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NMDA receptor-mediated transmission of carotid body chemoreceptor input to expiratory bulbospinal neurones in dogs

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1. This study tested the hypothesis that excitatory amino acid receptors mediate the excitatory response of expiratory bulbospinal neurones to carotid body chemoreceptor inputs.

2. Studies were carried out in thiopental sodium anaesthetized, paralysed, ventilated, vagotomized dogs.

3. Brisk, short-duration chemoreceptor activation was produced by bilateral bolus injections of CO_2-saturated saline ($P_{CO_2} > 700$ mmHg) into the autoperfused carotid arteries. A pressurized-reservoir–solenoid valve system was used to deliver the CO_2 bolus injections just prior to the onset of the neural expiratory phase, as determined from the phrenic neurogram, about once per minute.

4. Multibarrelled micropipettes were used to record neuronal unit activity and deliver neurotransmitter agents. Net responses of expiratory bulbospinal neurones to peripheral chemoreceptor activation were determined by subtracting the mean discharge frequencies (F_n) during three control expiratory cycles from the F_n during administration of a CO_2 test bolus. The role of excitatory amino acid receptors in mediating this response was determined by comparing the baseline and bolus expiratory neuronal F_n before, during and after the pressure microejection of the NMDA receptor antagonist 2-amino-5-phosphonovalerate (AP5) or the non-NMDA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(*f*)quinoxaline (NBQX). Ejection rates of AP5 and NBQX were measured by monitoring the movement of the pipette meniscus.

5. AP5 reduced F_n during both the control and bolus cycles, as well as reducing the change in F_n between control and bolus cycles. NBQX had no effect on either baseline or bolus responses.

6. AP5 did not prevent excitation of expiratory bulbospinal neurones by AMPA. Co-administration of AMPA with AP5 prevented the AP5-mediated decrease in F_n but not the dose-dependent reduction in the CO_2 bolus response.

7. Taken together, these data strongly suggest that the carotid chemoreceptor-mediated excitation of expiratory bulbospinal neurones is dependent on NMDA but not non-NMDA glutamate receptors.

Expiratory bulbospinal neurones represent the vast majority of the neuronal population in the caudal ventral respiratory group (VRG) region of the brainstem (Feldman, 1986). These neurones provide phasic excitatory input patterns to motoneurones innervating expiratory muscles of the chest wall and abdomen, as well as inhibition (via interneurones) of thoracic inspiratory motoneurones. In dogs, most of the expiratory bulbospinal neurones exhibit a decrementing firing pattern during the expiratory phase and respond strongly to afferent inputs arising from chemoreceptors and pulmonary mechanoreceptors (Bainton & Kirkwood, 1979; Bajic, Zuperku, Tonkovic-Capin & Hopp, 1994). Characterization of the responses of expiratory bulbospinal neurones to these afferent inputs suggests that

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these neurones are involved in the reflex control of expiratory airflow rate and end-expired lung volume (Bajic, Zuperku, Tonkovic-Capin & Hopp, 1992; Tonkovic-Capin, Zuperku, Bajic & Hopp, 1992; Bajic *et al.* 1994).

It is well known that adjustments in alveolar ventilation necessary for maintaining steady blood gas levels rely on chemoreceptor input from both central and peripheral sources. Carotid body chemoreceptor stimulation results in an increase in the rate of augmentation of inspiratory activity and significant changes in respiratory phase durations (Eldridge, 1972, 1976; Koepchen, Klussendorf & Philipp, 1973; Hopp, Seagard, Bajic & Zuperku, 1991). It has been shown that the magnitudes of the change in inspiratory activity, inspiratory duration (T_I) and expiratory duration (T_E), are greatly affected by the timing of this stimulus relative to the respiratory cycle (Eldridge, 1972; Lipski, McAllen & Spyer, 1977; Marek, Prabhakar & Loeschcke, 1985). Koepchen *et al.* (1973) first demonstrated a strong excitation of expiratory medullary neurones from peripheral chemoreceptor stimulation. However, the reflex role of the chemoreceptors in the control of expiratory neurones is not well known.

In an effort to understand better the integration of chemoreceptor inputs at the caudal VRG, expiratory bulbospinal neurones, this study was done to determine the neurotransmitter receptors responsible for the carotid chemoreceptor-mediated excitation of these neurones. In a preliminary study, Đogaš *et al.* (1993) suggested that the main source of the baseline excitatory drive to expiratory bulbospinal neurones was through NMDA receptor activation; the ionotropic non-NMDA receptors appeared not to be involved. Those studies were carried out under hyperoxic conditions where the contribution of the carotid chemoreceptors would have been minimized. Thus, the purpose of this study was to determine whether NMDA or non-NMDA receptors, or possibly both, are involved in the carotid body chemoreceptor-mediated excitation of expiratory bulbospinal neurones.

METHODS

Experiments were performed on fifteen mongrel dogs of either sex, weighing from 8 to 15 kg. Thiopental sodium was used for induction of anaesthesia (15 mg kg^{-1} I.V.) and halothane anaesthesia (1·0–1·4% end-tidal concentration) was used during the surgical preparation. During the experimental protocol an I.V. infusion of 8–12 mg kg^{-1} h^{-1} of thiopental sodium was used for maintenance of anaesthesia, since halothane has been reported to depress the carotid chemoreceptors (Mitchell & Selby, 1988). The animals were monitored for signs of inadequate anaesthesia, including salivation, lacrimation, and/or increases in blood pressure and heart rate. The anaesthetic depth was increased immediately if such signs were present.

Surgical procedure

The animals were intubated with a cuffed endotracheal tube and mechanically ventilated with an air–O_2 mixture. End-tidal CO_2 concentration was continuously recorded with an infrared analyser (POET II, Criticare Systems, Inc.). Tracheal pressure (P_t) 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, as well as drug and anaesthetic administration. Blood gas samples were obtained periodically and additional sodium bicarbonate was given to correct metabolic acidosis, if required. Oesophageal 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 deg. The vertebral column was maintained straight through caudal tension applied via a hip-pin clamp. Bilateral, dorsolateral neck dissection was performed to expose the cervical vagi, thyroid and common carotid arteries and the right C5 phrenic nerve rootlet. The phrenic nerve 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 neurogram (PNG) was recorded and used to obtain inspiratory and expiratory timing pulses.

An occipital craniotomy was performed and the dura mater opened along the mid-line and reflected laterally in order to expose the dorsal surface of the medulla oblongata. To minimize brainstem movements during neuronal unit recording and feedback from extravagal afferents, a bilateral pneumothorax was created, and the animal was paralysed with a 0·1 mg kg^{-1} I.V. bolus of pancuronium bromide and supplemental doses of 0·05 mg kg^{-1}, as required. During paralysis, the level of anaesthesia was assessed as described above. Bilateral cervical vagotomy was performed to eliminate afferent vagal input from pulmonary stretch receptors except in one dog from which the data are shown in Fig. 7.

Multibarrelled micropipettes, consisting of one recording barrel containing a carbon filament and three drug barrels, were used for extracellular neuronal recordings and microejections. The ejected solutions consisted of the vehicle, an artificial cerebrospinal fluid (ACSF), the NMDA antagonist 2-amino-5-phosphonovalerate (AP5, 2 mM and 5 mM; Research Biochemicals Inc.), the non-NMDA antagonist 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(*f*)-quinoxaline (NBQX, 320 μM; Novo Nordisk A/S, Novo Nordisk Park, 2760 Måløv, Denmark) and the non-NMDA agonist α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA, 2·5 μM; Research Biochemicals Inc.) all of which were dissolved in artificial cerebrospinal fluid (ACSF). The ACSF consisted of (mM): 124 NaCl, 2 KCl, 2 $MgCl_2$, 1·3 KH_2PO_4, 0·9 $CaCl_2$, 26 $NaHCO_3$, and 11 glucose. The pH of each solution was adjusted to 7·2–7·4 by aeration with 5% CO_2.

