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Pentobarbital Tolerance and Withdrawal: Correlation With Effects on the $GABA_A$ Receptor

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SAUNDERS, P. A., Y. ITO, M. L. BAKER, A. S. HUME AND I. K. HO. *Pentobarbital tolerance and withdrawal: Correlation with effects on the $GABA_A$ receptor.* PHARMACOL BIOCHEM BEHAV **37**(2) 343–348, 1990.—A model for the development of pentobarbital tolerance and dependence was characterized and correlated with changes in radioligand binding to the $GABA_A$-benzodiazepine receptor chloride channel complex. While one day of pentobarbital exposure decreased the duration of loss of righting reflex, tolerance to the hypothermic effects of thiopental and barbital took 7 days to develop, indicating that pharmacokinetic and pharmacodynamic tolerance are separable. Increased sensitivity to pentylenetetrazol-induced seizures was first observed after 3 days of pentobarbital exposure, suggesting brain areas involved in seizure control develop tolerance to, and dependence on pentobarbital faster than those involved in temperature regulation. Acute exposure to pentobarbital in vivo did not affect cortical binding of [^{3}H]muscimol in vitro, while tolerance caused a decrease in binding due to an increase in the low-affinity site K_D. Pentobarbital tolerance also caused a decrease in the cortical binding of the benzodiazepine, [^{3}H]flunitrazepam. These observations suggest that the acute effects of barbiturates on the $GABA_A$ receptor complex are reversible, while tolerance causes receptor modifications which may be related to the development of physical dependence.

Barbiturate Tolerance Dependence $GABA_A$ receptor Hypothermia Seizure

ALTHOUGH the clinical symptoms of barbiturate tolerance and withdrawal have been well documented since the early 1950's (8), relatively little is known about the mechanisms which cause them. A great deal of evidence in the past few years has suggested that the pharmacologic effects of barbiturates may be related to an enhancement of the activity of a gamma-aminobutyric acid (GABA) receptor-activated chloride channel (19). This membrane-bound protein complex has also been shown to have binding sites for benzodiazepines and chloride channel-blocking convulsants such as picrotoxin (16). Our laboratory has previously shown that barbiturate dependence causes parallel changes in seizure sensitivity and [^{35}S]TBPS binding to the convulsant site (10), suggesting a correlation between changes in the $GABA_A$ receptor and the symptoms of barbiturate withdrawal. The effects of barbiturate tolerance on the GABA binding site, however, have not been consistently reported. Increases in GABA binding (18), no changes in GABA binding (20), and decreases in GABA binding (14) have been published. This inconsistency may be due to differences in treatment protocols and assay methods. The following experiments were undertaken to determine if changes in $GABA_A$ receptor binding take place in an animal model rigorously proven to produce pentobarbital tolerance and withdrawal.

METHOD

Assessment of Pentobarbital Tolerance and Withdrawal

Male Sprague-Dawley rats weighing 225 to 250 grams at the beginning of the experiment were obtained from Charles River, Wilmington, MA and housed in the animal quarters on a 12-hour light, 12-hour dark photoperiod with food and water ad lib for a week before use. The rats were then implanted with pellets subcutaneously by methods similar to those previously described (4). Two pellets, each containing either 75 mg of pentobarbital free acid or the placebo excipient, were implanted every other day for up to 7 days. Serum pentobarbital levels from rats sacrificed by decapitation were determined by HPLC with a Fischerbrand Resolvex C8 column, an acetonitrile:1 M potassium phosphate: water 4.93:3:1.8 mobile phase, pH 4.4, and an internal standard of aprobarbital. The lowest level of pentobarbital detected by the ultraviolet spectrophotometer on the HPLC was 0.4 μg/ml. The implanted pellets were also removed from the decapitated rats for determination of residual pentobarbital. The pellets were homogenized in 0.1 N NaOH and the insoluble material removed by passage through a Whatman No. 1 filter. The filtrate was diluted 1:100 with 0.01 N NaOH and a further 1:10 dilution was made

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into either 0.01 N NaOH or 0.1 N HCl. Using a Cary 219 dual beam spectrophotometer (Varian, Palo Alto, CA) with a path length of 1 cm, the difference in absorbance between the pentobarbital in the acid and base solutions was measured at 240 nm and the baseline absorbance was defined as the absorbance at 300 nm. The ultraviolet measurements were able to detect a minimum of one microgram of pentobarbital per ml solution.

