Role of Inhibitory Neurotransmission in the Control of Canine Hypoglossal Motoneuron Activity In Vivo

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Hypoglossal motoneurons (HMNs) innervate all tongue muscles and thus are vital for maintenance of upper airway patency during inspiration. Partial or complete loss of inspiratory hypoglossal motoneuron (IHMN) activity during sleep (Remmers et al. 1978), during various stages of anesthesia (Eastwood et al. 2002), or during postanesthetic recovery (Dhonneur et al. 1999) and analgesia produced by opioids (Bartlett Jr and St John 1986) can lead to life-threatening upper airway obstruction. It has been long known that volatile anesthetics cause significant airway compromise at doses relevant for surgical anesthesia (Nishino et al. 1985; Ochiai et al. 1989), but even subanesthetic concentrations of volatile anesthetics, which may continue to be present in patients for many hours during postanesthesia recovery, can cause airway related morbidity.

The generation and control of HMN activity and motoneuronal activity in general are mediated by multiple neurotransmitters and neuromodulators in conjunction with intrinsic membrane properties (Rekling et al. 2000). The marked depressive effects of clinically used anesthetic agents on IHMNs may be produced by direct activation of specific receptors, such as $\gamma$-aminobutyric acid type A (GABA$_{A}$) receptors (Franks and Lieb 1998) or, indirectly, by altering the synaptic inputs and/or transmission of the endogenously released neurotransmitters or modulators that contribute to the underlying HMN discharge. Sleep/wake state-dependent changes in upper airway patency are also thought to be mediated by changes in synaptic input to IHMNs (Chan et al. 2006; Fenik et al. 2005; Horner 2008). Thus a primary goal to understanding the mechanisms underlying the depressive effects of clinically used agents or changes in the sleep/wake state is to identify the key neurotransmitters/modulators that contribute to the spontaneous discharge patterns of HMNs in vivo under conditions that have clinical and physiological relevance. The next step is then to determine the amount by which a clinical agent alters the endogenous release and/or the effectiveness of those neurotransmitter/modulators on motoneuronal activity.

Several in vivo studies have identified a role for various neurotransmitters/modulators in the hypoglossal motor nucleus using whole XII nerve activity or genioglossal electromyography in conjunction with microinjection or microdialysis of antagonists into the hypoglossal motor nucleus (Jelev et al. 2001; Liu et al. 2005; Morrison et al. 2003a; Steenland et al. 2006), but only a few attempts have been made to determine the magnitude of endogenous neurotransmission to single IHMNs in vivo. For example, in an in vivo vagotomized canine decerebrate preparation, the highly localized block of the serotonergic input to single IHMNs with the antagonist ketanserin led to a 68% reduction in neuronal peak frequency of IHMNs (Brandes et al. 2006b), suggesting that serotonin acting at 5-HT$_{2A}$ receptors strongly enhances the ongoing activity. Vagotomy appears to remove inhibition from genioglossal activity (Sica et al. 1984) and possibly increases the serotonergic contribution to IHMN activity (Sood et al. 2005, 2006) Thus Sood et al. (2005) showed that a significant serotonergic drive augmenting genio-

INTRODUCTION

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glossal activity is present only in vagotomized but not in nonvagotomized anesthetized rats. For further discussion of the experimental limitation imposed by vagotomy see Discussion. With respect to additional inputs only preliminary results for the contribution of endogenous glutamatergic (Brandes et al. 2006a), GABAergic, and glycinenergic inputs to the IHMN discharge pattern in vivo have been presented (Sanchez et al. 2007).

An important role for GABA_A and glycine receptors has been suggested by in vitro studies that showed that both receptor types are colocalized (Donato and Nistri 2000) on IHMN postsynaptic surface with cotransmission of GABA and glycine (O’Brien and Berger 1999). Both in awake and naturally sleeping rats (nonrapid-eye-movement [non-REM] sleep), block of GABA_A receptors with bicuculline led to increased genioglossal activity during normocapnia and hypercapnia (Morrison et al. 2003b), whereas block of glycine receptors with strychnine produced an increase in naturally sleeping rats (non-REM sleep) only during normocapnia but not hypercapnia (Morrison et al. 2003a). Similarly, in urethane anesthetized vagotomized rats bicuculline led to a dose-dependent increase in genioglossal activity both during normocapnia and hypercapnia (Liu et al. 2003). Results of the latter studies are based on antagonistic application to the whole hypoglossal motor nucleus via microdialysis. The possible simultaneous block of motoneurons, interneurons, and neurons in the region surrounding the nucleus may confound the true role of these inhibitory receptor systems at the neuronal level. A more conclusive result may be obtained by the highly localized application of the antagonists to single recorded IHMNs in vivo without the need to affect whole XII nerve activity.

Accordingly, the purpose of the current studies was to characterize inhibitory synaptic neurotransmission to IHMNs in a vagotomized decerebrate canine model under moderate hypercapnic hyperoxia, in vivo. These studies suggest that the spontaneous activity of IHMNs is actively controlled by a bicuculline-sensitive GABAergic mechanism, but IHMNs are not inhibited by endogenous activation of picrotoxin-sensitive GABA_A receptors or strychnine-sensitive glycine receptors.