Single unit neuronal activities were recorded from expiratory bulbospinal neurones in the VRG, typically 2–5 mm caudal to the obex, 2·5–4·5 mm lateral to the mid-line and 2·5–4·5 mm below the dorsal medullary surface. The composite tip diameter of the microelectrode ranged from 10 to 50 μm. The amplified output of the microelectrode 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 digital-to-analog 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 spike frequency, phrenic nerve activity, tracheal pressure (P_t), proximal airway CO_2 concentration, a carotid artery injection indicator and a pressure microejection marker.

Carotid body stimulation

Both common carotid arteries were cannulated via the thyroid arteries for delivery of bolus injections of 100% CO_2 in saline ($P_{CO_2} > 700$ Torr) via a pressurized-reservoir–solenoid valve system. The injection rate, which was determined by the reservoir pressure (~104 kPa) and cannula flow resistance, was approximately 0·8–1·0 ml s^{-1}, and the infusion durations most commonly used were 1–2 s, resulting in bolus sizes of 1–2 ml. Using the inspiratory and expiratory timing pulses and appropriate timing circuitry, the triggering time of the solenoid valves was adjusted to the time during the respiratory cycle in which the most obvious effect on expiratory neurones could be elicited. Most commonly this was at the onset of the expiratory phase or late in the inspiratory phase.

Pressure microejection from multibarrelled micropipettes

Compressed nitrogen gas was used for pressure microejection of neurotransmitter analogues. Ejection was done by using the 'picospritzer' technique that allowed well-controlled drug volume application by regulation of three parameters: (1) ejection pressure (0–690 kPa), (2) duration of the pressure pulse (0·1–990 ms), and (3) frequency of the ejection pressure pulses (0–20 Hz). Ejected volume/time was measured via height changes of the meniscus in the pipette barrel with a ×50 magnification microscope equipped with a reticule. To minimize vibration effects during pressure application, 1 m long, soft catheter tubings were connected between the microejector solenoid valves and the micropipette barrels.

Protocol

Once a stable extracellular recording of a VRG expiratory neuronal unit was established, carotid body stimulations were performed. At least ten to fifteen control respiratory cycles, or a 1–1·5 min period of time, preceded and followed each test bolus stimulation cycle to allow for complete recovery of respiratory

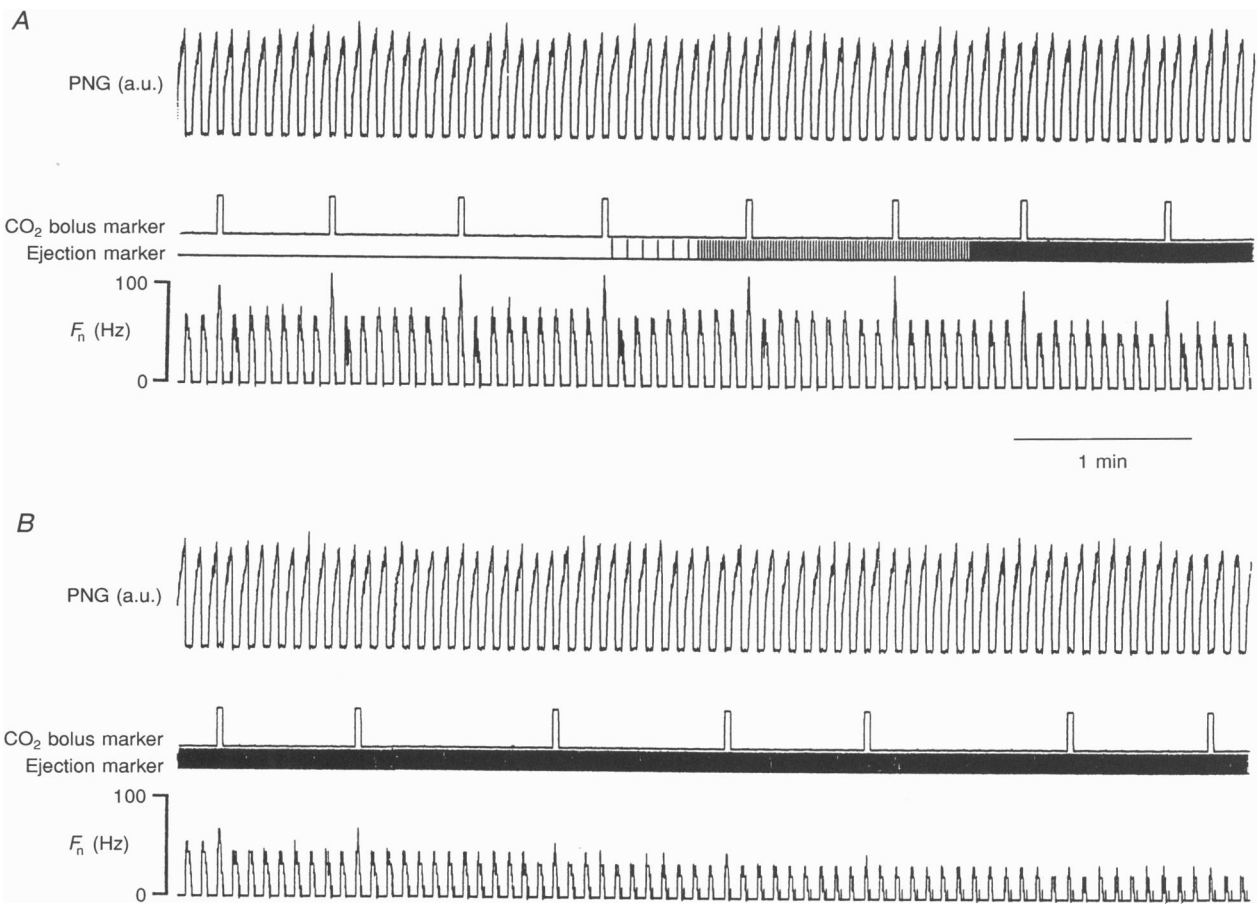

Figure 1. Example of the AP5-induced depression of spontaneous and carotid chemoreceptor-evoked increases in expiratory neuronal activity

Traces in *B* are a continuation of the traces in *A*. Top to bottom in each panel: phrenic neurogram (PNG in arbitrary units), CO_2 bolus infusion marker (see text for further details), pressure microejection marker (AP5 dose, 5 and 75 pmol min^{-1}), expiratory neuronal firing rate (F_n).

drive parameters. After three to five test bolus cycles had been obtained during the pre-ejection control, drug or vehicle ejection followed with 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. Prior to antagonist (AP5 or NBQX) applications, ACSF was ejected to verify that the vehicle and the ejected volume had little or no effect on neuronal discharge frequency.

Data analysis

The tape-recorded data were digitized (20 Hz per channel) and analysed using a Hewlett-Packard model 370 computer. One to six minute periods of neuronal spikes per 100 ms, PNG, CO_2 injection indicator and a pressure microejection marker were stored in files. During each respiratory cycle, peak PNG, peak expiratory neuronal discharge frequency (peak F_n), mean expiratory neuronal discharge frequency during the expiratory phase (mean F_n), T_I, T_E