To test for barbiturate tolerance, rats implanted with placebo and pentobarbital pellets for up to seven days were injected intraperitoneally with either sodium pentobarbital (60 mg/kg), sodium thiopental (45 mg/kg), or sodium barbital (200 mg/kg) dissolved in distilled water. The duration of loss of righting reflex was defined as the interval between the time the rats lost and regained the ability to spontaneously right themselves. Body temperature was measured with a digital rectal thermometer (Sensortek, Clifton, NJ). The ambient temperature in the room in which the body temperature measurements were made was maintained at 22 to 25°C. During the pellet implantation regimen, basal body temperature was measured on a daily basis in the morning between 8:30 and 10:00 a.m. The basal body temperatures in the placebo- and pentobarbital pellet-implanted groups were not significantly different from each other (data not shown).

In experiments in which barbiturate dependence was to be assessed, pentobarbital and placebo pellets were removed under lidocaine local anesthesia 24 hours before their use as dependent and placebo + sham groups, respectively. The pellets were wrapped in a piece of nylon stocking before implantation to facilitate their removal. To test for physical dependence, the rats were injected subcutaneously with 50 mg/kg of pentylenetetrazol and the onset of first twitch and convulsions was determined.

Membrane Preparation and Binding Assays

To prepare brain membrane fractions for binding assays, rats were treated with placebo and pentobarbital pellets as described above. The animals were sacrificed by decapitation, the brains were rapidly removed from the skull, and the frontal cortex was dissected as described by Glowinski and Iversen (6). In experiments where the comparison of control, acute pentobarbital exposure, and pentobarbital tolerance were to be performed, placebo-implanted rats were injected intraperitoneally with 60 mg/kg of sodium pentobarbital for one hour (the acute group) or the distilled water vehicle (the control group), and a pentobarbital pellet-implanted group received the water vehicle (the pentobarbital-tolerant group). All membrane preparation steps were performed at 4°C as previously described (9). The brain tissues were homogenized in 15 volumes of 0.32 M sucrose for 1 minute and centrifuged at $1,000 \times g$ for 10 minutes. The supernatant was decanted and centrifuged at $20,000 \times g$ for 20 minutes. The supernatant was discarded and the remaining pellet was homogenized for 15 seconds in 40 volumes of ice-chilled distilled water and centrifuged at $8,000 \times g$ for 20 minutes. The supernatant and the soft upper buffy layer of the pellet were combined and centrifuged at $48,000 \times g$ for 20 minutes. The supernatant was discarded and the pellet was homogenized 15 seconds in 40 volumes of 50 mM Tris-citrate buffer, pH 7.1, and was centrifuged at $48,000 \times g$ for 20 minutes. The pellet obtained was then homogenized for 15 seconds in 10 volumes of Tris-citrate buffer and frozen at −70°C for at least 48 hours.

On the day a binding assay was to be performed, the membranes were thawed, diluted to 40 volumes with Tris-citrate buffer, homogenized for 15 seconds, and centrifuged at $48,000 \times g$ for 20 min. The pellet was homogenized for 15 seconds in 40 volumes of Tris-citrate buffer and incubated for 30 min at 25°C. The incubated membranes were then centrifuged at $48,000 \times g$ for 20 min, and the pellets were homogenized for 15 seconds in enough Tris-citrate buffer to give a protein concentration of about 1 mg/ml. Two hundred micrograms of membrane protein were incubated in 1 ml of 50 mM Tris-citrate buffer, pH 7.1, at 0°C for 30 and 60 min with 10 or 100 nM [^{3}H]muscimol or 1 nM [^{3}H]flunitrazepam, respectively. For Scatchard plots of [^{3}H]muscimol binding, the muscimol concentrations used were from 0.5 to 200 nM. Concentrations of [^{3}H]muscimol greater than 25 nM were obtained by dilution of the specific activity of the radioligand with nonradioactive muscimol. Nonspecific binding was defined as binding in the presence of 1 mM GABA and 1 μM clonazepam for [^{3}H]muscimol and [^{3}H]flunitrazepam, respectively. Specific binding was 96% of total for [^{3}H]flunitrazepam binding and 90% of total for [^{3}H]muscimol binding. The incubations were terminated by aspirating the reaction mixtures through GF/B filters using a Brandel cell harvester model M24R (Gaithersburg, MD). The radioactivity trapped on the filters was washed twice with 5 ml of buffer. Scatchard plots of [^{3}H]muscimol were fitted to a two binding site model using an iterative curve fitting program written for IBM PC compatible computers (13). Protein was determined by the method of Lowry *et al.* (12), and the number of binding sites was expressed as pm/mg protein.

