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Effects of neuromuscular blocking agents on central respiratory chemosensitivity in newborn rats

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ABSTRACT

Neuromuscular blocking agents suppress central respiratory activity through their inhibitory effects on preinspiratory neurons and the synaptic drive from preinspiratory neurons to inspiratory neurons. Central CO_2-chemosensitive areas, which partly consist of CO_2-excited neurons, in the rostral ventrolateral medulla are thought to provide tonic drive to the central respiratory network and involve cholinergic mechanisms, which led us to hypothesize that neuromuscular blocking agents can inhibit CO_2-excited neurons and attenuate respiratory CO_2 responsiveness. To test this hypothesis, we used isolated brainstem-spinal cord preparations from newborn rats. The increase of C4 burst frequency induced by a hypercapnic superfusate, i.e. respiratory CO_2 responsiveness, was suppressed by the application of neuromuscular blocking agents, either d-tubocurarine (10, 100μM) or vecuronium (100μM). These agents (40μM) also induced hyperpolarization and decreases in firing frequency of CO_2-excited neurons in the rostral ventrolateral medulla. Our results demonstrate that neuromuscular blocking agents inhibit CO_2-excited tonic firing neurons and attenuate respiratory CO_2 responsiveness.

Key terms: brainstem, chemosensitivity, CO_2 responsiveness, muscle relaxant, nicotinic acetylcholine receptor

INTRODUCTION

Central CO_2 chemosensitivity plays a crucial role in central respiratory control. Chemosensitive neurons, i.e. CO_2-excited neurons, in the rostral ventrolateral medulla (RVLM) are thought to provide tonic drive to the neuronal network that generates the respiratory rhythm (Eugenin & Nicholls, 1997; Richerson, 1998; for review, see Eugenin et al., 2001) partly by cholinergic mechanisms (for review, see Loeschcke, 1982; Eugenin & Nicholls, 1997; Eugenin et

al., 2001; Okada et al., 2001). A clear relationship between endogenous cholinergic mechanisms and central chemoreception has been found using the isolated brainstem-spinal cord preparation of neonatal rat (Monteau et al., 1990) and of opossum (Eugenin & Nicholls, 1997). In these preparations, the increase in respiration induced by acetylcholine (ACh) and acetylcholinesterase inhibitors show a similar pattern as that induced by low pH stimulation (Monteau et al., 1990; Eugenin & Nicholls, 1997). ACh- and low pH-

sensitive areas in the RVLM overlapped (Eugenin & Nicholls, 1997).

The cholinergic nature of chemosensitive neurons in the RVLM has been investigated extensively. Chemosensitive neurons sensitive to H^+ are excited by ACh and depressed by muscarinic and nicotinic cholinergic blocking agents in the medulla slice preparation of rat (Fukuda & Loeschcke, 1979; for review, see Okada et al., 2001). The application of atropine attenuates respiratory CO_2 responsiveness in the *in vivo* anesthetized cat (Dev & Loeschcke, 1979) and in the *in vitro* preparation of neonatal rat (Monteau et al., 1990). The topical application of nicotine on chemosensitive areas in the RVLM induces hyperventilation, and intravenous injection of hexamethonium, a nicotinic ACh receptor (nAChR) antagonist, diminishes nicotine-induced hyperventilation in the *in vivo* anesthetized rat (Dev & Loeschcke, 1979). More interestingly, muscarine reverses the opioid-induced suppression of respiratory (C4) activity in parallel with chemosensitive CO_2-excited neuron activity in the isolated brainstem-spinal cord preparation of neonatal rat (Ballanyi et al., 1999), indicating the direct relationship between chemosensitive CO_2-excited neurons in the RVLM and the efferent respiratory activity.

