Differential Roles of Ionotropic Glutamate Receptors in Canine Medullary Inspiratory Neurons of the Ventral Respiratory Group

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Krolo, M., E. A. Stuth, M. Tonkovic-Capin, Z. Dogas, F. A. Hopp, D. R. McCrimmon, and E. J. Zuperku. Differential roles of ionotropic glutamate receptors in canine medullary inspiratory neurons of the ventral respiratory group. J. Neurophysiol. 82: 60–68, 1999. The relative roles of ionotropic N-methyl-d-aspartate (NMDA) and non-NMDA glutamate receptors in supplying excitatory drive to inspiratory (I) augmenting pattern neurons of the ventral respiratory group were studied in anesthetized, ventilated, paralyzed, and vagotomized dogs. Multibarrel micropipettes were used to record simultaneously single-unit neuronal activity and pressure microej ect the NMDA antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX; 0.25 mM), and an artificial cerebrospinal fluid vehicle. Ejected volume-rates were measured directly via meniscus level changes. The moving time average of phrenic nerve activity was used to determine respiratory phase durations and to synchronize cycle-triggered histograms of the discharge patterns. Both AP5 and NBQX produced dose-dependent reductions in peak spontaneous I neuronal discharge frequency \( (F_s) \). The average \( \pm \text{SE} \) maximum reduction in peak \( F_s \) produced by AP5 was 69.1 \( \pm \) 4.2\% and by NBQX was 47.1 \( \pm \) 3.3\%. Blockade of both glutamate receptor subtypes nearly silenced these neurons, suggesting that their activity is highly dependent on excitatory synaptic drive mediated by ionotropic glutamate receptors. Differential effects were found for the two glutamatergic antagonists. AP5 produced downward, parallel shifts in the augmenting pattern of discharge, whereas NBQX reduced the slope of the augmenting discharge pattern. These results suggest that time-varying excitatory input patterns to the canine I bulbospinal neurons are mediated by non-NMDA glutamate receptors and that constant or tonic input patterns to these neurons are mediated by NMDA receptors.

Introduction

Inspiratory (I) premotor neurons, which are located within the ventral respiratory group (VRG) of the ventrolateral medulla, provide the main source of phasic excitatory drive to motoneurons innervating the diaphragm and inspiratory muscles of the chest wall as well as inhibition (via interneurons) of thoracic and abdominal expiratory (E) motoneurons (Cohen 1979; Euler 1986; Feldman 1986; Sears 1977). Most of these I bulbospinal neurons exhibit an augmenting firing pattern during the I phase. They are not involved with rhythm generation, but rhythm is imposed on them by presynaptic excitatory and inhibitory inputs from neurons located more rostrally within the medulla (Smith et al. 1991). Their discharge frequency is highly dependent on the level of PaCO\(_2\) (Cohen 1979) acting via central and peripheral chemoreceptors (St. John and Bianchi 1985; St. John and Wang 1977).

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 either N-methyl-d-aspartate (NMDA) or non-NMDA ionotropic glutamate receptors produced reductions in the discharge frequency of single respiratory neurons (Pierrefiche et al. 1991, 1994). When the NMDA and non-NMDA antagonists were given together, further reductions were found. It was suggested that the sequential activation of non-NMDA and NMDA receptors possibly may shape the respiratory burst (Pierrefiche et al. 1991).

On the other hand, we have found that the excitatory drive to E bulbospinal neurons in dogs is almost exclusively through NMDA receptor activation (Dogas et al. 1995). Picoejection of the highly selective, ionotropic non-NMDA receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX), had no effect on the spontaneous discharge patterns of these E bulbospinal neurons (Dogas 1996; Dogas et al. 1995), while it blocked the excitation induced by picoejection of the specific non-NMDA receptor agonist, \( \alpha \)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA). These results suggest that endogenous activation of the non-NMDA receptors is not involved in the excitation of canine E bulbospinal neurons in the anesthetized dog. In contrast, preliminary studies have implicated both NMDA and non-NMDA receptor activation in the control of baseline I neuron discharge (Krolo et al. 1996).

The purpose of the present study was to determine the extent to which NMDA and non-NMDA receptors are involved in the production of the spontaneous activity of canine VRG I neurons.