Statistics

For comparisons of the duration of righting reflex induced by pentobarbital, two-way analysis of variance (ANOVA) was used. The durations of thiopental- and barbital-induced loss of righting reflex were compared using the Mann-Whitney U-test. The onset of first twitch, and convulsions were compared using the Mann-Whitney U-test. Two-way analysis of variance was applied to binding data, followed by Student's *t*-test for unpaired groups. The barbiturate-induced hypothermia at each time point was compared by Student's *t*-test.

RESULTS

Figure 1 illustrates the time course of serum pentobarbital levels and the amount of residual pentobarbital left in the implanted pellets. The highest levels determined were at 6 hours after the implantation of a single pair of pellets. The serum pentobarbital levels at 1, 3 and 7 days of pentobarbital exposure were not significantly different from each other. In the 3-day pentobarbital pellet-implanted group, wrapping the pellets did not make a significant difference in the serum pentobarbital levels (data not shown). Approximately 50% of the pentobarbital in the pellets was remaining after 2 days. After 3 days of pellet implantation, the wrapped pellets contained the same amount of residual pentobarbital as after 2 days of implantation, indicating that no further pentobarbital is released after 2 days of implantation.

Treatment of rats with pentobarbital pellets for as short as one day significantly reduced the duration of loss of righting reflex in rats given an acute dose of pentobarbital (Fig. 2). The plasma pentobarbital levels on regaining righting reflex were 11.29 ± 1.05 μg/ml and 11.37 ± 1.26 μg/ml in the 3- and 7-day placebo-implanted groups and 11.43 ± 0.65 μg/ml and 8.95 ± 0.90 μg/ml in the 3- and 7-day pentobarbital-implanted groups, respectively, and were not significantly different from each other. This indicates that the primary determining factor in the shorter duration of loss of righting reflex was the rate of metabolism and clearance.

In order to avoid issues of pharmacokinetics in our experiments, two barbiturates with special characteristics, barbital and thiopental, were used as challenge drugs to test for tolerance. Although low in potency, barbital is excreted unchanged (3). Thiopental is a thiobarbiturate analogue of pentobarbital. Due to

