Relative magnitude of tonic and phasic synaptic excitation of medullary inspiratory neurons in dogs

M. KROLO, E. A. STUTH, M. TONKOVIC-CAPIN, F. A. HOPP, D. R. MCCRIMMON, AND E. J. ZUPERKU. Relative magnitude of tonic and phasic synaptic excitation of medullary inspiratory neurons in dogs. Am J Physiol Regulatory Integrative Comp Physiol 279: R639–R649, 2000.—The relative contribution of phasic and tonic excitatory synaptic drives to the augmenting discharge patterns of inspiratory (I) neurons within the ventral respiratory group (VRG) was studied in anesthetized, ventilated, paralyzed, and vagotomized dogs. Multibarrel micropipettes were used to record simultaneously single-unit neuronal activity and pressure microejected antagonists of GABAergic, glycinegic, N-methyl-D-aspartate (NMDA) and non-NMDA glutamatergic, and cholinergic receptors. The discharge patterns were quantified via cycle-trigger histograms. The findings suggest that two-thirds of the excitatory drive to caudal VRG I neurons is tonic and mediated by NMDA receptors and the other third is ramp-like phasic and mediated by non-NMDA receptors. Cholinergic receptors do not appear to be involved. The silent expiratory phase is produced by phasic inhibition of the tonic activity, and ~80% of this inhibition is mediated by γ-aminobutyric acid receptors (GABA<sub>A</sub>) and ~20% by glycine receptors. Phasic I inhibition by the I decrementing neurons does not appear to contribute to the predominantly step-ramp patterns of these I neurons. However, this decrementing inhibition may be very prominent in controlling the rate of augmentation in late-onset I neurons and those with ramp patterns lacking the step component.

control of breathing; central pattern generation; neurotransmitters; γ-aminobutyric acid receptors; glutamatergic receptors

SYNAPTIC EVENTS AND INTRINSIC neuronal properties are the main determinants of the discharge patterns of respiratory neurons that control the musculature for breathing. Inspiratory (I) and expiratory (E) neurons typically exhibit active (discharge present) and inactive (discharge absent, e.g., the E phase of an I neuron) phases. The absence of discharge during the silent or inactive phase of most respiratory neurons appears to be produced by phasic inhibition. During the active phase, the commonly observed discharge frequency (F<sub>n</sub>) patterns consist of augmenting ramps, decrementing ramps, and constant patterns (5, 18, 30). Although explanations for the mechanisms underlying the generation of these discharge patterns have been offered (e.g., Refs. 14, 27), clear evidence that validates such hypotheses is still lacking.

I premotor neurons, within the ventral respiratory group (VRG) of the ventrolateral medulla, provide the main source of phasic excitatory drive to spinal I motoneurons (8, 9, 17, 22). Most bulbospinal I neurons exhibit an augmenting firing pattern during the I phase (4). Based on synaptic connection studies, it has been hypothesized that augmenting patterns are produced by 1) recurrent excitation from other augmenting I neurons (15) in conjunction with 2) excitation from constant pattern I neurons (16), and 3) phasic inhibition from I interneurons with decrementing discharge patterns (16, 32).

Ionotropic glutamate receptors appear to mediate most of the excitatory drive to respiratory neurons during their active periods. In cats, microiontophoretic application of specific antagonists of both N-methyl-D-aspartate (NMDA) and non-NMDA ionotopic gluta- mate receptors produced reductions in the F<sub>n</sub> of single I and E respiratory neurons (25, 26). Coapplication of these antagonists resulted in further reductions. It was suggested that the sequential activation of non-NMDA and NMDA receptors may possibly shape the respiratory burst frequency pattern (26). The microiontophoretic application of acetylcholine (ACh) and its analogs has also implicated cholinergic receptors in the control of the discharge patterns of I neurons (20, 23, 36). Nevertheless, the nature of the potential cholinergic contribution remains controversial.

In a recent study we found that the main component of the excitatory drive to canine bulbospinal I neurons is mediated by NMDA and non-NMDA receptors (21). However, analyses of the discharge patterns showed that each receptor subtype provides a different and distinct function. During the I phase, NMDA receptors appear to mediate a constant level of excitation while the non-NMDA ionotopic glutamate receptors medi-
ate a phasic augmenting or a combined step and augmenting pattern of excitation. Bulbospinal I neurons also appear to receive an additional tonic excitatory input, which is active throughout the respiratory cycle (31) and is difficult to distinguish from the step-like input from the I constant neurons (14, 32). It is not yet known whether NMDA receptors mediate both of these tonic inputs. Also, the relative magnitudes of the tonic and phasic contributions to the overall discharge pattern are not known. Furthermore, phasic inhibition, mediated via I neurons with decrementing firing patterns, may also contribute to the shaping of the augmenting patterns (14). However, the relative importance of this inhibition is not known.