**Methods**

This research was approved by the subcommittee on animal studies of the Zablocki VA Medical Center (Milwaukee, WI), in accordance with provisions of the Animal Welfare Act, the PHS Guide for the Care and Use of Laboratory Animals, and VA policy. Experiments were performed on dogs of either sex, weighing from 8 to 12 kg. Inhalational anesthesia was induced by mask and maintained with isoflurane at 1.5–2.5% end-tidal concentration. The animals were monitored for signs of inadequate anesthesia such as salivation, lacrimation, and increases in blood pressure and heart rate. If required, anesthetic depth was increased immediately.

**Surgical procedures**

Dogs were intubated with a cuffed endotracheal tube and mechanically ventilated with an air/O_2/isoflurane mixture. The surgical procedures, monitoring, and maintenance of body homeostasis have been previously described in detail (Brandes et al. 2007). Briefly, after cannulating the femoral artery for blood pressure recording and blood gas sampling and cannulation of the femoral vein for continuous infusion of maintenance fluids and intravenous administration of drugs, a bilateral pneumothorax was performed to reduce motion artifacts. The animal was then decerebrated by midcollicular transection (Tonkovic-Capin et al. 1998) and isoflurane was discontinued. After decerebration the animal was ventilated with an air/O_2 mixture and maintained in hyperoxic hypercapnia (FiO_2 >0.6, ETCO_2 45–55 mmHg). ETCO_2 was elevated and kept within the range of 45–55 mmHg. We have previously shown that such a level of moderate hypercapnia under hyperoxic condition is necessary to provide sufficient chemodrive to hypoglossal inspiratory neurons so that they will show vigorous phasic activity. At this level of hyperoxic hypercapnia the hypoglossal nerve (XII) activity is well above the apneic threshold and peak hypoglossal activity is about 50% of the maximum achievable under hyperoxia (Brandes et al. 2006b). Continuous neuromuscular block was achieved with intravenous pancuronium (0.1 mg·kg⁻¹·h⁻¹) and used to reduce motion artifacts during neuronal recordings. A bolus (125 mg/kg) of e-amino-n-caproic acid (Sigma–Aldrich, St. Louis, MO) was administered intravenously after anesthetic induction and maintained at 15 mg·kg⁻¹·h⁻¹. Such an empiric dose has been found to limit fibrinolysis and control bleeding during extensive neurosurgery in humans (Nilsson et al. 1960; Risberg 1978). The dorsal surface of the medulla oblongata was exposed by an occipital craniotomy for neuronal recording. Phrenic nerve activity was recorded from the desheathed right C5 rootlet. The phrenic neurogram (PNG) was obtained from the moving-time average (100 ms) of the amplified phrenic nerve activity and was used to produce timing pulses corresponding to the beginning and end of the inspiratory phase. The right hypoglossal nerve was desheathed and embedded in silicon gel (Wacker Sil-Gel, Univ. of Iowa) and a hypoglossal neurogram was obtained in a similar fashion to the PNG.

**Picoejection technique and neuronal recording**

A minimum of 1 h was allowed after surgery for preparation stabilization before data collection. Extracellular single-unit recordings from IHMNs were obtained using multibarrel micropipettes (10–30-μm composite-tip diameter) consisting of a recording barrel containing a 1-μm-thick carbon filament and three drug barrels. These microelectrodes allowed extracellular recordings of neuronal action potentials before and during pressure ejection of picomolar amounts of drug onto the neuron. The following agents from Sigma (St. Louis, MO), except where noted, were dissolved in the artificial cerebrospinal fluid (aCSF) solution: the competitive GABA_A receptor antagonist bicuculline methochloride (BIC; 200 μM), the noncompetitive GABA_A receptor antagonist picrotoxin (PIC; 5 mM; Research Biochemicals International, Natick, MA), the glycine receptor antagonist strychnine HCl (STR; 200 μM), GABA (2 mM), glycine (2 mM), and serotonin HCl (5-HT; 500 μM). The aCSF consisted of (in mM) 124 NaCl, 2 KCl, 2 MgCl₂, 1.3 KH₂PO₄, 0.9 CaCl₂, 26 NaHCO₃, and 11 glucose. The picoejected dose rate was measured via height changes of the meniscus in the pipette barrel with a ×100 magnification microscope equipped with a reticule (resolution ~2 nl). The location of the recorded neurons was in the hypoglossal motor nucleus based on a histological study of the location of canine genioglossal motoneurons, which is a region extending from 0.5 mm caudal to 3.5 mm rostral to the obex, about 1 mm lateral to the midline, and about 1–2 mm below the dorsal medullary surface in adult dogs (Brandes et al. 2004). A time–amplitude window discriminator was used to generate a standard logic pulse for each spike. Neuronal discharge frequency (F₅ₐ) was continuously determined from the number of discharges in each 100-ms time increment.

