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Binding of [^{14}C]thiopental to rat cerebrocortical membranes

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The relationship between anesthetic potency and lipid solubility suggests a unitary mechanism of general anesthetic action [1]. However, strong evidence that general anesthetics may act by binding directly to proteins (ion channels, receptors) but not to lipid bilayers has been reported since the late 1980s [2]. Recent papers [2–4] suggest that the γ-aminobutyric acid $(GABA)_A$ receptor might represent another "unitary site" for anesthetic action. Clinically relevant concentrations of several anesthetic agents produce a stereoselective potentiation of Cl^- flux through the $GABA_A$ receptor. In addition, the existence of a barbiturate-binding site on the $GABA_A$ receptor [2,5,6] has been proposed. In this study, we examined the binding of a radiolabeled barbiturate, [^{14}C]thiopental, to rat cerebrocortical membranes to determine whether a specific anesthetic-binding site exists in the brain.

On each experiment day, three female Wistar rats (250–300 g) were killed by cervical dislocation and decapitation. The brain was removed rapidly, and the cerebrocortex was detached from its internal structures and then homogenized at 4°C using a tissue Tearor (setting 5.5 × 30-s bursts) in 50 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 18000 × *g* for 10 min and the pellet was resuspended in Tris-HCl buffer. This procedure was repeated three times [7]. These fresh crude membranes were made immediately prior to use every day. A total of 21 rats were used to complete all experiments.

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The [^{14}C]thiopental binding assays were performed in 1 ml of Tris-HCl buffer (pH 7.4) for 90 min at room temperature using fresh cerebrocortical membranes containing approximately 1 mg of protein and 3 μM of [^{14}C]thiopental (10 mCi·mmol^{-1}, American Radiolabeled Chemical, St. Louis, MO, USA). To define nonspecific binding (NSB), excess unlabeled thiopental would be needed. However, since unlabeled thiopental showed low solubility in Tris-HCl buffer, the equilibrium dissociation constant (K_d) and the maximal binding capacity (B_{max}) could not be determined. Displacement studies were performed to examine the effects of a range of ions, anesthetic agents, and or receptor ligands on total (i.e., uncorrected for NSB) [^{14}C]thiopental binding. The effects of Na^+ (100 mM), Ca^{2+} (2.5 mM), Mg^{2+} (10^{-4}–10^{-1} M), thiopental (10^{-6}–3×10^{-4} M), pentobarbital (10^{-6}–10^{-3} M), phenobarbital (10^{-6}–3×10^{-2} M), barbituric acid (10^{-6}–10^{-3} M), alphaxalone (3×10^{-6}–3×10^{-4} M), etomidate (3×10^{-6}–3×10^{-4} M), ketamine (10^{-5}–10^{-3} M), propofol (10^{-6}–10^{-3} M), midazolam (10^{-9}–10^{-4} M), GABA (10^{-8}–10^{-3} M), and picrotoxin (3×10^{-6}–10^{-8} M) were examined. The agents were dissolved in Tris-HCl buffer as follows: thiopental (100 mM stock in 0.1 M NaOH); pentobarbital, barbituric acid, GABA, and picrotoxin (50 mM stock in distilled water); phenobarbital (100 mM stock in distilled water); propofol (100 mM stock in DMSO); alphaxalone (50 mM stock in DMSO); etomidate (50 mM stock in 0.1 M HCl); ketamine (500 mM stock in distilled water); and midazolam (5 mM stock in distilled water). The highest anesthetic concentration used was limited by the solubility of the agent. Following incubation, each sample was filtered (and washed) under vacuum through Whatman GF/B filters (Brandel, Gaithersburg, MD, USA) using a Brandel cell harvester to separate bound and free radioactivity. Filter bound [^{14}C]thiopental was extracted with 4.5 ml of Optiphase Safe as a scintillant for at least 8 h prior to quantification using a scintillation spectrophotometer.