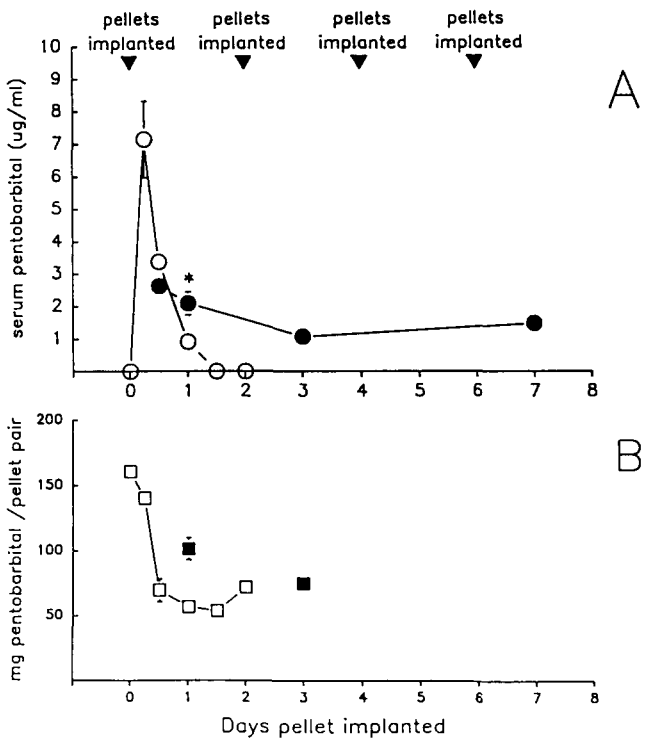


FIG. 1. Serum pentobarbital levels with pellet implantation and residual pentobarbital in the pellet. Rats were implanted with pentobarbital pellets every other day, as marked by downward triangles. Trunk blood was collected on decapitation and allowed to clot for the separation of serum. The pellets were excised for analysis as well. The serum and pellets were frozen for later analysis by HPLC and UV methods, respectively. (A) Serum pentobarbital levels: open circles, after a single implantation (n=7), solid circles, levels after multiple implantations (n=8 to 10). *Indicates that at 24 hours the single and multiple implantation groups had different serum pentobarbital levels (*t*-test, $p<0.05$). (B) Residual pellet levels: open squares, unwrapped pellets, single implantation (n=7), solid squares wrapped pellets, multiple implantations (n=4).

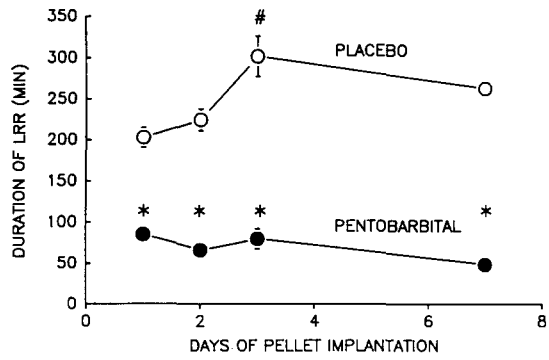


FIG. 2. Effect of pellet implantations on the duration of loss of righting reflex. Rats were implanted with two pellets containing either 75 mg of pentobarbital free acid each or the placebo every other day for up to 7 days, followed by an acute injection of sodium pentobarbital, 60 mg/kg, IP. The duration of loss of righting reflex was defined as the time from when the rat lost righting reflex to when it regained the ability to right itself spontaneously. The data were analyzed by a two-way analysis of variance, which showed a significant effect of pentobarbital vs. placebo on the duration of loss of righting reflex and an interaction with the duration of implantation. #Indicates that 3 days of treatment was different from 1 and 7 days of treatment by Bonferroni's post hoc test at $p<0.05$. *Indicates significantly different from placebo at $p<0.05$ from the pentobarbital group by one-way analysis of variance for each duration of pellet implantation. N=5 to 7 for all groups.

its high lipid solubility, an acute injection of thiopental rapidly produces high drug levels in well perfused lipid containing tissues such as the brain, followed by later redistribution to the rest of the body. Although thiopental is a metabolizable barbiturate, the initial high levels of thiopental have been observed to be relatively independent of the rate of metabolic clearance (2). Table 1 shows that pentobarbital tolerance decreased the duration of righting reflex of rats acutely injected with thiopental but not barbital.

Both thiopental and barbital produced a decrease in rectal temperature (Fig. 3). In both the thiopental- and the barbital-injected animals, there was no significant difference in the time required to reach the lowest body temperature between the placebo- and pentobarbital-tolerant groups. However, there was a significant decrease in the degree of thiopental- and barbital-induced hypothermia after 7 but not 3 days of pentobarbital pellet implantation. The degree of tolerance to barbiturate-induced hypothermia with 7 days of pentobarbital tolerance was strikingly similar between the thiopental- and barbital-challenged groups (Fig. 4).

Twenty-four hours after the pellets were removed, both 3- and 7-day dependent rats had a significantly shorter onset of both first twitch and convulsions induced by pentylenetetrazol (Fig. 5). The latency of pentylenetetrazol-induced seizures decreased with the duration of pentobarbital exposure.

