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Effects of Thiopental and Its Optical Isomers on Nicotinic Acetylcholine Receptors

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Background: **With the exception of γ-aminobutyric acid$_A$ (GABA$_A$) receptors, the major molecular targets underlying the anesthetizing actions of thiopental have yet to be established. Neuronal nicotinic acetylcholine receptors (nAChRs) are closely related to GABA$_A$ receptors and hence might also be major targets. If so, they might be expected to be substantially inhibited by surgical concentrations (EC_{50} = 25 μM) of thiopental and to display the same stereoselectivity as does general anesthesia.**

Methods: **Neuronal α4β2, neuronal α7 and muscle αβγδ nAChRs were expressed in Xenopus oocytes. Peak acetylcholine-activated currents were measured at −70 mV using the two-electrode voltage clamp technique. Racemic thiopental and its two optical isomers were applied with and without preincubation and at high and low concentrations of acetylcholine.**

Results: **Inhibition of all three nAChRs was enhanced by preincubation with thiopental, a protocol that mimics the pharmacologic situation *in vivo*. Using this protocol, inhibition was further enhanced by high concentrations of acetylcholine, with IC_{50} = 18 ± 2, 34 ± 4, and 20 ± 2 μM (mean ± SEM) thiopental for the neuronal α4β2, neuronal α7 and muscle αβγδ nAChRs, respectively, with Hill coefficients near unity. Neither the neuronal α7 nor the muscle αβγδ nAChR differentiated between the optical isomers of thiopental. However, *R*(+)-thiopental was significantly more effective than the *S*(−) isomer at inhibiting the neuronal α4β2 nAChR; interestingly, this is diametrically opposite to their stereoselectivity for general anesthesia.**

Conclusions: **Both central neuronal and peripheral muscle nAChRs can be substantially inhibited by thiopental at surgical EC_{50} concentrations but with either no stereoselectivity or one opposite to that for general anesthesia. Thus, nAChRs are probably not crucial targets for producing thiopental anesthesia, although nAChRs may play a part in the side effects produced by this agent. (Key words: Chiral anesthetics; enantiomers; ionotropic receptors.)**

HISTORICALLY, thiopental has been one of the most popular and important of the intravenous general anesthetics in routine clinical use. Nonetheless, with the exception of γ-aminobutyric acid$_A$ (GABA$_A$) receptors,[1–4] the molecular targets that underlie its anesthetizing actions remain unknown. We consider here, as possible and plausible targets, neuronal nicotinic acetylcholine receptors (nAChRs) because they belong to the same superfamily of fast neurotransmitter-gated receptors as do GABA$_A$ receptors.[5–7] We have expressed in *Xenopus* oocytes what are possibly the major vertebrate central nervous system (CNS) species[8] of neuronal heteromeric (α4β2) and neuronal homomeric (α7) nAChRs together with (for comparison) the muscle heteromeric (αβγδ) nAChR. Recent studies[9,10] with this expression system have found the neuronal α4β2 (but not the neuronal α7 nor the muscle αβγδ) nAChR to be extremely sensitive to inhibition by volatile anesthetics.

Thiopental is invariably administered surgically as an equimolar (racemic) mixture of its two optical isomers (enantiomers), *R*(+) and *S*(−), although the *S*(−) enantiomer appears to be approximately twice as potent as the *R*(+) enantiomer at abolishing the righting reflex in mice.[11–13] The scarcity of experimental data on the effects of the pure thiopental enantiomers is largely a result of their being unavailable commercially and their chemical syntheses being difficult. Fortunately, it is now feasible to prepare pure *R*(+)- and *S*(−)-thiopental by separation of racemic thiopental on a chiral column.[2] Indeed, these enantiomers have been prepared and used in this laboratory to study the stereoselective interactions of thiopental with GABA$_A$ receptors. Using pure enantiomers of not only thiopental, but also of pentobarbital and hexobarbital, it was found[2] that the rank orders and degrees of chirality observed for potentiating the

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activity of $GABA_A$ receptors matched those for barbiturate general anesthesia. (A similar study using $GABA_A$ receptors and chemically synthesized thiopental enantiomers was subsequently published.[14])

Many criteria can be used to judge the relevance of a putative target to the anesthetizing actions of a particular agent.[1,15] Here we use two particular criteria: anesthetic sensitivity and stereoselectivity. For a given neuronal nAChR and thiopental, these criteria lead to the following tests for relevance: (1) The activity of the nAChR should be substantially inhibited by a clinical EC_{50} concentration of thiopental (approximately 25 μM[1]). (2) This inhibition should not only be stereoselective, but *S*(−)-thiopental should be substantially more effectual than *R*(+)-thiopental. Here we describe the design and results of experiments that test these predictions for different classes of nAChR channels.

Materials and Methods

This study conforms to the United Kingdom Animals (Scientific Procedures) Act of 1986.