Accordingly, the purpose of this study was to determine the relative contributions of the phasic and tonic excitatory synaptic inputs in shaping the discharge pattern of canine VRG I neurons. The role of I phase decrementing inhibition and the contribution of cholinergic inputs to the generation of the augmenting patterns were also examined. Pressure microejections of GABAergic and glycinergic antagonists on single I neurons in vivo were used to block the silent phase inhibition. This allowed us to determine the amount of tonic excitation that these neurons receive. Subsequent pressure microejections of glutamatergic antagonists were used to determine the magnitude of the phasic excitation that these neurons receive.

**METHODS**

Most of the methods have been previously described in detail (11,21). The increased size and spacing of canine respiratory neurons make this species suitable for these picoejection studies. All experimental protocols were approved by the Animal Studies Subcommittee of the Zablocki Veterans Affairs Medical Center and the Animal Care Committee of the Medical College of Wisconsin. Data were obtained from 31 halothane-anesthetized mongrel dogs of either sex, weighing from 8 to 15 kg. The animals were maintained on 31 halothane-anesthetized mongrel dogs of either sex, weighing from 8 to 15 kg. The animals were mechanically ventilated (Dräger AV anesthesia machine) with an air-O2 mixture. The following variables were continuously monitored: end-tidal CO2, O2, and halothane concentrations (Pentrafic, Criticare systems), tracheal pressure (P0), and femoral arterial blood pressure (Gould-Statham P23 ID transducers and Grass model 7 polygraph). A triple-lumen catheter was placed in the femoral vein and used for continuous infusion of maintenance fluids (0.9% NaCl) and intermittent administration of muscle relaxants (pancuronium). Blood gas samples were obtained periodically, and sodium bicarbonate was added to the maintenance fluids to correct metabolic acidosis if required. Esophageal temperature was monitored and maintained at 37.5–38.5°C with a servo-controlled heating pad (custom built).

The dogs were positioned in a Kopf (model 1530) stereotaxic apparatus. Bilateral cervical vagotomies were performed to eliminate afferent vagal input from pulmonary stretch receptors. The moving time average of phrenic nerve activity [4th order Bessel linear averaging filter (custom built), averaging interval 100 ms], i.e., the phrenic neurogram (PNG) was recorded from the right C5 phrenic nerve rootlet [amplifier (custom built) band pass 0.1–3 kHz] and used to obtain I and E timing pulses.

The dorsal surface of the medulla was exposed via an occipital craniotomy. To minimize brain stem movements during neuronal unit recording and to eliminate feedback from extravagal chest wall afferents, a bilateral pneumothorax was created and the animal was paralyzed with a 0.1 mg/kg intravenous bolus of pancuronium bromide and supplemental doses of 0.05 mg/kg as required.

**Picoejection Procedure**

Multibarrel micropipettes (composite tip diameter 10–30 μm), consisting of one recording barrel containing a carbon filament and three drug barrels, were used for extracellular neuronal recordings and picoejections. The ejected solutions consisted of the vehicle, an artificial cerebrospinal fluid (aCSF), a γ-aminobutyric acid receptor (GABA_A) channel blocker, picrotoxin (Pic, 2 mM, Research Biochemicals), a non-NMDA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo-(D)quinoxaline (NBQX, 250 μM; Novo Nordisk), a NMDA receptor antagonist 2-amino-5-phosphonovalerate (AP-5, 2 mM; Research Biochemicals), glycine (1 mM, Research Biochemicals), a glycine receptor antagonist strychnine (1 mM, Research Biochemicals), and ACh (2 mM, Research Biochemicals), all of which were dissolved in aCSF. The aCSF consisted of (in mM) 124 NaCl, 2 KCl, 2 MgCl2, 1.3 KH2PO4, 0.9 CaCl2, 26 NaHCO3, and 11 glucose. The pH of each solution was adjusted to 7.2–7.4 by aeration with a 5% CO2-95% O2 mixture.

A four-channel pressure microejection system (custom built) was used for continuous local application of the drug solutions with dose rates in the picomole per minute range. Thus the term “picoejection” will be preferably used in place of pressure microejection. The ejected volume per time was measured via height changes of the meniscus in the pipette barrel with the reticle of a ×50 magnification microscope (resolution = 2 nl). To obtain steady-state dose-response data, constant-rate picoejection was used, and dose rates were increased via increases in ejection pulse rate.