Methods

Experiments were performed on 25 mongrel dogs of either sex, weighing from 8 to 15 kg. Halothane anesthesia was used for induction of anesthesia, during the surgical preparation (1.0–1.4% end-tidal concentration), and during the experimental protocol (0.8–1.2% end-tidal concentration). The animals were monitored for signs of inadequate anesthesia, including salivation, lacrimation, and/or increases in blood pressure and heart rate. The anesthetic depth was increased immediately when such signs were present.

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Surgical procedure

The animals were intubated with a cuffed endotracheal tube and mechanically ventilated with an air-O₂ mixture. End-tidal CO₂ concentration was monitored continuously with an infrared analyzer (POET II, Criticare Systems). Tracheal pressure (Pₜ) was measured from an airway side-port with an air-filled catheter connected to a Gould-Statham P23 ID transducer. The femoral arteries were cannulated for arterial blood sampling and continuous blood pressure monitoring (Gould-Statham P23 ID transducer). A triple-lumen catheter was placed in the femoral vein and used for continuous infusion of maintenance fluids (0.9% NaCl) and drugs. Blood gas samples were obtained periodically, and additional sodium bicarbonate was given to correct metabolic acidosis if required. Esophageal temperature was monitored and maintained at 37.5–38.5°C with a servo-controlled heating pad.

The dogs were positioned in a Kopf (model 1530) stereotaxic apparatus with the head flexed ventrally by 30°. The vertebral column was maintained straight through caudal tension applied via a hip-pin clamp. Bilateral, dorsolateral neck dissection was performed and bilateral cervical vagotomies performed to eliminate afferent vagal input from pulmonary stretch receptors. The right C5 phrenic nerve rootlet was cut distally, desheathed, and placed on bipolar platinum electrodes in a mineral oil pool formed from a neck pouch. The moving-time average of phrenic nerve activity or the phrenic neuronalogram (PNG) was recorded and used to obtain I and E timing pulses.

An occipital craniotomy was performed and the dura mater opened along the midline and reflected laterally to expose the dorsal surface of the medulla oblongata. To minimize brain stem movements during neuronal unit recording and to eliminate feedback from extravagal chestwall afferents, a bilateral pneumothorax was created, and the animal was paralyzed with a 0.1 mg/kg iv bolus of pancuronium bromide and supplemental doses of 0.05 mg/kg as required.

Pressure microejection procedure

Multibarrel micropipettes (composite tip diameter: 10–30 μm), consisting of one recording barrel containing a carbon filament (7 μm diam) and three drug barrels, were used for extracellular neuronal recordings and pressure microejections. The ejected solutions consisted of the vehicle, an artificial cerebrospinal fluid (ACSF), the NMDA antagonist 2-amino-5-phosphonovaleate (AP5; 2 mM, Research Biochemicals), the non-NMDA antagonist NBQX (250 μM, Novo Nordisk), the non-NMDA agonist AMPA (10 μM, Research Biochemicals), and the NMDA agonist NMDA (200 μM, 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 5% CO₂-95% O₂.

The pressure microejection system is similar to the ‘Picospritzer’ (General Valve) in that each timed pressure pulse ejects a volume in the 50- to 300-pl range. By delivering repeated picospritzes of microtonomous solutions, typical dose rates in the picomole/minute range can be obtained. The term picoejection will be used preferably in place of pressure microejection. The picoejection system was pressurized with compressed nitrogen and the parameter ranges typically used were ejection pressure: 10–100 psi; duration of the pressure pulse: 10–100 ms; and frequency of the ejection pressure pulses: 0.5–4 Hz. Ejected volume/time was measured via height changes of the meniscus in the pipette barrel with a ×50-magnification microscope equipped with a reticle (resolution ~ 2 nl). To minimize vibration effects during pressure application, 3-foot-long, soft catheter tubings were connected between the picoejector solenoid valves and the micropipette barrels. To obtain steady-state dose-response data, constant-rate picoejection was used. The doses were increased via increases in ejection rate.

