ELSEVIER

European Journal of Pharmacology 280 (1995) 257–275

ejp

Respiratory effects produced by microinjection of L-glutamate and an uptake inhibitor of L-glutamate into the caudal subretrofacial area of the medulla

John E. McManigle [b], William H. Panico [a,b], Angelo M. Taveira DaSilva [a,b], Richard A. Gillis [a,b,*]

[a] *Departments of Pharmacology and Medicine (Pulmonary Division), Georgetown University School of Medicine, Washington, DC 20007, USA*
[b] *Department of Medicine (Pulmonary Division), Uniformed Services University of Health Sciences, Bethesda, MD 20814, USA*

Received 23 January 1995; accepted 28 March 1995

Abstract

The purposes of our study were to determine the type of respiratory changes that would occur when either an excitatory amino acid receptor agonist or an uptake inhibitor was administered into the caudal subretrofacial area. This was done by microinjecting either L-glutamate or L-pyrrolidine-2,4-dicarboxylate (L-*trans*-2,4-PDC) into the caudal subretrofacial area while monitoring tidal volume, respiratory rate, mean arterial blood pressure and heart rate. Bilateral microinjection of 2.5 nmol of L-glutamate into the caudal subretrofacial area produced apnea in eight of eight animals tested, and the duration of apnea was 27 ± 2 s. To determine the type of L-glutamate receptor responsible for mediating the apneic response, antagonists of the *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor, namely, 3-[(*RS*)-carboxypiperazin-4-yl]-propyl-phosphonic acid (CPP), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), respectively, were tested. Neither antagonist in doses that blocked NMDA (in the case of CPP) and amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) (in the case of CNQX) blocked apnea elicited by L-glutamate. In addition, kynurenic acid, an antagonist of NMDA and non-NMDA ionotropic receptors, failed to block the effect of L-glutamate. Microinjection of the metabotropic receptor agonist drug, *trans*-L-1-amino-1,3-cyclopentone-dicarboxylic acid (L-*trans*-ACPD), into the caudal subretrofacial area failed to have any effect on respiratory activity. Because of the inability to block the effect of L-glutamate in the caudal subretrofacial area, and the lack of effect of L-*trans*-ACPD, the data suggest that the apneic response produced by L-glutamate is mediated by an as yet undefined receptor. Microinjection of the L-glutamate uptake inhibitor, L-*trans*-2,4-PDC, was found to produce apnea. Using the dose of 0.5 nmol of L-*trans*-2,4-PDC, we examined the type of excitatory amino acid receptor that mediated the response. Neither pretreatment with the NMDA receptor antagonist, CPP, nor the non-NMDA receptor antagonist, CNQX, affected L-*trans*-2,4-PDC-induced apnea. However, combined use of these two antagonists prevented L-*trans*-2,4-PDC-induced apnea. These data suggest that the effect of synaptically released exitatory amino acid at the caudal subretrofacial area on breathing is apnea, and that this effect is mediated by simultaneous activation of both NMDA and non-NMDA ionotropic receptors.

Keywords: Ventrolateral medulla; Glutamate receptor; L-Glutamate uptake inhibitor; Respiratory function; Excitatory amino acid receptor antagonist; Blood pressure; Heart rate

1. Introduction

We recently documented a role for an excitatory amino acid in the central nervous system control of breathing in an area located in the ventrolateral medulla (Abrahams et al., 1991). We found that bilateral microinjection of either kynurenic acid or a combination of 3-[(*RS*)-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (CPP) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) into the ventrolateral medulla at a site 7.6 mm caudal to the foramen cecum, 4 mm lateral to the midline and 1.5 mm below the ventral medullary surface produced apnea. In the rostral-caudal plane the site is located approximately 3.0 mm rostral to

* Corresponding author. Department of Pharmacology, Georgetown University, School of Medicine, 3900 Reservoir Road, N.W., Washington, DC 20007, USA.