When possible, on-line spike-triggered averaging of XII nerve activity was used to confirm that the recorded action potentials originated from a hypoglossal motoneuron. The presence of an axon spike potential within the hypoglossal nerve and the neuron firing in phase with the phrenic nerve activity confirmed that the recorded brain stem neuron was an IHMN. Alternatively, inspiratory neurons in the hypoglossal motor nucleus were considered hypoglossal motoneurons if they responded to picoejected serotonin (Brandes et al. 2006b).
Protocols

Three sets of studies were performed, one for each of the antagonists (BIC, PIC, and STR). Only one antagonist was used per study to avoid possible cross-contamination. The three barrels of the micropipette contained the selective antagonist, its corresponding agonist, and serotonin. This allowed for separate sequential or simultaneous ejections of the drugs. The micropipette was advanced into the hypoglossal motor nucleus and, after establishing a stable recording of single neuronal activity, control activity was recorded for 2–4 min (15–30 respiratory cycles). During this control period, transient inhibitions of discharge frequency produced by single picoejections of GABA or glycine, time-synchronized with the onset of the PNG, were recorded. The short-acting agonist was applied every third to fifth respiratory cycle and repeated such that 10–12 test cycles were obtained. The longer-acting antagonists were then picoejected with step increases in rate until no further increase in effect to the antagonist could be noted, indicating that a maximum block of receptors occurred. In the case where no antagonist-induced changes in neuronal activity occurred, picoejection was gradually increased to a predetermined maximum rate. This predetermined rate was established by prior experience based on the ability of the antagonist to completely block a robust agonist-induced inhibition. For all protocols, the duration of the ejections was typically 10 min. Immediately following termination of the antagonist picoejection, the effectiveness of the antagonist was again tested with picoejections of the agonist as during the control period. A recovery from the antagonist effects of approximately 30 min was allowed before recording end-control data. Since 5-HT2A receptors are present on the soma of canine IHMNs, we confirmed that the recorded neuron responded to picoejected 5-HT, which produces excitation of IHMNs, after its activity had returned to baseline levels postantagonist application. The neuron was considered “serotonin responsive” if the activity increased by ≥50% or became tonic, discharging both during the active and previously silent phase (see Fig. 1).

Data analysis

Cycle-triggered histograms (CTHs; bin width, 50 ms), triggered from the onset of the inspiratory phase as defined by the phrenic neurogram, and averaged over 11–20 respiratory cycles, were used to quantify the peak neuronal discharge frequency (peak $F_n$) and discharge pattern. Values were obtained for the control period and during the maximum effect of the respective drug. Peak $F_n$ values were normalized and expressed as a percentage of their corresponding control values to allow comparisons between neurons.

The relationship of the control discharge pattern ($F_{con}$) and the discharge pattern during drug application ($F_{drug}$) was analyzed using time-synchronized $F_{drug}$ versus $F_{con}$ plots. These plots are typically linear and least-squares linear regression analysis was used to quantify the slope and y-intercept during maximal drug effect. A plot of the control pattern against itself results in the line of identity (LOI) with a slope of 1 and an intercept of 0. Changes in slope relative to the LOI indicate that the two patterns are proportionally related, but the gain of the $F_{drug}$ pattern has changed. A change in the y-intercept indicates a parallel shift in the baseline (tonic) activity. This offset is expressed in terms of percentage of peak $F_{con}$. Statistical procedures were carried out using SigmaStat 3.5 (Systat Software, Richmond, CA). For all data sets, tests for normality of the normalized data (Kolmogorov–Smirnov test) were performed before parametric procedures were used. For normally distributed data, a one-way ANOVA was used with the Holm–Šidák method for pairwise multiple comparisons with a familywise error rate of 0.05. For nonnormally distributed data, a Kruskal–Wallis ANOVA on ranks was used with Dunn’s method for pairwise multiple comparisons. Values are expressed as means ± SE.

RESULTS

Antagonism of GABA A receptors by bicuculline

The data of 20 IHMNs were obtained from 10 dogs during hypercapnic hyperoxia ($\text{ETCO}_2 52 ± 2 \text{ mmHg}$). For illustrative purposes of our analysis we show in Fig. 2 an IHMN with a particularly pronounced increase in discharge during BIC picoejection, where BIC was picoejected at a high dose rate that resulted in maximal effects. The CTH analysis of
the discharge pattern for this neuron shows that BIC increased the peak discharge frequency $F_n$ from 27 to 47 Hz (Fig. 3, top). The BIC-induced increases in $F_n$ occurred only during the active inspiratory phase and no activity was evoked during the expiratory phase. Linear regression analysis of the $F_{BIC}$ versus $F_{CON}$ plot shows a 53% increase in the slope with a small increase in offset (2.6 Hz) indicative of a significant increase in the gain of the motoneuron input-output relationship (Fig. 3, bottom). For 15 of 20 IHMNs, spike-triggered averaging (STA) of XII nerve activity detected the presence of an action potential. Four of 20 neurons could not be tested due to inadequate XII nerve activity due to technical limitations of the nerve preparation and one of 20 was STA negative. However, since all neurons responded to serotonin picoejection and were from the region of the canine hypoglossal motor nucleus, all 20 were included in the statistical analysis. At maximally effective dose rates of bicuculline (mean of 0.97 pmol/min; 4.8 nl/min) the peak discharge frequency increased from 27 Hz (preejection control) to 33 Hz (preejection control) to 33 Hz or by 30% relative to control. The time-averaged discharge frequency showed a similar increase of 31% (Fig. 6). Six of 20 neurons did not show any increase in discharge with BIC. The CTH analysis revealed a significant increase in the gain of the pattern by 25% without a significant change in offset (Fig. 7).