Respiratory CO_2 responsiveness is partly inhibited by the non-selective nAChR antagonist mecamylamine and a4b2 nAChR selective antagonist dihydro-b-erythroidine in the isolated brainstem-spinal cord preparation of neonatal rat (Kuwana et al., 2000a,b). This result indicates that the a4b2 nAChR subunit plays a role in central chemosensitivity as above. In a recent study, atracurium, one of the neuromuscular blocking agents (NMBAs), blocks a4b2 nAChRs expressed in *Xenopus* oocytes (Chiodini et al., 2001), although the subunit composites of neuronal and muscle nAChRs vary (Lindstrom et al., 1995). Furthermore, our previous study has revealed that NMBAs suppress central respiratory control by their inhibitory effects on preinspiratory neurons and on the excitatory connection from preinspiratory neurons to inspiratory neurons (Sakuraba et al., 2003). Taken together, it is possible that NMBAs suppress central

chemosensitivity by blocking excitatory input to central respiratory control (Eugenin & Nicholls, 1997; Richerson, 1998; for review, see Eugenin et al., 2001), via a4b2 nAChR in the RVLM. Therefore, we hypothesized that NMBAs suppress respiratory CO_2 responsiveness by their inhibitory effects on chemosensitive CO_2-excited neurons of the RVLM.

The purpose of this study is to investigate the effect of NMBAs on respiratory CO_2 responsiveness and chemosensitive CO_2-excited neurons in the RVLM.

MATERIALS AND METHODS

Isolated brainstem-spinal cord preparation

All experiments were approved by the Animal Experimentation Ethics Committee of the Keio University, Tokyo, Japan. Data were obtained from 86 neonatal Wistar rats (1-4 days old) using the isolated brainstem-spinal cord preparation as previously described in detail elsewhere (Kuwana et al., 1998; Sakuraba et al., 2003). In brief, the brainstem with the cervical spinal cord was isolated under deep ether anesthesia. The cerebellum and pons were ablated in a chamber filled with oxygenated, artificial cerebrospinal fluid (ACSF) consisting of (in mM) 126 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 2 $MgSO_4$, 26 $NaHCO_3$ and 30 glucose. Then, the preparation was transferred to a recording chamber of 2 ml volume and was fixed with miniature pins on a silicon rubber base with the ventral side up. The preparation was superfused at 26°C with control ACSF (2% CO_2 in O_2; pH = 7.8). With such alkaline ACSF, tissue pH of the superficial (< 400 μm) medullary layer could be maintained in the physiological range (Okada et al., 1993a). The activity of C4 ventral roots was recorded using a glass suction electrode, amplified with a conventional AC amplifier (AVH 11, Nihon Kohden, Tokyo, Japan) and integrated (time constant 100 msec). The signals were recorded on a thermal array recorder and stored on digital tape for

subsequent analysis. The C4 burst frequency was measured as the respiratory frequency (f_R). The pH of the superfusate was continuously monitored with a conventional glass pH electrode calibrated using two different calibration solutions with known pH values. The control ACSF was replaced by several ACSF solutions in accordance with the protocol.

Neuronal recording

Activities of tonic firing neurons in the superficial (< 400 µm) RVLM were recorded intracellularly using a blind patch clamp configuration (Kuwana et al., 1998; Sakuraba et al., 2003). Briefly, a glass pipette (GC100TF-10, Clark Electromed, Reading, UK) was pulled with a horizontal puller (PA-91, Narishige, Tokyo, Japan) to a tip size of approximately 2 µm. Electrode resistance ranged from 12 to 16 MΩ when it was filled with a solution containing (in nM) 130 K-gluconate, 10 EGTA, 10 HEPES, 1 $CaCl_2$, 1 $MgCl_2$, and nystatin (100 µg/ml); pH was adjusted to 7.2-7.3 by using KOH. The micropipette was inserted into the RVLM with a manual hydraulic micromanipulator. Membrane potentials were recorded with a whole cell patch amplifier (CEZ 3100, Nihon Kohden, Tokyo, Japan). Neurons were searched for by applying positive pressure (10-20 cm H_2O) inside the pipette. After giga-ohm seal was obtained, the recorded membrane potential became gradually negative and was stabilized in about 10 min. The membrane potential (E_m) was presented without correcting the liquid junction potential. The firing frequency was also calculated as the number of spikes per second. This perforated patch recording remained stable for more than 60 min.

