ethanol. This study investigated the effects, in mice, of the dihydropyridine Ca^{++} antagonist, nitrendipine, on acute tolerance to nitrous oxide after 60 min exposure to anesthetizing concentrations, and also the withdrawal syndrome which occurred following removal from nitrous oxide. Control mice were anesthetized by nitrous oxide concentrations in the range 1.28–1.51 atmospheres. Nitrendipine 10, 50, and 100 $\text{mg} \cdot \text{kg}^{-1}$, i.p., produced a dose-dependent potentiation of nitrous oxide anesthesia ($P < 0.05$ for nitrendipine 50 and 100 $\text{mg} \cdot \text{kg}^{-1}$). Tolerance to nitrous oxide anesthesia developed over 60 min (13% increase in ED_{50} , $P < 0.05$). Concurrent administration of nitrendipine at all doses prevented the development of nitrous oxide tolerance. After 60 min exposure to nitrous oxide 1–1.5 atmospheres, all control mice showed handling seizures. Nitrendipine diminished or prevented nitrous oxide withdrawal seizures, in a dose-dependent manner ($P < 0.05$ for nitrendipine 50 and 100 $\text{mg} \cdot \text{kg}^{-1}$). These results support the importance of the role of dihydropyridine-sensitive Ca^{++} channels in the mechanism of tolerance and dependence to central depressant drugs. They also suggest that acute and chronic tolerance to sedative drug action may share some common pathways, and that tolerance and physical dependence may share a common mechanism through voltage-operated Ca^{++} channels. (Key words: Anesthetics, gases: nitrous oxide. Ions: calcium. Pharmacology: nitrendipine. Tolerance, physical dependence: nitrous oxide.)

ALTERED NEURONAL CALCIUM (Ca^{++}) fluxes have long been considered to play a central role in the mechanism of general anesthesia. A variety of Ca^{++} related neuronal events, such as neurotransmitter release,¹ postsynaptic function,² and intracellular messenger function,³ have been shown to be affected by anesthetic drugs. However, no particular neuronal event has been shown to be crucial for the production of general anesthesia.

Evidence for the importance of altered intracellular Ca^{++} movements in the development of general anesthesia is considerable but indirect. A number of anesthetic drugs, including barbiturates,⁴ ethanol,⁵ and benzodiazepines,⁶

have been shown to diminish depolarization-dependent Ca^{++} uptake into synaptosomal preparations. In contrast, it has been suggested that anesthetic drugs may cause an elevation in intracellular Ca^{++} concentrations, leading to activation of Ca^{++} -sensitive potassium and chloride conductances, resulting in reduced neuronal excitability.⁷ Also, it has been proposed that anesthetic drugs may act by increasing the sensitivity of endogenous Ca^{++} binding proteins in the absence of altered intracellular Ca^{++} concentrations.⁸

Chronic exposure to sedative/anesthetic drugs results in neuronal adaptation, which is expressed behaviorally as tolerance, i.e., diminished effect of a constant drug dosage, and physical dependence, i.e., hyperexcitability when the drug is withdrawn. This has been shown to occur with drugs with simple chemical structures, e.g., ethanol,⁹ bromide, and nitrous oxide,¹⁰ and also with drugs known to undergo specific receptor-mediated interactions, such as barbiturates,¹¹ benzodiazepines, and opiates.

We have previously reported¹² that calcium (Ca^{++}) antagonists increased the potency of a variety of structurally dissimilar anesthetic drugs, including ethanol, pentobarbital, midazolam, and the inert gas argon. In addition, we reported that Ca^{++} antagonists diminished or prevented the seizure component of the ethanol withdrawal syndrome.⁹ In further biochemical studies, we have demonstrated that increased dihydropyridine Ca^{++} channel number and function were associated with ethanol physical dependence,¹³ and proposed that these changes may be part of the mechanism underlying the development of tolerance and physical dependence. Concurrent administration of the Ca^{++} antagonist, nitrendipine, with ethanol, prevented the development of ethanol tolerance.