**Antagonism of picrotoxin-sensitive GABA$_A$ receptors**

Seventeen IHMNs in five dogs were subjected to the picrotoxin protocol during hypercapnic hyperoxia (ETCO$_2$: 58 ± 1 mmHg) to explore whether picrotoxin-sensitive GABA$_A$ receptors were endogenously active in these neurons. The presence of PIC-sensitive GABA$_A$ receptors on IHMNs was confirmed by demonstrating that inhibitory effects caused by picoejection of GABA could be blocked by PIC as described in METHODS (e.g., Fig. 4A). The picoejection of PIC on the spontaneous discharge pattern of a typical IHMN shown by CTHs in Fig. 4B produced no noticeable change. The $F_{PIC}$ versus

**FIG. 2.** Example of a highly responsive IHM neuron to antagonism of GABA$_A$ receptors with bicuculline (BIC). A: a high picoejection pressure had to be used to open up the barrel tip resulting in a high initial BIC dose rate with almost a doubling in discharge frequency $F_n$ (rate-meter recording). B: time-expanded views of neuronal activity during control conditions and at maximally effective BIC dose rates. PNG, phrenic neurogram; NA, raw neuron action potential spikes; $F_n$, rate meter recording of discharge.

**FIG. 3.** Cycle-triggered histogram (CTH) analysis of the activity of the IHMN in Fig. 2 shows the effect of BIC on the control discharge frequency ($F_n$) pattern. Top: CTH. Bottom: linear regression analysis of the plot of $F_n$ discharge pattern with BIC ($F_{BIC}$) vs. the control $F_n$ pattern ($F_{CON}$) shows that the slope (gain) of the neuronal discharge pattern increases with GABAergic block. PNG, phrenic neurogram; LOI, line of identity; $F_n$, discharge frequency; CON, control.
FCON plot shows a small, nonsignificant decrease in slope. The pooled data show that at maximally effective PIC dose rates (174 ± 13 pmol/min; 34.8 ± 0.6 nl/min) the peak and average firing frequency increased by 5 ± 5 and 2 ± 6%, respectively (Fig. 6). PIC had no effect on neuronal activity during the expiratory phase. There was a small decrease in the gain (slope change: −7 ± 7%) and no change in offset (y-intercept: 8 ± 6%) of the discharge patterns (Fig. 7).

Antagonism of glycinergic receptors with strychnine

Twenty-four neurons were tested in six dogs during hypercapnic hyperoxia (ETCO₂ 56 ± 1 mmHg) to explore whether strychnine-sensitive glycine receptors were endogenously active in these neurons. The presence of strychnine-sensitive glycine receptors on IHMNs was confirmed by demonstrating that picoejection of glycine inhibited these neurons and that the inhibitory glycine effects could be blocked by strychnine at the dose rates used (e.g., Fig. 5A, second and third respiratory cycles). Picoejection of strychnine on a typical IHMN did not change the spontaneous discharge pattern (e.g., Fig. 5B). The FSTR versus FCON plot shows a small, nonsignificant decrease in slope (Fig. 5C). For the pooled data, picoejection of strychnine at maximally effective dose rates (1.7 ± 0.1 pmol/min; 8.5 ± 0.5 nl/min) on a typical IHMN did not affect either the peak firing frequency or the average firing frequency (Fig. 6). Changes in slope and y-intercept of the FSTR–FCON plots were not significant: 3 ± 4 and 0 ± 4%, respectively; Fig. 7).

DISCUSSION

Our study shows that antagonism of GABA_A receptors on single IHMNs with BIC increased the discharge during hypercapnic hyperoxia in vivo by blocking a gain modulatory attenuation during the inspiratory phase. This type of response is qualitatively similar to that produced by BIC in respiratory bulbospinal premotor neurons, in which tonic GABAergic inhibition best describes this phenomenon (McCrimmon et al. 1997; Zuperku and McCrimmon 2002). Furthermore, neither PIC nor strychnine had an effect on the discharge of these neurons during their normally active or silent phases, suggesting that PIC-sensitive GABA_A and glycine receptors are not endogenously active during our experimental conditions (decerebration, vagotomy, and hyperoxic hypercapnia). However, during the expiratory phase, it is possible that any antagonized inhibition may not have been observed because of insufficient excitatory drive that is required to depolarize the neuron above its firing threshold. Nonetheless, our results are consistent with a prior study (Woch and Kubin 1995) that suggests that the silent phase of inactivity of these neurons does not appear to be produced by phasic GABAergic or glycinergic synaptic inhibition.