Protocols

After the preparation was superfused with control ACSF (2% CO_2 in O_2; pH = 7.8) for more than 30 min and the stable recording of the C4 activities was established, experiments were conducted using the following protocol.

Protocol-1: Neuromuscular blocking agent and respiratory CO_2 responsiveness

Respiratory CO_2 responsiveness was tested first without NMBA by switching superfusate from control ACSF to high CO_2/low pH ACSF (8% CO_2 in O_2; pH = 7.2) (7 min), followed by a washout period using control ACSF again for more than 10 min. Then, respiratory CO_2 responsiveness was tested by superfusion with d-tubocurarine (D-TC; Sigma, St. Louis, MO, USA) (1, 10, 100 µM) or vecuronium bromide (VB; Organon, Amsterdam, the Netherlands) (10, 100 µM). Respiratory CO_2 responsiveness was defined as the difference of f_R between 2% CO_2 (pH = 7.8) and 8% CO_2 (pH = 7.2) (Df_R). Preparations in which respiratory activity was completely abolished by NMBA or complete recovery was not obtained were excluded from the study.

Protocol-2: Neuromuscular blocking agent and the activity of tonic firing neurons in the rostral ventrolateral medulla

Control ACSF was changed to high CO_2/low pH ACSF (8% CO_2 in O_2; pH = 7.2) and then intracellular recording of chemosensitive neurons by blind patch clamp techniques was tried because the firing of chemosensitive neurons was increased by hypercapnic superfusate (Kawai et al., 1996), which made the search for chemosensitive neurons easier. After this was performed, the preparation was superfused by high CO_2/low pH ACSF for at least 10min until the intracellular recording of tonic firing neurons became stable. Then, the superfusate was changed to control ACSF (2% CO_2) for 8 min to assess CO_2 sensitivity of tonic firing neurons. If the membrane potential of neurons was hyperpolarized by changing superfusate from high CO_2/low pH ACSF to control ACSF, we considered these neurons to be chemosensitive and continued the protocol. Then, the superfusate was changed to control ACSF (10 min). Next, high CO_2/low pH ACSF was changed to high CO_2/low pH ACSF containing D-TC 40 µM or VB 40 µM for 3-5 min to analyze

effects of NMBAs on chemosensitive neurons, followed by high CO_2/low pH ACSF (10 min). We examined the change of E_m (mV) and firing frequency (Hz) of tonic firing neurons. All parameters were measured for 2-3 minutes at the end of each period.

Statistical Analyses

The original peak values of Df_R with and without NMBAs were analyzed by using a paired *t*-test. Effects on tonic firing neurons were analyzed with a paired *t*-test. All data were expressed as mean ± SEM. *P* value < 0.05 was considered to be significant.

RESULTS

Effects of neuromuscular blocking agents on respiratory CO_2 responsiveness

The superfusion of hypercapnic ACSF induced an increase in f_R. This increase of f_R, respiratory CO_2 responsiveness, was suppressed by D-TC 10 μM (p<0.05; Figure 1) and 100 μM (p<0.05), but not by D-TC 1 μM (Table I).

VB 10 μM did not affect respiratory CO_2 responsiveness, but VB 100 μM suppressed respiratory CO_2 responsiveness (p<0.01; Table I). In 5 out of 26 preparations, VB 100μM induced C4 tonic discharge. These

C4 tonic discharges disappeared with an increase of the CO_2 fraction from 2% to 8% in the superfusate (Figure 2).

After switching from control ACSF to ACSF containing D-TC 100 μM or VB 100 μM, respiratory activity was completely suppressed in 4 out of 19 preparations or 6 out of 26 preparations, respectively. In these 10 preparations, respiratory activities completely silenced by NMBAs were not restored by 8% CO_2. We excluded these preparations in which VB induced C4 tonic discharge or NMBAs induced complete disappearance of respiratory activities from the evaluation of respiratory CO_2 responsiveness.