In this study, we have investigated the effects of the dihydropyridine Ca^{++} antagonist nitrendipine on nitrous oxide anesthesia, the development of tolerance to the anesthetic effects of nitrous oxide over 1 h, and the seizure component of the withdrawal syndrome seen after removal of mice from a nitrous oxide environment. The aim of these experiments was to determine whether or not the observed interactions between Ca^{++} antagonists and ethanol applied to other anesthetic drugs, such as nitrous oxide.

Materials and Methods

The methods for the experiments on nitrous oxide anesthesia, tolerance, and withdrawal were based on pre-

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viously reported studies on mice.¹⁴ Male T.O. mice were obtained specific pathogen free, provided with water and rat chow, and maintained in a light-cycled environment.

Nitrendipine (Bayer U. K. Ltd.) was dissolved in water and Tween 80 (0.5%) at 1, 5, and 10 mg · ml⁻¹, respectively. Three doses of nitrendipine were used: 10, 50, and 100 mg · kg⁻¹. Concurrently tested control animals received the vehicle injection only. Nitrendipine and vehicle injections were given by the intraperitoneal route. Nitrendipine was given either 15 min or 2 h prior to nitrous oxide. Two hours corresponded to the plateau of nitrendipine's anticonvulsant action and its effects on blood pressure, while 15 min approximated the peak of these actions.^{15,16} To assess the effects on the nitrous oxide withdrawal syndrome, nitrendipine was given 15 min before nitrous oxide administration. The effects of nitrendipine on tolerance development were assessed after this pretreatment time and after nitrendipine administration 2 h prior to nitrous oxide. Once injected, the mice were kept warm by the use of heating mats and overhead illumination, maintaining rectal temperatures between 36–37° C. Temperatures were monitored on some animals prior to nitrous oxide administration.

Nitrous oxide (B.O.C.) is a gas that produces full general anesthesia only at pressures greater than atmospheric. A 20-l steel pressure chamber was used to assess tolerance to nitrous oxide anesthesia. Physical dependence was determined by assessing withdrawal seizures following 1 h exposure to anesthetizing concentrations (pressures) of nitrous oxide.

Animals were placed in a four-division rotating cage, one mouse in each division. The cage could be rotated by remote control and the mice observed through a window in the pressure chamber. Mice from different groups, including concurrent controls, were placed in different divisions of the rotating cage, on a random basis. The mice were coded so that the treatment schedule of any individual mouse was unknown during the observation. Two mice, one control and the other treated with the highest dose of nitrendipine, 100 mg · kg⁻¹, were placed in a separate non-rotating cage within the pressure chamber. These two acted as temperature controls; each had a rectal thermistor temperature probe *in situ*. The rectal temperatures were maintained at 36–37° C during the course of the experiment, by adjusting the ambient temperature within the pressure chamber. This was achieved by the use of an internal water heating coil and an external electric coil around the chamber. An ambient temperature of 32–33° C was required within the chamber as nitrous oxide at anesthetic concentrations produced a lowering of the body temperature.

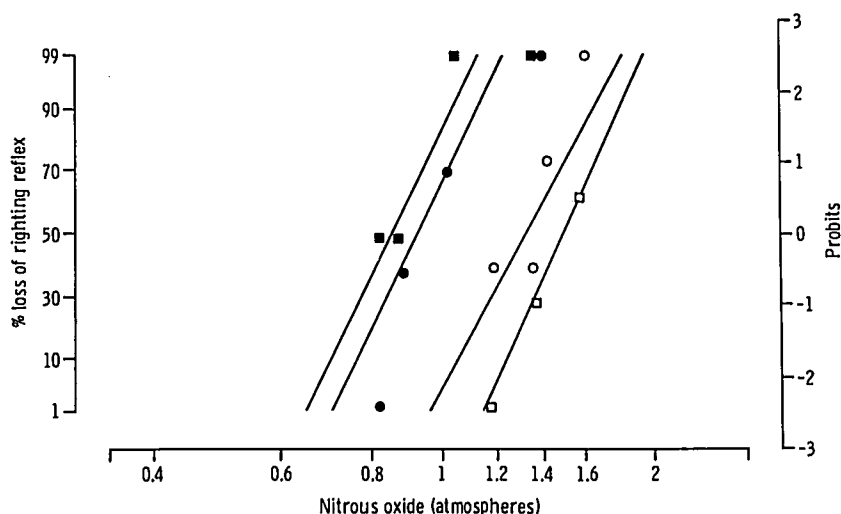
Once the animals were in the pressure chamber, 0.4 atmospheres of oxygen was added, so that oxygen would