Scatchard plots of [^{3}H]muscimol binding to frontal cortical membranes (Table 2) showed that acute pentobarbital treatment in vivo had no effect on in vitro binding. In contrast, 3 days of pentobarbital pellet implantation caused an 85% increase in the low affinity K_D without changing B_{max}. In single concentration assays (Fig. 6) which do not distinguish between changes in K_D and B_{max}, 2-way ANOVA indicated that there was no significant effect of pentobarbital treatment on high-affinity [^{3}H]muscimol

TABLE 1

EFFECT OF PENTOBARBITAL TOLERANCE ON THE DURATION OF LOSS OF RIGHTING REFLEX

	Duration of Loss of Righting Reflex (min)	
	Thiopental Challenged	Barbital Challenged
3-Day placebo	156.23 (10/10) $p<0.05$	441.66 (7/7) (NS)
3-Day pentobarbital	110.23 (7/10)	472.66 (7/7)
7-Day placebo	186.6 (7/7) $p<0.01$	394.3 (8/8) (NS)
7-Day pentobarbital	118.87 (3/7)	455.4 (8/8)

Numbers in parentheses = number losing righting reflex/number of animals injected.

Probabilities determined by Mann-Whitney U-test with animals failing to lose righting reflex given a value of zero. The averages reflect only those animals which lost righting reflex. (NS) not significant.

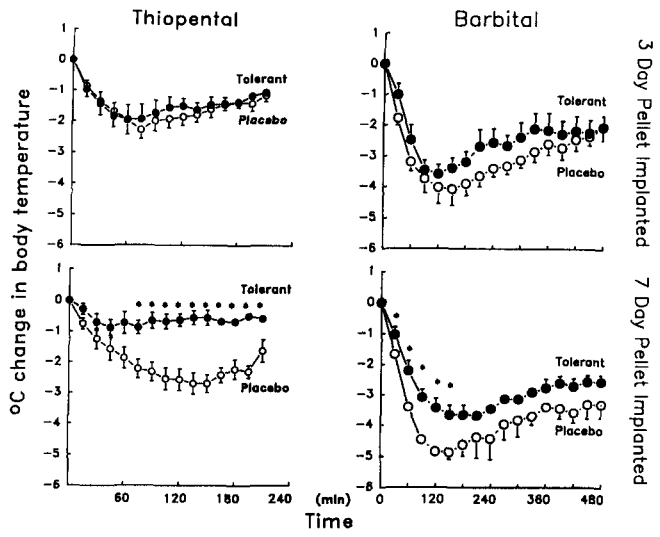


FIG. 3. Effect of pentobarbital tolerance on barbiturate-induced hypothermia. Rats implanted with pentobarbital or placebo pellets for 3 or 7 days were acutely injected with either 45 mg/kg of sodium thiopental or 200 mg/kg of sodium barbital. The change in body temperature from the preinjection basal level was measured over time and *indicates a significant difference from placebo at $p<0.05$ by unpaired t-test. N=6 for thiopental groups and N=4 for barbital groups.

binding to cortical membranes. For the low-affinity [^{3}H]muscimol binding, there were significant effects of both pentobarbital pellet implantation ($p<0.01$), duration of implantation ($p<0.01$), and an interaction between pentobarbital implantation and duration of implantation ($p<0.05$). Student's t-tests indicated that both 3 and 7 days of pentobarbital pellet implantation caused significant decreases in [^{3}H]muscimol binding. By solving the equilibrium binding equations at a [^{3}H]muscimol concentration of 10 nM, the concentration used to measure high-affinity binding, the radioligand bound to high- and low-affinity sites, was calculated. It was determined that approximately 29% of the specific binding was

