Inhibitory Effects of Barbiturates on Nicotinic Acetylcholine Receptors in Rat Central Nervous System Neurons

Yoshinori Kamiya, M.D.,* Tomio Andoh, M.D., Ph.D.,† Itaru Watanabe, M.D.,‡ Tomoko Higashi, M.D.,‡ Hideki Itoh, M.D.*

Background: Neuronal nicotinic acetylcholine receptors (nAChRs) are widely expressed in the central and autonomic nervous systems. The authors have previously shown that depressant and convulsant barbiturates both inhibit the ganglion-type nAChRs in PC12 cells. However, the central and ganglion-type receptors have different subunit composition and pharmacologic properties. In this study, the authors investigated the effects of thiopental, depressant [$R(-)$] and convulsant [$S(+)$] stereoisomers of 1-methyl-5 phenyl-5-propyl barbituric acid (MPPB) on neuronal nAChRs in the rat central nervous system to explore significance of these effects in barbiturate anesthesia.

Methods: Whole-cell currents were measured in acutely dissociated rat medial habenula (MHb) neurons by applying 10 or 100 μM nicotine in the absence or presence of thiopental 3–100 μM. Effects of $R(-)$- and $S(+)$-MPPB on the nicotine-induced current were also studied.

Results: Thiopental suppressed the nicotine-elicited inward current and accelerated the current decay dose-dependently at the clinical relevant concentrations. $R(-)$- and $S(+)$-MPPB both inhibited the nicotine-induced current dose-dependently without augmenting the current decay. There was no significant difference in the magnitudes of inhibition by $R(-)$- and $S(+)$-MPPB.

Conclusions: Although thiopental suppressed the current mediated through native nAChRs in rat MHb neurons at the clinically relevant concentrations, the depressant and convulsant stereoisomers of MPPB both inhibited the current in the same extent. These findings are consistent with the results previously obtained in the ganglion-type receptors of PC12 cells and suggest that inhibition of nAChRs in MHb neurons is not directly relevant to the hypnotic or anticonvulsive actions of barbiturates.

NEURONAL nicotinic acetylcholine receptors (nAChRs) are widely expressed in the central and autonomic nervous systems.[1,2] They are thought to modulate synaptic transmission in the central nervous system (CNS) by regulating the release of neurotransmitters mainly through presynaptic mechanisms.[3–6] Although roles of neuronal nAChRs in the CNS are poorly understood, these receptors are reported to be involved in cognitive performance, locomotor activities, nociception, and psychoneurologic disorders such as Alzheimer and Parkinson disease.[7–9] It is known that both central and peripheral neuronal nAChRs are very sensitive to various types of anesthetics, including volatile anesthetics, ketamine, and barbiturates.[10–13] Therefore, neuronal nAChRs are candidates for potential target sites of these anesthetics.

We reported that thiopental strongly suppresses the current mediated through neuronal nAChRs at clinically relevant concentrations in PC12 cells, a rat pheochromocytoma cell line,[11] and that both stereoisomers of 1-methyl-5-phenyl-5-propyl barbituric acid (MPPB) suppress neuronal nAChRs in PC12 cells.[14] Since $R(-)$-isomer of MPPB induces loss of righting reflex, but $S(+)$-MPPB causes hyperactivity and convulsion *in vivo*,[15] our results suggest that inhibition of neuronal nAChRs by barbiturates does not correlate with their hypnotic effects *in vivo*. However, central receptors may possibly show different sensitivity to thiopental and MPPB isomers compared with ganglionic receptors because of differences in subunit composition and pharmacologic properties.[3,16] Thus, we intended to study the effects of these barbiturates on native nAChRs in the CNS.

A very recent study investigated the effects of thiopental stereoisomers on the recombinant receptors with subunit composition putatively expressed in the brain[17] and showed that α_7 homomeric receptors and $\alpha_4\beta_2$ receptors are both sensitive to clinical concentrations of racemic thiopental. It also revealed that α_7 homomeric receptors do not discriminate the stereoisomers with different anesthetic potencies and $\alpha_4\beta_2$ receptors are more sensitive to the isomer less potent as an anesthetic. However, effects of barbiturates on native nAChRs in CNS neurons have not been clarified. Because it is known that native neuronal nAChRs show different electrophysiological behaviors from those observed in the recombinant receptors with putatively corresponding subunit composition,[1,18] we cannot automatically extrapolate the findings obtained from the recombinant nAChRs to the native receptors.

The medial habenula nucleus (MHb), located in the medial part of the dorsal thalamus, receives cholinergic input from the basal forebrain and the brain-stem tegmentum. It is one of the regions in which nAChRs are strongly expressed, and electrophysiological properties of nAChRs in this region have been extensively studied using acutely dissociated neurons[19–21] and slice preparations.[22,23] In the current study, we evaluated whether thiopental inhibits native nAChRs in rat MHb neurons at clinically relevant concentrations. We also compared the

---

694

effects of stereoisomers of MPPB on the nAChR-mediated current in these neurons to clarify if inhibition of these receptors correlates with anesthetic actions of barbiturates.

Material and Methods

Acute Isolation of Medial Habenula Neurons

This study was approved by the Animal Care and Use Committee at Yokohama City University School of Medicine (Yokohama, Japan). MHb neurons were isolated from the MHbs of rats using a modification of methods described elsewhere.[20,21] Briefly, 10- to 25-day-old Sprague-Dawley rats were anesthetized with diethylether and decapitated, then the brains were rapidly placed in ice-cold and equilibrated with 100% O_2 PIPES saline containing 150 mm NaCl, 5 mm KCl, 5 mm $MgCl_2$, 25 mm PIPES, and 25 mm D-glucose (pH was adjusted to 7.4 with NaOH). The transverse slices containing the MHb nuclei were made with Vibratome tissue slicer (DTK-1000; Dosaka, Kyoto, Japan) and maintained in well-oxygenated PIPES saline for 50–60 min at room temperature (22–26°C). Thereafter, the slices were incubated in PIPES saline containing papain (7.5 U/ml), bovine serum albumin (1 mg/ml), and L-cystein (0.2 mg/ml) for 25–35 min at 35°C. During incubation, the slices were constantly agitated using a gentle rocking motion. The slices were washed twice with PIPES saline containing 1 mg/ml each of bovine serum albumin and trypsin inhibitor. The MHbs were then dissected under a binocular microscope and triturated using fire-polished Pasteur pipette in Dulbecco modified Eagle medium containing 25 mm HEPES and supplemented with Ultroser G (2%). Dissociated neurons were plated onto polyornitine-coated coverslips in a 35-mm petri dish and maintained in a humid atmosphere of 95% air and 5% CO_2 at 37°C for more than 1 h and used up to 8 h after isolation.

Cell Culture

PC12 cells were cultured in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum and 5% horse serum, as previously described.[11] For the experiment, cells were plated on collagen and poly-L-lysin–coated coverslips and used after additional 2–4 day culture.

Electrophysiology

Membrane currents were measured by conventional whole-cell voltage clamp method.[24] Cells on the coverslips were placed in a recording bath, mounted on the stage of an inverted microscope (IX50, Olympus, Tokyo, Japan) with an approximate volume of 1.5 ml, and continuously perfused at the rate of 1–2 ml/min with external solution containing 140 mm NaCl, 5.4 mm KCl, 1.8 mm $CaCl_2$, 1 mm $MgCl_2$, 10 mm HEPES, and 11.1 mm D-glucose

(pH was adjusted to 7.4 with NaOH). Recordings were made from small MHb neurons (< around 10 μm cell body diameter), which consistently responded to nicotinic agonists. Bicuculine methbromide (50 μM) was included in the external solution for the experiments using acutely dissociated neurons to prevent γ-aminobutyric acid receptor type A (GABA$_A$)-mediated chloride current. Heat-polished patch pipettes had tip resistance of 2–10 MΩ when filed with an intracellular solution. An intracellular solution contained 140 mm CsF, 10 mm CsCl, 10 mm HEPES, 5 mm EGTA, 2 mm Mg-ATP (pH was adjusted to 7.3 with CsOH), which brought the reversal potential for Cl close to the holding potential. We compensated the junction potential between intracellular and external solutions but not the series resistance. The series resistance changed within approximately 20% from the initial value. In the experiments using PC12 cells, which lack GABA$_A$ receptors, we used an intracellular solution containing 150 mm CsCl, 10 mm HEPES, 5 mm EGTA, and 2 mm Mg-ATP (pH was adjusted to 7.3 with CsOH). Cells were voltage clamped at −60 mV with a patch clamp amplifier (CEZ 2400, Nihon Koden, Tokyo, Japan and Axopatch 200A, Axon Instruments, Foster City, CA). Whole-cell currents were filtered at 0.5 kHz with Bessel filter and digitized at 2 kHz. The currents were stored on a computer using pClamp software (Axon Instrument) and analyzed using Axograph 3.5 software (Axon Instruments). All experiments were performed at room temperature (22–26°C).