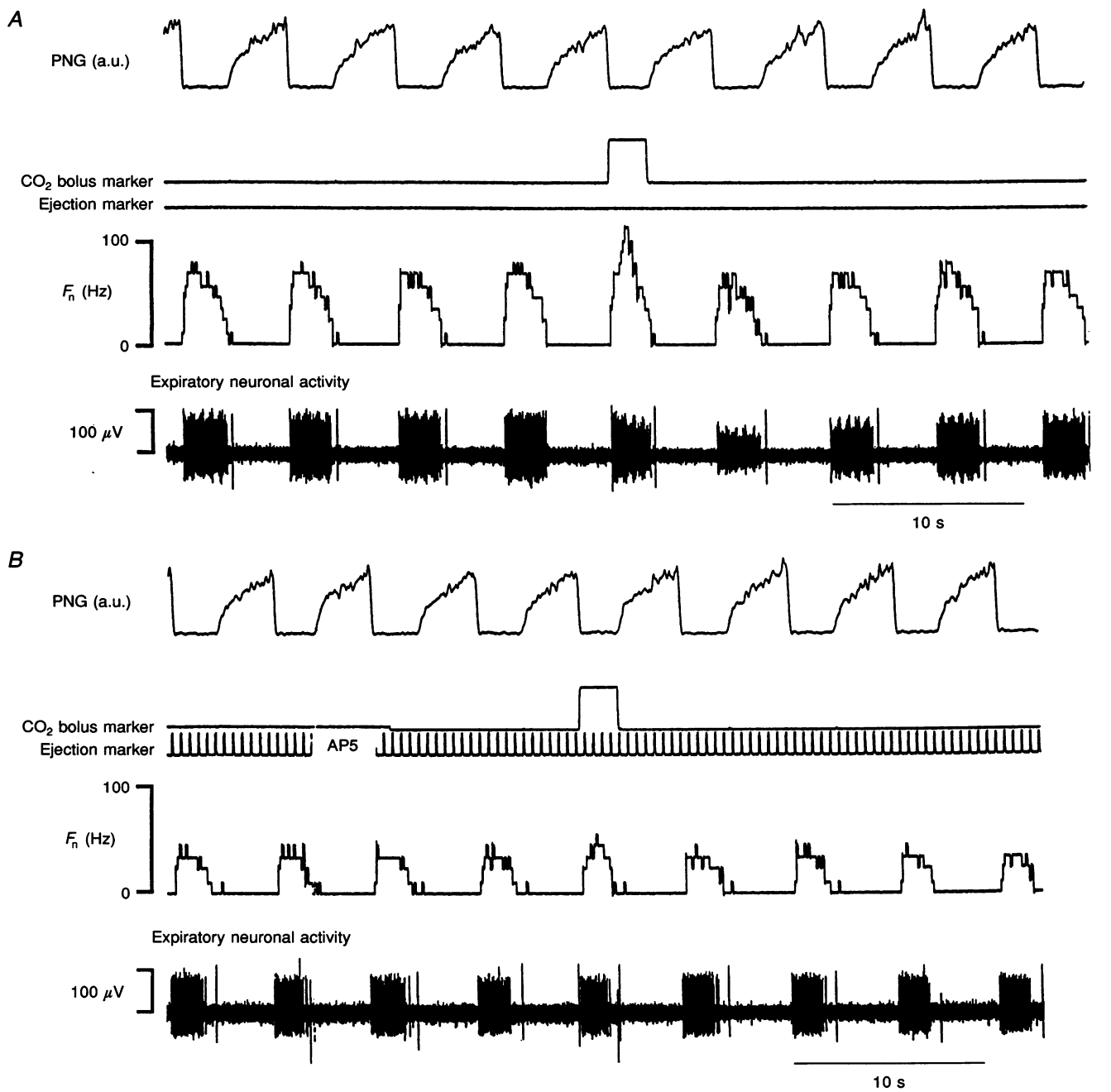

Figure 2. Higher resolution depiction of the depression of expiratory neuronal activity shown in Fig. 1

A, pre-ejection control with four respiratory cycles preceding and four following chemoreceptor stimulation (CO_2 bolus). *B*, same protocol with AP5 ejection (75 pmol min^{-1}). Top to bottom in each panel: PNG (in arbitrary units), CO_2 bolus infusion marker, pressure microejection marker, expiratory neuronal firing rate (F_n) and raw neuronal activity.

and peak PNG/T_I values were generated for four different conditions for each experimental run: (1) non-bolus pre-ejection control, (2) CO_2 bolus pre-ejection control, (3) non-bolus drug-ejection and (4) CO_2 bolus drug-ejection. Three respiratory cycles preceding the chemoreceptor test cycle were averaged to obtain control values of the variables. Differences between mean F_n of the chemoreceptor test cycle and the mean of the three preceding non-bolus cycles for both pre-ejection and ejection conditions were expressed as the net response due to the CO_2 bolus injections. The reflex shortening of T_E was analysed and used to evaluate the effectiveness of the CO_2 in saline boli throughout the protocol.

The values of mean F_n from each neurone were expressed as a percentage of the non-bolus, pre-ejection values and values for each neurone were then pooled. The statistical significance of differences in mean F_n was determined using a two-way, repeated measures, analysis of variance (ANOVA), with main factors of dose and CO_2 stimulus (i.e. non-bolus and bolus). 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 (Wallenstein, Zucker & Fleiss, 1980). Differences were considered significant for $P < 0.05$.

RESULTS

AP5 ejection

Pressure microejection of the NMDA receptor competitive antagonist AP5 produced strong decreases (78 ± 16%; mean ± S.D.) in expiratory neuronal firing rate (F_n). The effect always outlasted the ejection period, usually recovering to 70–100% of the baseline level in 30–60 min. The incomplete recovery is partially due to the fact that some protocols were discontinued after 30 min of recovery. Control ejections performed with ACSF to test for a possible vehicle effect did not produce significant decreases in F_n. Figure 1 shows an example of the effect of 5 mM AP5 microejection on the F_n of an expiratory neuronal unit. The ejection rate was increased during the run from an initial

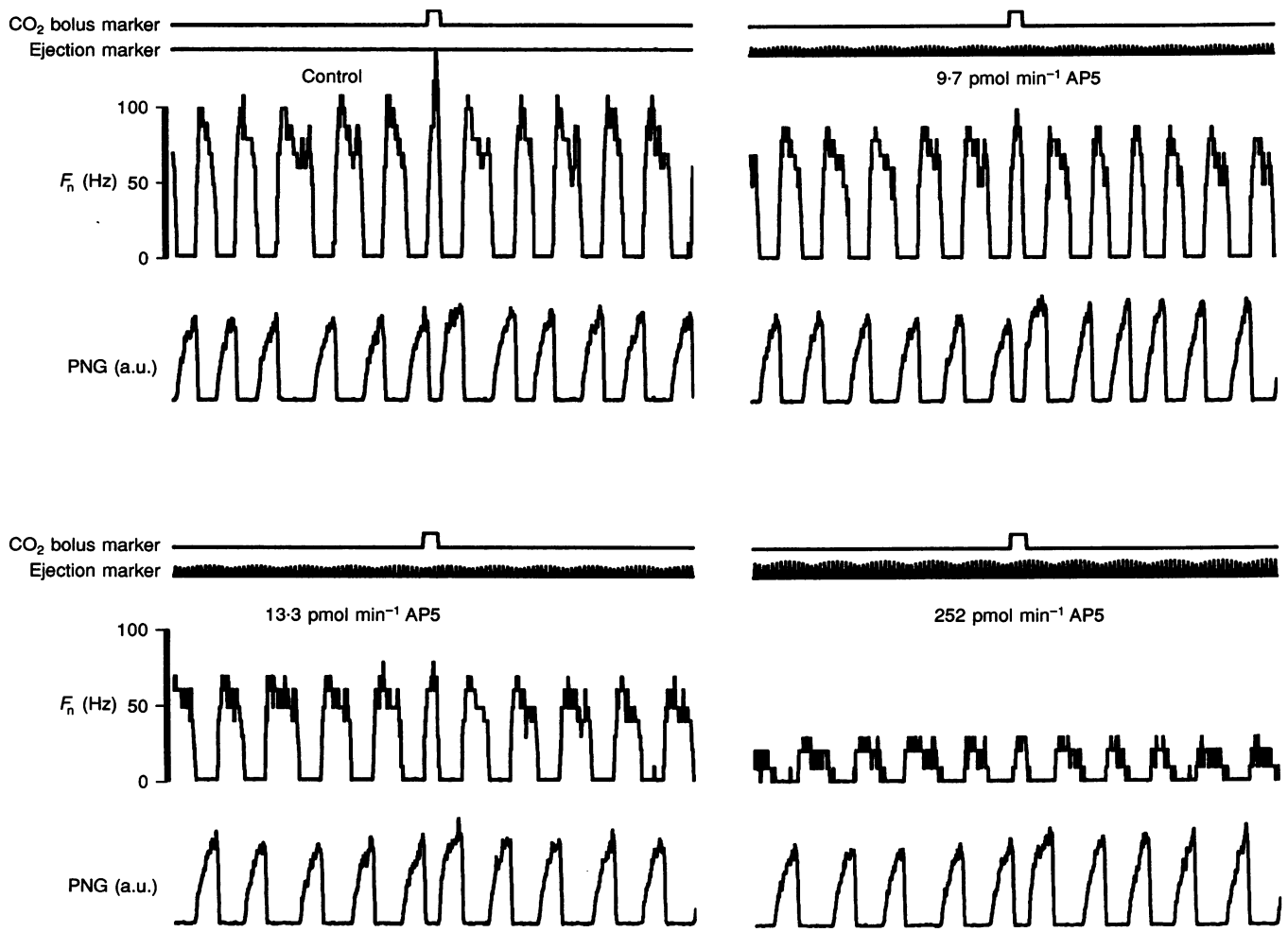

Figure 3. AP5 dose-dependent depression of expiratory neuronal activity and responsiveness to chemoreceptor activation

Steady-state, 1 min periods, during each of three indicated AP5 doses. On each segment, top to bottom: CO_2 bolus marker, pressure microejection marker, expiratory neuronal firing rate (F_n), PNG (in arbitrary units).