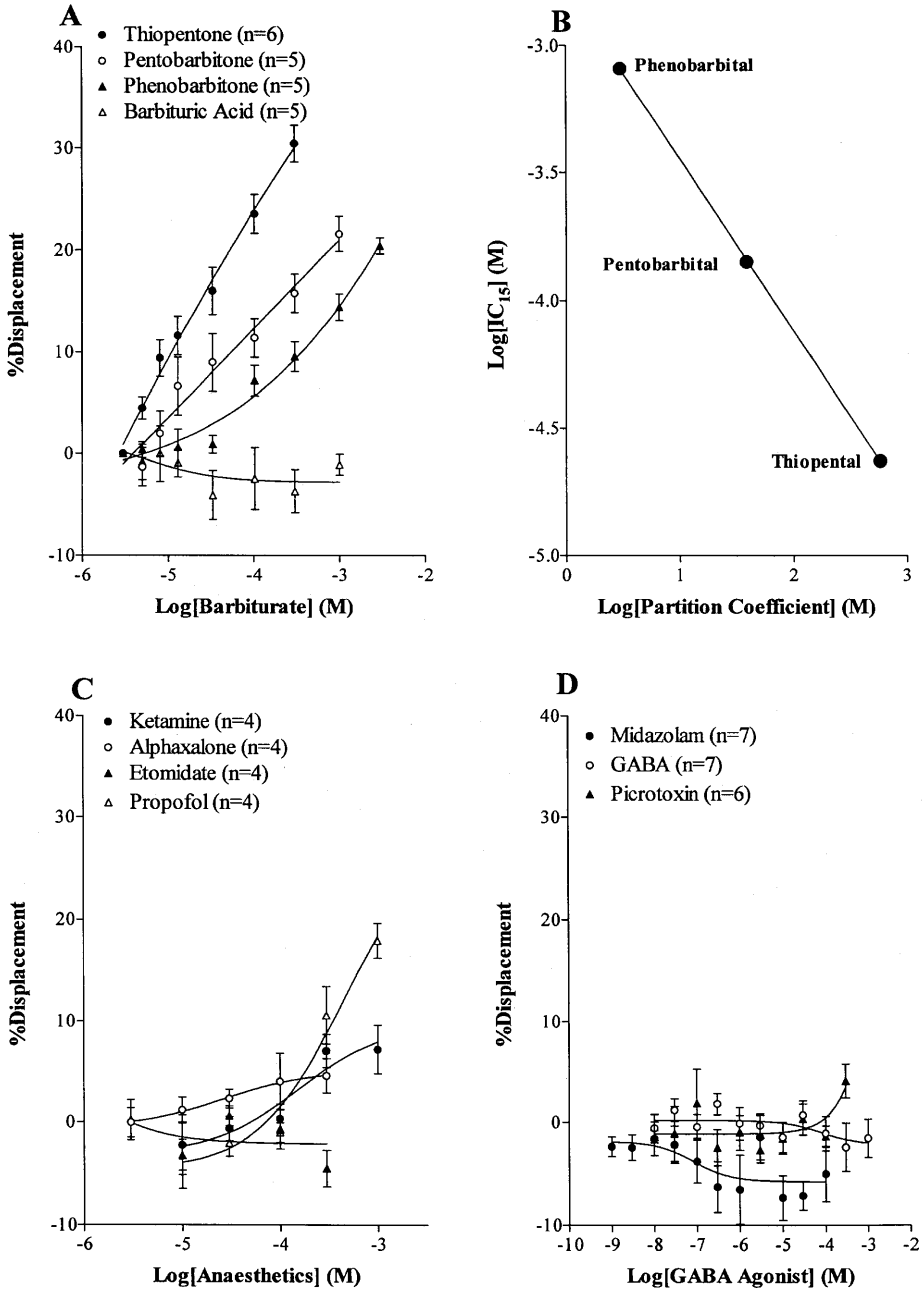

Fig. 1. Thiopental, pentobarbital, and phenobarbital, but not barbituric acid, displaced [^{14}C]thiopental binding to rat cerebrocortical membranes (**A**). The concentration producing 15% displacement of binding correlates with the lipid : water partition coefficient (**B**) ($r^2 = 0.9999$). Nonbarbiturate anesthetic agents (**C**) ($n = 4$ each) and GABA receptor ligands (**D**) ($n = 6$–7) did not displace. Data are means ± SEM

All data are expressed as means ± SEM from at least four independent experiments. The concentration (IC_{15}) of barbiturate producing 15% displacement of total binding was obtained by computer-assisted curve fitting GRAPHPAD-PRISM, assuming a theoretical maximum displacement of 100%.

The binding of [^{14}C]thiopental was time-dependent, reaching apparent saturation at 90 min (Table 1). [^{14}C]Thiopental binding was enhanced by Mg^{2+} (pEC_{50} = 2.23 ± 0.04: 5.9 mM, maximum enhancement 23 ± 4%) but not Ca^{2+} (2.5 mM) or Na^{+} (100 mM). Three anesthetic barbiturates, but not barbituric acid, pro-

Table 1. Time course of [^{14}C]thiopental (3 μM) binding to rat cerebrocortical membranes

Time (min)	[^{14}C]Thiopental binding (DPM·mg^{-1} protein)
15	726 ± 7
30	837 ± 21
45	933 ± 10
60	953 ± 6
75	1028 ± 12
90	1069 ± 6

DPM, Disintegration per minute. Data are means ± SEM ($n = 5$).

duced dose-dependent displacement of [^{14}C]thiopental binding (Fig. 1A), and there was a good correlation between the concentration of active barbiturates producing 15% displacement (IC_{15}, thiopental 22 μM, pentobarbital 155 μM, phenobarbital 537 μM) and the lipid–water partition coefficient [6] (Fig. 1B) (r^2 = 0.9999). With the exception of propofol at high concentrations (18 ± 2% displacement at 1 mM) (Fig. 1C), no other anesthetic agents (Fig. 1C) receptor ligands (Fig. 1D) displaced [^{14}C]thiopental.

In the present study, although we could not define specific [^{14}C]thiopental binding, total binding was dose-dependently displaced by three active barbiturates at concentrations that produce anesthesia [8,9]. In addition, there was a good correlation between IC_{15} and the lipid–water partition coefficient. Because the highest concentration (3×10^{-4} M) of thiopental used displaced 30 ± 2% of the total [^{14}C]thiopental binding, IC_{15} was used for the correlation. Consistent with previous reports, our data suggest that the barbiturate-binding site may be distinct from the benzodiazepine- [10], GABA- [11], and picrotoxin- [12] binding sites on the $GABA_A$ receptor, because midazolam, GABA, and picrotoxin did not displace [^{14}C]thiopental. However, it is important to bear in mind that this binding ([^{14}C]thiopental) occurs at very high concentrations and hence at very low affinity. The present study suggests that the [^{14}C]thiopental-binding site does not represent a common site of anesthetic action. The possible correlation between IC_{15} and lipid solubility is interesting but may not reveal any new information in the search for a unitary hypothesis.

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