Drug Application

A Y-tube method described elsewhere[11] was used for delivery of the agonists and the barbiturates. The tip of the Y-tube was made by a glass micropipette (Microcaps, 2 μl, Drummond, Broomall, PA) with about a 100-μm opening and was positioned about 500 μm from the recorder cell. This method enabled the complete exchange of the external solution surrounding the cell around 100 ms, as estimated by recording the liquid junction current produced at an open patch pipette. To obtain concentration–response relation for nicotine, we recorded the whole-cell currents induced by 1–300 μM nicotine in MHb neurons. Nicotine was applied for 2.5 s, and each application was separated by 4 min. To study the effects of barbiturates on the nicotine-induce current, the agonist with or without the barbiturates was applied in the same manner. For preincubation with the barbiturates or the antagonists, the external solution containing the drugs was perfused at the rate of 5 ml/min for 4 min before rapid application. Cells were perfused with the plain external solution at the same rate for 4 min to wash out the drugs from the bath after the measurement. We applied both MPPB isomers to a given cell to compare the effects of the isomers on the nicotine-induced current in the same population of MHb neurons. One half of the recorded cells received

R(−)-isomer first and the other half received *S(+)*-isomer first to eliminate time-dependent bias.

Drugs

Drugs used in the current study included (−)-nicotine, hexamethonium (Wako, Osaka, Japan), Mg-ATP, acetylcholine bromide (Sigma), thiopental sodium (Tanabe, Osaka, Japan), Ultroser G (IVF, Villneuve-la-Garenne, France), cytisine, and bicuculine methbromide (Research Biochemicals Inc., Cambridge, MA). *S(+)-R(−)*-MPPB were provided by Professor J. Knabe (Saaland University, Saarbrücken, Germany). Acetylcholine bromide and cytisine were dissolved in distilled water to make 60 and 30 mM stock solution and stored at −80°C. Thiopental sodium was dissolved in 0.1N NaOH to make 100 mM stock solution and stored at −80°C. *S(+)*- and *R(−)*-MPPB were dissolved in 0.1N NaOH to make 30 mM stock solution just before experiments. They were diluted with the external solution to the designated concentration. The addition of the barbiturates to the external solution caused pH changes less than 0.05.[25]

Data Analysis

We measured the peak and the nondesensitized current, which was defined as the average amplitude from 2.45 to 2.5 s during agonist application. Because nicotine-induced currents declined slightly with each application of nicotine, the response in the presence of barbiturates was compared with the average amplitude of elicited currents before and after application of the barbiturates. This procedure was rationalized by the finding that the second response was almost the same as the average of the first and third responses when nicotine was applied successively three times at an interval of 4 min (data not shown). Concentration–inhibition curves were fitted to the following equation by a least-squares fit:

$$I = 1 - C^n/(C^n \pm IC_{50}{}^n)$$

where I is the relative current normalized to the control currents, C is the concentration of barbiturates, n is the Hill coefficient, and IC_{50} is the concentration for 50% inhibition. The decaying phases of the nicotine-induced current were fitted either to a single or a double exponential function of the following form by simplex method:

$$I = I_{final} + \Sigma I_i \times exp(-t/t_i)$$

where I is the total peak current, I_{final} is the residual current at the steady state condition, I_i is the peak current amplitude of the each component, and t_i is the time constant of the corresponding component. Goodness of fit was compared by chi-square test between single and double exponential models. This analysis was conducted for the experiments using barbiturates as well as the ones in which nicotine alone was applied successively. Time constant ratio was defined as the ratio of t_i in the
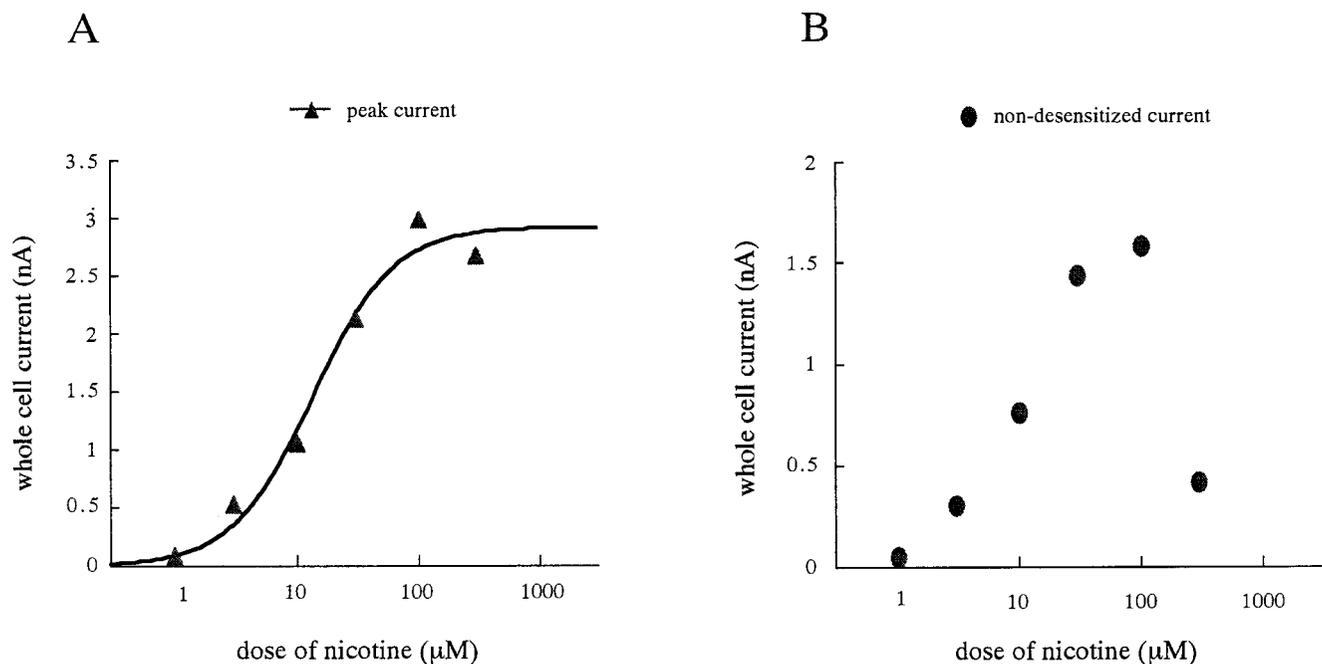