Single-unit neuronal activities were recorded from I neurons in the VRG, from 1 mm rostral to 3 mm caudal to the obex, 2–4 mm lateral to the midline, and 3–4.5 mm below the dorsal surface. The amplified output of the micropipette was monitored on a cathode ray oscilloscope, and an amplitude-time window discriminator 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 a polygraph (Grass model 7) via a D/A converter. The data were recorded on an eight-channel digital tape system (A. R. Vetter Digital PCM recording adaptor, Model 3000A). The recorded signals consisted of neuronal unit activity, phrenic nerve activity, tracheal pressure (Pₜ), proximal airway CO₂ concentration, and pressure picoejection marker.

Protocol

Once a stable extracellular recording of a VRG I neuronal unit was established, 1–2 min (10–15 respiratory cycles) of prejection control data were recorded. Vehicle or drug ejections then were made using step increases in dose (i.e., ejection 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 agonist (AMPA or NMDA) or antagonist (AP5 or NBQX) applications, ACSF was ejected to verify that the vehicle and ejected volume had little or no effect on neuronal discharge frequency.

Data analysis

Cycle-triggered histograms (CTHs; binwidth: 50 ms), triggered from the onset of the I phase and, based on 5–15 respiratory cycles, were used to quantify the discharge frequency (Fₚ) patterns at each dose-rate. The values of Fₚ for each bin were calculated as the number of spikes per bin/bin duration in seconds. For each time increment (bin) within the triggered cycle, these values were averaged over the number of cycles used to generate the CTH. Values of the peak discharge frequency (peak Fₚ) during the active (I) phase of the neuron were obtained from the CTHs.

To distinguish between antagonism of a constant input (i.e., constant level throughout the I phase) and antagonism of a time-varying input during the I phase, we analyzed plots of the Fₚ pattern (CTH) during antagonist application versus the control Fₚ pattern (CTH). Linear regression techniques were used to obtain the slope (unitless) and intersects (Hz) of these plots. A plot of the control pattern against itself would have a slope of 1.0 and a y intercept of 0.0. Antagonism of constant inputs would reduce the intercept but not the slope of these plots, whereas antagonism of a time-varying input would reduce the slope and increase the intercept (a further description is given in Results, Fig. 6). Antagonism of both types of excitatory inputs to the same extent would reduce the slope without altering the intercept. This method of analysis has the advantage of being insensitive to the geometric shape of the neuronal discharge pattern. For example, the analysis can detect the amount by which a pattern is parallel-shifted and the amount by which a pattern is attenuated (proportionally reduced), regardless of whether a pattern is ramp-like or has some type of a curved trajectory.

Plots of peak Fₚ versus dose-rate were used with linear interpolation to estimate ED₅₀ values for each neuron. The dose-response data then were pooled in terms of multiples of ED₅₀, and included the maximum effective dose (ED₉₀) above which further increases in dose produced no additional effect. A repeated-measures two-way ANOVA, with drug type and dose level as main treatment factors, was used to test for possible differential effects between the two antagonists at each dose rate on peak Fₚ and the slope and intercept of the Fₚ(antagonist) versus Fₚ(control) plots. 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 (Wal-
lenstein et al. 1980). Differences were considered significant for $P < 0.05$. Variables are expressed as means ± SE.

RESULTS

Antagonist effects on peak discharge frequency

Picoejections of the NMDA receptor antagonist, AP5, produced dose-dependent reductions in the peak $F_n$ of I neurons (e.g., Fig. 1). The onset of the response typically became visible ~1 min after the start of picoejection (Fig. 1, top), and full recovery usually required 30–40 min. In this example, the peak $F_n$ was reduced from 78 to 26 Hz, or ≈68%, at the maximum effective dose of 206 pmol/min. Time-expanded views of neuronal activity (NA), rate meter output ($F_n$), and the phrenic neurogram (PNG) are shown for the pre-ejection period and during the maximum dose rate (Fig. 1, bottom).

![FIG. 1. Picoejection of 2-amino-5-phosphonovalerate (AP5) dose-dependently reduces the spontaneous discharge activity of an inspiratory (I) neuron. Bottom: time-expanded records of neuronal activity (NA), discharge frequency ($F_n$), and phrenic neurogram (PNG) taken from segments indicated (↓).](image1)

![FIG. 2. Picoejection of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) dose-dependently reduces the spontaneous discharge activity of an I neuron (same neuron shown in Fig. 1). Bottom: time-expanded records of neuronal activity (NA), discharge frequency ($F_n$), and phrenic neurogram (PNG) taken from segments indicated (↓).](image2)
overall neural breathing pattern, in terms of respiratory rate and phrenic nerve amplitude, was essentially unaltered by this procedure as indicated by the PNG record.