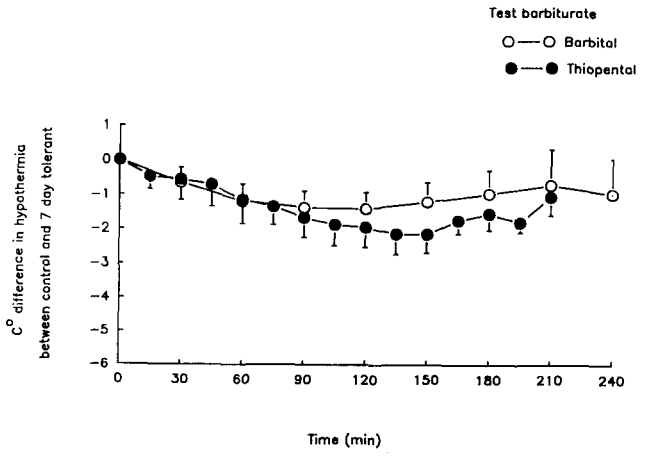
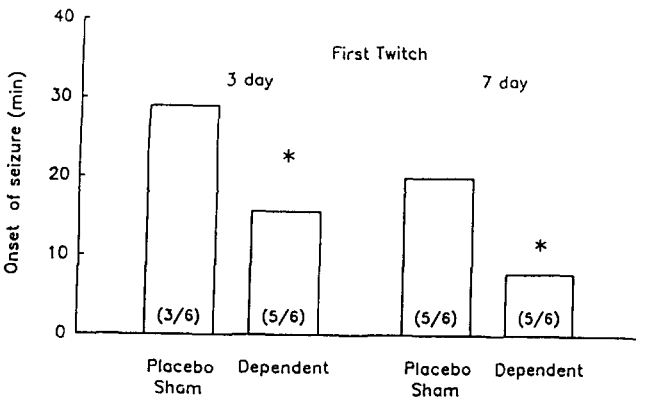


FIG. 4. Difference in degree of hypothermia caused by 7 days of pentobarbital pellet implantation. The difference in sodium thiopental- and sodium barbital-induced hypothermia from Fig. 3. Standard error bars represent the pooled standard error of the placebo and tolerant groups for each drug injected.



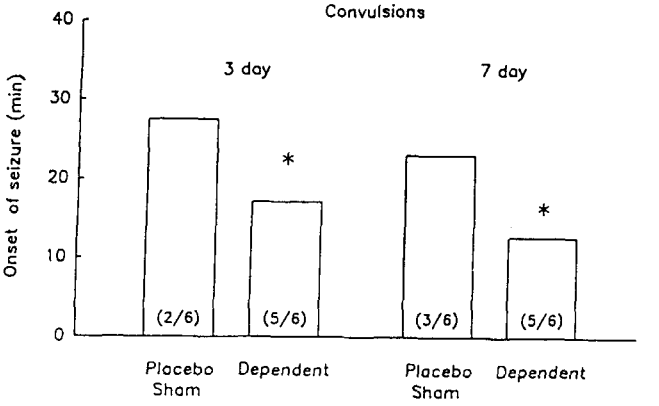


FIG. 5. Effect of pentobarbital dependence on pentylenetetrazol-induced seizures. After 3 or 7 days of placebo or pentobarbital pellet implantation, the pellets were removed under local lidocaine anesthesia. One day later, the rats were injected with 50 mg/kg of pentylenetetrazol and the onset of first twitch and convulsions observed. *Represents $p<0.05$ by Mann-Whitney U-test. Numbers in parentheses = number of animals with a seizure/number of animals injected.

due to low-affinity binding in the placebo control group. The increased low affinity site K_D with 3 days of pentobarbital exposure was not enough to cause a detectable change in specific binding. A further increase in the K_D of the low-affinity binding site, e.g., to 200 nM or greater, may be responsible for the significant decrease in specific binding with 10 nM of [^{3}H]muscimol observed with 7 days of pentobarbital exposure (Fig. 6).

Pentobarbital exposure caused significant changes in cortical [^{3}H]flunitrazepam binding (Fig. 7). Two-way ANOVA indicated that there were significant effects of both pentobarbital pellet implantation ($p<0.01$), duration of implantation ($p<0.01$), and an interaction between pentobarbital implantation and duration of implantation ($p<0.01$). Student's t-tests indicated that both 3 and 7 days of pentobarbital exposure caused significant decreases in [^{3}H]flunitrazepam binding.