Fig. 1. The dose–response curves for nicotine in rat medial habenula (MHb) neurons. Neurons were voltage clamped at −60 mV. Whole-cell currents in response to 1–300 μM nicotine were recorded from 4–5 different neurons. Each symbol represents the average from 4–5 experiments, and the vertical line represents SEM if it is larger than the symbol. The solid line in the graph represents the fit of the Hill equation. (*A*) The peak current was maximized at 100 μM nicotine. The concentration for 50% maximal response (EC$_{50}$) was 13.7 ± 3.4 μM, and the Hill coefficient was 1.31 ± 0.35. (*B*) The nondesensitized current was also maximized at 100 μM nicotine, and it declined at the higher concentration because of desensitization.
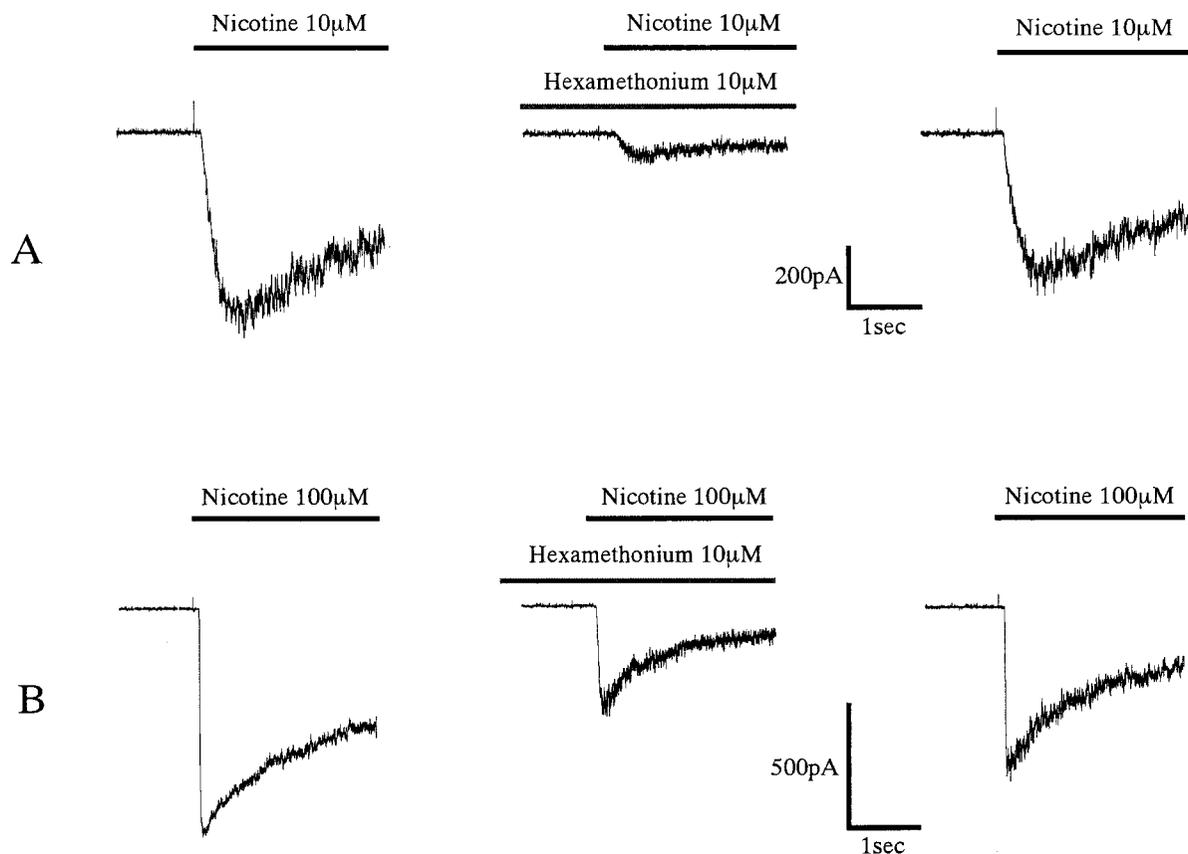
Fig. 2. Inhibition of the nicotine-induced current by hexamethonium in rat medial habenula (MHb) neurons. Neurons were held at −60 mV, and the horizontal bar indicates drug application. Neurons were preincubated with 10 μM hexamethonium for 4 min before hexamethonium was coapplied with 10 or 100 μM nicotine. (*A*) Hexamethonium at 10 μM strongly inhibited the currents evoked by 10 μM nicotine, indicating that those currents are mediated through nAChRs. (*B*) The blocking effect of hexamethonium was reduced with the higher nicotine concentration, suggesting that hexamethonium acts as a competitive inhibitor on these currents.

presence of the drugs relative to the average of precontrol and postcontrol. Desensitization was also evaluated by calculating the percent decay of the current during agonist application (% current decay) defined by the following equation[26]:

% current decay = (peak current − nondesensitized

current)/peak current × 100

Statistical Analysis

The data were expressed as mean ± SEM. The significance of differences was analyzed using one-way analysis of variance followed by Dunnett test for comparison among different doses of thiopental. A paired t test was performed to analyze differences between $R(-)$- and $S(+)$-isomers. A P value < 0.05 was considered to indicate a significant difference.

Results

Nicotine-induced Current in Acutely Dissociated Rat Medial Habenula Neurons

Nicotine elicited inward currents, which decayed rapidly during nicotine application because of desensitiza-

tion, at the membrane potential of −60 mV in acutely dissociated rat MHb neurons. The dose–response relation for the peak current showed that EC_{50} for nicotine was 12.9 μM and that the peak response was saturated at the doses equal to or more than 100 μM of nicotine (fig. 1A). For the nondesensitized current, the response was also saturated at 100 μM, and 10 μM nicotine produced near half maximal response. The nondesensitized current decreased at 300 μM because of augmented desensitization (fig. 1B). The currents elicited by 10 and 100 μM nicotine were suppressed by 10 μM hexamethonium, a competitive antagonist for neuronal nAChRs. When 10 μM hexamethonium was preincubated and coapplied, the peak and nondesensitized currents were 30.5 ± 7.9 and 27.7 ± 12.0 % of control at 10 μM nicotine (n = 5), and they were 62.7 ± 7.6 and 51.4 ± 16.0% of control at 100 μM nicotine (n = 5), respectively (fig. 2).

Effect of Thiopental on the Nicotine-induced Current

Thiopental alone produced no current response in the tested neurons at 100 μM. This finding confirmed that the direct activation of $GABA_A$ receptors by thiopental was effectively blocked in the current condition. Three
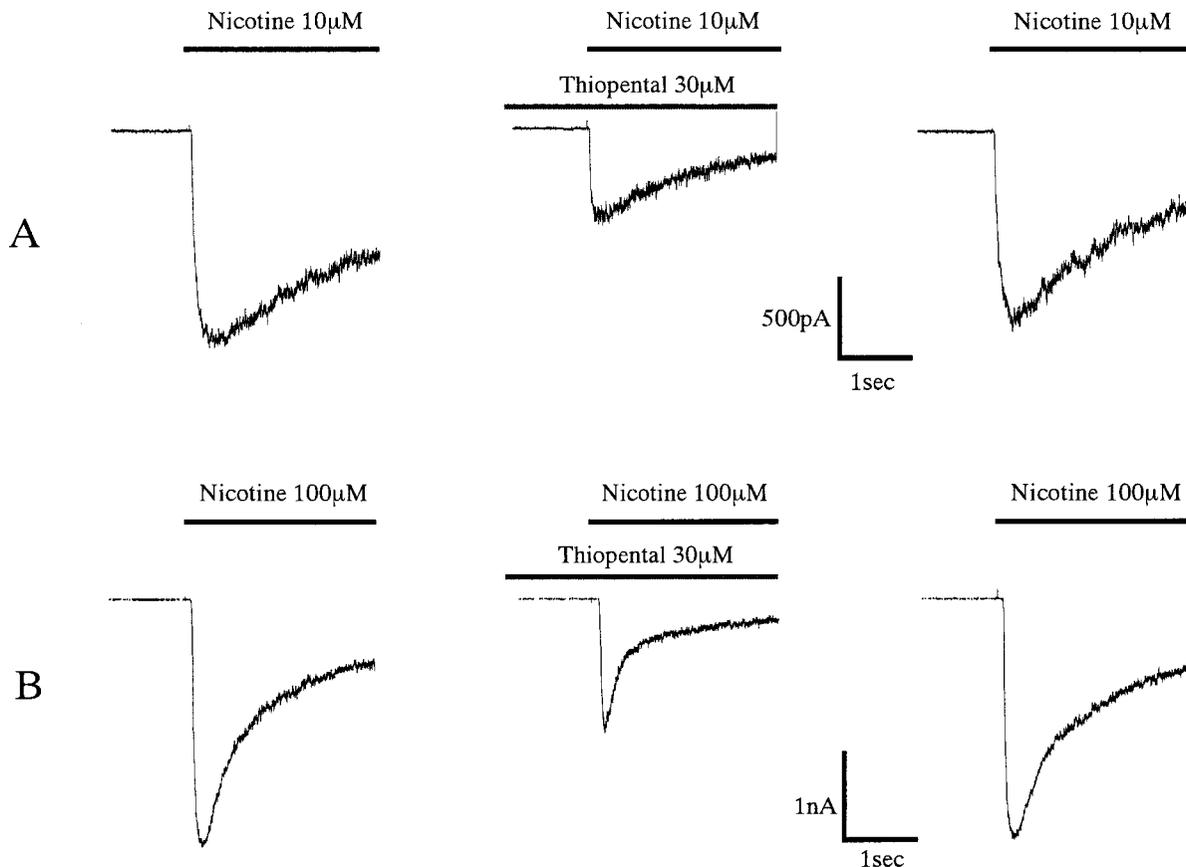
Fig. 3. Inhibition of the nicotine induced current by 30 μM thiopental in rat medial habenula (MHb) neurons. The recordings were made in the same way as in figure 2. Thiopental at 30 μM inhibited the currents elicited by 10 (*A*) and 100 μM (*B*) nicotine, and it accelerated the current decay. These effects were reversible and augmented with the higher concentration of nicotine.