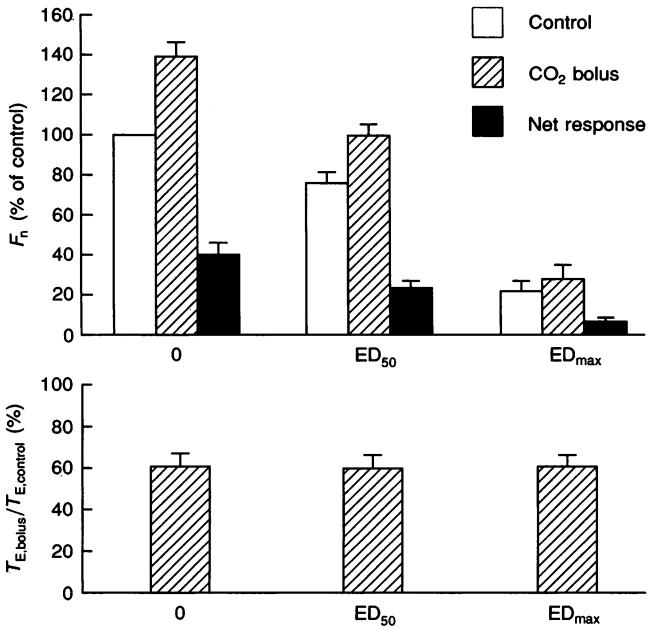

Figure 4. Summary of the dose-dependent AP5 depression of expiratory neuronal activity and responsiveness to carotid chemoreceptor activation

A, mean F_n values ($n = 10$) for control, CO_2 bolus and net response. 0, pre-ejection control; ED_{50}, AP5 dose causing a 50 % decrease in the net response; ED_{max}, AP5 dose causing a maximum decrease in the net response. *B*, group mean data for the $T_{E,bolus}/T_{E,control}$ ratio, indicating the consistency of the chemoreceptor-mediated change in respiratory timing throughout the protocol.

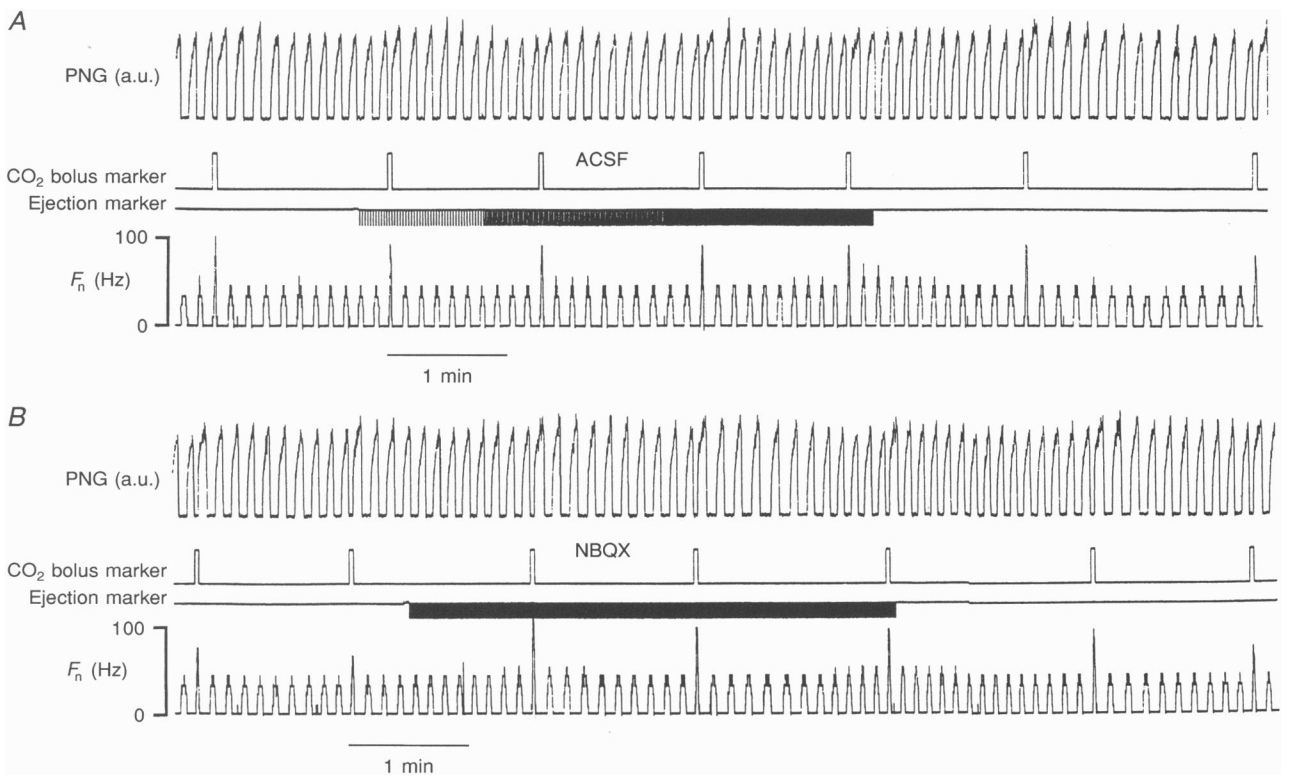

Figure 5. Example of the lack of influence of either ACSF or a non-NMDA receptor antagonist on expiratory neuronal activity

A, ACSF application (55 nl min^{-1}). *B*, NBQX (26 pmol min^{-1}) on the same neurone using the same protocol. Top to bottom in each panel: PNG (in arbitrary units), CO_2 bolus infusion marker, pressure microejection marker, expiratory neuronal firing rate (F_n).

level of 5 pmol min^{-1} to 75 pmol min^{-1}, or an ejection rate from 1 to 15 nl min^{-1}. Over a 7–8 min period, mean F_n gradually decreased from 44·1 to 9·5 Hz for non-bolus control cycles and from 57·4 to 10·6 Hz for chemoreceptor test cycles. Thus, not only was F_n of the control cycles strongly reduced by AP5, but also the strong excitation by carotid chemoreceptor stimulation was abolished. Figure 2 shows two time-expanded segments of the experimental run presented in Fig. 1: pre-ejection control (*A*) and AP5 ejection at 75 pmol min^{-1} (15 nl min^{-1}; *B*). The PNG recordings indicate that the T_E of the test cycle is shortened to the same extent for both conditions, but the CO_2 bolus-mediated increase in F_n is clearly depressed by AP5. For the analysis of the dose-dependent effects of AP5 on the pooled neuronal responses, three different conditions were defined: (1) pre-ejection control (0, zero dose), (2) ED_{max}, the AP5 dose at which the maximum reduction in the response to the CO_2 bolus occurred, and (3) ED_{50}, the AP5 dose at which a 50% reduction in the response occurred. Several ejection doses were commonly used to estimate ED_{50} levels, which were obtained by linear interpolation from steady-state, dose–response plots. Figure 3 shows an example of this type of data in which sufficient time (several minutes) was allowed at each ejection rate to obtain steady-state levels of neuronal activity. A summary bar graph (Fig. 4, top) shows the response of ten neurones under each of the three experimental conditions (pre-AP5, ED_{50}, and ED_{max}). The mean F_n (mean ± S.E.M.) values are expressed as a percentage of pre-ejection control: non-bolus control cycles (□), chemoreceptor test (CO_2 bolus) cycles (▨), and the difference or net response (■). Microejection of AP5 produced statistically significant ($P < 0·0001$) dose-dependent decreases in all variables. The average increase in mean F_n due to the CO_2 bolus, which was ~40% in the pre-ejection period, decreased with increasing AP5 dose, and was non-significant at the ED_{max}. Accordingly, there was a dose-dependent reduction in the net response. ED_{50} and ED_{max} (mean ± S.E.M.) values were 23·6 ± 5·1 pmol min^{-1} (7·40 ± 2·2 nl min^{-1}) and 121·7 ± 36·3 pmol min^{-1} (44·5 ± 18·4 nl min^{-1}), respectively.