Effects of neuromuscular blocking agents on chemosensitive neurons

Tonic firing neurons were divided on the basis of their response of E_m to the increase of CO_2 fraction of the superfusate from 2% to 8% into three groups – excited (chemosensitive), inhibited and insensitive (Table 2). In the RVLM, three types of neurons were located, but we did not test the effects of NMBAs on CO_2-inhibited and insensitive neurons in this study. In all of recorded CO_2-excited neurons (n = 5), D-TC or VB induced hyperpolarization of E_m (p<0.01, p<0.05 respectively) and decreases in firing frequency (p<0.05 respectively; Figure 3; Table 3).
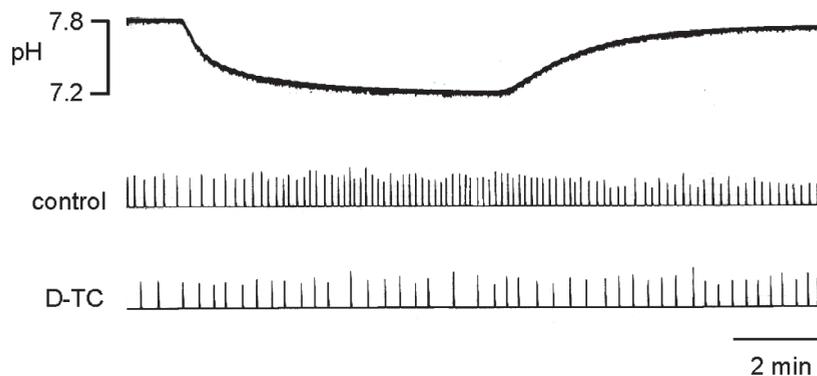

Figure 1. *Effects of d-tubocurarine (D-TC) on respiratory CO_2 responsiveness*. Switching the superfusate from control ACSF (CO_2 2%; pH = 7.8) to hypercapnic ACSF (CO_2 8%; pH = 7.2) led to an increase of f_R. The respiratory CO_2 responsiveness was attenuated by D-TC 10 μM. The pH of the superfusate was continuously monitored, and is presented at the top of the figure. Bottom traces show integrated C4 activity.

TABLE I

Effects of neuromuscular blocking agents (NMBAs) on respiratory CO_2 responsiveness

			Before application of NMBAs			During application of NMBAs		
			f_R (min^{-1})		Df_R (min^{-1})	f_R (min^{-1})		Df_R (min^{-1})
		n	2% CO_2	8% CO_2		2% CO_2	8% CO_2	
d-tubocurarine	1μM	7	5.2 ± 1.0	7.2 ± 1.0	1.9 ± 0.6	5.3 ± 0.7	7.7 ± 1.1	2.4 ± 0.9
	10μM	10	5.3 ± 0.5	6.9 ± 0.4	1.6 ± 0.2	4.3 ± 0.8	5.0 ± 0.8	0.8 ± 0.4*
	100μM	15	6.3 ± 0.6	8.7 ± 0.6	2.4 ± 0.4	5.4 ± 0.6	6.6 ± 0.5	1.3 ± 0.5*
vecuronium bromide	10μM	15	4.1 ± 0.5	6.6 ± 0.5	2.5 ± 0.5	3.7 ± 0.4	5.9 ± 0.5	2.3 ± 0.4
	100μM	15	4.2 ± 0.7	6.5 ± 0.7	2.3 ± 0.3	4.0 ± 0.6	4.8 ± 0.6	0.8 ± 0.3**

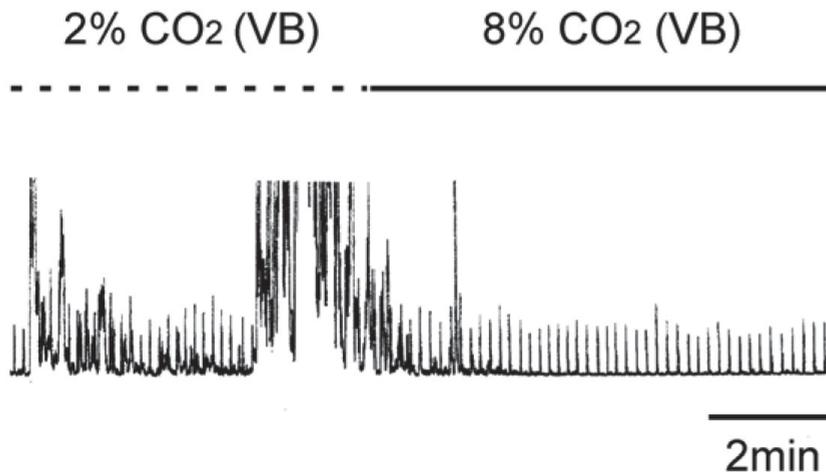Data are presented as mean ± SEM.
*p<0.05
**p<0.01