to 100 μM thiopental inhibited the peak and nondesensitized current in a dose-dependent manner. The inhibition was much stronger for the current elicited by nicotine 100 μM than that for the current elicited by nicotine 10 μM. Inhibitions of the nondesensitized current were much greater than that of the peak current (figs. 3A and 3B). The IC50 values for the peak and nondesensitized currents were 30.1 ± 7.5 and 19.0 ± 3.3 μM for 10 μM nicotine. Those were 21.9 ± 6.5 and 6.2 ± 1.1 μM for 100 μM nicotine, respectively (figs. 4A and 4B).

Percent current decay represents the fraction of the current that decays during nicotine application. Thiopental augmented the current decay in a dose-dependent manner. Increases in percent current decay caused by 100 μM thiopental were significantly larger than those caused by 3 μM thiopental. However, the difference between 10 and 100 μM thiopental did not reach statistical significance (table 1). The decaying phases of the elicited current were well fitted to single exponential functions for nicotine 10 μM and better fitted to double exponential functions for nicotine 100 μM. Thiopental at 100 μM significantly reduced time constant ratios of the currents induced by 10 μM nicotine. The fast decaying phases of the current induced by 100 μM nicotine were

significantly accelerated by thiopental at 30 and 100 μM. Although thiopental tended to decrease time constant ratios of the slow phases, the changes did not reach statistical significance (table 2).

We compared the inhibitory effects of 30 μM thiopental on the nicotine-induced current in MHb neurons with those in PC12 cells (fig. 5). Thiopental at 30 μM suppressed the nondesensitized currents elicited by 100 μM nicotine more strongly than those elicited by 10 μM nicotine in MHb neurons. However, there was no significant difference in the magnitudes of inhibition of the current elicited by 100 μM nicotine between MHb neurons and PC12 cells.

Effect of MPPB Isomers on the Nicotine-induced Current

Both *R*(−)- and *S*(+)-MPPB reversibly inhibited the current induced by 10 μM nicotine in a dose-dependent manner (figs. 6A and 6B). There was no significant difference in the magnitudes of inhibition by these isomers at either doses (fig. 6C). Neither isomers of MPPB changed percent current decay at 30 or 100 μM (table 1). Both isomers tended to increase time constants of the decaying phases of the responses at 100 μM; however, the differences in time constant ratios did not reach
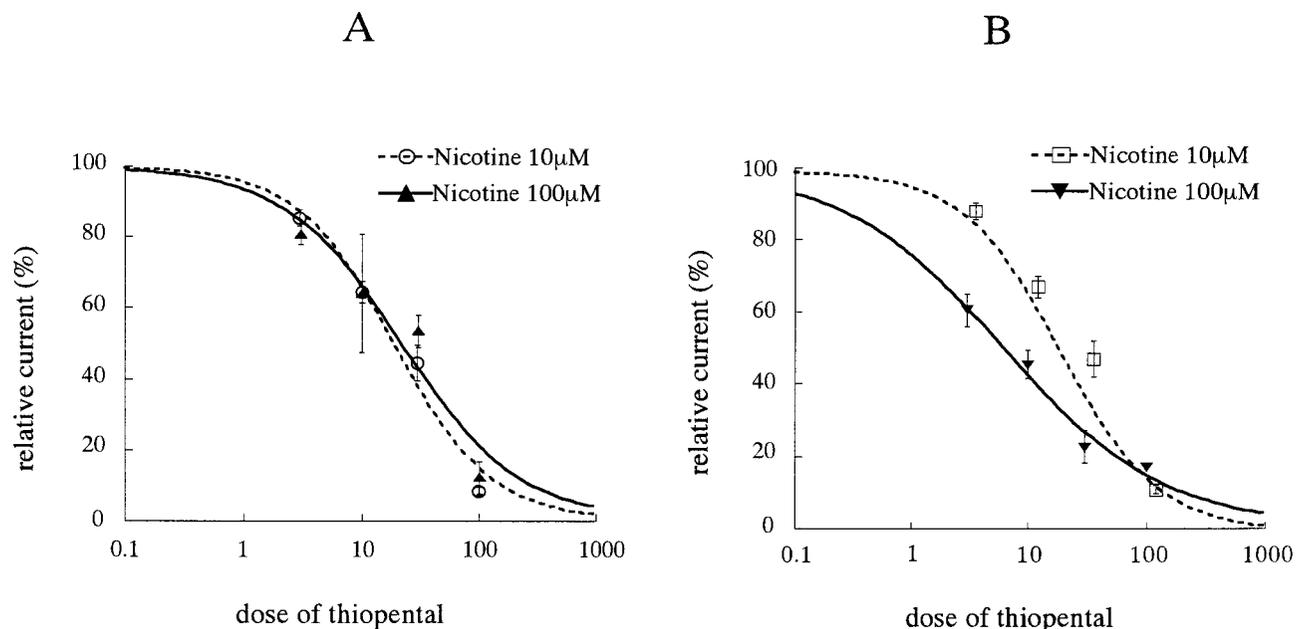
Fig. 4. The concentration–inhibition curves of thiopental for the peak current (*A*) and the nondesensitized current (*B*) elicited by 10 and 100 *μ*M nicotine in rat medial habenula (MHb) neurons. The currents in the presence of thiopental were normalized to the average of the control currents before and after the addition of thiopental and were plotted against the concentration of thiopental. A least-squares fit was performed using the equation described in materials and methods. The fitting procedure gave an IC$_{50}$ value of 30.1 ± 7.5 *μ*M and a Hill coefficient of 1.06 ± 0.29 for the peak currents elicited by 10 *μ*M nicotine, and an IC$_{50}$ value of 21.9 ± 6.5 *μ*M and a Hill coefficient of 0.87 ± 0.25 for the peak current elicited by 100 *μ*M nicotine (*A*). The IC$_{50}$ value and Hill coefficient were 19.0 ± 3.3 *μ*M and 1.05 ± 0.19 for the nondesensitized current induced by 10 *μ*M nicotine, and 6.2 ± 1.1 *μ*M and 0.63 ± 0.09 for the nondesensitized current induced by 100 *μ*M nicotine, respectively (*B*). The number of the experiments was 6–12 for each condition. The inhibition of thiopental on the nondesensitized current was greater for 100 *μ*M nicotine than that for 10 *μ*M nicotine.

statistical significance because of large variances in the values for 100 *μ*M MPPB. There was no significant difference in time constant ratios between the isomers (table 3). These results showed that although the effects of MPPB isomers *in vivo* were completely opposite, both isomers showed non-stereoselective inhibition of native nAChRs expressed in rat CNS neurons.