Although our data clearly show that IHMNs are inhibited by both exogenous glycine and by exogenous GABA, the antagonist studies with strychnine and BIC suggest that glycine was not coreleased endogenously with GABA under our experimental conditions. It is possible that synapses also exist that corelease both neurotransmitters, but are not activated under our experimental conditions, which is a more intact and more mature preparation than the neonatal rat slice in vitro preparation used by O’Brien and Berger (1999). In the same type of in vitro preparation, but measuring spontaneous, miniature, and electrically evoked inhibitory postsynaptic currents, Donato and Nistri (2000) found that glycinergic synaptic events differed from GABAergic events in terms of kinetics, frequency, and sensitivity to tetrodotoxin and antagonism of glutamatergic metabotropic receptors. Their data suggest a limited role for corelease of glycine and GABA.
There are other examples of inhibitory neurons that corelease glycine and GABA, but their target neurons differentially express postsynaptic receptors (e.g., Dugue et al. 2005). However, this type of arrangement cannot explain our results, where we demonstrate that both exogenous GABA and exogenous glycine inhibit the IHMNs.

**Differential pharmacology**

One explanation for the differential pharmacology of the two GABA_A receptor antagonists may be possible nonspecific effects of bicuculline. In in vitro preparations, methyl derivatives of BIC have been shown to reduce the amplitude of the slow afterhyperpolarization (AHP) that follows an action potential, which is mediated by small-conductance calcium-activated potassium (SK) channels (Debarbieux et al. 1998; Khawaled et al. 1999; Seutin and Johnson 1999). A reduction in the...
AHP increases neuronal excitability. However, in canine bulbospinal premotor neurons under similar experimental conditions, we have previously demonstrated that bicuculline methochloride, at the same concentration as that used in the current study (200 μM), was a specific GABA_A receptor antagonist (Tonkovic-Capin et al. 2001). Blockade of the SK channels with apamine increases the gain of the neurons, but does not prevent their response to BIC. Both GABA_A receptors and SK channels produce gain modulation and appear to act in a cascade manner at different functional sites on the premotor neurons (Tonkovic-Capin et al. 2003). We did not test the specificity of BIC methochloride on IHMNs, but assumed its pharmacology is similar to that on canine respiratory premotor neurons (Tonkovic-Capin et al. 2003). We did not test the specificity of BIC methochloride on IHMNs, but assumed its pharmacology is similar to that on canine respiratory premotor neurons due to the qualitative similarity of the responses.

The differential pharmacology of GABA_A receptors to BIC and PIC on respiratory neurons is thus more likely due to the molecular heterogeneity of GABA_A receptors. GABA_A receptors form pentameric complexes assembled from ≥21 subunits and the most common combination for native GABA_A receptors contains 2α/2β/1γ-subunit variants (Fritschy and Brunig 2003). The different subunit combinations alter ligand affinity and pharmacology. The BIC-sensitive, PIC-insensitive pharmacology in canine IHMNs and canine ventral respiratory column neurons (Dogas et al. 1998; Zuperku and McCorminck 2002) has also been found in other preparations. Retinal ganglion and amacrine cells in chicken express a GABA receptor that can be blocked by BIC and positively modulated by barbiturates and benzodiazepines, but is insensitive to PIC (Shen et al. 2002). Guinea pig hippocampal interneurons are PIC insensitive but are sensitive to BIC, pentobarbital, and zolpidem (Semyanov and Kullmann 2002). Recently, in glutamateergic hippocampal neurons, Eisenman et al. (2006) described a novel tonically active, BIC-sensitive chloride conductance that modulates neuronal excitability and is insensitive to gabazine and to PIC and thus not mediated by conventional GABA receptors.

Conversely, the retinal GABA_C receptor comprised of ρ subunits is an example of an ionotropic GABA receptor that is insensitive to BIC and the GABA_B receptor agonist baclofen (Bormann 2000), but is antagonized by PIC. Even the ratio of ρ1/ρ2 subunits strongly affects the effectiveness of PIC (Enz and Cutting 1999). More recently, ρ subunits have been found to be widely expressed in several areas of the CNS including the brain stem and pons (Lopez-Chavez et al. 2005). Furthermore, evidence for inhibition mediated by coassembly of GABA_C and GABA_A receptor subunits in native central neurons has been found (Milligan et al. 2004). In rats, mRNA encoding the ρ1 subunit is highly expressed in brain stem neurons and appears to be colocalized with GABA_A receptor α1 and γ2 subunits (Frazao et al. 2007). Bath application of a highly selective GABA_C receptor agonist, which requires the ρ1 subunit to elicit responses, was blocked by both BIC and PIC (Milligan et al. 2004).