SSDI 0014-2999(95)00203-0

obex. This places the site about 1 mm caudal to the subretrofacial nucleus and just rostral to the lateral reticular nucleus. Relative to the inferior olive, it is located about 1.0 mm rostral to the halfway point of this nucleus. In terms of the hypoglossal nucleus, the site is located at a rostral-caudal level corresponding to the rostral tip of this nucleus. Relative to the C1 and A1 neurons, the site is rostral to the densest region of the A1 neurons and caudal to the densest region of the C1 neurons (i.e., in the region of overlap of C1 and A1 neurons). In the dorsal-ventral plane, the site is located ventral to nucleus ambiguus and dorsal to the region of overlap of C1 and A1 neurons. In the medial-lateral plane, the site is dorsolateral to both the inferior olive and the lateral wings of the B_1/B_3 group of serotonin neurons. Because the site is located immediately caudal to the subretrofacial nucleus, we will refer to it as the 'caudal subretrofacial area'.

As indicated above, by the use of kynurenic acid, an agent that blocks both *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors (Jackson et al., 1985; Stone and Connick, 1985), and a combination of CPP and CNQX, both NMDA and non-NMDA receptors appeared to be involved in eliciting apnea. Apnea was never observed upon blockade of only NMDA receptors and only non-NMDA receptors. Indeed, microinjection of the non-NMDA receptor antagonist, CNQX, into the caudal subretrofacial area had very little effect on respiratory activity when given alone. On the other hand, blockade of just NMDA receptors with CPP at the caudal subretrofacial area evoked a large decrease in tidal volume and an increase in respiratory rate (f). In conclusion, blockade of NMDA receptors was responsible for most of the respiratory effects observed with kynurenic acid but blockade of non-NMDA receptors in the presence of NMDA receptor blockade resulted in apnea (Abrahams et al., 1991).

One purpose of the present study was to determine the type of respiratory changes that would occur when an excitatory amino acid receptor agonist was administered into the caudal subretrofacial area. The agonist that we focused on in our studies was L-glutamate, an endogenous occurring amino acid that is thought to be the primary excitatory neurotransmitter in the CNS (Di Chiara and Gessa, 1981). A few studies were also carried out using specific agonists for the NMDA and non-NMDA receptors.

Another purpose of our study was to determine the effect of tonically released excitatory amino acids at the caudal subretrofacial area on respiratory activity. The approach taken to examine this issue was based on the premise that inactivation of synaptically released excitatory amino acid neurotransmitters occurs through uptake processes into both synaptic terminals and surrounding glia (Curtis et al., 1970; Benjamin and Quastel, 1976; Schousboe, 1981). Thus, an inhibitor of uptake should increase the extracellular concentration of an endogenously released excitatory amino acid, and enhance its postsynaptic action. By microinjecting an inhibitor of the uptake of excitatory amino acids into the caudal subretrofacial area we set out to: (1) determine the effect of synaptically released excitatory amino acid neurotransmitter on breathing, (2) determine the type of excitatory amino acid receptor(s) that mediate the response, and (3) obtain data that would allow us to speculate as to whether L-glutamate could be the excitatory amino acid neurotransmitter that is synaptically released at the caudal subretrofacial area.

2. Materials and methods

Experiments were performed on adult cats (2.5–4.0 kg) unselected as to sex. Anesthesia was induced with α-chloralose, 70–80 mg/kg i.v. A femoral artery and vein were cannulated for measurement of arterial blood pressure and systemic administration of drugs, respectively. Lead II of the electrocardiogram was recorded, and heart rate was determined by measurement of the R–R interval. Rectal temperature was monitored and maintained between 37° C and 38° C by an infrared heating lamp.