Comparison of Pharmacologic Properties of Neuronal Nicotinic Acetylcholine Receptors in PC12 Cells and Rat Medial Habenula Neurons

We compared the responses to three different nAChRs agonists in PC12 cells and rat MHb neurons to charac-terize agonist pharmacology of nAChRs in these prepa-rations. We used nicotine, acetylcholine, and cytisine at 30 *μ*M. For the peak current, the rank order of the amplitudes of the responses was cytisine > nicotine > acetylcholine in both types of neuronal cells. For the nondesensitized current, it was nicotine = cytisine > acetylcholine in MHb neurons, and cytisine > nicotine > acetylcholine in PC12 cells. The normalized responses relative to the nicotine-induced response were not sig-nificantly different between these two types of neuronal cells (fig. 7). These results indicated that PC12 cells and rat MHb neurons share similar agonist pharmacology.

Table 1. Changes in Percent Current Decay by Thiopental and MPPB Isomers

| Thiopental (*μ*M) | 10 *μ*M Nicotine | | | 100 *μ*M Nicotine | | | MPPB | 10 *μ*M Nicotine | | |
	Control (%)	Thiopental (%)	Thiopental/ Control	Control (%)	Thiopental (%)	Thiopental/ Control		Control (%)	MPPB (%)	MPPB/ Control
3	57.6 ± 4.4	57.8 ± 3.6	1.01 ± 0.02	76.8 ± 4.1	81.4 ± 3.8	1.06 ± 0.02	R(−) 30	36.6 ± 3.1	41.3 ± 4.0	1.13 ± 0.07
10	42.3 ± 3.0	51.7 ± 2.6*	1.28 ± 0.06†	56.0 ± 6.2	69.4 ± 5.5*	1.36 ± 0.01†	S(+) 30	45.1 ± 4.8	44.5 ± 4.3	1.01 ± 0.03
30	53.6 ± 4.8	63.3 ± 4.7*	1.22 ± 0.04†	67.3 ± 7.4	84.4 ± 4.5*	1.30 ± 0.09†	R(−) 100	39.7 ± 2.7	37.9 ± 3.5	0.96 ± 0.05
100	46.7 ± 3.6	68.5 ± 3.5*	1.51 ± 0.04†	56.9 ± 5.9	89.9 ± 1.9*	1.75 ± 0.16†	S(+) 100	40.1 ± 1.8	42.9 ± 4.0	1.03 ± 0.07

Percent current decay = (peak current − nondesensitized current)/peak current × 100.

Values are mean ± SEM. There were 6–12 experiments for each condition. Control: values for the average responses before and after administration of thiopental or 1-methyl-5-phenyl-5-propyl barbituric acid (MPPB) isomers. Thiopental and MPPB: values for the responses in the presence of thiopental or MPPB isomers. Thiopental/control and MPPB/control: ratios of percent current decay in the response of the barbiturates to that at control.

* *P* < 0.05 *versus* control. † *P* < 0.05 *versus* 3 *μ*M thiopental in the same group.

Table 2. Changes in the Time Constant of the Decaying Phase of the Elicited Current Produced by Thiopental

	Time Constant (s)	Time Constant Ratio				
	Baseline	Nicotine	3 μM Thiopental	10 μM Thiopental	30 μM Thiopental	100 μM Thiopental
10 μM Nicotine	2.56 ± 0.002 (n = 24)	0.93 ± 0.05 (n = 4)	0.85 ± 0.09 (n = 6)	0.80 ± 0.11 (n = 6)	0.82 ± 0.09 (n = 6)	0.42 ± 0.08* (n = 6)
100 μM Nicotine—fast	0.35 ± 0.001 (n = 22)	1.34 ± 0.52 (n = 5)	0.75 ± 0.13 (n = 6)	0.56 ± 0.1 (n = 6)	0.37 ± 0.05* (n = 5)	0.29 ± 0.05* (n = 5)
100 μM Nicotine—slow	2.69 ± 0.003 (n = 22)	1.05 ± 0.16 (n = 5)	0.61 ± 0.08 (n = 6)	0.66 ± 0.04 (n = 6)	0.82 ± 0.14 (n = 5)	0.61 ± 0.15 (n = 5)

Time constant ratio: the ratio of the time constant in the presence of thiopental relative to the average of pre- and postcontrol. Baseline: values for the responses immediately before thiopental. Nicotine: the ratio of the time constant of the second response relative to the average of the time constants of the first and third responses, derived from the experiments in which nicotine alone was applied repeatedly. Fast and slow: the fast and slow components of double exponential functions fitted to the responses elicited by 100 μM nicotine. Values are mean ± SEM.

* $P < 0.05$ *versus* nicotine.

Discussion

To our knowledge, the current study is the first to demonstrate that thiopental inhibits native nAChR-mediated current at clinically relevant concentrations (20–50 μM)[27] in rat CNS neurons. Studies using native receptors have importance because recombinant nAchRs do not necessarily reflect characteristics of corresponding native receptors.[1,18] The discrepancies may be raised by differences in subunit stoichiometry, presence of unidentified subunits, and distinct modulation by cellular elements of host cells.[18] We also found that convulsant and depressant isomers of MPPB both inhibited native nAChRs in rat CNS neurons and that the inhibitory actions of MPPB isomers were almost the same in their potencies, despite their opposite behavioral effects.[15] These results suggest that there is no correlation between inhibitory actions on central nAChRs and the hypnotic effects of the barbiturates *in vivo*. This finding suggests that inhibition of neuronal nAChRs in MHb neurons is not directly relevant to the hypnotic or anticonvulsive actions of barbiturates. Lack of correlation between inhibitory effects on neuronal nAChRs and anesthetic potencies of barbiturate stereoisomers is consistent with the results of the recent study investigating effects of thiopental stereoisomers on recombinant $\alpha_4\beta_2$ and α_7 homomeric receptors.[17]

Comparison of Inhibitory Effects of the Barbiturates in PC12 cells and Medial Habenula Neurons

We previously reported that depressant [thiopental and $R(-)$-MPPB] and convulsant [$S(+)$-MPPB and -5-(2-cyclohexilidene-ethyl)-5-ethyl barbituric acid] barbiturates both suppress neuronal nAchRs in PC12 cells.[14] We have confirmed that thiopental and MPPB isomers also inhibit nAchR-mediated response in rat MHb neurons independent from their abilities to produce hypnosis. We have verified our assumption in the previous study that nAchRs in PC12 cells and the CNS have similar sensitivities to barbiturates. Thiopental at 30 μM reduced the current elicited by 100 μM nicotine to the same extent in PC12 cells and MHb neurons, and it accelerated the current decay in the similar manner. IC$_{50}$ values for thiopental are comparable in these two populations, even though agonist concentrations and application times were different. Although we studied effects of MPPB isomers using different nicotine concentrations, characteristics of inhibition by the isomers were slightly different between two preparations. Although MPPB isomers accelerated the current decay in PC12 cells, they did not significantly increase percent current decay nor decrease the time constant of the decaying phases in