not be depleted by six mice in the chamber over the 60 min that the experiment lasted. Miller *et al.*¹⁷ reported that oxygen concentrations between 0.2 and 2 atmospheres did not affect the anesthetic potencies of a variety of gases when measurements were made on three mice in a 200-ml pressure chamber, over 60 min. It was assumed that 0.4 atmospheres of oxygen in a much larger chamber would be more than adequate. Nitrous oxide was then added to a predetermined pressure between 0.5 and 1.5 atmospheres. Carbon dioxide and water produced by the mice were absorbed by fresh soda lime and silica gel, respectively, placed within the chamber in excess quantity as assessed by color changes at the end of each experiment. An electric fan was used to circulate all gases within the chamber throughout the experiment. This also ensured that soda lime and silica gel were well ventilated.

Loss of righting reflex was the behavioral endpoint used to determine the presence of anesthesia. This technique has been widely used for assessment of anesthesia in mice, and there has been good agreement among various papers for a variety of gaseous anesthetic agents.¹⁷ Each animal was rolled onto its back in the rotating cage and allowed 60 s to regain the upright posture. Failure to do so within the allotted time was taken as the criterion for anesthesia. Loss of righting reflex was assessed at 5, 15, 30, and 60 min at each pressure of nitrous oxide and for each dose of nitrendipine. Each treatment group at each pressure of nitrous oxide contained eight mice, and a minimum of four different pressures of nitrous oxide were used to construct each dose-response curve. Each group of mice was tested once only. Probit analysis was used to construct dose-response lines of loss of righting reflex against pressures of nitrous oxide. ED₅₀ values were derived from probit analysis. The effects of nitrendipine on nitrous oxide anesthetic potency were assessed by comparison of ED₅₀ values from nitrendipine treated with concurrent controls. Tolerance to the effects of nitrous oxide on loss of righting reflex was assessed by comparing ED₅₀ values at 60 min with those at 5 min, within each treatment and control group.

At the end of 60 min exposure to nitrous oxide, the chamber was decompressed and flooded with oxygen to prevent diffusion hypoxia,¹⁸ and mice were removed from the nitrous oxide environment. Mild withdrawal seizures were evoked by holding each mouse up by its tail. These could be reliably reproduced in control mice following a 1-h exposure to nitrous oxide at pressures of 1 atmosphere or greater. They were characterized by mild clonic seizures of several seconds duration. The mouse adopted a stereotypic pose consisting of crossed forepaws, splayed back legs, and ears pointed backwards. These seizures could be consistently elicited for up to 30 min after withdrawal from nitrous oxide. These nitrous oxide seizures

FIG. 1. Dose-response lines after 60 min exposure to nitrous oxide in mice, derived by probit analysis. Each point represents percent anesthetized (out of 10). Nitrendipine was injected 2 h prior to nitrous oxide administration; controls received vehicle injection. \square = Controls, \circ = nitrendipine $10 \text{ mg} \cdot \text{kg}^{-1}$, \bullet = nitrendipine $50 \text{ mg} \cdot \text{kg}^{-1}$, \blacksquare = nitrendipine $100 \text{ mg} \cdot \text{kg}^{-1}$.



were similar to those described by Goldstein¹⁹ as handling convulsions due to ethanol withdrawal in mice. In addition, an auditory stimulus (an electric bell, held above the cage for 45 s) was applied to the mice after they had been held by the tail to elicit handling seizures.

It is known that high pressure is itself a convulsant (at pressures of helium of around 100 atmospheres),^{20,21} and in addition that high pressure can potentiate ethanol withdrawal seizures.²² A separate control group was therefore used to assess the effects of pressure on nitrous oxide withdrawal seizures. This control consisted of groups of eight mice treated with the vehicle injection at the same time as the nitrous oxide groups. The pressure control group was placed in a separate pressure chamber, 0.4 atmospheres of oxygen was added, and helium was added to equal the pressure of nitrous oxide in the other chamber. Helium is a gas without anesthetic properties.²³ Temperatures were maintained in an identical way to the previous experiments. After 60 min of helium, the mice were removed from the pressure chamber and held up by the tail to determine whether any seizure activity could be elicited. The auditory stimulus was applied for 45 s in an attempt to elicit audiogenic seizures.