Single I neuronal activities were recorded in the VRG [amplifier (custom built) bandwidth 0.2–3 kHz] from 1 mm rostral to 3 mm caudal to the obex, 3–5 mm lateral to the midline, and 3–4.5 mm below the dorsal surface. Past experiments have shown that over 88% of the I neurons in this region have spinal projections (34). Thus most of the neurons of the present study are likely to be bulbospinal neurons. The amplified output of the microelectrode was monitored on a cathode ray oscilloscope (Kikusui COS 6150), and an amplitude-time window discriminator (custom built) was used to generate a standard pulse for each neuronal spike. These pulses were counted during 100-ms intervals, and the resulting spike frequency was displayed on the polygraph. Neuronal unit activity, phrenic nerve activity, Pco2, proximal airway CO2 concentration, picoejection marker, and arterial blood pressure were recorded on an eight-channel digital tape system (Vetter Digital PCM Recording Adapter, model 3000A) for further off-line analysis.

**Protocol**

Once a stable extracellular recording of a VRG I neuron was established, 1–2 min (10–15 respiratory cycles) of preejection control data were recorded. Vehicle or drug
ejection then followed, with step increases in dose (i.e., ejection pulse rate). To permit calculation of effective doses, each ejection rate was maintained until a steady-state response was achieved. Step increases in dose rate were continued until no further effect was observed. Before antagonist applications, aCSF was ejected to verify that the vehicle and ejected volume had little or no effect on neuronal $F_n$.

**Data Analysis**

Cycle-triggered histograms (CTHs) were generated by custom hardware interfaced via an IEEE 488 bus system to a Hewlett Packard 360 workstation and custom software and were used to quantify the $F_n$ patterns at each dose rate. The CTHs, based on 9–32 (average 18 cycles/CTH) respiratory cycles, were triggered from the onset of the I phase. The values of $F_n$ for each 50-ms bin (time increment) were calculated as the number of spikes per bin per bin duration (seconds). For each bin within the triggered cycle, these values were averaged over the number of cycles used to generate the CTH. In addition, the standard deviation (SD) and standard error (SE) for each bin were calculated; these values were then averaged for the bins within the I phase and the average coefficient of variation of the mean was determined (SE/peak $F_n$). From the CTH data, values of time-averaged $F_n$ and peak $F_n$ during the active (I) phase and average discharge activity during the inactive (E) phase that was relatively constant were calculated.

The average $F_n$-to-peak $F_n$ ratio ($R$) was also calculated for each CTH and served as a geometric index of the discharge pattern. For a pure ramp pattern the average peak ratio is 0.5, while for a pure step pattern this ratio is 1.0. Patterns composed of step and ramp components would have an $R$ value between 0.5 and 1.0, where $R$ is linearly related to magnitude of the step component (i.e., $R = [1 + \text{step/peak}]/2$). Because the $R$ value is sensitive to changes in the geometry of the pattern, it also was used to detect the presence of I phasic inhibition, as demonstrated by the effects of the GABA$_A$ receptor antagonist Pic on the discharge pattern of a late-onset I neuron (Fig. 1). Thus changes in $R$ reflect changes in the discharge pattern and are used in this study as an additional method to detect the effects of phasic inputs, especially when these effects are small.

Plots of peak $F_n$ (I phase) and average $F_n$ (E phase) vs. dose rate were used with linear interpolation to estimate the dose of an antagonist producing 50% of the maximal change in $F_n$ (ED$_{50}$) for each neuron. The dose-response data were then pooled in terms of ED$_{50}$ and the maximum effective dose (ED$_{max}$). ED$_{max}$ was defined as the dose rate above which further increases produced no additional effect.

A one-way, repeated-measures ANOVA, with drug-type/dose-level as the main treatment factor, was used to test for

![Image](http://ajpregu.physiology.org/)

**Fig. 1.** Example demonstrating the utility of the average $F_n$-to-peak $F_n$ ratio ($R$) as an index for detecting changes in shape of discharge patterns. **A-C:** data show the effects of picrotoxin (Pic) on the discharge activity of a late-onset inspiratory (I) neuron. **A:** control; PNG, phrenic neurogram; NA, neuronal activity; $F_n$, ratemeter output of discharge frequency (100 ms/bin). **B:** Pic increased activity during the I phase at an intermediate dose rate (87.1 pmol/min). **C:** Pic also induced activity during the expiratory (E) phase at a higher dose rate (118.3 pmol/min). **D-F:** cycle-triggered histograms (CTHs) were obtained for 10–28 central respiratory cycles; 50 ms/bin. **D:** lower dose rates of Pic converted a late-onset pattern to a typical early-onset I neuronal pattern. The $R$ increased from 0.34 to 0.53 to 0.71. **E:** effective maximal Pic dose rate induced activity during the E phase without further increases in I phase activity. **F:** difference CTH illustrating the magnitude and time course of the effect of phasic I inhibition on the discharge pattern. Lower amplitude CTH (thick line), difference of partially blocked pattern ($R = 0.53$) and fully blocked pattern ($R = 0.71$). Higher amplitude CTH (medium line), difference of unblocked, control pattern ($R = 0.34$) and fully blocked pattern ($R = 0.71$); left portion of this CTH (thinner line), when the control pattern was subthreshold, was extrapolated from the time course of the lower amplitude CTH. Note the decrementing nature of this form of inhibition, which not only controlled the rate of augmentation but also peak $F_n$. 
significant drug-induced effects during both the I and E phases for each of the antagonists. If the ANOVA revealed a significant difference between treatments, the treatment means were compared using the modified t-values and the Bonferroni procedure for multiple comparisons (37). Differences were considered significant for \( P < 0.05 \). Variables are expressed as means ± SE.