The trachea of each cat was cannulated and fitted with a No. 0 Fleisch pneumotachograph connected to a respiratory flow transducer (HP47630A, Hewlett-Packard, Waltham, MA, USA). The airflow signal obtained was integrated (HP8815A respiratory integrator) to provide V_t. Respiratory rate was obtained from fast tracings of the flow signal. End-tidal CO_2 was measured with a CO_2 infrared analyzer (Datex Airway Gas Monitor PB Model 252, Datex Medical Instrumentation, Tewksbury, MA, USA). All indices of respiratory and cardiovascular function were recorded continuously on an eight-channel recorder (HP model 7758B).

The animal was placed in a stereotaxic holding device with the ventral side uppermost, and the ventral surface of the medulla was exposed as previously described (Gillis et al., 1988). Briefly, the trachea and esophagus were exposed and retracted, and the prevertebral muscles scraped from the basal plate of the skull. A portion of this plate was removed, creating a window 15 mm by 9 mm over the medullary surface. The dura was then cut and reflected to expose the surface of the medulla and allowed the cerebrospinal fluid to drain.

Double-barreled (ID 0.3 mm; FHC, New Brunswick, ME, USA) micropipettes were pulled, the tip cut to approximately 15 μm inside diameter, and were filled by vacuum pressure through a length of PE-90 tubing. Double-barreled micropipettes were used for multiple microinjections in the following experiments: (1) L-

glutamate (50 nl, 19.5 pmol to 5.0 nmol) microinjected bilaterally for dose-responses studies; (2) L-glutamate (50 nl, 2.5 nmol) combined with either CPP (125 nl, of either 0.62 nmol or 5.6 nmol) or CNQX (125 nl, 125 pmol) for bilateral microinjection studies; and (3) L-glutamate (50 nl, 2.5 nmol) combined with either CPP/CNQX 'cocktail' (125 nl, 5.6 nmol CPP/125 pmol CNQX) or kynurenic acid (125 nl, 31.2 nmol) for unilateral microinjection studies. All drugs were dissolved in 0.9% saline containing 1% fast green dye. The pH of all drug solutions ranged from 7.3 to 7.4.

Experiments were performed as follows:

(1) *Bilateral L-glutamate dose-response studies.* Micropipettes were inserted bilaterally into the left and right caudal subretrofacial area. A volume of 50 nl of L-glutamate was injected on one side (using pressure injection) until the meniscus moved the appropriate distance using formaline tape (type 9006 B, Graphic Products Corp., Rolling Meadows, IL, USA) as a guide. Once the injection was complete the process was immediately repeated on the contralateral side. The L-glutamate uptake inhibitor, L-*trans*-2,4-PDC, was also studied in a similar manner.

(2) *Bilateral L-glutamate microinjection before and after microinjection of either CPP or CNQX.* Micropipettes were inserted bilaterally into the left and right caudal subretrofacial area. L-Glutamate (50 nl, 2.5 nmol) was microinjected bilaterally. Typically, 2–3 control responses were obtained with time allowed between each dose for complete recovery from respiratory depression. Then either CPP (125 nl, either 0.62 nmol or 5.6 nmol) or CNQX (125 nl, 125 pmol) was microinjected bilaterally using the same technique. L-Glutamate (50 nl, 2.5 nmol) was then microinjected bilaterally 3–5 min after microinjecting either CPP or CNQX.

(3) *Unilateral L-glutamate microinjection into caudal subretrofacial area before and after either CPP / CNQX 'cocktail' or kynurenic acid.* A micropipette was inserted unilaterally into either the right or left caudal subretrofacial area. L-Glutamate (50 nl, 2.5 nmol) was microinjected. Two to three control responses were obtained with time allowed between each dose for complete recovery from respiratory depression. Then either the 'cocktail' (125 nl, 5.6 nmol CPP/125 pmol CNQX) or kynurenic acid (125 nl, 31.2 nmol) was microinjected at the same site. Three to five minutes after microinjection of either the 'cocktail' or kynurenic acid, L-glutamate (50 nl, 2.5 nmol) was microinjected.