The results of the withdrawal experiments were compared as numbers in each group that demonstrated seizure activity, out of the total numbers. Statistical analysis was by Fisher's exact probability test.

Results

Dose-response lines were constructed by probit analysis, at 5, 15, 30, and 60 min of nitrous oxide exposure. Examples are shown in figure 1. The nitrous oxide dose-

response lines from those animals treated with nitrendipine were shifted to the left in a dose-dependent manner. The dose-response lines were parallel, and residual variation was consistent with a binomial distribution.

The ED_{50} values for loss of righting reflex was derived from the probit transformation of the dose-response curves, and was the endpoint used to measure tolerance to nitrous oxide anesthesia. ED_{50} values are shown in figures 2 and 3. Nitrendipine produced a dose-dependent increase in nitrous oxide anesthetic potency that occurred from the start of anesthesia and continued for the 1 h that the experiment lasted. The increase in anesthetic potency was statistically significant for those mice treated with the higher doses of nitrendipine 50 and $100 \text{ mg} \cdot \text{kg}^{-1}$. When nitrendipine was given 15 min prior to nitrous oxide, the effect on anesthetic potency increased steadily over the 60 min of observation, except for the lowest dose, $10 \text{ mg} \cdot \text{kg}^{-1}$ (fig. 2). When nitrendipine was given 2 h prior to nitrous oxide (fig. 3), the effects on anesthetic potency were stable over 60 min.

Tolerance to nitrous oxide anesthesia was assessed by comparing the ED_{50} values at 5 min with ED_{50} values at 60 min, both in control and nitrendipine-treated mice (table 1). In control mice, significant tolerance to nitrous oxide loss of righting reflex occurred between 5 and 60 min ($P < 0.05$). While nitrendipine decreased the ED_{50} for nitrous oxide anesthesia, it also prevented the development of tolerance to the anesthetic effects of nitrous oxide.

The results of the effects of nitrendipine on withdrawal seizures are shown in table 2. Nitrous oxide, at pressures greater than 1 atmosphere, produced a reliable withdrawal syndrome characterized by handling seizures. Nitrendipine given 15 min before the beginning of the ni-