Preparation and Injection of Xenopus *Oocytes*

Adult female *Xenopus laevis* frogs (Blades Biological, Cowden, Kent, United Kingdom) were maintained in fresh-water holding tanks with a 12-h light–dark cycle in a temperature-controlled room at 20–22°C. Animals were anesthetized by immersion in a 0.2% (wt/vol) solution of tricaine (3-aminobenzoic acid ethyl ester methanesulfonate). A surgical incision into the abdomen was made, and portions of the ovaries containing oocytes were removed and teased apart with forceps. The ovarian fragments were then briefly washed in calcium-free oocyte incubation buffer (Ca^{2+}-free OIB; composition: 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 15 mM HEPES, titrated to pH 7.5 with NaOH). Individual oocytes were obtained by digestion of these fragments in Ca^{2+}-free OIB containing 2 mg/ml of type 1A collagenase (Sigma Chemical Co., Poole, Dorset, United Kingdom), for 3 h at room temperature. The oocytes were then carefully washed in Ca^{2+}-free OIB to remove all traces of collagenase, before being transferred into normal OIB (composition 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.3 mM $Ca(NO_3)_2$, 15 mM HEPES, titrated to pH 7.5 with NaOH). Oocytes were then scrutinized under a dissection microscope, and healthy eggs at stages 5–6 of development were set aside for injection with either DNA (chick α7 subunit; rat α_4 and β2 subunits) or RNA (mouse α, β, γ, and δ subunits). Injections of cDNA were made with 10 nl of diethyl pyrocarbonate–treated water containing 0.1–1.0 pg of the appropriate cDNA directly into the nucleus of the oocyte, using the "blind" method. In the case of the $\alpha4\beta2$ combination, a premixed solution with an equimolar ratio of the $\alpha4$ and $\beta2$ cDNAs was used. Injections of cRNA were made into the cytosol of the eggs with 50 nl of diethyl pyrocarbonate–treated water containing a total of 10–40 ng of the cRNAs premixed at an equimolar ratio of the α, β, γ, and δ subunits. All injections were conducted with use of calibrated micropipettes (10–16-μm tip diameter) in combination with a Picospritzer II valve (General Valve Corp., Fairfield, NJ), which provided short pressure pulses of nitrogen gas to expel known volumes of the nucleic acid solutions from the micropipettes. Injected oocytes were maintained in a cooled incubator at 19–20°C in normal OIB supplemented with antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml; Life Technologies, Paisley, United Kingdom) for 2–7 days before use. All chemicals, unless otherwise stated, were obtained from Sigma Chemical Company (Poole, Dorset, United Kingdom).

The nAChR cDNAs used here were kindly supplied as follows. Rat neuronal nAChR cDNAs for the $\alpha4$ and $\beta2$ subunits (in the pSM expression vector) were from Jim Patrick (Baylor College of Medicine, Houston, TX). Mouse muscle nAChR cDNAs encoding the α, β, γ, and δ subunits were from Jim Boulter (Salk Institute, San Diego, CA), contained in either the pSP64 (γ) or pSP65 (α, β, and δ) vectors. The chick α7 cDNA was supplied by Marc Ballivet (Université de Genève, Switzerland) in the "flip" expression vector.[16] The pSM- and "flip"-derived plasmids contain SV40 promoter sequences, which allows direct use of these cDNAs by the nuclear injection method. However, in the case of the mouse muscle subunits, individual cRNA transcripts suitable for injection were prepared from the cDNAs supplied with use of a Riboprobe System cRNA production kit (Promega, Southampton, United Kingdom).

Recording Saline and Drugs Used

The extracellular saline used for electrophysiological recordings had the following composition: 110 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $BaCl_2$, and 10 mM HEPES (titrated to pH 7.5 with NaOH). Additionally, atropine (100 nM) was added to all solutions to suppress the activity of endogenous muscarinic receptors. At this con-

centration atropine did not affect the acetylcholine-evoked nicotinic currents. The *R*(+) and *S*(−) enantiomers of thiopental were prepared from racemic thiopental stock (94.3% sodium salt–sodium carbonate mixture) to a purity level of 99.0% or more, with use of high-performance liquid chromatography with a permethylated β-cyclodextrin column, as described elsewhere.[2] Acetylcholine chloride and thiopental solutions were prepared freshly from stock salts on the day of the experiment.

Recording Technique for Xenopus *Oocytes*

Inward currents evoked by bath application of acetylcholine chloride (0.3 μM to 10.0 mM) were recorded at −70 mV from oocytes expressing the desired subunit combinations and situated in a bath (volume ≈ 50 μl), where they were continuously perfused at a rate of 2 ml/min with appropriate solutions. Currents were recorded with use of an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) in the two-electrode voltage clamp mode. Recording electrodes were made from thin-walled filamented borosilicate glass capillary tubes (GC150TF, Clark Electromedical Instruments, Reading, Berkshire, United Kingdom); the passive electrode was filled with 3 M KCl and the current-passing electrode with 3 M KCl supplemented with BAPTA [1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, tetrapotassium salt, 100 mM]. These electrodes typically had resistances of 0.4–0.8 MΩ. Currents were filtered at 10 Hz (−3 dB, 8-pole Bessel filter; Model 902, Frequency Devices, Haverhill, MA) before being digitized (sampling rate 20 Hz) and stored on a computer for offline analysis. All experiments were performed at room temperature (21–23°C).

Current responses were evoked by continuous application of acetylcholine-containing solutions until a well-defined peak in the response was observed (usually at 20–30 s); this peak was then used as the measure of receptor activity. During data collection a standard acetylcholine concentration was chosen and applied alternately throughout the experiment, to allow the data to be normalized and to correct for any fluctuation in the control response. Two protocols were used concurrently to assess the effects of thiopental. In the first instance, a protocol was used where appropriate concentrations of acetylcholine were simply coapplied with the various concentrations of thiopental. In the second, or preapplied protocol, the oocyte was bathed in the appropriate concentration of thiopental for 1 min before its coapplication with acetylcholine. (The effect of preincubation was complete after approximately 30 s.) In both cases, application of acetylcholine was maintained until an unambiguous peak in the response was observed. Such applications were repeated to ensure consistency of the response. Thiopental by itself had no significant effect on the resting current. Inhibition by thiopental of the response to acetylcholine was shown to be fully reversible at the thiopental concentrations used here. Inhibition of the acetylcholine-evoked current by thiopental was calculated by averaging controls before and after application of anesthetic and comparing these mean control values with the current responses in the presence of thiopental.