RESULTS

A total of 34 neurons was studied: 12 neurons with the Pic-NBQX-AP-5 protocol, 9 neurons with the strychnine protocol, and 13 neurons with the ACh protocol. The average number of cycles per CTH was 18 ± 1 (\( n = 54 \)) with 10 and 90 percentile values of 10 and 29 cycles, respectively. The 50-ms bin SD, averaged over the I phase, was 10.7 ± 0.35 Hz. The coefficient of variation of the mean (SE/F peak) was 3.7%.

Responses of I Neurons to GABAergic and Glutamatergic Antagonists

In the first set of studies the sequence in which picoejections were given was 1) Pic, 2) NBQX, and 3) AP-5. Picoejection of the noncompetitive GABA\(_A\) receptor antagonist Pic produced a dose-dependent increase in \( F_n \) during the E phase but with the exception of late-onset I neurons (Fig. 1) had little or no effects during the I phase (e.g., Figs. 2 and 3A). At the maximum effective dose, this block of GABA\(_A\) receptors is expected to reveal most of the tonic excitation that these I neurons receive. The effects of Pic were very long lasting with very little or no recovery observed within 1–2 h. This allowed the study of the glutamate antagonists in sequence. Thus subsequent picoejection of the competitive non-NMDA glutamate receptor antagonist NBQX produced a dose-dependent reduction in peak \( F_n \) during the I phase with little or no effect on \( F_n \) during the E phase (Figs. 3B and 4B). The effects of NBQX were also long lasting. Picoejection of competitive NMDA receptor antagonist AP-5 subsequent to NBQX produced a dose-dependent downward shift in \( F_n \) during both phases (Figs. 3C and 4C).

The summary data from 12 I neurons are shown in Fig. 5. Completion of the full protocol including NBQX application was obtained for 10 of the 12 neurons, whereas completion of the protocol through the AP-5 application was obtained for 6 of the 12 neurons. The data obtained from analysis of the CTHs were normalized relative to the peak \( F_n \) of the control period before Pic application (i.e., \( F_n \) control 100%). The average-to-peak ratio of the control patterns was 0.78 ± 0.03 and ranged from 0.56 to 0.87.

Block of GABA\(_A\) receptors with Pic increased average \( F_n \) (\( F_{ave} \)) during the E phase from 4.3 ± 2.2% to 56.2 ± 3.4% at the ED\(_{max} \) whereas peak \( F_n \) (\( F_{peak} \)) of the I phase only increased by 6.5% at ED\(_{max} \) (Fig. 5A). In addition, Pic had no effect on the average \( F_n \) of the I phase and R (\( F_{ave} \) and \( F_{ave}/F_{peak} \), Fig. 5B). Subsequent application of NBQX reduced the peak \( F_n \) of the I phase from 104.9 ± 1.9% to 69.6 ± 5.1% and the average \( F_n \) from 78.2 ± 2.9% to 66.4 ± 4.4% (Fig. 5B), whereas E phase activity was unaffected by NBQX (Fig. 5A). There was a small, but nonsignificant, increase in R. Subsequent application of AP-5 produced a dose-dependent reduction in average and peak \( F_n \) during both phases. At ED\(_{max} \), I phase peak \( F_n \) was 8.1 ± 5.3% and average \( F_n \) during the E phase was 3.0 ± 2.1%. An analysis of the differences between \( F_n \) of the I and E phases showed no differential effect of AP-5. Neuronal activity was

![Fig. 2. Effect of Pic on the activity of a typical ventral respiratory group (VRG) I neuron. A: preejection control activities. B: picoejection of Pic-induced activity during the normally silent E phase. C: ensemble averages and CTHs of the data from A and B show that the block of GABA\(_A\) receptor-mediated inhibition with Pic-induced activity in the E phase with little effect on the I phase activity. Control pattern, thick line, 19 cycles/CTH, R 0.86. Pattern during Pic application, thin line, 18 cycles/CTH, R 0.83. Data are shown for the maximum effective dose rate of 56.8 pmol/min.](http://ajpregu.physiology.org/doi/abs/10.1152/ajpregu.00428.2017)
Effects of Strychnine