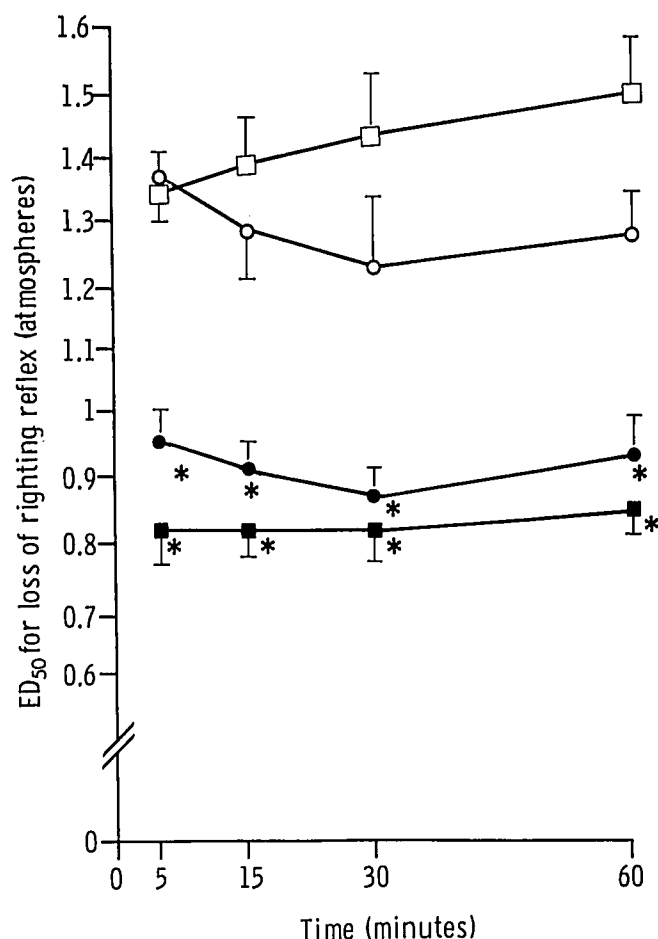


FIG. 2. Time course of the effects of nitrendipine on nitrous oxide anesthesia in mice, when nitrendipine was given 15 min prior to nitrous oxide. ED_{50} values for loss of righting reflex were obtained from dose-response lines (fig. 1). Control mice were given a vehicle injection. * $P < 0.05$, ** $P < 0.01$ compared with concurrent controls, Chi-squared analysis following probit analysis. □ = Controls, ○ = nitrendipine 10 $mg \cdot kg^{-1}$, ● = nitrendipine 50 $mg \cdot kg^{-1}$, ■ = nitrendipine 100 $mg \cdot kg^{-1}$.

trous oxide exposure reduced the incidence of withdrawal seizures in a dose-dependent manner. This achieved statistical significance at the higher doses, 50 and 100 $mg \cdot kg^{-1}$. Audiogenic seizures did not occur following removal of mice from the nitrous oxide environment.

As a control for pressure, the effects of 60 min exposure to helium were compared with nitrous oxide at the same pressures. The results are shown in table 3. Helium, 1.0 and 1.5 atmospheres, for 60 min, was not followed by any withdrawal seizures. Therefore, the effect of pressure alone did not contribute to the nitrous oxide withdrawal seizures.

Discussion

Tolerance can be defined as the diminution of the responses to a given concentration of drug after a period of administration of the drug. The drug effect examined in this paper was the anesthetic effect of nitrous oxide. Tolerance was assessed over 60 min and may be described as acute tolerance. The exact relationship between acute and chronic tolerance to sedative/hypnotic drugs is unclear, as is the relationship between tolerance and physical dependence. In the present work, the Ca^{++} antagonists have been used as a tool to investigate the role of voltage-operated Ca^{++} channels in tolerance to anesthetic drugs, and perhaps help to define some common ground between tolerance and physical dependence.

The methods for these experiments, designed to investigate the effects of Ca^{++} antagonists on rapidly developing tolerance to nitrous oxide, were based on studies on mice by Smith *et al.*^{10,24,25} Our results largely reproduced the results of Smith *et al.* in the control groups (*i.e.*, those not receiving nitrendipine). The lowest ED_{50} for loss of righting reflex was after 5 min of nitrous oxide, and peak was at 60 min. This is in slight contrast with

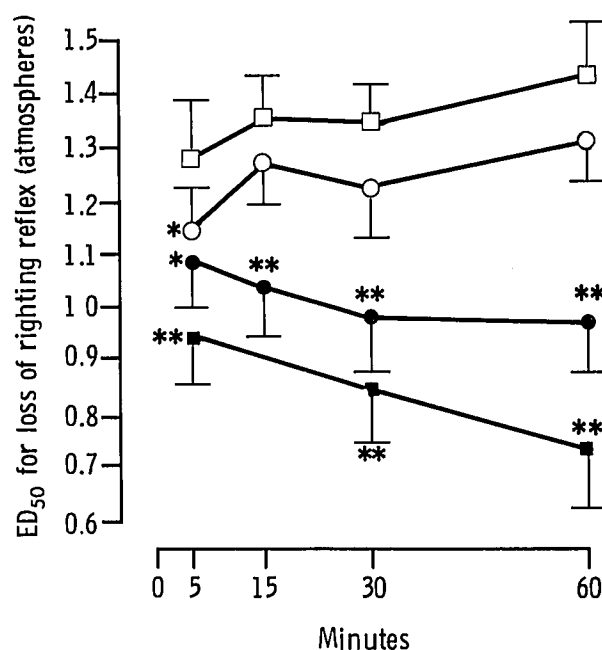


FIG. 3. Time course of the effects of nitrendipine on nitrous oxide anesthesia in mice, when nitrendipine was given 2 h prior to nitrous oxide. ED_{50} values for loss of righting reflex were obtained from dose-response lines (fig. 1). Control mice were given a vehicle injection. * $P < 0.05$ compared with concurrent controls, Chi-squared analysis following probit analysis. □ = Controls, ○ = nitrendipine 10 $mg \cdot kg^{-1}$, ● = nitrendipine 50 $mg \cdot kg^{-1}$, ■ = nitrendipine 100 $mg \cdot kg^{-1}$.