DISCUSSION

One of the major issues in developing a model of barbiturate

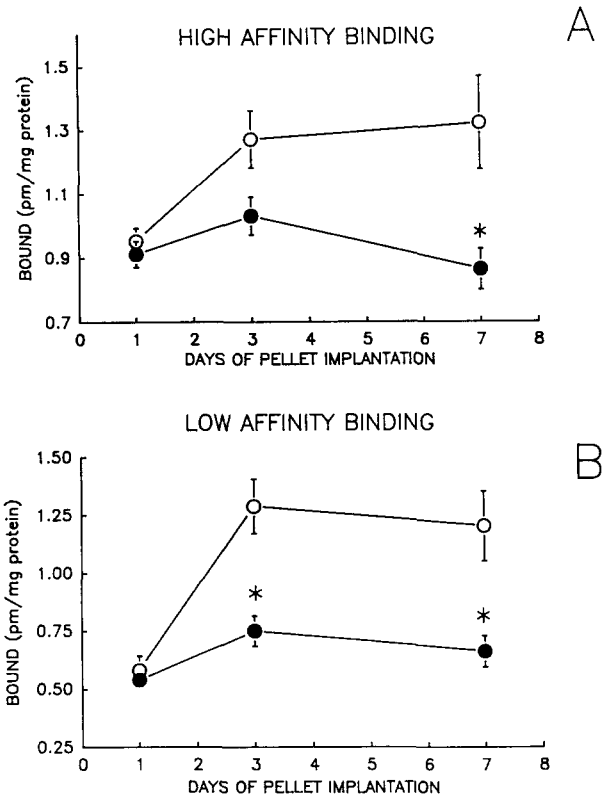


FIG. 6. Binding of [^{3}H]muscimol in cortical membranes from placebo- and pentobarbital-implanted animals. (A) [^{3}H]Muscimol binding to rat cortical membranes. High-affinity GABA sites as determined using 10 nM [^{3}H]muscimol. (B) Low-affinity binding as determined using 100 nM [^{3}H]muscimol and after subtracting out the high-affinity binding at 10 nM. Open circles represent the placebo group, and solid circles denote the pentobarbital-implanted group. *Indicates $p<0.05$ by grouped t-test. N = 4 for all points.

tolerance is separating the development of pharmacokinetic tolerance, due to increased clearance rates, from pharmacodynamic tolerance, due to changes in the central nervous system. In our experiments, it was clearly shown that the two phenomena are separable. Reduced duration of loss of righting reflex began one day after pellet implantation was initiated. Since serum levels of pentobarbital on regaining righting reflex in the pentobarbital-pelleted group were not significantly different from the placebo-implanted group, this was pharmacokinetic tolerance, due to

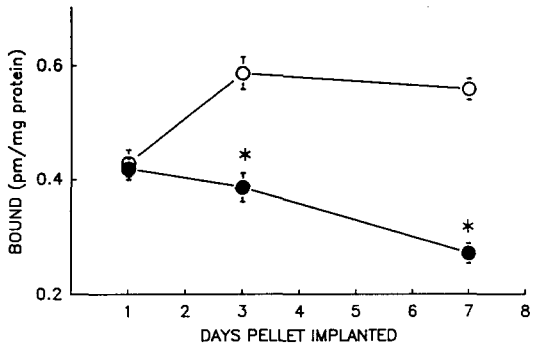


FIG. 7. Binding of [^{3}H]flunitrazepam in cortical membranes from placebo- and pentobarbital-implanted animals. Binding of 1 nM [^{3}H]flunitrazepam to rat cortical membranes. Open circles represent the placebo group, and solid circles denote the pentobarbital-implanted group. *Indicates $p<0.05$ by grouped t-test. N = 4 for all points.

increased clearance. This is consistent with our previous data in mice (1).