The reflex shortening of T_E due to CO_2 bolus infusion was analysed throughout the protocol, and the $T_{E,bolus}/T_{E,control}$ ratios (Fig. 4, bottom) were not different from one another, indicating that the CO_2 bolus effectiveness was consistent throughout the experimental run. In addition, there were no changes in the overall respiratory pattern (T_I, T_E and PNG) at the dose rates used (e.g. Figs 1–3), indicating that AP5 was acting locally.

ACSF and NBQX ejection

A typical experimental run with ACSF ejection is shown in Fig. 5*A*. There is a consistent shortening of T_E during the CO_2 bolus infusion, while the expiratory neurone exhibits a brisk, strong increase in discharge frequency. Ejection of ACSF (55 nl min^{-1}) at rates equal to or greater than those for AP5 ejection did not change mean F_n during either the control, or the chemoreceptor test cycles, suggesting that any volume or intrinsic effect of the vehicle is minimal. Similar results were obtained when using the AMPA receptor antagonist NBQX. The example in Fig. 5*B* shows the same neuronal unit tested using NBQX and there was no reduction in F_n for non-bolus and bolus cycles during the ejection at a rate of 26 pmol min^{-1} (volume, 80 nl min^{-1}). The pooled data obtained from six neurones using ACSF and four neurones using NBQX are shown in the bar graphs of Fig. 6*A* and *B*, respectively. Ejection rates (mean ± S.E.M.) were 44·1 ± 8·5 nl min^{-1} for ACSF and 67·4 ± 13·5 nl min^{-1} (or 22·2 ± 4·4 pmol min^{-1}) for NBQX. Neither ACSF nor NBQX had an effect on the mean F_n of the control and chemoreceptor test cycle data or on the net responses (i.e. bolus minus control). The effectiveness of NBQX on these neurones was tested via its ability to antagonize the excitatory responses produced by microejection of AMPA as shown by the example in Fig. 7. The microejection of NBQX alone produced only a minor reduction in peak F_n and effectively antagonized the AMPA

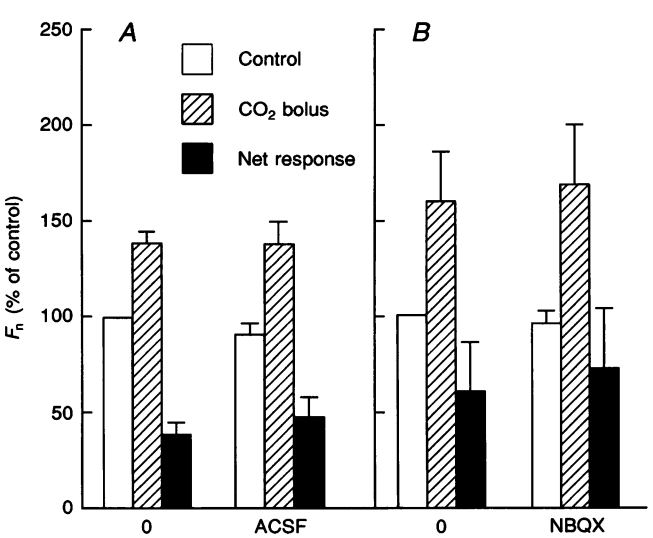

Figure 6. Summary of the lack of effect of ACSF and NBQX on expiratory neuronal activity

Mean F_n values for control, CO_2 bolus and net response variables during the pre-ejection and ejection periods. *A*, ACSF data for 6 neurones; *B*, NBQX data for 4 neurones.

response (Fig. 7*A*, 10 min after NBQX). In this particular example, a larger NBQX dose (60 pmol min^{-1}) was used in an attempt to see if a potentially greater degree of antagonism at non-NMDA receptors would have any effect on the spontaneous neuronal activity. Repeating the AMPA application after ACSF ejection (wash-out) and 40 min after NBQX produced a ~100% increase in peak F_n (Fig. 7*B*) showing that AMPA excites these neurones via NBQX sensitive receptors. In addition, the microejection of a mixture of 10 μM AMPA (0·75 pmol min^{-1}) and 320 μM NBQX (24 pmol min^{-1}), contained in the same micropipette barrel, was without effect (Fig. 7*B*, right), demonstrating

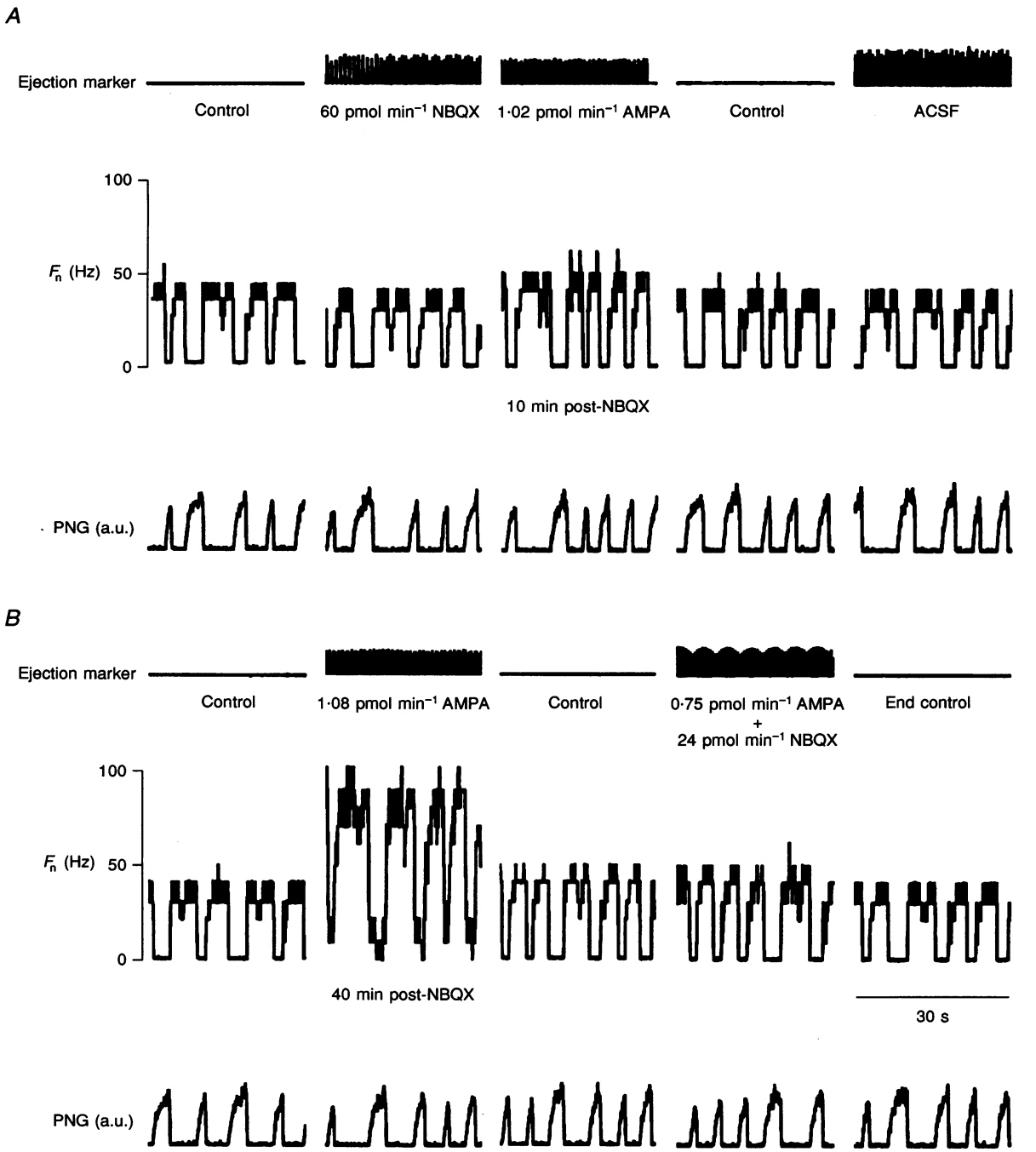

Figure 7. Antagonism of excitatory AMPA responses in an expiratory neurone by NBQX