It is unlikely that the lack of effect by PIC is due to its concentration relative to that of BIC, since we used a concentration that was >20-fold greater than the BIC concentration. In addition, studies have shown that effective inhibitory concentrations for picrotoxin are typically in the same low micromolar range as for bicuculline (Baufreton et al. 2001; Krishkev et al. 1996). In bulbospinal respiratory neurons, 5 mM PIC was able to completely antagonize the silent-phase inhibition (Dogas et al. 1998; Krholo et al. 2000). The duration of picroejections was 2–10 min, which should have provided sufficient time to overcome any activation dependence of PIC. Furthermore, PIC was able to block the inhibitory response of IHMNs to exogenous GABA (Fig. 4), which suggests not only that PIC was ejected at effective concentrations but that there are also GABA_A receptors on IHMNs that are not endogenously activated but can be blocked by picrotoxin.

In conclusion, the subunit diversity of ionotropic GABA receptors is a likely explanation for the novel pharmacology observed in canine IHMNs and bulbospinal premotor neurons (Mohler 2006).

This is the first study that examines the inhibitory neurotransmitter physiology at the level of single inspiratory hypoglossal motoneurons in an in vivo mammalian decerebrate animal model. Our findings are consistent with data from urethane-anesthetized rodent preparations where bicuculline microdialysis doubled baseline genioglossal activity during both CO2-stimulated and quiet breathing (Liu et al. 2003). GABA_A receptor antagonism with bicuculline similarly increased genioglossal activity in awake and in naturally sleeping rats during nonREM sleep, but not during tonic REM sleep (Morrison et al. 2003b). Similarly to our findings for glycine, no increase in genioglossal activity was seen in hypocapnic, naturally sleeping rats with the glycine antagonist strychnine during non-REM sleep, although increases were seen in these rats in non-REM sleep during normocapnia and during wakefulness in normocapnia and hypercapnia (Morrison et al. 2003a). In addition, increases were observed with strychnine during hypocapnic hyperoxia in vagotomized anesthetized rats (Morrison et al. 2002).

The spontaneous activity of IHMNs during moderate hypocapnic hyperoxia was attenuated about 25% by a BIC-sensitive GABAergic gain modulatory mechanism. This attenuation was weaker than the GABAergic modulation that we previously reported in respiratory bulbospinal premotor neurons (Dogas et al. 1998). This suggests that a similar modulatory process also regulates IHMNs, where the discharge frequency is two- to threefold lower (20–30 vs. 60–90 Hz) than that of the bulbospinal neurons under comparable baseline conditions. Tonic GABAergic gain modulation has been described as a mechanism by which the phasic discharge frequency pattern of a neuron is an attenuated replica of the underlying pattern that would be present without this tonic GABA input (McCracken et al. 1997; Zuperku and McCorminck 2002). For caudal ventral respiratory group bulbospinal neurons, tonic GABAergic gain modulation, mediated via BIC-sensitive GABA_A receptors, produces a 50–65% attenuation of the discharge frequency pattern. Our results suggest that a similar mechanism controls the discharge of IHMNs. However, since we have not observed discharge during the normally silent expiratory phase, it is possible that GABAergic inhibition may be phasic. For example, if the time course of the inhibitory input matches the underlying discharge pattern of the neuron, the relationship between the discharge pattern before and during block of inhibition would be linear (Parkins et al. 1999; Saywell and Feldman 2004), similar to that obtained with block of tonic inhibition. However, this requirement for the inhibitory input to match the underlying discharge pattern is much more restrictive, since any other synaptic inputs that alter a neuron’s...
Discharge pattern would require that the inhibitory input will be similarly altered to maintain the observed linear relationship.

Evidence supportive of phasic inhibition of IHMNs has been previously reported. In voltage-clamped HMNs in a neonatal rat slice preparation, Saywell and Feldman (2004) found three subpopulations based on their inhibitory inputs: noninhibited (~36%), inspiratory-inhibited (~18%), and late-inspiratory-inhibited (~45%). The inhibition was antagonized by application of BIC, implicating GABA_A receptors. In a small sample of IHMNs in anesthetized cats, a late-inspiratory inhibition was also shown using Cl^- injection (Withington-Wray et al. 1988). However, the linear relationship between control and BIC discharge patterns of canine IHMNs is not supportive of late-inspiratory inhibition, at least in the population of neurons that we studied. As mentioned earlier, phasic inspiratory inhibition, if present in canine IHMNs, would have to match the underlying discharge pattern.

In contrast to the effects of BIC, we previously showed that PIC-sensitive GABA_A receptors phasically inhibit the discharge of inspiratory and expiratory bulbospinal motoneurons and are responsible for the silent phases of these neurons (Dogas et al. 1998; Krolo et al. 2000). However, lack of endogenous expiratory-phase inhibition of IHMN is consistent with the data from other mammalian models of IHMNs, which also showed that these neurons lack reciprocal inhibition in contrast to most respiratory-related neurons (Woch and Kubin 1995). On the other hand, our protocols clearly demonstrated that IHMNs express functional glycineric and picrotoxin-sensitive GABAergic receptors. Certainly, XII motoneurons in adult humans express glycine receptors on their cell bodies and dendrites (Baer et al. 2003). These receptors may therefore participate in other oropharyngeal behaviors, including mastication (Roda et al. 2002). In addition, the studies by Morrison et al. (2003a) suggest that hypercapnia may occlude tonic glycineric inputs during non-REM sleep and a similar effect cannot be excluded under our experimental conditions.