In a separate series of studies, picoejection of the glycine receptor antagonist strychnine on I neurons produced dose-dependent increases in the $F_n$ during the E phase with little effect on peak $F_n$. Complete recovery from strychnine typically required 20–30 min. In contrast, picoejection of glycine produced a rapid, dose-dependent reduction in the peak $F_n$ of I neurons, which promptly recovered with cessation of picoejection (e.g., Fig. 6A). Picoejection of strychnine produced a small increase in E phase activity (Fig. 6B, left), had no effect on peak $F_n$ of the I phase, but strongly antagonized the effects of glycine as would be expected (Fig. 6B, Gly post STR). The pooled data from 9 neurons, normalized to the preejection control peak $F_n$ (84.3 ± 6.3 Hz), show that strychnine produced only small increases in average $F_n$ ($≈8.5\%$) and also in peak $F_n$ of the I phase ($≈7.5\%$), but produced a greater increase in the average $F_n$ of the E phase ($≈12\%$, Fig. 7). Strychnine application did not change $R$, which had an average value of 0.75 (range 0.64 to 0.81) for the control patterns.

Effects of ACh

Picoejection of ACh on caudal VRG I neurons was without effect (Fig. 8A). However, when ACh from the same pipette was applied to a more rostral I neuron, increases in peak $F_n$ were observed (Fig. 8B), demonstrating that this agent at the indicated dose rates was effective. The pooled data from 13 I neurons in the VRG caudal to the obex show that ACh had no discernible effect on these neurons [peak $F_n$ (Con) 65.6 ± 6.42 Hz; peak $F_n$ (ACh) 65.4 ± 6.68 Hz; ACh dose 61.1 ± 7.72 pmol/min].

DISCUSSION

The results of this study suggest that 56–68% of the excitatory drive to canine I neurons in the caudal VRG is mediated by tonic inputs while phasic inputs contribute another 34–44% of this drive. NMDA receptors appear to mediate the tonic component, whereas non-NMDA glutamate receptors appear to mediate the I phasic component. Cholinergic receptors do not appear to contribute to or modulate the excitatory drive.

Methodological Considerations

Functional role of recorded neurons. Although antidromic activation of these VRG I neurons from the spinal cord was not performed in this study, our previous studies found that 88% of the I neurons in the same region of the caudal VRG were bulbospinal (34). Thus it is likely that most of the neurons we studied were bulbospinal I neurons.

Quantitative analyses. One factor that may affect the accuracy of estimating drive levels is related to the cycle-to-cycle variability of the $F_n$ pattern and the number of cycles used to construct the CTHs. To increase the probability of obtaining complete protocols during sequential picoejections of the study agents, the number of central respiratory cycles was limited at each dose rate for each agent. The average number of
cycles per CTH was 18. During the generation of the CTH, the SD and SE of each 50-ms bin were calculated. Plots of the CTH SE revealed that the bin SE was uniform throughout the I phase, independent of the Fn level. Consequently, we averaged the SD and SE of the bins throughout the I phase for each CTH. The average SD of 54 CTHs was 10.7 Hz with 10th and 90th percentile values of 8.3 and 13.5 Hz, respectively, indicating that the cycle-to-cycle variability of the discharge pattern was relatively consistent among neurons and throughout protocols (e.g., Fig. 3). The average coefficient of variation of the mean peak Fn was 3.7%, indicating that small differences (10–12%) between peak Fn values could be detected as being statistically significant (P < 0.01). In addition, the use of a repeated-measures ANOVA on the pooled data further increases the ability to distinguish small differences.

Limitations of picoejection method. Indirect effects of the picoejected antagonist are another factor that may affect the magnitude of the responses. For example, it is possible that part of the neuronal responses to the picoejected antagonists may be due to effects of other neurons in the vicinity of the recorded cell. However, such effects appear to be small, if present, because 1) the qualitative character of the responses was consistent for all neurons studied. If indirect effects due to the spread of antagonist to other surrounding antecedent neurons were important, more variability in the results would be expected due to different combinations of altered presynaptic activities. 2) The response direction was always consistent with the expected effect of the antagonist. Pic produced increases in E phase Fn consistent with antagonism of inhibitory inputs and both NBQX and AP-5 produced decreases in Fn, which would be expected when excitatory inputs are antagonized. If, for example, the latter antagonists disfacilitated a nearby inhibitory interneuron, then a relative increase in activity of the recorded premotor neuron would be expected. In this regard, the dose-dependent responses indicated no obvious change in

Fig. 4. Quantification via CTHs of the neuronal response patterns to antagonist application to the I neuron shown in Fig. 3. Central respiratory cycles per histogram (11–37) and 50 ms/bin were used. Superimposed CTHs indicate E phase specific effects for picoejection of Pic and I phase specific effects of NBQX, but a parallel reduction in the activities of both phases for picoejection of AP-5. Thick lines, control and preejection reference patterns.