Segments (20–30 s) taken from a long run of one expiratory neurone. Prolonged inspiratory durations (see PNG) are due to non-inflation cycles used to visualize neuronal activity in the absence of pulmonary receptor feedback in a vagally intact dog. Top to bottom in each panel: pressure microejection marker, expiratory neuronal firing rate (F_n), PNG (in arbitrary units). Note the different responses to AMPA at 10 and 40 min post-NBQX (after wash-out of NBQX with ACSF); also the combined ejection of AMPA and NBQX was without effect.

the effectiveness of NBQX at a dose similar to the mean dose used in these studies. The microejection of ACSF at a rate greater than those of the pharmacological agents was also without effect (Fig. 7*A*, right).

AP5–AMPA mixture ejection

An additional series of experiments was done to test the possibility that the reduction in the net CO_2 bolus excitation of these expiratory neurones by AP5 was due to a reduction in neuronal excitability rather than by direct antagonism of the receptors mediating the CO_2 bolus excitation. AMPA was applied to prevent the AP5-mediated decrease in F_n of control cycles. Even though NBQX has no significant effect on the spontaneous activity of expiratory bulbospinal neurones, these cells appear to have non-NMDA receptors since they are excited by AMPA. Furthermore, AP5 appears not to antagonize the responses produced by microejection of AMPA, as indicated in the example shown in Fig. 8. Ejection of AMPA at a rate of 155 fmol min^{-1}, caused peak F_n to increase from about 60 to 120 Hz, a net increase of 60 Hz (Fig. 8, left). A mixture of AMPA (10 μM) and AP5 (4 mM), ejected at a comparable dose rate for AMPA (175 fmol min^{-1}), produced an increase in peak F_n from about 60 to 90 Hz. The latent AP5 effect can be seen well after the rapid-acting AMPA response has decayed (6 min post-mixture) and is associated with a peak F_n of about 30 Hz, which is about 60 Hz less than that during microejection of the mixture. Thus, the net increase in F_n due to AMPA is approximately the same with and without AP5. Complete recovery from the AP5 effect occurred after about 30 min.

Accordingly, to prevent the reduction in peak F_n of the control cycles during the microejection of AP5, a mixture of 2 mM AP5 and 2·5 μM AMPA was used in additional studies, and an example of these results is given in Fig. 9. Figure 9*A* shows the pre-ejection spontaneous activity and CO_2 bolus response, while *B* shows the same data during microejection of 87 pmol min^{-1} AP5 and 109 fmol min^{-1} of AMPA (volume, 43·5 nl min^{-1}). Note that there was a slight increase in the peak F_n of control cycles over the pre-ejection levels (Fig. 9*A*), but the CO_2 bolus response was markedly reduced. With a further increase in dose (Fig. 9*C*; 142 pmol min^{-1} AP5, 177 fmol min^{-1} AMPA; volume, 71 nl min^{-1}), the response to the CO_2 bolus was reduced further. Ten minutes after termination of the microejection (Fig. 9*D*), the effects of AP5 are unmasked, consisting of a reduction in F_n of both the control and chemoreceptor test cycles, and hence the net response. The reflex shortening of T_E is similar in all four panels. These data suggest that the expiratory neuronal response due to chemoreceptor activation is similarly and markedly reduced by AP5 even though the neurone may be at two different levels of excitability.

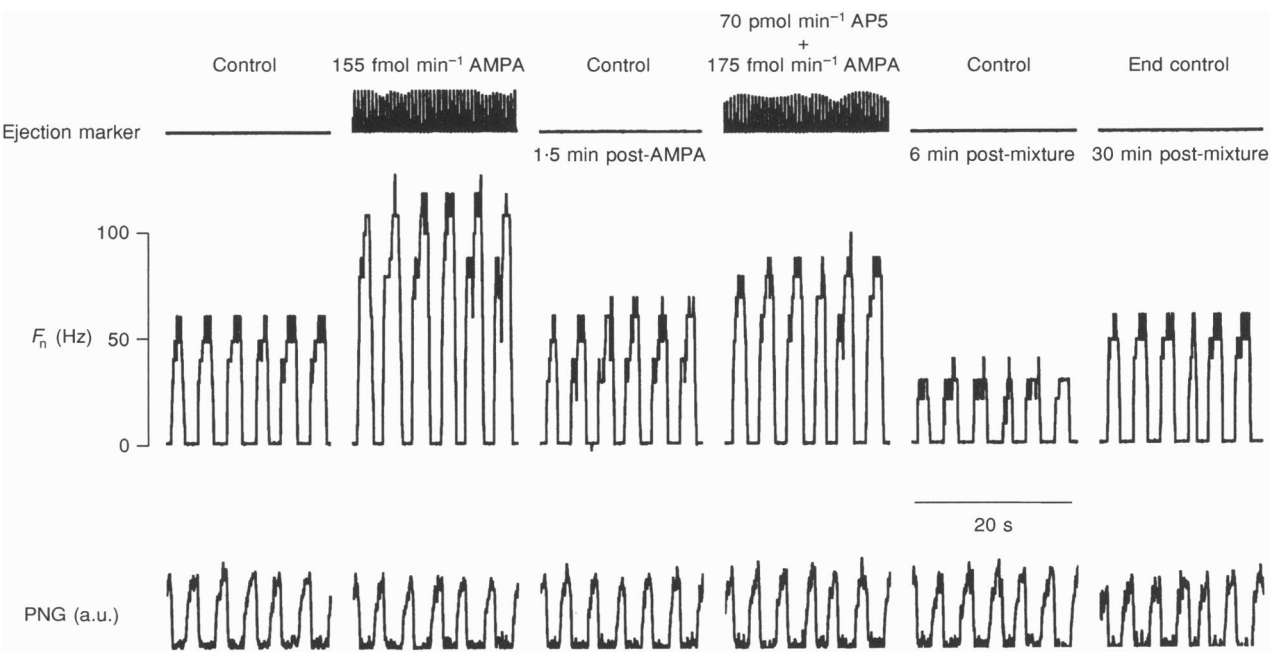

Figure 8. The effect of AP5 on the expiratory bulbospinal neuronal response to AMPA

Segments were from the same neurone at times indicated. The mixture was given 4·2 min after discontinuation of the AMPA microejection. Note that the net increase in neuronal discharge frequency (F_n) due to AMPA was approximately the same in the absence and presence of AP5. The post-mixture control indicates the depression of neuronal activity by AP5 when AMPA is not present. Top to bottom: pressure microejection marker, excitatory neuronal firing rate (F_n), PNG (in arbitrary units).