Figure 2. *Representative C4 tonic discharge induced by high concentration of vecuronium bromide (VB) and its restoration by hypercapnia*. High concentration of VB (100 μM) induced C4 tonic discharge at low CO_2 (2%) superfusate in 5 out of 26 preparations. However, these C4 tonic discharges disappeared, and normal regular respiratory C4 activity was restored by elevating CO_2 fraction of the superfusate to 8% in all 5 preparations. Trace shows integrated C4 activity.

TABLE II

Characteristics of tonic firing neurons in the rostral ventrolateral medulla

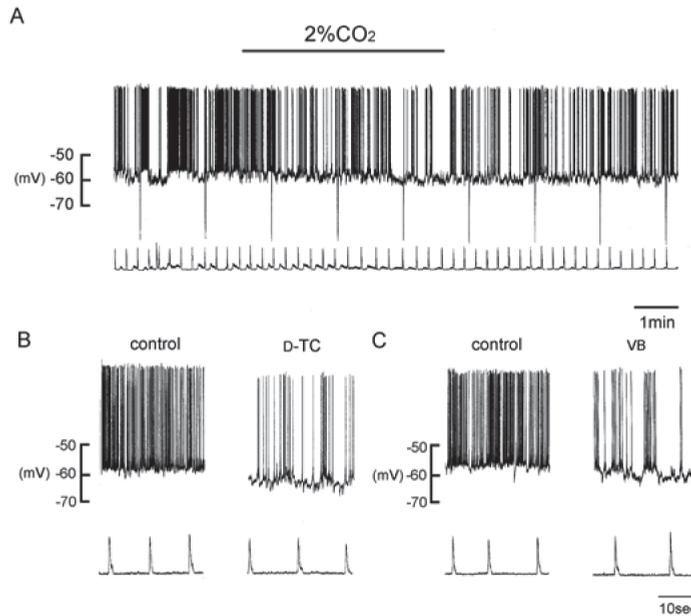|		E_m (mV)		Firing frequency (Hz)	
	n	2% CO_2	8% CO_2	2% CO_2	8% CO_2
CO_2-insensitive	3	-51.1 ± 8.1	-51.1 ± 8.1	0.44 ± 0.43	0.47 ± 0.46
CO_2-depressed	1	-55	-62	0.3	0.3
CO_2-excited	5	-50.6 ± 3.6	-47.7 ± 3.7	0.49 ± 0.19	1.0 ± 0.45

Data are presented as mean ± SEM.

Figure 3. *Effects of neuromuscular blocking agents on chemosensitive neurons*. Representative activity of CO_2-excited neuron (A) and the effects of d-tubocurarine (D-TC) 40 μM (B) and vecuronium bromide (VB) 40 μM (C) on this chemosensitive neuron. Top traces of each figure show the membrane potential of CO_2-excited neuron. In this recording, -5 nA current pulse (300 ms duration) was applied every 20 seconds for measurement of input resistance. Bottom traces of each figure show integrated C4 activity.