The sequence of experiments was as follows: In our initial studies we focused on determining a dose-response relationship of L-glutamate at the caudal subretrofacial area. L-Glutamate is known to excite at least five excitatory amino acid receptors: NMDA, AMPA, kainate, metabotropic, and L-AP4 receptors. We then selected a dose of L-glutamate which gave a reproducible response (2.5 nmol) and attempted to block the response with excitatory amino acid antagonists. The excitatory amino acid antagonists we evaluated were CPP, CNQX, a combination 'cocktail' of CPP and CNQX, and kynurenic acid. The rational basis for the choice of doses of these agents is provided in the Results section. Finally, to test for the presence of a metabotropic excitatory amino acid receptor at the caudal subretrofacial area, we had to first find a dose of the metabotropic receptor agonist drug, *trans*-L-1-amino-1,3-cyclopentone-dicarboxylic acid (L-*trans*-ACPD) (Palmer et al., 1989), that would exert an effect in the brainstem. This was done by testing L-*trans*-ACPD at the caudal ventrolateral medulla.

The coordinates for placing the micropipette tip into the caudal subretrofacial area were as follows: 7.6 mm caudal to the foramen cecum, 4.0 mm lateral to midline and 1.5 mm below the ventral surface of the medulla. These coordinates were established in our previous study (see Abrahams et al., 1991). The coordinates for placing the micropipette tip into the caudal ventrolateral medulla were as follows: 9.0 mm caudal to the foramen cecum, 4.0 mm lateral to midline and 1.0–1.5 mm below the ventral surface of the medulla. These coordinates were established in our previous study (Gatti et al., submitted).

On completion of the experiment, brains were removed and fixed for at least 48 h in 6% buffered paraformaldehyde. The tissue was then blocked and transferred to 20% sucrose in phosphate-buffered saline 24 h before sectioning at 50 μm. Sections were counterstained with neutral red to facilitate identification of nuclear groups, and the site of microinjection was easily determined as a green spot, with tissue damage marking the pipette tract in some cases.

The values presented in the tables (and text) are the means $\pm$ the standard error of the mean and were taken at the time of peak quantitative changes and/or just before apnea occurred. Statistical analysis was performed using the Student's paired t-test with $P < 0.05$ being the criterion for statistical significance.

The following drugs were used: α-chloralose (ICN Biomedical, Cleveland, OH, USA); L-glutamate (Sigma Chemical Co., St. Louis, MO, USA); 4-hydroxyquinoline-2-carboxylic acid (kynurenic acid) (Sigma Chemical Co., St. Louis, MO, USA); 3-[(±)-2-carboxy-piperazin-4-yl]-propyl-1-phosphonic acid (CPP) (Tocris Neuramin, Essex, UK); 6-cyano-7- nitroquinoxaline-2,3-dione (CNQX) (Tocris Neuramin, Essex, UK); *N*-methyl-D-aspartate (NMDA) (Sigma Chemical Co., St. Louis, MO, USA); amino-3-hydroxy-5-methyl-isoxazole-4-4-propionic acid (AMPA) (Tocris Neuramin, Essex, UK); and *trans*-L-1-amino-1,3-cyclopentone-dicarboxylic acid (L-*trans*-ACPD) (Tocris Neuramin, Essex, UK), and L-pyrrolidine-2,4-dicarboxylate (L-*trans*-2,4-PDC) (Tocris Neuramin, Essex, UK).