Advantages and limitations of the experimental conditions

The current study serves as a transition and fills a gap between those carried out in highly reduced, immature rodent models and those in the freely moving adult models that are used to study state-dependent effects on synaptic inputs to HMNs. Our approach has the advantage of studying single IHMNs, where the magnitude of a synaptic input can be estimated in a population of neurons that are heterogeneous in terms of their target muscles and most likely their synaptic inputs. This type of data is not possible from XII nerve recordings, where the data are interpreted as if the population was homogeneous. In addition, the picoejection method has the advantage of delivering a highly localized drug application, thereby limiting the spread of the drug to more distant presynaptic neurons whose inputs may be altered by global or nuclear drug applications, which may confound data interpretation. Indeed, the amount of picoejected drug has no effect on whole XII nerve activity or any other parameters of global respiratory output, which can be observed with nuclear injections.

Although the physiological relevance due to our experimental conditions has limitations, it was necessary to optimize conditions to be able to obtain meaningful single-neuron data. Decerebration was necessary due to severe anesthetic-induced depression of HMN activity. The resulting loss of excitatory drive compared with awake animals was offset by an increase in CO2 drive and vagotomy. It may be argued that these and other conditions (see following text) result in altered neurochemical environment and modulatory mechanisms controlling XII MN excitability, but this may affect only the quantitative rather than qualitative nature of the synaptic inhibition. Previously, we have found that peak inspiratory XII nerve activity increases monotonically from a zero threshold to a maximum level as a function of end-tidal CO2 without altering its activity profile (Brandes et al. 2006b). Similarly in decerebrate, vagal nerve intact cats, Hwang and St John (1987) found that the number of IHMNs recruited increased with increased CO2 drive. This occurred both with and without lung inflation, where the number of neurons suppressed during lung inflation (i.e., during vagal inhibitory input) remained relatively constant even as CO2 increased. With regard to the current study, it is also possible that the magnitude of the GABAergic inhibitory input may have changed as a function of arterial CO2, although it is likely that it continued to play a role in controlling IHMN activity.

Decerebration

Hypoglossal motoneurons are exquisitely sensitive to anesthetics, which exert a strong depressant effect on inspiratory hypoglossal motor activity (Brandes et al. 2007; Nishino et al. 1985). Decerebration circumvents the confounding effects of anesthesia, although decerebration appears to be distinct from the awake state and may resemble most closely non-REM sleep. Carbachol injections can mimic REM-sleep–induced atonia in decerebrate animals and the carbachol-induced REM sleep in decerebrate and intact animals seems to resemble natural REM sleep in intact animals (Kimura et al. 1990). Compared with the non-REM sleep state, intact awake rats show stronger inspiratory phasic genioglossal activity and in addition marked tonic activity. This suggests that wakefulness contributes major excitatory inputs to genioglossal activity (Morrison et al. 2003a) and these have been proposed to be mainly glutamatergic in intact rodents (Sood et al. 2005). However, in our decerebrate canine model, inspiratory hypoglossal motor neurons do not show any tonic activity under baseline conditions, which closely resembles the genioglossal activity in intact rodents during non-REM sleep (Morrison et al. 2003a,b).

Vagotomy

Vagotomy appears to remove inhibition from genioglossal activity (Sica et al. 1984) and possibly increases the serotonergic contribution to IHMN activity (Sood et al. 2005, 2006). After vagotomy, peak genioglossal activity increases up to threefold in anesthetized rodents (Sood et al. 2005) and decerebrate cats (Kubin et al. 1992) and we have also found increases in hypoglossal nerve activity in dogs (Brandes et al. 2006a). Some of the increase in genioglossal activity with vagotomy may be related to disinhibition of serotonergic raphe neurons (Blair and Evans 1991; Evans and Blair 1993). Thus Sood et al. (2005) showed that a significant serotonergic drive augmenting genioglossal activity is present only in vagotomized but not in nonvagotomized anesthetized rats. Although a
large amount of the increase in XII nerve activity due to the removal of vagal afferent input may be due to increased excitation of the IHMNs, we cannot exclude that vagotomy also releases inspiratory motor neurons from direct GABAergic, and possibly glycnergic, inhibition. This may partially explain why microdialysis of bicuculline directly into the hypoglossal motor nucleus of vagal nerve–intact rats leads to a much larger increase in genioglossal activity (about twofold) both in wakefulness and during non-REM sleep (Morris et al. 2003b) than the 25–30% increase of IHMN activity that we observed in vagotomized decerebrate dogs.