Data Analysis

Control acetylcholine concentration–response data were fitted to a standard activation Hill equation of the form

$$E = \frac{100\,A^{n_H}}{A^{n_H} + (EC_{50})^{n_H}} \quad (1)$$

where E is the peak acetylcholine-induced current expressed as a percentage of the maximal current, A is the concentration of acetylcholine, n_H is the Hill coefficient, and EC_{50} is the acetylcholine concentration that gives a half-maximal effect.

Similarly, data describing the inhibition of the acetylcholine-evoked response by thiopental were fitted to an inhibition Hill equation of the form

$$y = \frac{100\,(IC_{50})^{n_H}}{I^{n_H} + (IC_{50})^{n_H}} \quad (2)$$

where y is the percentage of the control acetylcholine current remaining in the presence of thiopental at a concentration I, n_H is the Hill coefficient, and IC_{50} is the concentration of thiopental required to inhibit the control response by 50%.

Statistical Analysis

Numerical fits to these Hill equations were obtained using the method of unweighted least-squares (Marquardt-Levenberg algorithm, KaleidaGraph version 3.0.2, Abelbeck Software, Reading, PA) applied to individual (not mean) data points. Values throughout are given as mean ± SEM, and statistical significance was assessed with use of the Student *t* test.

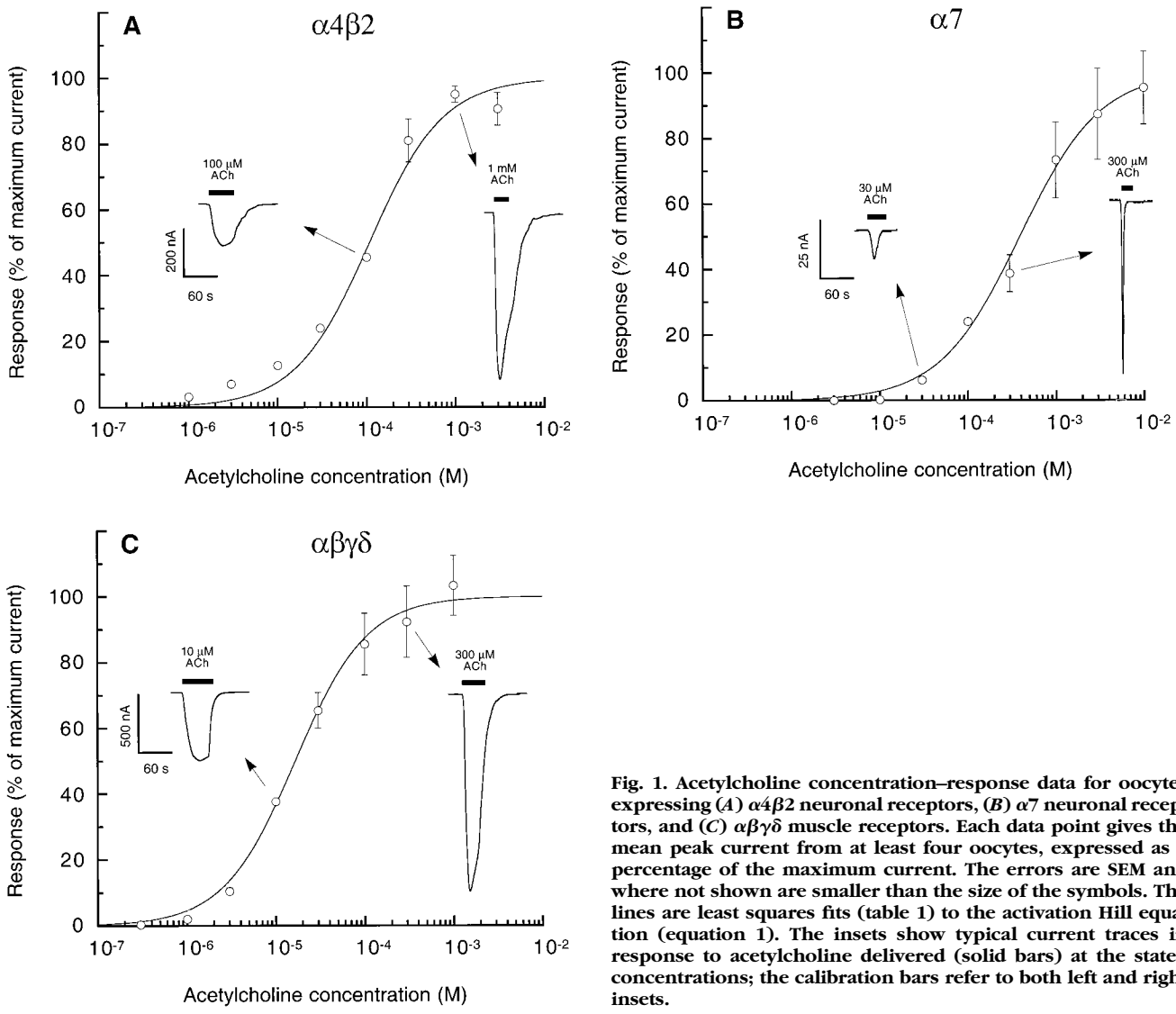

Fig. 1. Acetylcholine concentration–response data for oocytes expressing (*A*) $\alpha4\beta2$ neuronal receptors, (*B*) $\alpha7$ neuronal receptors, and (*C*) $\alpha\beta\gamma\delta$ muscle receptors. Each data point gives the mean peak current from at least four oocytes, expressed as a percentage of the maximum current. The errors are SEM and where not shown are smaller than the size of the symbols. The lines are least squares fits (table 1) to the activation Hill equation (equation 1). The insets show typical current traces in response to acetylcholine delivered (solid bars) at the stated concentrations; the calibration bars refer to both left and right insets.