Picoejections of the non-NMDA glutamate receptor antagonist, NBQX, also produced marked reductions in peak $F_n$ (e.g., Fig. 2). In this example from the same neuron as shown in Fig. 1, NBQX picoejection was initiated following a 40-min recovery period after the AP5 application. At the maximum effective dose of 12.6 pmol/min, peak $F_n$ was reduced from 65 to 21 Hz, a 68% reduction, again with no noticeable effect on the overall neural breathing pattern. However, the effects of NBQX are long-lasting and only a small degree of recovery was observed after 1 h.

Before the antagonist applications, picoejection of the ACSF vehicle alone, at ejection volume rates equal to or greater than those typically used for the antagonists, had very little or no noticeable effect on the neuronal discharge pattern (e.g., Fig. 3). This example is also from the same neuron shown in Figs. 1 and 2.

The pooled data from 32 I neurons, from 11 of which both AP5 and NBQX data were obtained, indicate that AP5 reduced peak $F_n$ significantly more ($P < 0.001$) than NBQX at maximally effective dose-rates. At maximally effective dose-rates, picoejection of AP5 produced a 69.1 ± 4.2% reduction in peak $F_n$ relative to preejection control values, whereas NBQX picoejections produced only a 47.1 ± 3.3% reduction in peak $F_n$.

Differential effects of the glutamate antagonists

For both antagonists, the peak $F_n$ was reduced but apparently via different mechanisms. Cycle-triggered histogram analysis of the time courses of the discharge patterns of the I neurons clearly demonstrate the differential effects of AP5 and NBQX. This analysis applied to the data for the I neuron of Figs. 1–3, shows that increasing doses of AP5 produced downward parallel shifts in the step-ramp discharge pattern of the I neuron (same neuron shown in Figs. 1–3). This example is also from the same neuron shown in Figs. 1 and 2.

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changes in slope with only small changes in intercept, whereas picoejection of AP5 produced large dose-dependent changes in intercept and only modest changes in slope (Fig. 5, bottom). This pattern also was confirmed by the pooled data from 15 neurons. From 7 of these 15 neurons both AP5 and NBQX data were obtained (Fig. 6). Significant differences between the effects of AP5 and NBQX were found for both the slope and intercept parameters at each of the indicated doses. These data are consistent with the hypothesis that antagonism of non-NMDA receptors reduces a time-varying augmenting excitatory input and antagonism of NMDA receptors reduces a constant excitatory input.

The individual and combined effects of both antagonists are sequentially demonstrated for an I neuron in Fig. 7. Picoejection of AP5 produced a downward parallel shift in the discharge pattern (Fig. 7A). After partial recovery from NMDA receptor antagonism, NBQX reduced the augmenting slope to nearly zero (Fig. 7B). No recovery from the effects of NBQX was seen 26 min post-ejection and subsequent picoejection of AP5 markedly reduced the remaining neuronal activity (Fig. 7C). After recovery from the effects of AP5 (29.5 min post-ejection), the repeated application of NBQX produced only a small decrease in activity, indicating little recovery from the original NBQX application of 1 h earlier (Fig. 7D).

**Selectivity of the glutamate antagonists**

The selectivity of each of the antagonists also was verified for these canine I neurons. Picoejection of AP5 resulted in a decrease of spontaneous activity and preservation of the excitatory response to AMPA, but elimination of the response to NMDA (Fig. 8B). The responses to NMDA, pre- and post-AP5 application, are shown in Fig. 8, A and C. In contrast, picoejection of NBQX reduced the spontaneous activity, but the neuronal responses to NMDA were not affected (Fig. 9A). In neuron (Fig. 4, left). At the higher dose-rates, the onset of the discharge pattern relative to the onset of the I-phase was progressively delayed. In contrast, the CTHs for the NBQX run show that the slope of the ramp portion of the discharge pattern was dose-dependently reduced to zero. The step portion of the pattern also was reduced at the lowest dose rate, but no further reduction occurred at the higher dose rates, which continued to decrease the ramp slope. The onset time was not delayed.