Fig. 5. Summary of the pooled data of the I neuronal responses to each of the antagonists. Data were normalized with respect to the peak activity during the I phase of the initial preejection control period (i.e., before Pic picoejection). A: Con, control activity before picoejection of each antagonist; ED50, interpolated dose rate at which 50% of the maximum response occurred; EDmax, measured maximum effective dose rate. Comparisons were made relative to the respective control values. **P < 0.001, Pic effect on E phase activity. &&P < 0.001, NBQX effect on I phase activity. ††P < 0.001, AP-5 effect on I phase activity. Means ± SE for ED50 and EDmax dose-rate values are given below graph. B: pooled data for average Fave, peak Fpeak, and the R for the control and at EDmax for each of the antagonists. Significant differences of each parameter from their respective preejection control (Con) values: *P < 0.05, **P < 0.001.
the rate or direction of response as doses increased. The concentration of the picoejected antagonist is expected to be highest near the recorded neuron and to decrease rapidly with distance from the electrode tip. Thus concentrations are expected to be much less near any neighboring presynaptic neurons. Further evidence of localized effects is the observation that no changes in the PNG amplitude or phase timing occurred during picoejections of the antagonists.

Another factor that may affect the accuracy of the measurement is completeness of the block of the desired type of receptors. This relates to the effectiveness of the distribution of the picoejected antagonist to the receptor sites on the neuron under study and to the selectivity of the antagonist. In this study, the dose rates were increased until no further response could be observed. It is possible that picoejected antagonists did not adequately reach receptors on distal dendrites. However, as will be discussed shortly, the results of the combined antagonism of both inhibitory and excitatory inputs appear to account for the relative roles of each input in the generation of the total discharge pattern.

Estimation of Drive Levels

Tonic drive component. In these studies, it was assumed that tonic excitation to the VRG I neurons was obscured by E phasic inhibition, mediated mainly by GABA_A receptors and to a lesser extent by glycine receptors. This is illustrated by the combined step and ramp control pattern depicted in Fig. 9, top left, with tonic and phasic components. The noncompetitive GABA_A receptor antagonist Pic was used rather than the competitive antagonist bicuculline to block the GABA_A receptors that mediate E phasic inhibition. At low-dose rates, bicuculline amplifies the phasic F_n patterns of these neurons (11). At higher dose rates, bicuculline can also induce activity during the normally silent E phase. Conversely, Pic blocks the tonic GABAergic inhibition during the E phase without altering the activity during the I phase, except in the case of the late-onset I neurons. This selectivity of Pic in blocking the tonic (E phase) inhibition made it a more appropriate antagonist for examining the receptors mediating the tonic excitatory drive.

In the current study, picoejection of Pic induced activity in the normally silent E phase (Fig. 9, top, Picrotoxin), and at maximal effective dose rates this level of activity amounted to 56.2 ± 3.4% of the overall peak I phase activity. However, Pic provides a noncompetitive blockade of Cl^- channels, thus it is possible that channels associated with glycine receptors were also blocked (38, but see Ref. 7). To estimate the contribution of glycine receptors to E phasic inhibition, strychnine was locally applied to a group of I neurons that were not part of the Pic study. Strychnine in-
creased E phase activity by an average of $11.8 \pm 3.4\%$ ($n = 9$). If Pic blocked both GABA$_A$ and all glycine receptors, our estimate of tonic drive would be $\approx 56\%$. On the other hand, if Pic blocked only GABA$_A$ receptors and strychnine blocked only glycine receptors, then the estimated tonic drive would be $\approx 68\%$. It is likely that the level of tonic drive lies between 56 and 68%.

**Phasic drive component.** In the presence of Pic block of E phasic inhibition, antagonism of the ionotropic non-NMDA glutamate receptors with NBQX attenuated I activity by $\approx 34\%$, but not the E phase activity (Fig. 9, top, NBQX). The resulting average difference between peak I phase activity and E phase activity was $\approx 10\%$, resulting in a near tonic pattern throughout the respiratory cycle. This small difference may represent incomplete blockade of either or both GABA$_A$ and non-NMDA receptors. It could also be due to glycine-mediated E phase inhibition that was not antagonized by Pic.