TABLE 1. Tolerance to Nitrous Oxide Anesthesia: ED₅₀ Values \pm SEM (Atmospheres) for Loss of Righting Reflex

Drug	5 Min	60 Min	% Difference
Control	1.35 (0.04)	1.51* (0.07)	+13
Nitrendipine 10 mg \cdot kg ⁻¹	1.36 (0.04)	1.31 (0.05)	-4
Nitrendipine 50 mg \cdot kg ⁻¹	0.97 (0.04)	0.94 (0.03)	-3
Nitrendipine 100 mg \cdot kg ⁻¹	0.83 (0.04)	0.85 (0.03)	+2

* $P < 0.05$, compared with results at 5 min. Dose-response lines were compared by Chi-squared analysis for position following probit analysis.

Smith *et al.*,²⁴ who found that ED₅₀ was at a maximum by 30 min, and did not increase thereafter. Also anesthetic requirements did not increase after 30 min.²⁶ However, the difference between ED₅₀ at 30 and 60 min in our results was not significantly different. The increase in ED₅₀ for loss of righting reflex caused by nitrous oxide occurring between 5 and 60 min was 13% ($P < 0.05$), which compared favorably with results from Smith *et al.*,²⁴ who reported 22% increase in ED₅₀ between 5 and 60 min. Smith *et al.*^{23,24} used a different strain of mice from those in the present results.

Several different doses of a single Ca⁺⁺ antagonist were used in these experiments. Nitrendipine was chosen because we found previously that it had a greater effect on anesthesia and withdrawal seizures, at the doses used, than other Ca⁺⁺ antagonists we tested.^{9,12} Two pretreatment times were chosen: 15 min and 2 h before administration of anesthetic, to correspond with peak and plateau anti-convulsant effects of nitrendipine.¹⁶

When nitrendipine was given 15 min before nitrous oxide, the ED₅₀ fell progressively over 60 min after the two higher doses of nitrendipine (50 and 100 mg \cdot kg⁻¹). This suggested that the effects of nitrendipine, in potentiating nitrous oxide anesthesia, had not reached a plateau. When nitrendipine was given 2 h before nitrous oxide, the potency of the anesthetic was stable over 60 min. In both experiments, nitrendipine, 50 and 100 mg \cdot kg⁻¹, significantly potentiated nitrous oxide anesthesia, as expected from previous results using different anesthetics.¹² The potentiation of nitrous oxide anesthesia by nitrendipine, 100 mg \cdot kg⁻¹, was in the order of 38–42%, which was similar to that produced by ethanol, argon, and pentobarbital.¹²

Comparison of the ED₅₀ values at 5 and 60 min after nitrous oxide administration demonstrated development

TABLE 2. Nitrous Oxide Withdrawal Seizures (Incidence as Numbers, Out of Total, Showing Handling Seizures)

Drug	Nitrous Oxide (Atmospheres)			
	1.0	1.22	1.36	1.5
Control	8/8	8/8	8/8	7/7
Nitrendipine 10 mg \cdot kg ⁻¹	7/8	6/8	5/8	7/7
Nitrendipine 50 mg \cdot kg ⁻¹	0/8*	2/8*	1/8*	0/8*
Nitrendipine 100 mg \cdot kg ⁻¹	0/8*	0/8*	0/8*	0/8*

* $P < 0.05$, Fisher's exact probability test, compared with concurrently tested controls.

of tolerance; a higher ED₅₀ value at 60 min indicated tolerance. The pattern and degree of tolerance (+13%, $P < 0.05$) was the same in both groups that were given nitrous oxide alone. While the control ED₅₀ value rose steadily over the period of nitrous oxide administration, the presence of nitrendipine, at all doses, prevented the development of tolerance to nitrous oxide anesthesia. It is interesting to note that, at 2 h, nitrendipine 10 mg \cdot kg⁻¹ prevented the development of tolerance to nitrous oxide, but did not potentiate anesthesia, suggesting that the dihydropyridine-sensitive Ca⁺⁺ channels may be more important to the development of tolerance and physical dependence than to the mechanism of anesthesia.