FIG. 6. Pooled data contrasting the differential effects of AP5 and NBQX on the discharge patterns of I neurons. Top: dose-dependent reduction in slope was significantly larger for NBQX than for AP5 (**p < 0.001). Bottom: dose-dependent reduction in intercept was significantly greater for AP5 than for NBQX (**p < 0.01, ***p < 0.001). ED<sub>50</sub> and ED<sub>max</sub> dose rates (means and SE) are given below the bottom panel (  ), n: number of neurons. See text for further explanation.

Plots of F<sub>n</sub> during antagonist application [F<sub>n</sub>(antag)] versus preejection control F<sub>n</sub> [F<sub>n</sub>(con)] were used to quantify the amount of downward baseline shift in the discharge pattern and the amount of slope reduction (attenuation of the time-varying pattern). Linear regression was used to obtain estimates of the slope and ordinate intercept of these plots for each dose-rate and each antagonist. Data obtained from the CTHs of the neuron shown in Fig. 4 were used for the example of this analysis in Fig. 5. The plots of F<sub>n</sub>(antag) versus F<sub>n</sub>(con) for AP5 (only 2 of 4 dose rates are shown) clearly show a downward shift without slope change. Similar plots for picoejection of NBQX indicate a reduction in the slope (Fig. 5, top right). As previously indicated (METHODS), this type of analysis is insensitive to the time course of the discharge pattern, which in some I neurons is curvilinear and thus less amenable to linear regression analysis. NBQX produced large dose-dependent...
another I neuron where a smaller dose-rate of NBQX was used to allow for a faster recovery, the response to AMPA was blocked effectively once the effects of NBQX were established (Fig. 9B, bottom). A marked recovery of the response to AMPA was obtained 19.5 min post-NBQX ejection.

Effects of agonist/antagonist mixtures

To avoid the possible effects of dilution near the pipette tips and uneven distribution of the agonist and antagonist, which could occur during simultaneous picoejections from separate pipette barrels, studies were done using a mixture of the agonist and antagonist within the same barrel. The mixture concentration of the agonist and antagonist was the same as that for these agents given separately from their respective barrels. Results from this procedure using an NMDA-NBQX mixture and an AP5-AMPA mixture are shown in Fig. 10, A and B. Picoejection of NMDA alone produced a marked dose-dependent linear increase in peak $F_n$, whereas NBQX alone produced a smaller dose-dependent reduction in peak $F_n$. The NMDA-NBQX mixture produced responses similar to NMDA alone, suggesting that the non-NMDA antagonist, NBQX, had no effect on the NMDA response. The effect of NBQX in the mixture on the peak spontaneous activity became apparent at the higher dose rates, where a small departure can be seen from the NMDA alone response. For another I neuron, picoejection of AMPA alone produced a large dose-dependent increase in peak $F_n$, whereas AP5 alone produced a marked dose-dependent decrease in peak $F_n$ (Fig. 10B). Picoejection of the AMPA-AP5 mixture resulted in a modest dose-dependent increase in peak $F_n$. The net AMPA-induced excitation produced by the mixture, measured as the difference between the mixture and AP5 curves (bottom 8), at any chosen dose-rate, is comparable in magnitude with the response produced by AMPA alone (distance from the AMPA curve to the preejection level:}
100%). These data indicate that the selectivity of each antagonist is sufficient to distinguish the effects of the blockade of each receptor subtype.

**DISCUSSION**

The main finding of this study is that the roles of NMDA and non-NMDA ionotropic glutamate receptors in contributing to the discharge patterns of VRG I neurons are different. Together these two receptor types appear to mediate most of the excitatory drive to I neurons because simultaneous blockade of these two receptor types appears to mediate most of the excitation. This study also confirmed the selectivity of AP5 and NBQX as antagonists at NMDA and non-NMDA ionotropic glutamate receptors, respectively, on VRG I neurons.

**Inspiratory neurons of the caudal VRG**

Although antidromic activation and collision studies were not performed in this study, our previous studies found that approximate 88% (15 of 17) of the I neurons in the same region of the caudal VRG yielded positive collision tests with antidromic stimulation from the spinal C3 level (Stuth et al. 1994). Thus assuming that the probability of recording from an I bulbospinal neuron is 0.88 and using the binomial distribution, the probability that $\geq 75\%$ of the neurons that we studied were bulbospinal neurons exceeds 98%.