Fig. 5. Comparison of inhibitory effects of 30 μM thiopental on the nicotine-induced current in rat medial habenula (MHb) neurons and PC12 cells. The relative peak (*A*) and nondesensitized (*B*) currents were 57.3 ± 7.9 and 44.4 ± 7.5%, respectively, in the presence of thiopental and 10 μM nicotine in MHb neurons (n = 9). In the presence of thiopental and 100 μM nicotine, they were 53.2 ± 6.6 and 20.1 ± 5.7%, respectively, in MHb neurons (n = 4), and 60.4 ± 12.4 and 18.8 ± 3.0, respectively, in PC12 cells (n = 4). There was no significant difference between the inhibitory effects of thiopental on the current elicited by 100 μM nicotine in these two types of neuronal cells. Nic = nicotine; n.s. = not significantly different.
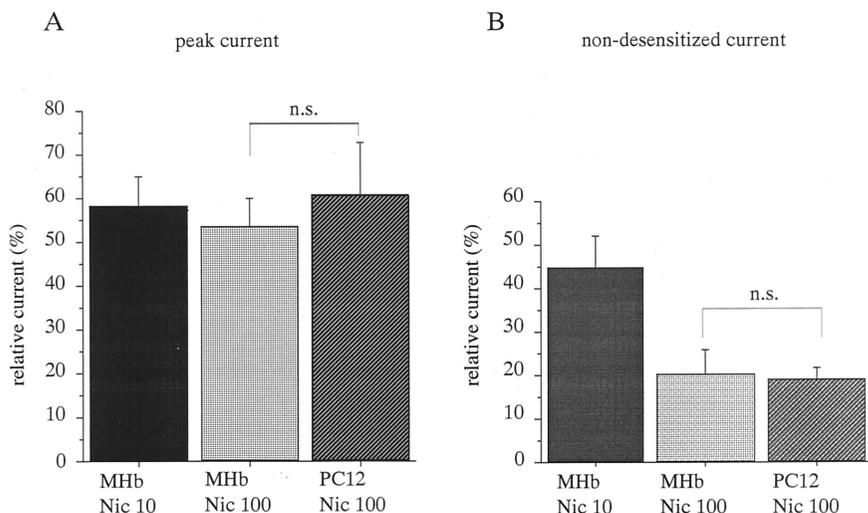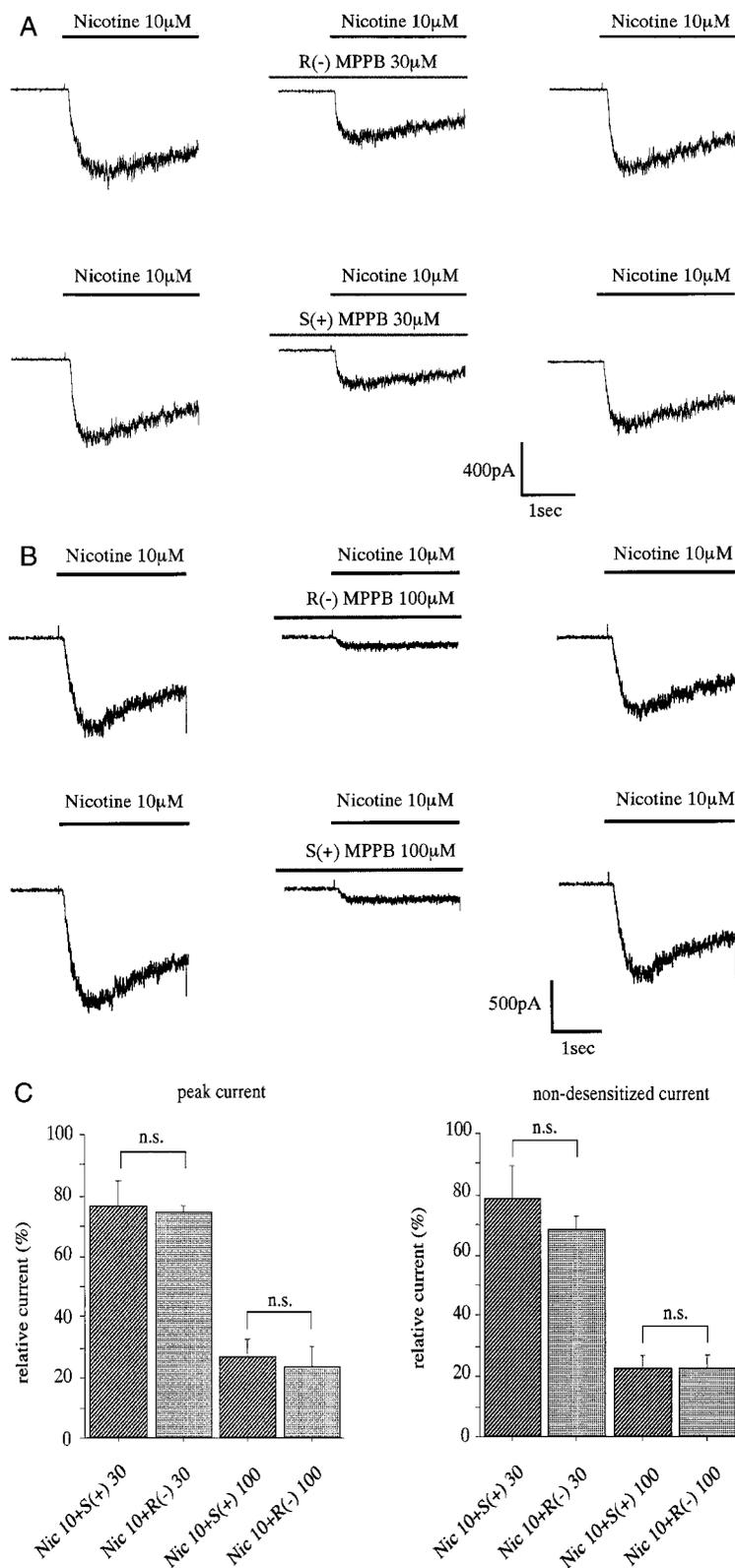
Fig. 6. Inhibition of the nicotine-induce current by 30 (*A*) and 100 μм (*B*) of 1-methyl-5-phenyl-5-propyl barbituric acid (MPPB) isomers in rat medial habenula (MHb) neurons. The recordings were made in the same way as in figures 2 and 3. Both *R*(−)- and *S*(+)-MPPB inhibited the nicotine-induced current reversibly in a dose-dependent manner. (*C*) Summary data for inhibition by MPPB isomers. In the presence of 30 μм *S*(+)-MPPB, the relative peak and nondesensitized currents were 75.7 ± 9.0 and 78.8 ± 10.7% of control (n = 12), respectivley. In the presence of 30 μм *R*(−)-MPPB, they were reduced to 73.6 ± 2.7 and 67.8 ± 5.0% of control (n = 12), respectively. *S*(+)-MPPB at 100 μм reduced the peak and nondesensitized currents to 26.6 ± 6.0 and 22.5 ± 4.6% of control (n = 6), respectivley. *R*(−)-MPPB at the same dose reduced them to 23.4 ± 6.5 and 22.1 ± 4.9% of control (n = 6), respectively. There was no significant difference in the magnitudes of inhibition by *S*(+)- and *R*(−)-MPPB at either concentration. Nic = nicotine; n.s. = not significantly different.

MHb neurons. This might be a result of different agonist concentrations used and differences in characteristics of the receptors. It may be possible that the modes of inhibition by MPPB are different in these two populations. Taken together, it seems that thiopental showed the similar effects on two populations of nAchRs, but characteristics of inhibition by MPPB isomers were slightly different between these populations.

Table 3. Changes in the Time Constant of the Decaying Phase of the Elicited Current Produced by MPPB Isomers

	Time Constant (s)	Time Constant Ratio		
	Baseline	Nicotine	30 μM MPPB	100 μM MPPB
10 μM Nicotine—$R(-)$-MPPB	3.06 ± 0.01 (n = 19)	0.93 ± 0.05 (n = 4)	0.98 ± 0.15 (n = 7)	2.99 ± 1.0 (n = 12)
10 μM Nicotine—$S(+)$-MPPB	3.73 ± 0.01 (n = 19)	0.93 ± 0.05 (n = 4)	1.07 ± 0.16 (n = 7)	3.54 ± 1.53 (n = 12)

Time constant ratio: the ratio of the time constant in the presence of 1-methyl-5-phenyl-5-propyl barbituric acid (MPPB) relative to the average of pre- and postcontrol. Baseline: values for the responses immediately before MPPB. Nicotine: the ratio of the time constant of the second response relative to the average of the time constants of the first and third responses, derived from the experiments in which nicotine alone was applied repeatedly. Values are mean ± SEM.