Results

Control Acetylcholine Concentration–Response Characteristics

Acetylcholine concentration–response relationships were determined for *Xenopus* oocytes expressing rat neuronal heteromeric $\alpha4\beta2$, chick neuronal homomeric $\alpha7$, and mouse muscle heteromeric $\alpha\beta\gamma\delta$ nAChRs. For each nAChR, the data were fitted to the activation Hill equation (equation 1) with the results given in figure 1 and table 1. The insets to figure 1 show typical inward current responses to acetylcholine; note the differing degrees of desensitization, depending on receptor type and acetylcholine concentration. The EC_{50} concentrations and Hill coefficients (n_H) for the relatively slowly desensitizing heteromeric $\alpha4\beta2$ and $\alpha\beta\gamma\delta$ receptors are comparable to those reported previously from this laboratory.[9] For the homomeric $\alpha7$ receptor, our EC_{50} value is in reasonable agreement with previously published values,[16–18] although these are all, almost certainly, substantial overestimates (perhaps by a factor of 10). This is an unavoidable consequence of combining fast $\alpha7$ receptor desensitization with the slow-mixing rates characteristic of the *Xenopus* oocyte perfusion system, and it is because of the peak of the acetylcholine receptor response occurring before the steady state acetylcholine concentration is attained.[19] Experiments (not shown) comparing the time courses of receptor activation and

Table 1. EC_{50} Concentrations and Hill Coefficients for Activation of Nicotinic Acetylcholine Receptor Channels by Acetylcholine

Subunit Combination	EC_{50} (μM)	n_H	Degrees of Freedom
$\alpha4\beta2$ (Rat neuronal)	108 ± 11	1.04 ± 0.11	95
$\alpha7$ (Chick neuronal)	391 ± 128	0.95 ± 0.19	66
$\alpha\beta\gamma\delta$ (Mouse muscle)	17 ± 2	1.08 ± 0.16	45

Activation Hill equation parameters are given as mean ± SEM for the specified degrees of freedom and were obtained from fits of the data to equation 1.

solution exchange suggested that this was a problem for the homomeric $\alpha7$ but not for the heteromeric $\alpha4\beta2$ and $\alpha\beta\gamma\delta$ nAChRs.

Racemic Thiopental Concentration–Response Relationships for Inhibition of the Nicotinic Acetylcholine Receptors

For each nAChR, thiopental concentration-dependent inhibition of acetylcholine-activated inward currents was determined during four conditions: at a low and a high concentration of acetylcholine, both with and without preincubation with thiopental. The low and high ACh concentrations for each nAChR (table 2) differed by at least an order of magnitude, with the low concentration being selected for experimental convenience as one that activated conveniently large but essentially nondesensitizing currents (for the heteromeric $\alpha4\beta2$ and $\alpha\beta\gamma\delta$ receptors) or minimally desensitizing but still measurable currents (for the homomeric $\alpha7$ receptor). Taking into account a possible 10-fold overestimate of our EC_{50} for the $\alpha7$ receptor (see above), the low acetylcholine concentrations for all three nAChRs turned out to be near to their actual EC_{50} concentrations.

Preincubation with racemic thiopental of oocytes expressing nAChRs increased their sensitivity to inhibition by this agent (fig. 2 and table 2). This was true for all three nAChRs at both low and high concentrations of acetylcholine, with the effects of preincubation being greater at the high acetylcholine concentrations. These enhancements of inhibition could be surprisingly large. For example, consider the neuronal $\alpha4\beta2$ nAChR. The IC_{50} for thiopental at the high (1,000 μM) concentration of acetylcholine decreased from 204 ± 38 to 18 ± 2 μM thiopental because of preincubation, whereas the corresponding decrease at the low (100 μM) concentration of acetylcholine was from 81 ± 5 to 26 ± 2 μM thiopental. (For comparison, EC_{50} = 25 μM thiopental for clinical general anesthesia, using as the anesthetic end point a lack of response of patients to a painful stimulus.[1])

Using the thiopental preapplication protocol (which is arguably more relevant pharmacologically; see Discussion), for all three nAChRs the racemic thiopental concentration–response curves were shifted to the left by the high concentrations of acetylcholine (fig. 2). This effect, which corresponds to an acetylcholine-induced increase in thiopental sensitivity, was very much more marked for the muscle ($\alpha\beta\gamma\delta$) nAChR than for the neuronal ($\alpha4\beta2$ and $\alpha7$) nAChRs. All the nAChRs, however, could be sensitive to thiopental. For example, at the high concentrations of acetylcholine, the IC_{50} concentrations for inhibition of the three receptors ($\alpha4\beta2$, $\alpha7$, and $\alpha\beta\gamma\delta$) were 18 ± 2, 34 ± 4, and 20 ± 2 μM thiopental, respectively (table 2). Comparing these IC_{50} values with the EC_{50} value for general anesthesia (25 μM thiopental), a case can be made for the substantial inhibition of all three nAChRs during maintenance of anesthesia with racemic thiopental.

Effects of the Optical Isomers of Thiopental on Inhibition of the Nicotinic Acetylcholine Receptors

In addition to the aforementioned experiments with racemic thiopental, we separated the two optical isomers (enantiomers) of thiopental, *S*(−) and *R*(+), and determined their individual effects on each of the three nAChRs at a fixed concentration (25 μM, the EC_{50} of the racemate for general anesthesia). For each nAChR we used eight conditions: a low and high concentration of ACh, with and without preapplication of each thiopental enantiomer.