Strong phasic genioglossal activity can be observed in intact spontaneously breathing awake rats during normocapnic normoxia during quiet wakefulness (Morris et al. 2003a,b). In contrast, the lower level of phasic IHMN activity in our canine preparation may be due in part to decerebration and the removal of upper airway sensory input, since endotracheal intubation bypasses the upper airway. In addition, paralysis with positive-pressure ventilation completely removes any upper airway inputs. Thus we used hypercapnia to increase phasic hypoglossal nerve and IHMN activity (Brandes et al. 2006b) to allow us to study whether GABAergic and glycnergic endogenous inhibitions directly affect the activity of these neurons. It is likely that hypercapnic-induced increases in serotonergic drive substantially contribute to the increase in IHMN activity, both in decerebrate vagotomized dogs (Brandes et al. 2006b) and in intact awake rats and during non-REM sleep (Sood et al. 2005).

Neuromuscular blockade

It is possible that the cholinergic neuromuscular blocker pancuronium, which normally does not cross the blood–brain barrier, may have leaked during CNS surgery and possibly antagonized acetylcholine at the nicotinic receptors on hypoglossal motoneurons (Quitadamo et al. 2005). However, it appears highly unlikely that any significant amount of pancuronium diffused into the region of the hypoglossal motor nucleus. In patients undergoing cerebral aneurysm clipping, Fuchs-Buder et al. (2004) showed that very small amounts of the related neuromuscular blocker rocuronium (12.4 ng/ml or about 1% of the therapeutic plasma concentration of 1,123 ng/ml) could be regularly detected in CSF. This CSF concentration was 1,000-fold smaller than the concentration that inhibits human neuronal nicotinic receptors (Chiodini et al. 2001). In our preparation, strict hemostasis was maintained and, since we used a constant background infusion of pancuronium, steady-state plasma (~0.34 μM) levels and about 1,000-fold lower brain levels (if any) can be expected throughout our neuron protocols and may possibly contribute to baseline conditions.

Hyperoxia

We used hyperoxia (FiO₂ >0.6) for three reasons: 1) to promptly discover and minimize any untoward effects from potential venous air embolism during brain stem surgery and decerebration; 2) to functionally “denervate” the peripheral chemoreceptors; and 3) to minimize any risk of hypoxemia during the prolonged surgery and experimental protocols. The FiO₂ (0.6) we used resulted typically in PaO₂ ranges between 250 and 400 mmHg, which likely resulted in some limited oxygen toxicity from radical formation. This level of hyperoxia may stimulate baseline ventilation by ≤50%, possibly via central chemoreceptor stimulation (Dean et al. 2004; Mulkey et al. 2003). Since we maintained a steady state of moderate hyperoxic hypercapnic conditions throughout the protocols the background excitatory drive during a protocol would have remained elevated but constant. It is unlikely that the resultant respiratory stimulation would qualitatively alter our main findings concerning the inhibitory control of IHMNs in vivo, although it is possible that, quantitatively, the observed effects would have been different under normoxic normocapnic conditions.

Clinical relevance

Our decerebrate model allows us to study synaptic inputs to single inspiratory hypoglossal motor neurons in vivo during physiologically and clinically relevant conditions without confounding background anesthesia. Hyperoxic hypercapnia is routinely encountered in the clinical management of patients at risk of airway obstruction. Vagotomy and hypercapnia will lead to increased serotonergic and glutamatergic excitation of these neurons. This may overestimate the importance of excitatory mechanisms in our setup, compared with intact awake animals or intact animals during non-REM sleep, but our findings should be relevant in patients at risk for obstructive sleep apnea (OSA) and in patients postanesthesia. The degree of hypercapnia, although its time course may differ, is in a clinically relevant range that is seen in patients recovering from anesthesia and in patients with severe sleep apnea. Compensatory reflexes arising from the upper airway in OSA patients and OSA animal models due to compromised upper airway anatomy will lead to increased serotonergic genioglossal activation (Mezzanotte et al. 1992; Veasey et al. 1996), which may be similar in magnitude to the effects of vagotomy in decerebrate preparations.

Inhibitory GABAergic attenuation of genioglossal activity has been shown both in intact rats during wakefulness and non-REM sleep (Morrison et al. 2003a,b) and in anesthetized vagotomized rats (Liu et al. 2003). The inhibitory effects seem relatively larger in absolute terms in awake animals compared with non-REM sleep (Morrison et al. 2003a,b). Our data confirm that inhibitory GABAergic attenuation occurs at least in part directly at hypoglossal motor neurons in vivo.

Summary

Our study shows that the discharge of single IHMNs is attenuated by endogenously active GABAₐ receptors during hypercapnic hyperoxia in vivo. Our findings also confirm that, under our experimental conditions, glycine receptors are present on IHMNs but do not contribute to synaptic inhibition during respiratory-related activity, and that the inactivity of these neurons during the expiratory phase is not due to phasic inhibition by GABA or glycine.

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INHIBITORY CONTROL OF HYPOGLOSSAL MOTONEURONS IN VIVO