It should be noted that a tonic GABAergic inhibition also has been described that mediates a bicuculline-sensitive but Pic-insensitive proportional control of the $F_n$ patterns of these neurons (11). This bicuculline-sensitive inhibition has a substantial effect on the $F_n$ of respiratory neurons, but the magnitude of the influence appears to be equivalent for both the tonic and phasic excitatory drives (11). Hence, blockade of this
form of inhibition would not be expected to change the relative contribution of non-NMDA and NMDA-mediated drives to the discharge of these neurons. A detailed analysis of the contribution of this form of inhibition is beyond the scope of this investigation.

Subsequent application of the NMDA receptor antagonist AP-5 produced an equal reduction in both the I and E phase activities, confirming the tonic nature of the NMDA receptor-mediated input (Fig. 9, top, AP-5). The combined block of both types of glutamate receptors nearly silenced the neurons, suggesting that the main source of excitation to these VRG I neurons is mediated by ionotropic glutamate receptors.

These results also suggest that the I phasic component of the discharge pattern is mediated by the non-NMDA receptors. It is possible that NBQX also may have indirectly blocked NMDA receptor-mediated I phasic activity at a distal dendritic site by producing a relative hyperpolarization. This would allow for a Mg$^{2+}$ block of NMDA receptors. This hypothesis assumes that prevailing membrane potential at the distal location is relatively more hyperpolarized than the somatic potential, because NMDA receptors are functional at the level of the soma. The latter is confirmed by the AP-5-induced reduction in tonic activity in the presence of NBQX blockade. On the other hand, for current to flow from the dendrites to the soma, the dendritic potential would have to be relatively more depolarized than that of the soma. In a previous study, AP-5 produced a downward parallel shift in the $F_n$ patterns of bulbospinal I neurons with essentially no alteration in the slope or shape of the augmenting pattern (21). In contrast, when NBQX was applied to the same neurons following recovery from the NMDA receptor blockade, the slopes of the augmenting patterns were markedly reduced. Therefore, it appears likely that the phasic excitatory drive to these VRG I neurons is exclusively mediated by non-NMDA receptors and the tonic excitatory drive by NMDA receptors.

$I$ decrementation inhibition. Using spike-triggered averaging, propriobulbar I neurons with a decrementing discharge pattern have been found to inhibit I neurons of the VRG and the dorsal respiratory group (DRG) monosynaptically (16). It has been proposed that this declining pattern of inhibition is also an important mechanism in the generation of the augmenting patterns of the bulbospinal I neurons (14, 32).

Most of the canine VRG bulbospinal I neurons that we have studied have a step-ramp pattern (e.g., Fig. 2) as suggested by the $R$ of $0.76 \pm 0.036$ (10th percentile 0.64 and 90th percentile 0.86) for 21 neurons. Based on a theoretical step-ramp pattern, a $R$ of 0.76 indicates that the step component is 52% of the peak $F_n$. This is consistent with the observed high level of tonic drive to these neurons. In these types of neurons, neither Pic nor strychnine had much effect on neuronal activity during the I phase, and $R$ values were unaltered. This suggests that inhibitory synaptic inputs from decrementing I neurons are very small and make little direct contribution to the overall discharge pattern of these neurons. In addition, because the block of E phase inhibition did not alter $R$, the role of postinhibitory rebound in the generation of the discharge pattern appears to be minimal. However, the possibility that decrementing I neurons are involved with shaping the discharge pattern of propriobulbar neurons, which in turn synapse on bulbospinal I neurons, cannot be ruled out.

In contrast, the role of decrementing inhibition in the control of the discharge pattern of late-onset I neurons is very prominent (e.g., Fig. 1). This mechanism was proposed for late I neurons in the model by Richter et al. (28). In the example of Fig. 1B, Pic converted the late-onset ramp pattern to an early-onset step-ramp pattern, where $R$ increased from 0.34 to 0.70. In all other aspects, the response of this neuron to Pic, NBQX, and AP-5 was similar to the early-onset I neurons. Because late-onset I neurons were rarely found in the caudal VRG, the data of Fig. 1 are not included in the pooled data analysis, but serve to illustrate the utility of the $R$ value to detect changes in pattern. We hypothesize that I neurons, which exhibit mainly ramp patterns without much of a step feature, may be subject to I decrementing inhibition that suppresses the tonic excitatory drive to these neurons in the early part of the I phase. The neuronal model simulations of Balis et al. (3) also suggested this hypothesis.