For several reasons, pharmacokinetic considerations were not important in the interactions of nitrendipine with nitrous oxide. First, nitrous oxide is not metabolized,²⁷ and, second, these experiments were conducted at equilibrium, because nitrous oxide is very insoluble and reaches equilibrium in brain in less than 10 min.²⁶ Furthermore, nitrendipine alone does not cause anesthesia. In previous experiments, we showed that, at doses up to 1 g \cdot kg⁻¹, nitrendipine did not result in loss of righting reflex.¹² Nitrendipine produces vasodilatation, an effect

TABLE 3. The Effects of Pressure on Withdrawal Seizures (the Incidence of Withdrawal Seizures, as Numbers Out of the Total, Showing Handling Seizures)

Drug	Pressure (Atmospheres) of Each Gas	
	1.0	1.5
Nitrous oxide	8/8	7/7
Helium	0/8*	0/8*

* $P < 0.05$, Fisher's exact probability test, compared with results for nitrous oxide.

that must be considered as a possible explanation of these data. This explanation was, in part, excluded by a previous study¹⁵ in which a non-calcium antagonist vasodilator was shown not to affect ethanol tolerance, in contrast to nitrendipine. The question of whether or not calcium antagonists were producing their effects *via* neuronal calcium channels, rather than some other mechanism, was addressed by a study on ethanol withdrawal using stereoisomers of the calcium antagonist PN200-110.²⁸ The isomer with proven calcium antagonist properties was effective against ethanol withdrawal seizures, while the other isomer without calcium antagonist properties was ineffective.

The results from our studies suggest that voltage-dependent Ca^{++} channels may be involved in tolerance and dependence. While Ca^{++} uptake through voltage-dependent Ca^{++} channels has been shown to be decreased by anesthetic drugs, including pentobarbital and ethanol, tolerance develops to this effect after chronic drug exposure,^{5,6} in parallel with increased *in vivo* ED_{50} for loss of righting reflex. In contrast to these findings, Ca^{++} antagonists do not appear to affect Ca^{++} uptake *via* voltage-dependent channels in neuronal preparations under normal circumstances,^{29,30} in contrast to effects on vascular tissue. However, concurrent administration of nitrendipine prevented nitrous oxide tolerance, and dependence, as was seen with our previous results on ethanol tolerance.¹⁵ Just as the presence of anesthetic agents seemed to allow Ca^{++} antagonists to produce depressant effects on the CNS,¹² it appears that, in the presence of the "adapted" membrane, calcium antagonists may be permitted to act. One possibility is that the Ca^{++} channels have been altered, by the structural membrane adaptation to a configurational state that is favorable to binding by Ca^{++} antagonists,³¹ thereby allowing the Ca^{++} antagonists to inhibit Ca^{++} entry with resultant decrease in transmitter release. We have reported that, following chronic exposure to ethanol over a period of 7–10 days, there was an increase in the numbers of dihydropyridine binding sites in rat cerebral cortex.^{13,15} These binding sites have been shown to be in close functional relationship to one species of voltage-operated Ca^{++} channels in neurons, the "L" type calcium channel.^{32,33} An increase in the numbers of voltage-dependent Ca^{++} channels appears to be causally related to ethanol tolerance as concurrent treatment of ethanol and nitrendipine prevented ethanol tolerance,¹⁵ and also prevented the increase in Ca^{++} channel density. It is entirely possible that the same process occurs with short exposures to anesthetic drugs, such as nitrous oxide.

These results indicate that nitrous oxide tolerance and dependence are affected by the dihydropyridine Ca^{++} antagonist, nitrendipine. Taken in conjunction with our

previous results, it appears that acute tolerance seen with nitrous oxide, and chronic tolerance seen with ethanol, are both affected by Ca^{++} antagonists. This indicates that acute and chronic tolerance may be part of a continuum. In addition, as both nitrous oxide tolerance and dependence were prevented by nitrendipine, at the higher doses used, these two phenomena may share a common mechanistic pathway *via* neuronal voltage-sensitive Ca^{++} channels.

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