The reduction in sensitivity of the rats to the hypothermic effects of thiopental and barbital took 7 days to develop, much longer than the effects on loss of righting reflex. Whereas thiopental is a metabolizable barbiturate, barbital is excreted essentially unchanged, and its distribution pharmacokinetics have been previously reported to be unaffected by barbiturate tolerance (3). Since the time to maximal thiopental- and barbital-induced hypothermia was unchanged by pentobarbital tolerance, it can be argued that pharmacokinetics other than metabolism do not play an important role in the tolerance to the hypothermic effects of the barbiturates. The tolerance must therefore be due to a change at the site of barbiturate action, the central nervous system. Despite the difference in their potency, barbital and thiopental produced the same degree of tolerance to hypothermia in the 7-day pentobarbital-exposed group (Fig. 4), indicating that the tolerance was due to the same mechanism. Although the depth of barbital-induced hypothermia was reduced in the pentobarbital-tolerant group, the hypothermia still present in the tolerant group was much deeper with barbital than with thiopental. Although the lower lipid solubility may cause barbital to remain in the brain longer, it is also possible that barbital also produces some of its effects by mechanisms not held in common with the more potent thiopental and pentobarbital. Barbital has been observed to have greater cross-tolerance to ethanol-induced intoxication than pentobarbital (7), suggesting that barbital may have general depressant effects.

Since increased sensitivity to convulsants took less time to develop (3 days) than did tolerance to hypothermia (7 days), it is

TABLE 2

EFFECTS OF ACUTE PENTOBARBITAL AND 3 DAYS OF PENTOBARBITAL TOLERANCE ON [^{3}H]MUSCIMOL BINDING IN THE RAT CEREBRAL CORTEX

	K_{DH} (nM)	B_{maxH} (pm/mg)	K_{DL} (nM)	B_{maxL} (pm/mg)
Control	3.94 ± 0.65	1.00 ± 0.23	93.92 ± 14.84	3.17 ± 0.21
Acute	4.14 ± 0.12	1.20 ± 0.13	99.87 ± 17.14	3.50 ± 0.40
Tolerant	4.61 ± 0.33	1.29 ± 0.07	173.91 ± 4.41*	3.79 ± 0.54

N = 4 for all groups.
*$p<0.01$ compared to placebo control.

possible that the areas involved in regulating the spread of seizures develop tolerance to barbiturates at a faster rate than those involved in temperature control. To our knowledge, this is the first report of differential sensitivity of the brain to barbiturates in an animal model. The increased seizure sensitivity correlates with increased [^{35}S]TBPS binding to the convulsant site on the GABA-benzodiazepine receptor chloride channel complex, including in the substantia nigra (10), an area of the brain where $GABA_A$ receptors are believed to be involved in the control of the spread of seizures (5). This indicates a possible involvement of the GABA-benzodiazepine receptor chloride channel complex in the development of physical dependence on barbiturates.

The lack of an effect of acute in vivo exposure to pentobarbital on [^{3}H]muscimol binding in vitro suggests that the mechanisms involved are reversible by the washing procedure used in the membrane preparation and are allosteric. Incomplete removal of residual barbiturates in membrane preparation procedures may explain why increases (18,20) in GABA receptor binding have been previously reported. The effects of tolerance and dependence on [^{3}H]muscimol, [^{3}H]flunitrazepam, and [^{35}S]TBPS (10) binding are not reversible by the washing procedure, suggesting that a change in the properties of the receptor has taken place. Each of the binding sites on the receptor-channel complex exhibits a different type of change in tolerance or dependence: decreased GABA ligand affinity, decreased number of benzodiazepine binding sites (11), and increased convulsant binding (10). It is therefore likely that fundamental changes in the properties of the receptor-channel complex have taken place. The increased K_D of the low-affinity [^{3}H]muscimol binding to the GABA receptor we observed with 3 days of pentobarbital tolerance has also been reported with barbital abstinence (15). This suggests that changes in the $GABA_A$ receptor complex in barbiturate tolerance are adaptive in nature and withdrawal symptoms are due to a lag in the ability of the nervous system to return to its normal state. Both posttranslational modification, such as phosphorylation (21), and receptor subtypes with different properties (17) have been suggested to play a role in regulating the activity of the $GABA_A$ receptor complex. Whether changes in the receptor protein or its modification are involved in the mechanism of barbiturate tolerance is currently unknown. Research in our laboratory is currently under way to address these questions.

ACKNOWLEDGEMENTS

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