**Sources of excitatory synaptic drive**

Cross-correlation between extracellular spike trains from pairs of neurons and spike-triggered averaging techniques have been used to ascertain the synaptic connections between medullary respiratory neurons. In the cat, the primary inputs to I bulbospinal neurons, which have augmenting discharge patterns, are from propriobulbar I neurons with a constant discharge pattern during the I phase and from other I bulbospinal neurons (Ezure 1990; Segers et al. 1987). The I “constant” neurons, which may show either a slight augmenting or decrementing pattern, appear to be an important source of excitation because they excite various kinds of I neurons in both the dorsal and ventral respiratory groups. These neurons have extensive medullary projections and tend to fire at rates in excess of 100 Hz (Ezure 1990). Another main source of synaptic input to the I bulbospinal neurons is from I bulbospinal neurons of the contralateral (Ezure 1990) and ipsilateral (Segers et al. 1987) VRG. It has been proposed that this type of recurrent or self-reexcitation among I augmenting neurons together with inhibition from I neurons with a decrementing pattern are key factors in the generation of the augmenting pattern (Ezure 1990; Feldman and Cowan 1975; Segers et al. 1987).

The excitatory postsynaptic potentials (EPSPs), which were recorded in VRG I augmenting neurons during the spike-triggered averaging studies and evoked by inputs of both I constant and I augmenting neurons, were fast and decayed rapidly with a 3- to 6-ms time constant (Ezure and Manabe 1989; Ezure et al. 1989). Such short-duration EPSPs are consistent with those due to inputs mediated by the non-NMDA ionotropic glutamate receptors (Collingridge and Lester 1989).

Tonic inputs from intracranial chemoreceptors and other excitatory sources also may provide a significant amount of excitation to I bulbospinal neurons (Sears et al. 1982). Cross-correlational studies have demonstrated a high percentage of the corregolram peaks at zero lag, suggesting a common input to the neuron pairs (Feldman and Speck 1983; Segers et al. 1987). Also blockade of GABA\(_A\) receptors with picrotoxin induces activity in the normally silent E phase of these I neurons. This E-phase activity, which has a constant discharge frequency of approximate 38% of the peak I discharge rate, appears to be due to a tonic input that is inhibited actively during the normally silent phase (Dogas et al. 1998). Thus these I bulbospinal neurons appear to receive both phasic and tonic excitatory synaptic drives.

**Possible differential roles of NMDA and non-NMDA receptors**

On the basis of the differential responses of I neurons to the antagonists of NMDA and non-NMDA glutamate receptors.
and the putative sources of synaptic inputs discussed in the preceding text, it would appear that non-NMDA receptors mediate the augmenting phasic excitation from other I augmenting neurons. It is possible that excitation from I constant neurons also is mediated by the non-NMDA receptors because the CTHs show a downward shift along with the decrease in augmenting slope (e.g., Figs. 4, right, and 7B). The downward parallel shift in the CTHs produced by picoejections of AP5 suggests that tonic excitation is mediated by NMDA receptors, although the downward shift also may be associated with a reduction of the input from I constant neurons. In this regard, 4 of the 15 neurons, which were analyzed using CTHs, had a small level of activity (<20% of peak) during the E phase. The application of AP5 reduced the E-phase activity to the same extent as the I-phase activity, suggesting the existence of a NMDA-receptor-mediated tonic input. On the basis of dynamic considerations for the temporal summation of EPSPs, the more rapid dynamics of the non-NMDA receptors would be more optimal for phasic inputs, whereas the slower dynamics of the NMDA receptors would be well suited for tonic inputs. The temporal summation of slow EPSPs due to a volley of synaptic input during the I phase would produce a ramp-like discharge pattern. However, AP5 did not decrease the ramp slope but produced a downward parallel shift in the discharge pattern. With regard to tonic inputs, the spontaneous activity of E bulbospinal neurons, which is highly dependent on tonic excitation (Bainton and Kirkwood 1979), is reduced markedly by picoejections of AP5, whereas picoejections of NBQX are without effect (Dogas et al. 1995).