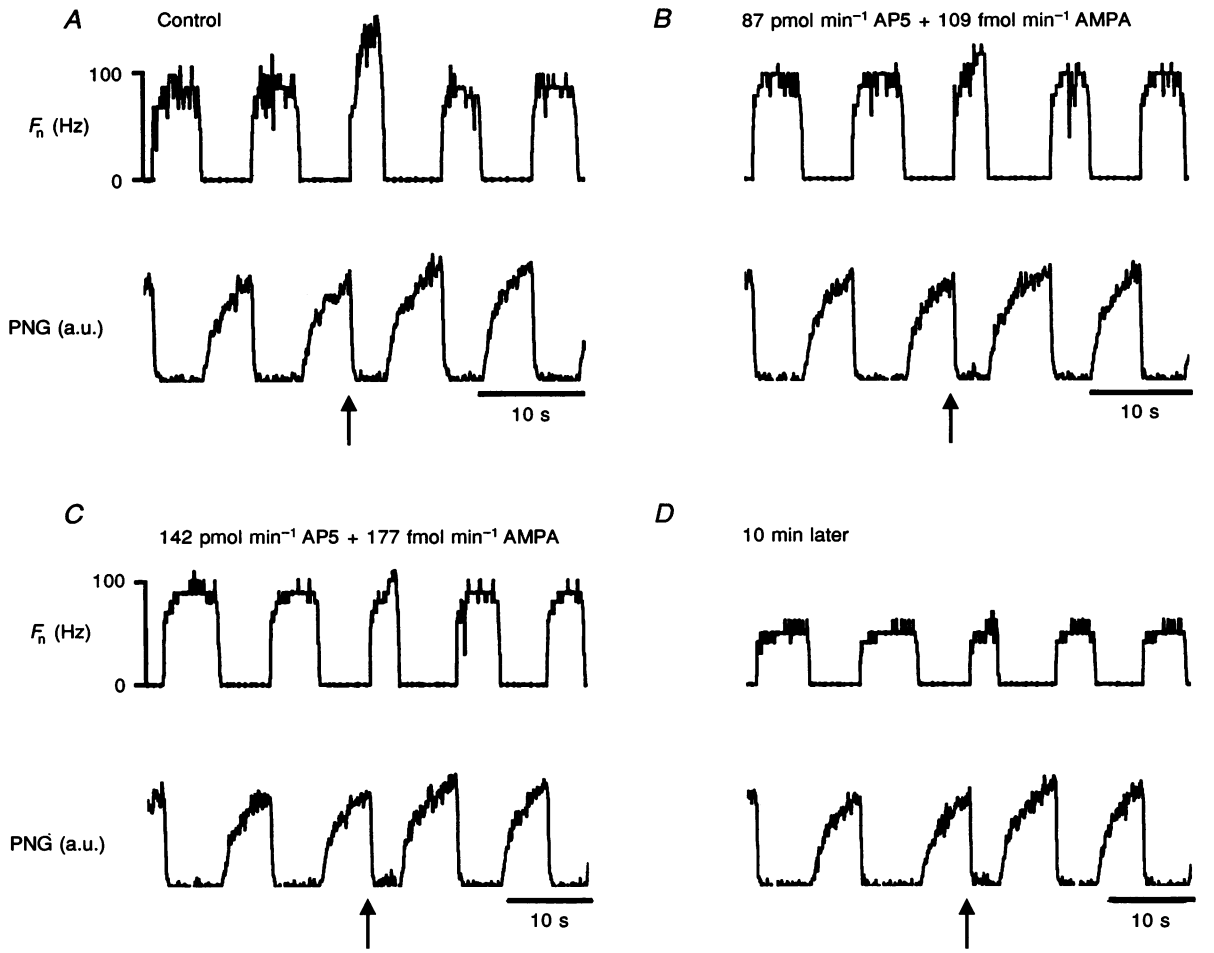

Figure 9. AP5 blockade of chemoreceptor-mediated neuronal excitation of an expiratory bulbospinal neurone is independent of the level of spontaneous activity

A, pre-ejection control; *B*, AMPA (109 fmol min^{-1}) + AP5 (87 pmol min^{-1}) ejection; *C*, AMPA (177 fmol min^{-1}) + AP5 (142 pmol min^{-1}) ejection; *D*, post-ejection control, 10 min later (see text for further details). Upper trace, PNG (in arbitrary units); lower trace, expiratory neuronal firing rate (F_n). Arrow indicates the onset of CO_2 bolus infusion.

DISCUSSION

The most significant finding of this study is that selective NMDA receptor blockade markedly reduced both the spontaneous discharge rate and the excitatory response of expiratory bulbospinal neurones to carotid chemoreceptor activation. These findings strongly suggest that NMDA excitatory amino acid receptors have an important role in the neurotransmission of carotid chemoreceptor-mediated excitation of expiratory bulbospinal neurones. In many systems NMDA receptor activation requires that neurones be depolarized to remove a voltage-dependent block of the channels (Mayer, Benveniste, Patneau & Vyklicky, 1992). This depolarization is frequently mediated via activation of non-NMDA excitatory amino acid receptors. However, the failure of NBQX to reduce the activity of expiratory bulbospinal neurones suggests that non-NMDA receptor activation is not required in these neurones.

The strength of the argument for an obligatory role for NMDA receptors in the mediation of peripheral (carotid) chemoreceptor input to these expiratory neurones is dependent upon the selectivity with which AP5 and NBQX block NMDA and non-NMDA receptors, respectively. While the selectivity of these agents has been documented for a number of systems, the diversity of excitatory amino acid receptors requires that selectivity be demonstrated in the current experimental protocol (McCrimmon, Mitchell & Dekin, 1994). In a previous preliminary study on expiratory neurones from this laboratory (Đogaš *et al.* 1993) we found that a mean NBQX dose of 12 pmol min^{-1} ($n = 5$) was capable of completely blocking AMPA-induced increases of 100% in F_n, where the mean AMPA dose which doubled F_n was $0{\cdot}38 \pm 0{\cdot}06$ pmol min^{-1} ($n = 7$). The same dose of NBQX had little or no effect on NMDA-induced responses. In the present study the mean NBQX dose was

22·2 ± 4·4 pmol min^{-1}, which is nearly twice the previously stated effective dose. The effectiveness of this dose is also demonstrated by the microejection of a mixture of NBQX (24 pmol min^{-1}) and AMPA (0·75 pmol min^{-1}) which resulted in a complete block of the AMPA effect (Fig. 7*B*, right). Conversely, AP5 at doses which were highly effective against the excitatory response to carotid chemoreceptor activation also abolished the excitatory response to NMDA but had little effect on the excitation elicited by AMPA (Fig. 8). This selectivity is in agreement with other reports in which AP5 and NBQX have been documented as highly selective competitive antagonists (Sheardown, Nielsen, Hansen, Jacobsen & Honore, 1990; Mayer *et al.* 1992; Randle, Guet, Cordi & Lepagnol, 1992; Stone, 1993). NBQX lacks the affinity of two earlier quinoxalinedione antagonists, CNQX and DNQX, for the glycine-binding site on the NMDA receptor (Harris & Miller, 1989; Sheardown *et al.* 1990; Randle *et al.* 1992) and has no affinity for the metabotropic glutamate receptors (Suzdak, Sheardown & Honore, 1993).

Since the microejection of AP5 produced a marked reduction in the spontaneous activity of the expiratory bulbospinal neurones during control (non-CO_2 bolus) cycles, as well as in the net chemoreceptor test (CO_2 bolus) response, it may be proposed that the reduction in the net response was related to the reduction of spontaneous activity, perhaps via a generalized reduction in neuronal excitability. In an attempt to address this possibility, we used AMPA in a mixture with AP5 to prevent the decrease in spontaneous F_n of control cycles (Fig. 9). AP5 was still capable of eliminating the net response to carotid chemoreceptor excitation. These results suggest that a change in peak F_n or neuronal excitability *per se* does not change the effectiveness of the AP5 block of carotid chemoreceptor input.

It may be argued that the level of F_n, in some cases, may not be a good index of excitability, and that changes in the input resistance of the membrane in the region of the synapse affect the responsiveness of the synaptic input of interest. Our methods do not allow us to address this problem directly. However, on the basis of theoretical considerations using a membrane chord conductance analysis (Nicholls, Martin & Wallace, 1992), the AP5 reduction of spontaneous activity does not necessarily imply that the neurone is less excitable. That is, the neurone should be capable of responding to an excitatory or inhibitory input just as effectively, or more so, since during AP5 application the membrane resistance should have increased as a result of a reduction in synaptic conductance of NMDA receptor channels. In addition, we have noted that a reduction in chemodrive (brought about by an increase in ventilation which reduced P_{a,CO_2}) reduced the spontaneous peak F_n of control cycles, but the F_n of the CO_2 bolus cycles was still significantly increased.