The results of these experiments with the enantiomers are given in figure 3. Neither the neuronal $\alpha7$ nor the muscle $\alpha\beta\gamma\delta$ nAChR differentiated between the *R*(+) and *S*(−) enantiomers of thiopental during any of the conditions used ($P > 0.5$). The $\alpha4\beta2$ nAChR, conversely, was consistently more sensitive to the *R*(+) enantiomer, and this difference was statistically significant ($P < 0.01$ or $P < 0.05$), except during the condition of high acetylcholine concentration and no preincubation with the anesthetic enantiomers. Overall, then, using the pharmacologically relevant preincubation protocol at both high and low concentrations of acetylcholine, thiopental was found to stereoselectively inhibit the neuronal $\alpha4\beta2$ but not the neuronal $\alpha7$ or the muscle $\alpha\beta\gamma\delta$ nAChR. Interestingly, the stereoselectively observed with the rat neuronal $\alpha4\beta2$ nAChR is the opposite of that observed for general anesthesia in mice.[11–13]

Table 2. IC_{50} Concentrations and Hill Coefficients for Inhibition of ACh-activated Nicotinic ACh Receptor Channels by Racemic Thiopental

Receptor Subunits	[ACh] (μM)	Thiopental Application Protocol	IC_{50} (μM)	n_H	Degrees of Freedom
$\alpha4\beta2$ (Neuronal)	100	Preapplied	26 ± 2	0.87 ± 0.07	35
	100	Coapplied	81 ± 5	0.83 ± 0.04	29
	1,000	Preapplied	18 ± 2	0.98 ± 0.08	31
	1,000	Coapplied	204 ± 38	0.72 ± 0.11	28
$\alpha7$ (Neuronal)	30	Preapplied	44 ± 4	1.11 ± 0.12	26
	30	Coapplied	302 ± 151	0.71 ± 0.18	28
	400	Preapplied	34 ± 4	1.02 ± 0.14	28
	400	Coapplied	NS	NS	29
$\alpha\beta\gamma\delta$ (Muscle)	10	Preapplied	77 ± 5	1.49 ± 0.12	21
	10	Coapplied	117 ± 7	1.50 ± 0.14	22
	300	Preapplied	20 ± 2	0.99 ± 0.08	21
	300	Coapplied	64 ± 14	0.61 ± 0.10	21

Inhibitory Hill equation parameters are given as mean ± SEM for the specified degrees of freedom and were obtained from fits of the data to equation 2. ACh = acetylcholine; NS = no significant fit.

Discussion

Actions of Intravenous General Anesthetics on Members of a Superfamily of Neurotransmitter-gated Receptor Channels

Nicotinic acetylcholine receptors are members of an anesthetic-sensitive superfamily of structurally and genetically related fast neurotransmitter-gated receptor channels.[5–7] This superfamily also includes $GABA_A$, glycine, and 5-hydroxytryptamine type 3 receptors (but not glutamate-gated receptors). Most members of this superfamily are very sensitive to minimum alveolar concentration levels of volatile general anesthetics but show varying susceptibilities to anesthetizing EC_{50} concentrations of intravenous general anesthetics such as propofol, etomidate, and thiopental. Low concentrations of propofol and etomidate, for example, substantially potentiate $GABA_A$ receptors,[20–22] while having relatively modest effects on glycine receptors,[20,21,23] 5-hydroxytryptamine type 3 receptors[24,25] and neuronal nAChRs.[9,26] In addition, thiopental and etomidate potentiate $GABA_A$ receptors in a stereoselective manner (propofol is achiral and cannot act stereoselectively), with the same rank orders of potency found for general anesthesia.[2,22] These results are consistent with major roles for $GABA_A$ receptors in the production of general anesthesia with propofol, etomidate, or thiopental.

Thiopental, however, appears to be a more promiscuous intravenous agent than either propofol or etomidate, substantially affecting not only $GABA_A$ receptors but some other superfamily members as well. For example, although 5-hydroxytryptamine type 3 receptors in N1E-115 neuroblastoma cells are insensitive to thiopental,[25,27] recombinant glycine receptors in the *Xenopus* expression system are sensitive.[28] Furthermore, a steady current (but not the peak current) through nicotine-activated ganglionic-type neuronal nAChRs in rat PC12 cells can be more than half-inhibited by surgical EC_{50} concentrations of thiopental.[29] The situation with respect to thiopental inhibition of CNS-type neuronal nAChRs is unknown and is addressed in the current study.

General Properties of the Major Central Nervous System Neuronal Nicotinic Acetylcholine Receptors

Neuronal nAChRs in vertebrates have a wide range of compositions.[30–33] Each functional receptor is a pentamer consisting of two to five subunits of the α type ($\alpha2$, $\alpha3$, $\alpha4$, α_5, α_6, $\alpha7$, $\alpha8$, $\alpha9$) and zero to three subunits of the β type ($\beta2$, $\beta3$, $\beta4$). (Muscle-type nAChRs are made up of other subunits: two α_1, one β_1, one δ, and either one γ or one ϵ.) The heteromeric $\alpha4\beta2$ (two $\alpha4$ and three $\beta2$ subunits) and homomeric $\alpha7$ (five $\alpha7$ subunits) nAChRs are representative of two important classes of neuronal CNS receptors that account for most of the high-affinity binding sites in the brain for nicotine and α-bungarotoxin, respectively. In addition, the $\alpha7$ nAChR is especially permeable to the physiologically important cation Ca^{2+}. Furthermore, the $\alpha4\beta2$ receptor is supersensitive to inhibition by volatile anesthetics,[9] whereas the $\alpha7$ receptor is insensitive to these agents.[10] For these reasons, and taking into account the availability or otherwise of specific subunit clones, we chose to study the rat neuronal heteromeric $\alpha4\beta2$