3. Results

3.1. L-Glutamate dose-ranging study

We selected a dose of 2.5 nmol of L-glutamate in our initial study because in earlier studies this dose microinjected at a nearby site, the subretrofacial nucleus, produced consistent, reproducible increases in arterial blood pressure. When this dose of L-glutamate was microinjected bilaterally into the caudal subretrofacial area of four animals, we observed, unexpectedly, apnea of 20, 40 and 42 s duration (mean = 34 ± 7 s) in three of the four animals. This response was unexpected because, in our earlier study, we also observed apnea from this site when we microinjected antagonists of receptors for L-glutamate (Abrahams et al., 1991). In view of this unexpected effect on respiratory function, we explored a wide range of doses of L-glutamate with the expectation that at some dose, L-glutamate would elicit an effect on respiratory function that would be opposite to the effect noted upon microinjection of excitatory amino acid antagonists into the caudal subretrofacial area. Doses of L-glutamate tested bilaterally at the caudal subretrofacial area were as follows: 19.5 pmol ($n = 1$), 38.8 pmol ($n = 2$), 77.5 pmol ($n = 1$), 0.156 nmol ($n = 1$), 0.312 nmol ($n = 1$), 0.62 nmol ($n = 1$), 1.25 nmol ($n = 1$), 2.5 nmol ($n = 4$) (as described above) and 5.0 nmol ($n = 4$). With the 19.5 pmol dose, a decrease in the V_t from 31 to 24 ml was noted. With 38.8 pmol, one animal did not show any respiratory effect while a second animal exhibited apnea for 20 s. Each animal receiving the 77.5 pmol, the 0.156 nnmol and the 0.312 nmol doses exhibited apnea ranging from 18 to 42 s, but the animal receiving the 0.62 nmol dose exhibited very little in the way of respiratory depression. With 1.25 nmol, apnea of 11 s duration was noted, and, as indicated above, in three of the four animals receiving the 2.5 nmol dose of L-glutamate, apnea of 34 ± 7 s occurred.

The fourth animal studied with the 2.5 nmol dose exhibited little effect on respiratory activity. Finally, all four animals receiving the 5.0 nmol dose of L-glutamate exhibited apnea and the durations varied from 39 to 58 s. With the longer duration apnea noted with the 5 nmol dose of L-glutamate, arterial pressure changes were observed that were delayed relative to the time of onset of apnea, and may have been a consequence of the apnea. In an attempt to avoid changes in arterial pressure from occurring secondarily from respiratory depression, we employed the 2.5 nmol dose of L-glutamate for all of our subsequent studies. An important feature of using this dose was the reproducibility of the response attained. For example, in one experiment the 2.5 nmol dose was given 3 times with time allowed between each dose for complete recovery from respiratory depression. In each of the three instances apnea occurred and the durations of apnea were 20 s, 30 s, and 24 s.

3.2. Effects of bilateral microinjections of L-glutamate into the caudal subretrofacial area on cardiorespiratory function

Bilateral microinjection of 2.5 nmol of L-glutamate into the caudal subretrofacial area produced apnea in each animal tested, and this effect can be observed in