An alternative explanation for the lack of effect by NBQX may involve inaccessibility to non-NMDA receptors. For example, it is possible that the carotid body chemoreceptor input uses both NMDA and non-NMDA receptors with different locations on the cell. The non-NMDA receptors may be located on distal dendrites and, as such, were inaccessible to the NBQX microejections, while the NMDA receptors may be more proximally located, and accessible to the AP5 microejections. In this study, however, the distribution of NBQX to distal regions of the neurones due to bulk-flow and diffusion, should have been greater than that of AP5, since the mean value of the maximum ejection rate for NBQX was 51 % greater than that for AP5 (67·4 *vs.* 44·5 nl min^{-1}). Since the steady-state response time for AP5 was of the order of 5–6 min (e.g. Fig. 1), this suggests that NMDA receptors at distal sights were being affected. Even in the event that distal sights were not reached, the fact that AP5 effectively blocked the chemoreceptor-mediated excitation suggests that: (1) the chemoreceptor input uses primarily NMDA receptors and/or (2) the portion of chemoreceptor input mediated by non-NMDA receptors was blocked due to non-specific effects of AP5 which reduced neuronal excitability. However, non-specific effects of AP5 appear to be insufficient to account for this block for the following reasons. (1) The net AMPA response is not altered by AP5 (e.g. Fig. 8), suggesting that the ability of the neurone to respond is not compromised. This point is also supported by the finding that the chemoreceptor-evoked excitation was eliminated by AP5, when AMPA in a mixture with AP5 prevented the decrease in spontaneous F_n of control cycles, as previously discussed. (2) AP5 effectively antagonizes NMDA-evoked responses in these expiratory neurones (Đogaš *et al.* 1993), but not AMPA-evoked responses, suggesting a specific effect of AP5. Non-specific effects due to locally high concentrations of drugs are not likely to be a problem with pressure ejection since the concentrations used were low and the tissue concentration of the drug would not be expected to exceed that in the pipette. From this perspective and in view of the results, it appears that the chemoreceptor-mediated excitation of the expiratory bulbospinal neurones is mediated, primarily and possibly exclusively, by NMDA receptors.

To date, based on many molecular biological, pharmacological and electrophysiological studies exploring the properties of both NMDA and non-NMDA (AMPA/kainate) receptors, it has been suggested that non-NMDA receptors are responsible for fast synaptic transmission, while NMDA receptors play a modulatory role and constitute a major factor in the computational ability of the CNS (learning, memory) and during development (Monaghan, Bridges & Cotman, 1989; Mayer *et al.* 1992; Nakanishi, 1992; Stone, 1993; McCrimmon *et al.* 1994). The complex NMDA receptor requires a co-agonist, glycine, and is modulated by polyamines and ions such as Mg^{2+} and Zn^{2+}. These factors, and especially the Mg^{2+} voltage-dependent block of the receptor channel, even at normal physiological concentrations, may give the impression that

NMDA receptors play only a minor role in fast synaptic transmission. The results of the present study suggest otherwise. However, the relative roles of non-NMDA *versus* NMDA receptors in the central control of breathing depend somewhat upon whether the experiments have been conducted *in vivo* or *in vitro* (McCrimmon *et al.* 1994). One of the major reasons for this difference may be the removal of the Mg^{2+} voltage-dependent block by the presence of sufficient synaptic drive *in vivo versus in vitro*. To understand better the functional roles of both receptor types in physiological reflexes, it is necessary to perform these experiments under *in vivo* conditions. Being a relatively large, approachable, homogeneous group of neurones, which integrate and strongly respond to many afferent inputs, expiratory bulbospinal neurones are ideally suited for these types of studies. In addition, at the dose rates used in this study, there was no alteration in T_I, T_E, or PNG, suggesting a relatively localized effect of the microejections.

The advantages of pressure microejection technique *versus* microiontophoretic injection have been somewhat ignored by many researchers since the method was introduced in 1963 by Krnjevic & Phillis as a control for possible current artifacts associated with the microiontophoresis of glutamate onto single neurones in cat brain (Krnjevic & Phillis, 1963). By using a few precautionary measures, some of the possible disadvantages of the method explained by Palmer (1982), Stone (1985) and Lipski, Bellingham, West & Pilowsky (1988) can be easily avoided, and several advantages emphasized. Such measures can be summarized as follows: (1) the direct monitoring of ejected volumes by observation of the meniscus; (2) the application of a less concentrated drug solution within the pH ranges of 7·2–7·4; (3) use of 1 m long soft catheter tubing that absorbs some of the applied pressure transients which can cause brief movements of the electrode tip; (4) the regular cleansing (with distilled water and 'ejection into the open') of the electrode tip after each penetration, to prevent it from clogging; (5) control ejections of ACSF to check for possible vehicle artifacts; (6) ejection rates always kept to a minimum (commonly 30–600 pl s^{-1} in dogs) during continuous ejection.

Due to the pharmacokinetics/dynamics of the microejected agents and the desire for steady-state conditions, the ejection of these agents was done in a slow continuous manner rather than as a bolus. On the basis of theoretical analysis, and considering that the extracellular volume space, which contains very little or none of the agent of interest, provides a relatively large dilution volume, a steady-state concentration gradient, which is proportional to the reciprocal of the distance from the electrode tip, can be achieved via diffusion from a constant point source (Stone, 1985, pp. 68–69). In this study, constant ejection was produced by the temporal summation of very small bolus doses; accordingly, the dose rate was directly related to the ejection frequency.

The identity of antecedent neurones supplying the carotid chemoreceptor, NMDA-mediated excitation to the expiratory bulbospinal neurones is unknown. Based on electrophysiological and neuroanatomical studies, there appears to be a rather direct projection from the carotid chemoreceptor afferents to the ventrolateral medulla. By recording field potentials and unit activity evoked by electrical stimulation of the carotid sinus nerve, and unit activity during chemical stimulation of the chemoreceptors in cats, Davies & Edwards (1975) found short-latency responses not only in the NTS, but also in and around the nucleus ambiguus, suggesting the existence of a possible direct projection. The inspiratory and expiratory neurones in this region increased their firing with both NaCN and hypoxia and had a respiratory-related pattern phase-locked with the PNG. Using microinjections of horseradish peroxidase into the vascularly isolated carotid body of rats *in situ*, Finley & Katz (1992) observed a prominent bilateral projection to the caudal ventrolateral medulla in the region of the nucleus retroambigualis, as well as bilaterally in the commissural and medial subnuclei of the caudal nucleus tractus solitarii (NTS) and other NTS subnuclei. In a recent study in rats, Vardhan, Kachroo & Sapru (1993) were able to block respiratory and cardiovascular responses to carotid chemoreceptor stimulation by a combined microinjection of antagonists for both NMDA and non-NMDA receptors in a mid-line region of the commissural NTS. Microinjections of specific agonists elicited responses similar to, but not identical, to those reported for chemoreceptor stimulation. While the latter findings suggest the existence of at least one interneurone in the pathway to the expiratory bulbospinal neurones, the number of synapses has yet to be determined. Nevertheless, a paucisynaptic connection is suggested by the observation that acute chemoreceptor stimulation, via carotid artery CO_2 bolus injection, produces very brisk, short-latency, excitatory responses in expiratory bulbospinal neurones of the caudal portion of VRG of dogs (Bajic *et al.* 1994).

In summary, these studies implicate NMDA receptors in the neurotransmission of reflexly evoked respiratory activity, such as chemoreceptor-mediated excitation of expiratory bulbospinal neurones. Thus, NMDA-mediated transmission *in vivo*, appears to be more important in the mediation of fast, 'point-to-point' neurotransmission than is generally thought.

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