Because the combined antagonism of both types of ionotropic glutamate receptors eliminates most of the activity of these I bulbospinal neurons (e.g., Fig. 7C), it appears that the activity of these neurons is primarily dependent on glutamatergic synaptic transmission. It is possible that intrinsic mechanisms, such as voltage-dependent calcium currents, also contribute to the generation of the augmenting discharge pattern (Ramirez and Richter 1996). Our techniques do not allow us to directly determine the amount of this contribution. The observation that AP5 produced dramatic reductions in peak activity without much change in the augmenting slope of the discharge pattern (e.g., Fig. 4, left) indicates that the role of intrinsic factors may be small in these particular respiratory neurons. Likewise the elimination of the augmenting portion of the discharge pattern by NBQX suggests that this component has a synaptic origin.

Possible indirect effects of the picoejection method

It is conceivable that part of the neuronal responses to the picoejected antagonists may be due to indirect effects via neurons other than 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. Both AP5 and NBQX produced decreases in discharge frequency, which would be expected when excitatory inputs are antagonized. If, for example, these 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 indicate no obvious change in the rate or direction of response as doses increase. 3) 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 consistent changes in the PNG amplitude or phase timing occurred during picoejections of the antagonists.

Work of others

The results of this study are consistent in part with those in which microiontophoretic application of NMDA and non-NMDA receptor antagonists on respiratory neurons in cats were used. For VRG inspiratory augmenting neurons, the NMDA receptor antagonist, AP7, produced a median decrease in peak $F_n$ of 24%, whereas the non-NMDA receptor antagonist, 6,7-dinoquinolinoline-2,3-dione (DNQX), produced a median decrease of 30% (Pierrefiche et al. 1991). In our study using picoejection, greater average reductions were obtained (69 and 47%, respectively). The picoejection method may be a more effective way to distribute the antagonists into the dendrite tree because it combines the effects of both diffusion and bulk flow. Similar to the findings of Pierrefiche et al. (1991), coapplication of both antagonists also produced a greater reduction in the activity of our neurons.

However, in terms of effects of these antagonists on the discharge pattern, our results show a clear differential effect whereas those using microiontophoresis showed similar effects for both AP7 and DNQX (Fig. 5 of Pierrefiche et al. 1991). A possible reason for this discrepancy may be the lack of selectivity of DNQX for the non-NMDA receptors. We have found that the closely related antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), is very effective at antagonizing NMDA receptors, possibly via the strychnine insensitive glycine site (Harris and Miller 1989) as well as non-NMDA receptors in canine E bulbospinal neurons (Dogas et al. 1996) and nucleus tractus solitarius neurons in rats (Bonham et al. 1993). Because iontophoretic application of NMDA produced greater increases than quisqualate in the active phase spontaneous discharge (Pierrefiche et al. 1991), it is possible that the antagonism of NMDA receptors by DNQX outweighed its effect on the non-NMDA receptors. Thus differential effects may have been obscured. In a more recent study by the same group, microiontophoretic application of the highly selective non-NMDA receptor antagonist, NBQX, produced about a 32% decrease in the peak $F_n$ of feline I augmenting neurons (Pierrefiche et al. 1994). Also the CTHs of both I and E neurons indicate that there is a reduction in the slopes of the discharge patterns, which accompanies the reductions in peak $F_n$. However, as previously mentioned, NBQX has no effect on the spontaneous activity of canine E bulbospinal neurons (Dogas et al. 1995). It is possible that a species difference exists.

Our in vivo data do not support the notion that excitation via the non-NMDA ionotropic glutamate receptors is required to overcome the voltage dependent block of the NMDA receptor channels by $Mg^{2+}$ (Pierrefiche et al. 1994). Both I and E bulbospinal neurons continue to discharge after the local application of
NBQX, and this discharge is highly dependent on NMDA-receptor-mediated excitation because subsequent application of AP5 can decrease the activity of these neurons to near zero.

In conclusion, the spontaneous activity of canine VRG I augmenting bulbospinal neurons is mainly dependent on glutamatergic neurotransmission acting at both NMDA and non-NMDA ionotropic glutamate receptors. Phasic excitation appears to be mediated by the non-NMDA receptors, whereas tonic excitation appears to be mediated by NMDA receptors.

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