TABLE III

Effects of neuromuscular blocking agents (NMBAs) on chemosensitive
non-respiratory-excited neurons

	Before application of NMBAs		During application of NMBAs	
	E_m (mV)	Firing frequency (Hz)	E_m (mV)	Firing frequency (Hz)
d-tubocurarine	-48.6 ± 3.0	0.7 ± 0.3	-50.1 ± 3.1**	0.5 ± 0.2*
vecuronium bromide	-48.9 ± 3.1	0.6 ± 0.4	-49.7 ± 2.9*	0.5 ± 0.3*

Data are presented as mean ± SEM.
*$p<0.05$
**$p<0.01$

DISCUSSION

In the present study, we have shown that the sole application of d-tubocurarine and vecuronium attenuates respiratory CO_2 responsiveness in an isolated brainstem-spinal cord preparation of the neonatal rat. Also, these agents induce hyperpolarization of E_m and decreases in firing frequency of CO_2-excited neurons in the RVLM. These results suppose that NMBAs suppress respiratory CO_2 responsiveness at least partly by inhibiting the activity of CO_2-excited neurons in the RVLM.

Respiratory CO_2 responsiveness in the isolated brainstem-spinal cord preparation of neonatal rat

In contrast to *in vivo* studies where hypercapnia causes an increase in tidal volume (Loeschcke, 1982; Millhorn &

Eldridge, 1986; Bruce & Cherniack, 1987), it is controversial how hypercapnia induces changes in C4 activities in the isolated brainstem-spinal cord preparation of neonatal rat. Most studies indicate that hypercapnia induces increases in respiratory frequency (Suzue, 1984; Issa & Remmers, 1992; Okada et al., 1993b; Kawai et al., 1996; Voipio & Ballanyi, 1997; Kuwana et al., 2000a), although some studies have shown that it induces increases in C4 amplitude (Monteau et al., 1990) or both (Harada et al., 1985). It is also controversial whether central chemosensitivity of respiratory control depends on pH (Suzue, 1984; Harada et al., 1985) or CO_2 (Harada et al., 1985). Therefore, we evaluated respiratory CO_2 responsiveness as the difference of f_R between 2% CO_2 (pH = 7.8) and 8% CO_2 (pH = 7.2) in the present study.

Chemosensitivity and cholinergic mechanism

Chemosensitive tonic firing neurons are present in the ventral surface of medulla in rat (Fukuda & Loeschcke, 1979; Fukuda, 1983; Jarolimek et al., 1990; Richerson, 1995; Okada et al., 2002) and in the isolated brainstem-spinal cord of neonatal rat (Okada et al., 1993b; Kawai et al., 1996; Kuwana et al., 1998). Several previous studies have shown that cholinergic mechanism is involved in the central chemoreception. (Fukuda & Loeschcke, 1979; for review, see Nattie, 1999, Okada et al., 2001). Fukuda and Loeschcke (1979) showed that these chemosensitive neurons excited by H^+ are excited by ACh and the cholinesterase inhibitor eserine. Furthermore, these H^+-elicited excitation are blocked by muscarinic Ach-receptor antagonist atropine and the nAChR antagonist hexamethonium and mecamylamine in the medulla slice preparation of rats. Furthermore, Dermietzel and colleagues (1983) showed the existence of nAChRs in the chemosensitive area of the rat ventral medulla by the acetylcholinesterase staining-technique. Hence, cholinergic mechanisms seem to be involved in central chemosensitivity.

It has been shown that chemostimulation induces increases in tonic firing frequency

(Fukuda & Loeschcke, 1979; Fukuda, 1983; Jarolimek et al., 1990; Okada et al., 1993b; Richerson, 1995; Kuwana et al., 1998; Okada et al., 2002) or induces depolarization of most chemosensitive neurons in the RVLM (Kawai et al., 1996; Kuwana et al., 1998). In the present study, D-TC and VB induced hyperpolarization of E_m and decreased firing frequency of CO_2-excited neurons. Our result thus supposes that nAChRs are involved in CO_2 excitation of central respiratory neuronal network. However, we also have to take into account the possibility of neuronal nAChRs in motoneurons, because it has been indicated that neuronal nAChR is also included in motoneurons (Chamberlin et al., 2002; Wang et al., 2002), and it is blocked by D-TC in the slice preparation of the neonatal rats (Wang et al., 2002).