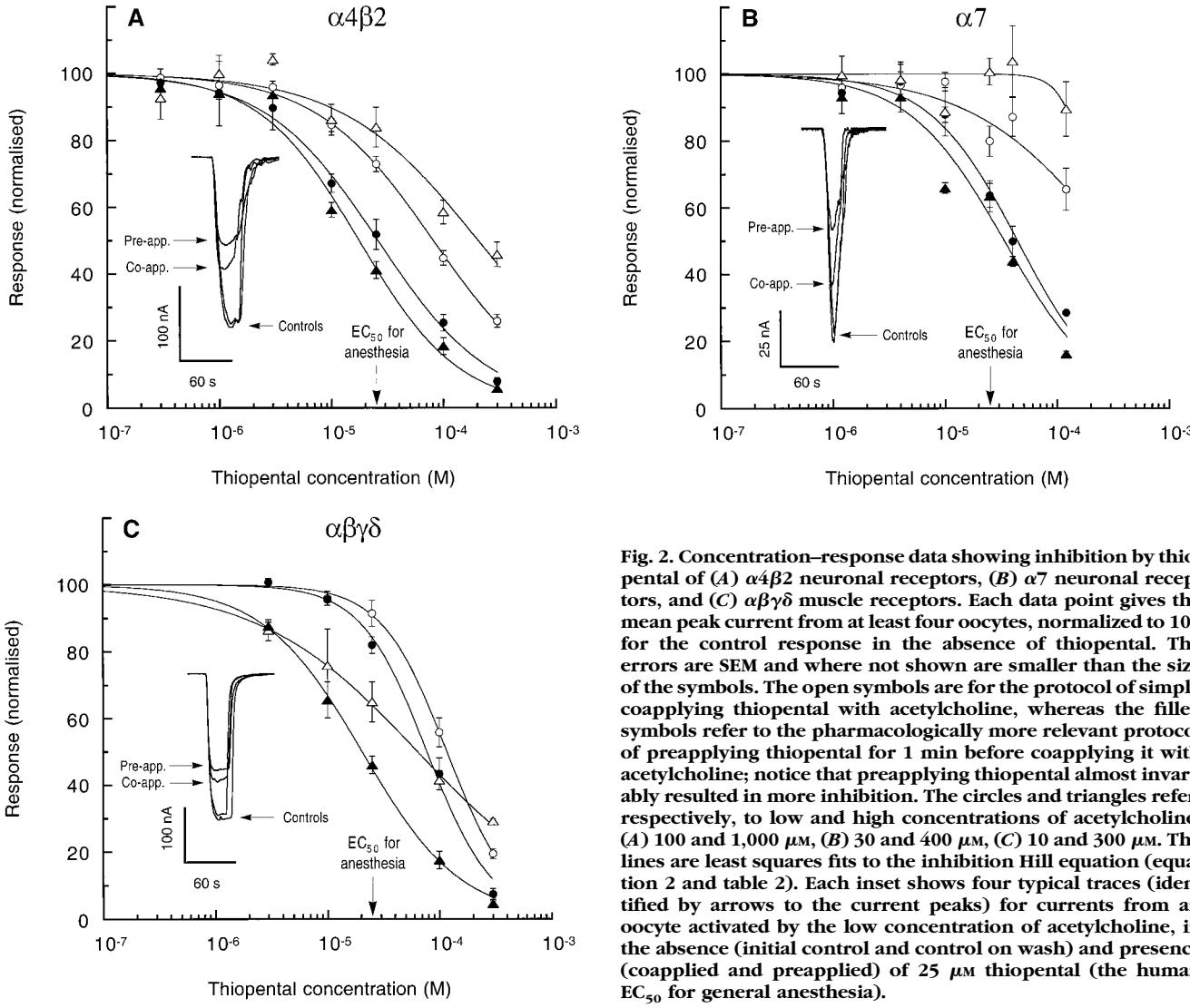

Fig. 2. Concentration–response data showing inhibition by thiopental of (*A*) $\alpha 4\beta 2$ neuronal receptors, (*B*) $\alpha 7$ neuronal receptors, and (*C*) $\alpha\beta\gamma\delta$ muscle receptors. Each data point gives the mean peak current from at least four oocytes, normalized to 100% for the control response in the absence of thiopental. The errors are SEM and where not shown are smaller than the size of the symbols. The open symbols are for the protocol of simply coapplying thiopental with acetylcholine, whereas the filled symbols refer to the pharmacologically more relevant protocol of preapplying thiopental for 1 min before coapplying it with acetylcholine; notice that preapplying thiopental almost invariably resulted in more inhibition. The circles and triangles refer, respectively, to low and high concentrations of acetylcholine: (*A*) 100 and 1,000 μM, (*B*) 30 and 400 μM, (*C*) 10 and 300 μM. The lines are least squares fits to the inhibition Hill equation (equation 2 and table 2). Each inset shows four typical traces (identified by arrows to the current peaks) for currents from an oocyte activated by the low concentration of acetylcholine, in the absence (initial control and control on wash) and presence (coapplied and preapplied) of 25 μM thiopental (the human EC_{50} for general anesthesia).

nAChR, the chick neuronal homomeric $\alpha 7$ nAChR, and (for comparison) the embryonic mouse muscle heteromeric $\alpha\beta\gamma\delta$ nAChR. We expressed all three nAChRs in the same system (the *Xenopus* oocyte) to make fair comparisons of their behaviors toward thiopental. However, the use of clones from different species, although necessary at the time this study began, may have introduced some variability into these comparisons.