Nicotinic Acetylcholine Receptors in PC12 cells and Medial Habenula Neurons

We found that the rank order of agonist potencies was almost the same for PC12 cells and MHb neurons. mRNAs detected in MHb neurons are for α_3, α_4, α_7, β_2, β_3, and β_4 subunits,[6,28] and the predominant subunit composition of nAChRs on somas is considered to be $\alpha_3\beta_4$.[23] However, multiple subtypes of the receptors exist, and the β_2 subunit is thought to be additionally contained in variable subpopulation of these neurons.[23] On the other hand, PC12 cells expressed mRNAs of α_3, α_5, β_2, β_3, and β_4 subunits,[29,30] and predominant subunit composition is estimated to be $\alpha_3\beta_4$ or $\alpha_3\beta_4\alpha_5$.[16,28,29] Therefore, it is likely that $\alpha_3\beta_4$-containing
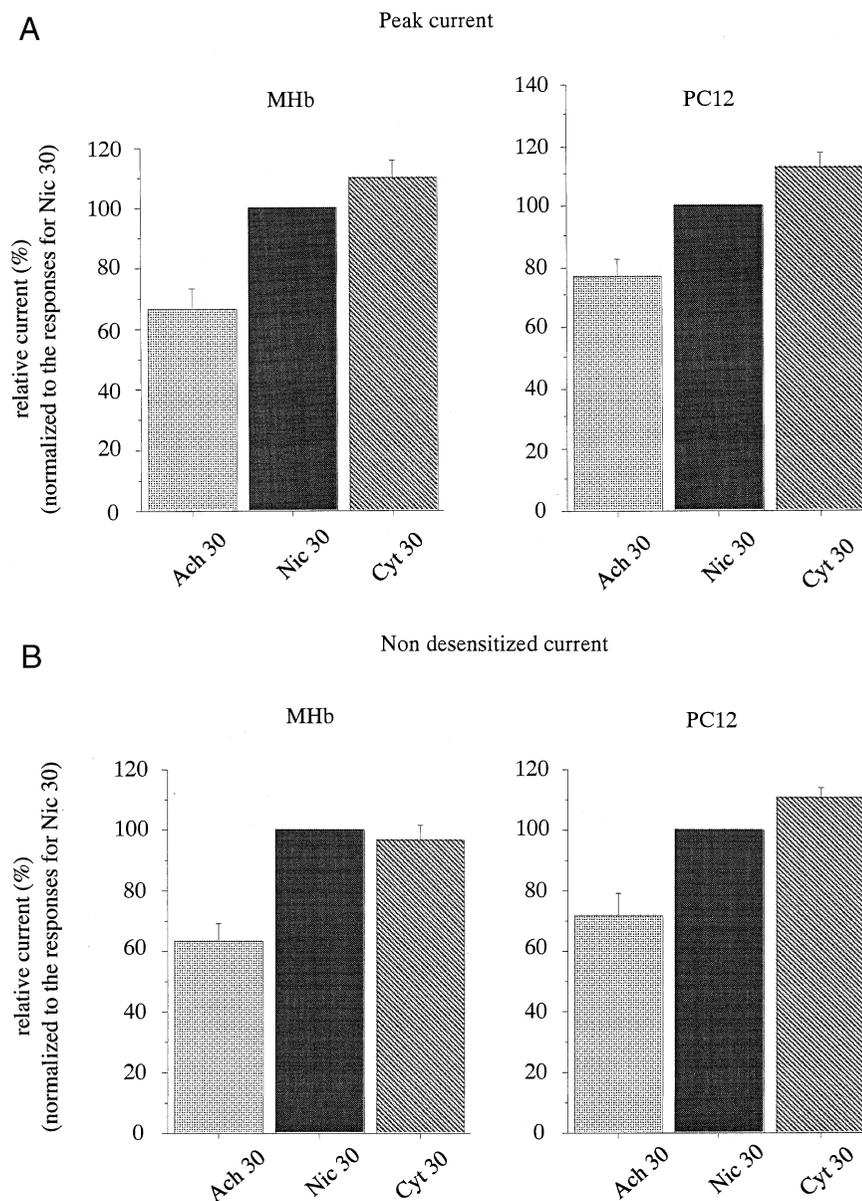

Fig. 7. Comparison of whole-cell responses to three different nicotinic agonists in rat medial habenula (MHb) neurons and PC12 cells. The responses induced by 30 μM acetylcholine (Ach) and 30 μM cytisine (Cyt) were normalized to the responses caused by 30 μM nicotine (Nic). (*A*) The normalized peak currents elicited by Ach were 67 ± 7% in MHb neurons and 76 ± 6% in PC12 cells. The normalized peak currents elicited by Cyt were 110 ± 6% and 113 ± 5% in MHb neurons and PC12 cells, respectively. (*B*) The normalized nondesensitized currents elicited by Ach were 63 ± 6% in MHb neurons and 71 ± 7% in PC12 cells. The normalized nondesensitized currents elicited by Cyt were 97 ± 5 and 111 ± 4% in MHb neurons and PC12 cells, respectively. The data were obtained from 15 MHb neurons and 5 PC12 cells for each agonist. These values were not significantly different between MHb neurons and PC12 cells.

receptors are predominant in both populations. However, the exact stoichiometry and combination of the subunits are not identified in either populations.

Early biochemical studies indicated that a major subtype of nAChRs responsible for high affinity nicotine binding sites in the CNS contains $\alpha_4\beta_2$ subunits.[3] However, consistent with the diversity in subunit gene expression,[31] a broad spectrum of nAChRs subtypes are identified by the recent electrophysiological studies in several regions of the brain, such as hippocampus,[32] MHb,[19,21,22] interpeduncular nucleus (IPN),[33] and midbrain nucleus (*e.g.*, Locus coeleus).[34] And in light of recent studies showing more extensive β_4 expression in the CNS than initially thought, β_4-containing nAChRs are also important in the CNS.[31,35]

Roles of the Medial Habenula

We have chosen to study MHb neurons because they respond to external application of nicotinic agonists robustly, and characteristics of the responses are relatively homogenous.[23,33] In addition, there are few models suitable for electrophysiological studies of native nAChRs with relatively homogenous properties in the brain. However, physiological roles of the MHb are poorly understood. MHb neurons give rise to a large cholinergic projection through the fasciculus retroflexus to the IPN and the median raphe.[33] The MHb-IPN system is thought to be involved in rapid-eye-movement sleep, avoidance response, arousal, and attentive behavior.[36–39] It has been reported that destruction of the MHb and its primary efferent pathway, the fasciculus retroflexus, impaired avoidance learning,[31,37] and decreased rapid-eye-movement sleep and hippocampal theta rhythm of electroencephalogram.[39] It is known that nAChRs with the same pharmacologic properties are present at the somas as well as the terminals of MHb axons connecting with the IPN.[33] Therefore, barbiturates are likely to affect activity of the MHb-IPN system by inhibition of these receptors, which may result in modulation of rapid-eye-movement sleep and avoidance learning.

Our study shows that thiopental and depressant and convulsant isomers of MPPB all inhibit native $\alpha_3\beta_4$-containing nAChRs in MHb neurons. This type of nAChR is not predominant in the brain, and MHb is not thought to be the primary responsible site governing consciousness. Therefore, these receptors are unlikely to represent the family of native central nAChRs. Taking account of the results of our experiment and the study by Downie *et al.*,[17] however, anesthetic potencies of barbiturate stereoisomers do not correlate with inhibitory effects on brain $\alpha_3\beta_4$-containing receptors, recombinant $\alpha_4\beta_2$ or α_7 homomeric receptors. On the contrary, it is known that potentiating effects on the activity of $GABA_A$ receptors matched well anesthetic actions of enantiomers of thiopental and MPPB.[25,40] Therefore, modulation of neuronal nAChRs is likely to be much less important for hypnotic action of barbiturates than that of $GABA_A$ receptors. In conclusion, since thiopental inhibits native nAChRs in rat MHb neurons at the clinically relevant concentrations, this effect may contribute some aspects associated with barbiturate anesthesia. However, the finding that convulsant and depressant isomers of a barbiturate both inhibited the nAChR-mediated currents in the same degree suggests that the inhibition of nAChRs in MHb neurons is not directly relevant to hypnotic or anticonvulsive actions of barbiturates.