Connection between chemosensitive area and central respiratory control

It is assumed that the tonic firing of these chemosensitive neurons in the RVLM induce an excitatory drive to the neuronal network that generates the respiratory rhythm (Eugenin & Nicholls, 1997; Richerson, 1998; for review, see Eugenin et al., 2001). Indeed, the local application of carbachol to H^+- and cholinergic-sensitive area in the RVLM induces a change of respiratory pattern similarly to low pH application, and these enhancements of respiration are diminished by muscarinic Ach-receptor antagonist scopolamine in the isolated brainstem-spinal cord of neonatal opossum (Eugenin & Nicholls, 1997). This study indicates that cholinergic mechanisms and chemosensitive CO_2-excited neurons in the RVLM possess the same pathway to respiratory activity. Furthermore, respiratory CO_2 responsiveness is partly inhibited by the non-selective nAChR antagonist mecamylamine and the a4b2 nAChR selective antagonist dihydro-b-erythroidine in the isolated brainstem-spinal cord preparation of neonatal rat (Kuwana et al., 2000a,b). Our previous study has revealed that two NMBAs (D-TC and VB) attenuate central respiratory control in the isolated brainstem-spinal cord of neonatal rat (Sakuraba et al., 2003). This occurs when these two NMBAs are used, irrespective of

their different molecular structure (ester-based versus aminosteroid-based) and possible different affinity for neuronal nAChR. We therefore speculate that NMBAs in general inhibit chemosensitive neurons, which send an excitatory drive to respiratory rhythm generating network, via `a4b2` nAChR, and hereby attenuate respiratory CO_2 responsiveness, even if the direct relationship between nAChR of chemosensitive areas and respiratory activity have not been investigated. Further studies are now needed to elucidate this direct relationship and what subunits of nAChR are affected by NMBAs.

Intrinsic chemosensitivity of preinspiratory neurons and neuromuscular blocking agents

It has shown that preinspiratory neurons, respiratory rhythm generator, in the RVLM have intrinsic pH sensitivities in the blockade of synaptic transmission (Onimaru et al., 1989; Ballanyi et al., 1999). Also, our previous study has shown that D-TC and VB suppress the central respiratory control partly by their inhibitory effects on preinspiratory neurons (Sakuraba et al., 2003). Taken together, D-TC and VB attenuated respiratory CO_2 responsiveness partly due to their inhibitory effects on the intrinsic chemosensitivities of preinspiratory neurons. However, this study provides no information as to whether the attenuation of respiratory CO_2 responsiveness is only dependent on the inhibitory effects on chemosensitive CO_2-excited neurons. Further studies are needed to clarify whether NMBAs inhibit intrinsic pH sensitivities of preinspiratory neurons and thus attenuate respiratory CO_2 responsiveness.

C4 tonic discharge

In the present study, high concentration of VB induces C4 tonic discharge and these activities disappeared when the CO_2 fraction in the superfusate was increased from 2% to 8%. It is consistent with our previous studies that high concentration of VB induces C4 tonic discharge due to the action of VB on the spinal cord, not on medulla (Sakuraba et al., 2003), and that

high concentration of calcium in the superfusate induces C4 tonic discharge even after most of medulla is ablated, and it is restored by elevating CO_2 fraction in the superfusate (Kuwana et al., 1998).

The exact mechanism underlying VB-induced C4 tonic discharge and their restoration with elevated CO_2 is not known. Intracerebral administration of VB into the CNS evokes acute excitation, shivering and myotonic contractions due to motoneuron excitation in rats (Szenohradszky et al., 1993). This may be caused by accumulation of cytosolic calcium due to VB-induced sustained activation of neuronal nAChRs with subsequent increase in calcium influx through calcium channels (Vernino et al., 1992; Cardone et al., 1994). Also, the restorative effect of CO_2 on VB-induced C4 tonic discharge may be explained as inhibition of calcium channels by hydrogen ions (Krafte & Kass, 1988; Takahashi et al., 1993).

In conclusion, the neuromuscular blocking agents, d-tubocurarine and vecuronium, attenuate respiratory CO_2 responsiveness partly by their inhibitory effects on chemosensitive neurons in the RVLM.

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