The physiologic functions of neuronal nAChRs in the CNS are still largely unknown,[32] and much of the interest in these nicotinic receptors stems from the agonist effects of tobacco-derived nicotine. There is little evidence for fast nicotinic cholinergic synaptic transmission in the brain, which is surprising in view of its important roles in peripheral ganglionic and neuromuscular transmission.[34] This points to a presynaptic rather than a postsynaptic role for central nAChRs. It is partly for this reason that presynaptic neuronal nAChRs in the brain are currently receiving so much attention,[34,35] the idea being that acetylcholine or nicotine binding to such receptors modulates the release of a number of different types of neurotransmitters (*e.g.*, dopamine, norepinephrine, glutamate, GABA, and acetylcholine) from presynaptic terminals. Although this is an attractive hypothesis that has received some experimental support, many questions remain to be answered. For example, although the source of nicotinic agonist poses no conceptual problems for cholinergic synapses or for experiments with

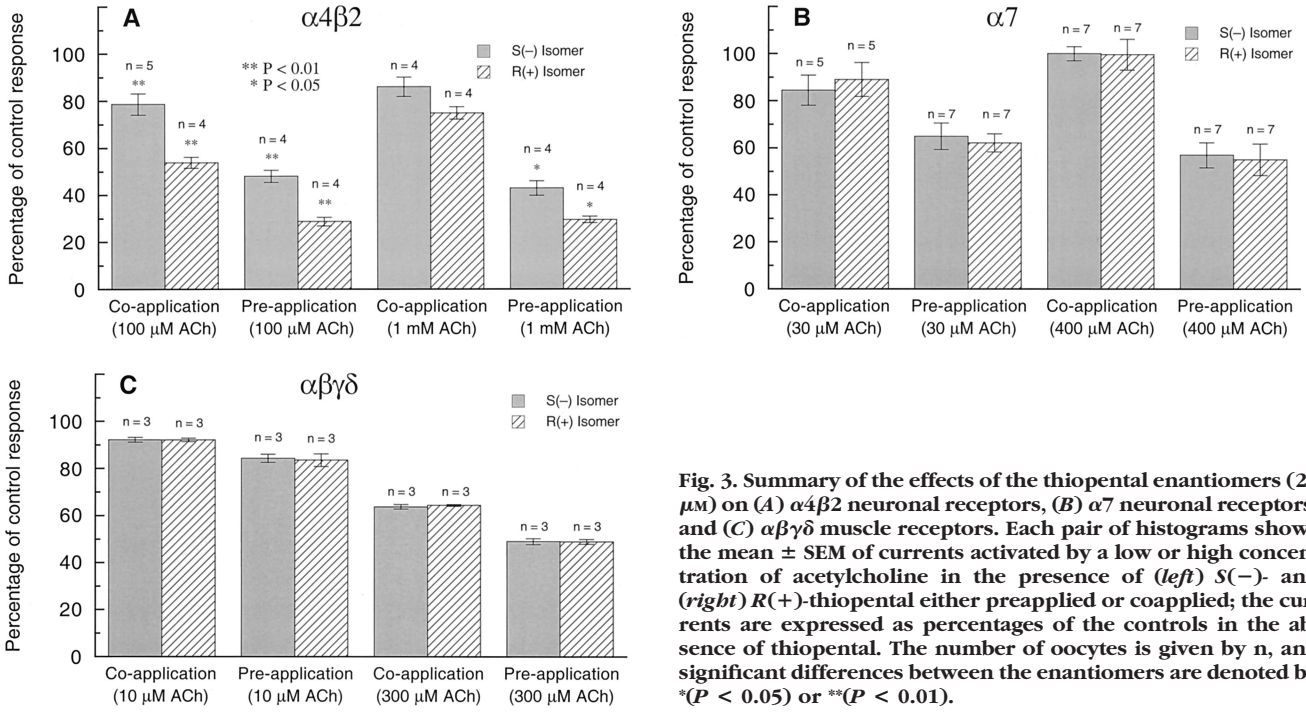

Fig. 3. Summary of the effects of the thiopental enantiomers (25 μM) on (*A*) α4β2 neuronal receptors, (*B*) α7 neuronal receptors, and (*C*) αβγδ muscle receptors. Each pair of histograms shows the mean ± SEM of currents activated by a low or high concentration of acetylcholine in the presence of (*left*) *S*(−)- and (*right*) *R*(+)-thiopental either preapplied or coapplied; the currents are expressed as percentages of the controls in the absence of thiopental. The number of oocytes is given by n, and significant differences between the enantiomers are denoted by *($P < 0.05$) or **($P < 0.01$).

exogenous nicotine, it is still not clear in most instances what are the physiologic sources of endogenous agonist (presumably acetylcholine) and what are the relevant concentrations at the presynaptic receptors. Whatever the answers, it seems likely that elucidation of the mechanisms and roles of neuronal nAChRs will produce more surprises.

Inhibition of Nicotinic Acetylcholine Receptors by Thiopental: Effects of the Different Experimental Protocols

Thiopental inhibited the peak current responses to acetylcholine in a protocol-dependent manner. We found that preincubation of oocytes with thiopental resulted in enhanced inhibition for all nAChRs (fig. 2 and table 2). This effect, which could be very large, was presumably a result of preincubation favoring the attainment of equilibrium between receptor and anesthetic before the application of the acetylcholine agonist. (This is the simplest explanation, although we cannot rule out slow second messenger effects.) Pharmacologically, preapplication of anesthetic would appear to be the protocol most relevant to the steady state maintenance of general anesthesia. For induction, the situation is less clear but would still seem to favor preapplication, because the thiopental concentration is at a quasi–steady state for times on the order of seconds, whereas acetylcholine release, presumably from nerve terminals, occurs at times on the order of milliseconds. For interpreting mechanisms at the molecular level, anesthetic–acetylcholine coapplication without anesthetic preapplication is the easier option but should be resisted, we believe, because it can be misleading. In our experiments, for example, we would have erroneously concluded from the results using the coapplication protocol alone that both the neuronal α4β2 and α7 nAChRs are relatively insensitive to thiopental.