References

1. Sargent PB: The diversity of neuronal nicotinic acetylcholine receptors. Annu Rev Neurosci 1993; 16:403–43

2. Lindstrom J: Neuronal nicotinic acetylcholine receptors, Ion Channels. Edited by Narahashi T. New York, Plenum Press, 1996, pp 377–450

3. Role LW, Berg DK: Nicotinic receptors in the development and modulation of CNS synapses. Neuron 1996; 16:1077–85

4. Alkondon M, Pereira EF, Barbosa CT, Albuquerque EX: Neuronal nicotinic acetylcholine receptor activation modulates gamma-aminobutyric acid release from CA1 neurons of rat hippocampal slices. J Pharmacol Exp Ther 1997; 283:1396–411

5. McGehee DS, Heath MJ, Gelber S, Devay P, Role LW: Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. Science 1995; 269:1692–6

6. Wonnacott S: Presynaptic nicotinic ACh receptors. Trends Neurosci 1997; 20:92–8

7. Brioni JD, Decker MW, Sullivan JP, Arneric SP: The pharmacology of (-)-nicotine and novel cholinergic channel modulators. Adv Pharmacol 1997; 37:153–214

8. Perry E, Walker M, Grace J, Perry R: Acetylcholine in mind: a neurotransmitter correlate of consciousness? Trends Neurosci 1999; 22:273–80

9. Marubio LM, del Mar Arroyo-Jimenez M, Cordero-Erausquin M, Lena C, Le Novere N, de Kerchove d'Exaerde A, Huchet M, Damaj MI, Changeux JP: Reduced antinociception in mice lacking neuronal nicotinic receptor subunits. Nature 1999; 398:805–10

10. Flood P, Ramirez-Latorre J, Role L: Alpha 4 beta 2 neuronal nicotinic acetylcholine receptors in the central nervous system are inhibited by isoflurane and propofol, but alpha 7-type nicotinic acetylcholine receptors are unaffected. ANESTHESIOLOGY 1997; 86:859–65

11. Andoh T, Furuya R, Oka K, Hattori S, Watanabe I, Kamiya Y, Okumura F: Differential effects of thiopental on neuronal nicotinic acetylcholine receptors and P2X purinergic receptors in PC12 cells. ANESTHESIOLOGY 1997; 87:1199–209

12. Violet JM, Downie DL, Nakisa RC, Lieb WR, Franks NP: Differential sensitivities of mammalian neuronal and muscle nicotinic acetylcholine receptors to general anesthetics. ANESTHESIOLOGY 1997; 86:866–74

13. Furuya R, Oka K, Watanabe I, Kamiya Y, Itoh H, Andoh T: The effects of ketamine and propofol on neuronal nicotinic acetylcholine receptors and P2x purinoceptors in PC12 cells. Anesth Analg 1999; 88:174–80

14. Watanabe I, Andoh T, Furuya R, Sasaki T, Kamiya Y, Itoh H: Depressant and convulsant barbiturates both inhibit neuronal nicotinic acetylcholine receptors. Anesth Analg 1999; 88:1406–11

15. Buch HP, Schneider-Affeld F, Rummel W: Stereochemical dependence of pharmacological activity in a series of optically active N-methylated barbiturates. Naunyn Schmiedebergs Arch Pharmacol 1973; 277:191–8

16. McGehee DS, Role LW: Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu Rev Physiol 1995; 57:521–46

17. Downie DL, Franks NP, Lieb WR: Effects of thiopental and its optical isomers on nicotinic acetylcholine receptors. ANESTHESIOLOGY 2000; 93:774–83

18. Sivilotti LG, McNeil DK, Lewis TM, Nassar MA, Schoepfer R, Colquhoun D: Recombinant nicotinic receptors, expressed in Xenopus oocytes, do not resemble native rat sympathetic ganglion receptors in single-channel behaviour. J Physiol (Lond) 1997; 500:123–38

19. Mulle C, Changeux JP: A novel type of nicotinic receptor in the rat central nervous system characterized by patch-clamp techniques. J Neurosci 1990; 10:169–75

20. Mulle C, Lena C, Changeux JP: Potentiation of nicotinic receptor response by external calcium in rat central neurons. Neuron 1992; 8:937–45

21. Lester RA, Dani JA: Acetylcholine receptor desensitization induced by nicotine in rat medial habenula neurons. J Neurophysiol 1995; 74:195–206

22. Connolly JG, Gibb AJ, Colquhoun D: Heterogeneity of neuronal nicotinic acetylcholine receptors in thin slices of rat medial habenula. J Physiol (Lond) 1995; 484:87–105

23. Quick MW, Ceballos RM, Kasten M, McIntosh JM, Lester RA: Alpha3beta4 subunit-containing nicotinic receptors dominate function in rat medial habenula neurons. Neuropharmacology 1999; 38:769–83

24. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 1981; 391:85–100

25. Kamiya Y, Andoh T, Furuya R, Hattori S, Watanabe I, Sasaki T, Ito H, Okumura F: Comparison of the effects of convulsant and depressant barbiturate stereoisomers on AMPA-type glutamate receptors. ANESTHESIOLOGY 1999; 90:1704–13

26. Valenta DC, Downing JE, Role LW: Peptide modulation of ACh receptor desensitization controls neurotransmitter release from chicken sympathetic neurons. J Neurophysiol 1993; 69:928–42

27. Franks NP, Lieb WR: Molecular and cellular mechanisms of general anaesthesia. Nature 1994; 367:607–14

28. Sivilotti L, Colquhoun D: Acetylcholine receptors: Too many channels, too few functions. Science 1995; 269:1681–2

29. Rogers SW, Mandelzys A, Deneris ES, Cooper E, Heinemann S: The expression of nicotinic acetylcholine receptors by PC12 cells treated with NGF. J Neurosci 1992; 12:4611–23

30. Ifune CK, Steinbach JH: Voltage-dependent block by magnesium of neuronal nicotinic acetylcholine receptor channels in rat phaeochromocytoma cells. J Physiol (Lond) 1991; 443:683–701

31. Winzer-Serhan UH, Leslie FM: Codistribution of nicotinic acetylcholine receptor subunit alpha3 and beta4 mRNAs during rat brain development. J Comp Neurol 1997; 386:540–54

32. Alkondon M, Albuquerque EX: Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. I. Pharmacological and functional evidence for distinct structural subtypes. J Pharmacol Exp Ther 1993; 265:1455–73

33. Mulle C, Vidal C, Benoit P, Changeux JP: Existence of different subtypes of nicotinic acetylcholine receptors in the rat habenulo-interpeduncular system. J Neurosci 1991; 11:2588–97

34. Nabekura J, Murata O, Ishibashi H, Akaike N: Use-dependent suppression of the nicotinic acetylcholine receptor response by the proadrenomedullin N-terminal 20-amino acid peptide in rat locus coeruleus neurons. J Neurochem 1998; 70:865–70

35. Dineley-Miller K, Patrick J: Gene transcripts for the nicotinic acetylcholine receptor subunit, beta4, are distributed in multiple areas of the rat central nervous system. Brain Res Mol Brain Res 1992; 16:339–44

36. Wirtshafter D: The role of interpeduncular connections with the tegmentum in avoidance learning. Physiol Behav 1981; 26:985–9

37. Thornton EW, Murray M, Connors-Eckenrode T, Haun F: Dissociation of behavioral changes in rats resulting from lesions of the habenula versus fasciculus retroflexus and their possible anatomical substrates. Behav Neurosci 1994; 108:1150–62

38. Murphy CA, DiCamillo AM, Haun F, Murray M: Lesion of the habenular efferent pathway produces anxiety and locomotor hyperactivity in rats: A comparison of the effects of neonatal and adult lesions. Behav Brain Res 1996; 81:43–52

39. Valjakka A, Vartiainen J, Tuomisto L, Tuomisto JT, Olkkonen H, Airaksinen MM: The fasciculus retroflexus controls the integrity of REM sleep by supporting the generation of hippocampal theta rhythm and rapid eye movements in rats. Brain Res Bull 1998; 47:171–84

40. Tomlin SL, Jenkins A, Lieb WR, Franks NP: Preparation of barbiturate optical isomers and their effects on GABA(A) receptors. ANESTHESIOLOGY 1999; 90:1714–22