Comparison with related respiratory neuron studies. The block of GABA$_A$ receptor chloride channels by Pic in the present study is comparable to previous studies (6, 11), which also found that GABA$_A$ receptor antagonists induce activity throughout the $E$ phase with no effect on $I$ phase activity. In the present study the neuronal responses to strychnine were qualitatively similar but much smaller in magnitude compared with those produced by Pic. Strychnine also affected feline I neurons that were studied with the microiontophoresis technique. However, the effects of strychnine were typically most evident in the early part of the $E$ phase (6, 29). Haji et al. (19) observed that strychnine increased the $I$ phase activity of I neurons. Schmid et al. (29) also found that strychnine was effective during the $I$ phase, but its effects were usually not evenly distributed throughout the phase and were most prominent near the end of the $I$ phase. The magnitudes of these effects were not large. Under the conditions of the present study, the effect of strychnine on $I$ phase activity of canine VRG I neurons was also small (Fig. 7).

Glutamate receptors appear to play a major role in providing excitation to bulbospinal I neurons (21, 25, 26). The present results confirm and further clarify that tonic excitatory drive to canine VRG I neurons appears to be exclusively mediated by NMDA receptors, whereas phasic excitatory drive appears to be exclusively mediated by the non-NMDA glutamate receptors (21). The findings are also consistent with the previous observation that NMDA receptor activation mediates the central excitatory drive of bulbospinal neurons, including the excitation mediated by the carotid body chemoreceptors (12). However, the non-NMDA receptor antagonist NBQX did not alter the ongoing or peripheral chemoreceptor-induced activity.
of E bulbospinal neurons (12), suggesting that the NMDA receptors are the exclusive mediators of this excitation to these neurons.

The reported effects of the local application of ACh on medullary respiratory neurons are equivocal. In this study picoejection of ACh was without effect on caudal VRG I neurons. To demonstrate that this lack of effect was not due to an ineffective agonist or inadequate dose rate, we tested a more rostral VRG I neuron and found that ACh produced an increase in I phase activity (Fig. 8). Likewise, Jordan and Spyer (20) found that microiontophoresis of ACh on VRG I neurons was without effect. However, Morin-Surun et al. (23) found that microiontophoresis of ACh produced excitation in about 27% and inhibition in about 36% of the feline respiratory neurons tested, and these responses could be antagonized by atropine but not d-tubocurarine. No clear differences were found for I and E neurons. In the presence of pentobarbital sodium anesthesia, fewer excitatory responses were found compared with decerebrate cats. Similarly, Takeda and Haji (36) found that microiontophoresis of ACh produced excitatory effects on 50% of the feline bulbar respiratory neurons they studied. Because their analysis included only nonannidromically activated neurons, the effects they reported are mainly on propriobulbar rather than on bulbospinal premotor neurons or cranial motoneurons. The ACh-induced excitations could be blocked by atropine but not by hexamethionium.

Perspectives

The findings of this study suggest the hypothetical model of Fig. 9, bottom, for the synaptic inputs to canine bulbospinal I neurons and their corresponding neurotransmitter receptors. The source of the prominent tonic drive may be due in large part to central and peripheral chemoreceptor activity.

During the hypocapnic apnea induced by hyperventilation, both E bulbospinal neurons and E motoneurons exhibit a CO2-dependent tonic discharge (1, 2). Above the apneic threshold, this tonic activity is rendered phasic by I-related inhibition, and the peak phasic activity increases with further increases in CO2 drive (35). Likewise, during hypocapnic apnea, progressive hypoxia was able to produce a graded tonic excitation of I motoneurons (31). Further lowering of O2 or increasing CO2 reestablished rhythm and produced a periodic E-phased inhibition of the tonic I motoneuron discharge. These studies suggested that the periodic discharge of both I and E motoneurons is sculpted from an underlying tonic discharge by reciprocal inhibition. From a theoretical point of view, neuronal network oscillators/pattern generators require a tonic input to each subsystem within the network (3, 13, 24, 28).

Synaptic connectivity studies suggest that the phasic component of excitation of bulbospinal I neurons is due to inputs from other I neurons with augmenting discharge patterns and I neurons with patterns of nearly constant discharge rate throughout the I phase (14, 32). The interconnection of excitatory inputs among augmenting I neurons may be the basis of a self-reexcitatory network, which has been postulated as a mechanism for the generation of the augmenting pattern. In contrast, the I constant neurons are propriobulbar neurons, mainly located in the pre-Bötzinger region of the VRG, with extensive medullary projections that form monosynaptic excitatory connections with various kinds of I neurons in both the VRG and DRG (14).

In summary, our studies confirm the key role of synaptic inputs in the generation of the discharge patterns of bulbospinal I neurons. The findings suggest that about two-thirds of the excitatory drive to canine bulbospinal I neurons of the caudal VRG is due to tonic inputs mediated exclusively by NMDA receptors and the remaining drive is due to phasic inputs mediated primarily by non-NMDA glutamate receptors.

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REFERENCES