Our results using the pharmacologically more relevant thiopental preincubation protocol, conversely, show that both neuronal and muscle nAChRs can be very sensitive to inhibition by thiopental. Indeed, at high concentrations of acetylcholine agonist IC_{50} = 18 ± 2, 34 ± 4, and 20 ± 2 μM thiopental for the neuronal α4β2, neuronal α7 and muscle αβγδ nAChRs, respectively, all with Hill coefficients near unity (table 2). These IC_{50} concentrations for inhibiting the nAChRs can be compared with the EC_{50} of 25 μM thiopental for surgical general anesthesia[1]; at this anesthetizing concentration of thiopental, the aforementioned IC_{50} concentrations translate into nAChR inhibitions of 58, 42, and 56%, respectively, for the neuronal α4β2, neuronal α7, and muscle αβγδ nAChRs. At the low concentrations of

acetylcholine, the neuronal nAChRs are slightly less sensitive to thiopental than at the high concentrations of acetylcholine (this is significant at the 5% level for the $\alpha4\beta2$ but not the $\alpha7$ nAChR), whereas the muscle nAChR is profoundly less sensitive to thiopental at low concentrations of acetylcholine. This result is consistent with substantial open-channel block for the muscle-type receptor[36,37] but not for the neuronal $\alpha4\beta2$ receptor,[9] as has been found for volatile anesthetics.

Testing for a Role of Neuronal Nicotinic Acetylcholine Receptors in the Anesthetizing Actions of Thiopental

Does thiopental inhibition of central neuronal nAChRs play an important role in producing general anesthesia? If it does, these receptor channels might be expected to be substantially inhibited at thiopental EC_{50} concentrations for surgical general anesthesia. Using as an anesthetic end point a failure of patients to respond purposefully to a painful stimulus equivalent to a surgical incision, we previously calculated[1] that this EC_{50} is approximately 25 μM thiopental. It can be seen from figures 2A and 2B that, using the thiopental preincubation protocol with both high and low concentrations of acetylcholine, the $\alpha4\beta2$ and $\alpha7$ neuronal nAChRs were indeed substantially (35–60%) inhibited at this anesthetizing EC_{50} concentration of thiopental (arrows in fig. 2). Although this suggests that the activities of neuronal nAChRs in the CNS are severely compromised during thiopental general anesthesia, it does not logically follow that thiopental disruption of nAChR function actually causes general anesthesia.

Clearly, another criterion (in addition to sensitivity) is needed to judge relevance. A convenient yet powerful test[1,7] exploits the fact that general anesthesia is stereoselective for many chiral (*i.e.*, optically active) anesthetics, including thiopental.[13] If a given receptor is one of at most a few important targets for the anesthetizing actions of a particular chiral agent, then the rank order and ratio of effectiveness of its optical isomers should be similar for producing general anesthesia and for modulating the activity of the receptor being tested. Using this test with optical isomers of not only thiopental but also of pentobarbital, hexobarbital, etomidate, and anesthetic neurosteroids, previous studies from this[2,22] and other[38] laboratories have identified potentiation of $GABA_A$ receptor activity as a major factor underlying the production of general anesthesia by these intravenous agents. Conversely, using this test with ketamine enantiomers identifies the glutamate *N*-methyl-D-aspartate receptor as a major target for this agent.[1,39,40]

What happens when this test is applied to our results for the *R*(+) and *S*(−) optical isomers of thiopental acting on neuronal nAChRs? *S*(−)-thiopental is more potent than *R*(+)-thiopental at abolishing the mouse righting reflex,[11,12] whereas it is less effective at inhibiting the neuronal heteromeric $\alpha4\beta2$ nAChR and equally effective at inhibiting the neuronal homomeric $\alpha7$ nAChR (fig. 3). (For a comparison, the muscle $\alpha\beta\gamma$ nAChR, like the neuronal $\alpha7$ nAChR, showed no discrimination between the thiopental enantiomers.) Thus both neuronal nAChRs fail to pass the stereoselectivity criterion for important roles in thiopental anesthesia.

A very recent study[41] using depressant and convulsant enantiomers of the barbiturate MPPB (1-methyl-5-phenyl-5-propyl barbituric acid) examined their effects on ganglion-type nAChRs in PC12 cells. The two MPPB enantiomers had opposite (inhibitory and excitatory) effects on animals, whereas they were found to have uniformly inhibitory effects on the activities of the neuronal nAChRs. The investigators concluded that inhibition of these ganglion-type neuronal nAChRs does not contribute to the anesthetic action of barbiturates.

Implications for Molecular Mechanisms of Thiopental General Anesthesia

What are the implications of our results for understanding the molecular mechanisms of thiopental general anesthesia? First, we conclude from our studies with the pure optical isomers of thiopental that neither of the neuronal nAChRs tested plays a major role in the anesthetizing actions of thiopental. Instead, from the positive results of our previous $GABA_A$ receptor study[2] with these same optical isomers, it would appear that potentiation of the activities of inhibitory $GABA_A$ receptors is the main factor. Second, although thiopental inhibition of nAChRs does not actually produce anesthesia, the fact that these receptors can be very sensitive to anesthetizing concentrations of this intravenous agent suggests that they may be involved in the generation of anesthetic side effects.

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