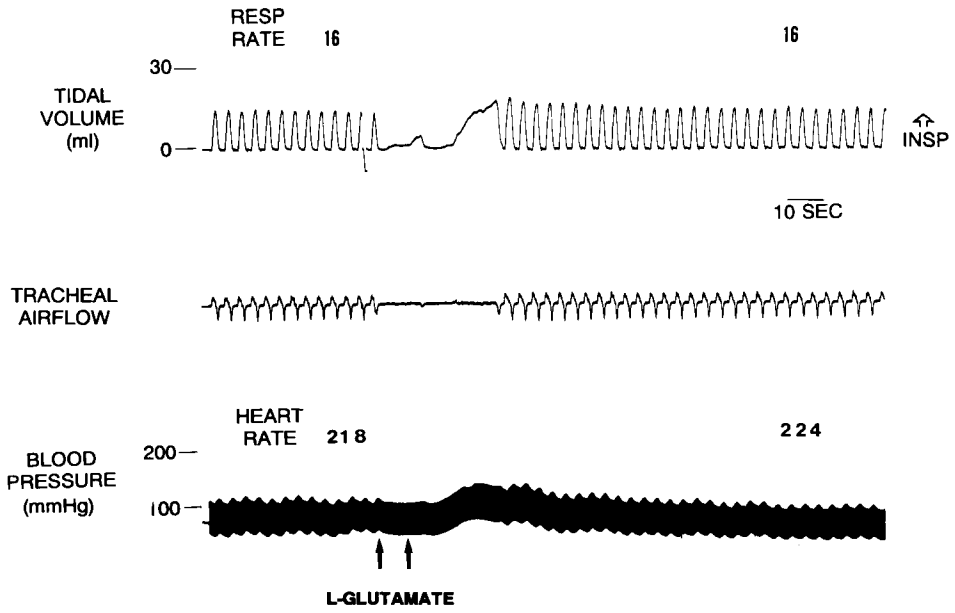

Fig. 1. Experiment showing cardiorespiratory effects of L-glutamate (L-GA) (2.5 nmol) microinjected bilaterally (as indicated by the arrows) into the caudal subretrofacial area.

the experimental traces depicted in Fig. 1. In this example, microinjection of 2.5 nmol of L-glutamate produced an immediate apnea. The duration of this apnea was 34 s. There was no immediate change in arterial blood pressure, but arterial blood pressure does increase following about 15 s of apnea. Respiratory activity and arterial blood pressure returned to baseline values about 2 min after L-glutamate microinjection into the caudal subretrofacial area.

Data obtained from eight animals (including data from the experiment shown as Fig. 1) can be summarized as follows: the most important finding is that in all eight animals, bilateral microinjection of 2.5 nmol of L-glutamate produced apnea. Apnea was characterized by a sudden onset with no sign of depressed breathing prior to cessation of breathing. That is, there was no indication of a decrease in V_t or f prior to cessation of breathing. The mean duration of apnea was 27 ± 2 s. L-Glutamate microinjection not only stopped breathing but increased mean arterial blood pressure ($+31 \pm 12$ mm Hg, $P < 0.05$; from a baseline of 118 ± 10 mm Hg) and decreased heart rate (-31 ± 10 beats/min, $P < 0.05$; from a baseline of 187 ± 9 beats/min). It is important to note, however, that in all cases the onset of apnea preceded by 5–15 s any change in arterial blood pressure or heart rate (see Fig. 1).

A photomicrograph of the cat medulla where bilateral microinjection of 2.5 nmol of L-glutamate produced apnea is shown in Fig. 2. The tips of the micropipette (visible by the green dye in the injectate) are located just ventrolateral to the rostral nucleus ambiguus, approximately 3.0 mm rostral to obex. These microinjection sites are located at the same sites where we earlier noted that blockade of an endogenous excitatory amino acid produces apnea (see Fig. 3, Abrahams et al., 1991).

3.3. Effects of bilateral microinjections of L-glutamate into the caudal subretrofacial area on respiratory function before and after blockade of NMDA receptors with CPP

The purpose of these experiments was to determine which type of glutamate receptor was responsible for the apnea noted after microinjection of L-glutamate into the caudal subretrofacial area. The first receptor we examined was the NMDA receptor. For this purpose, we found a dose of the NMDA receptor antagonist drug, CPP, that would block an effect of NMDA that was equivalent to the effect noted with L-glutamate microinjected into the caudal subretrofacial area (Fig. 3). As can be noted, NMDA was first microinjected bilaterally into the caudal subretrofacial area in a dose of 38 pmol and evoked a brief period of apnea similar to that seen with L-glutamate (Fig. 1). After obtaining consistent control responses to NMDA (data not shown), CPP, 0.62 nmol, was microinjected bilaterally into the same site in the caudal subretrofacial area. Five minutes later, the usual dose of NMDA was microinjected into the caudal subretrofacial area and relatively little effect on respiratory activity was obtained. This experimental protocol was performed in two additional animals and results obtained were the same as those shown in Fig. 3.

Next, the dose of CPP that was used to block NMDA was tested for its effectiveness in counteracting the respiratory effects of L-glutamate, and the data appear in Fig. 4. In the example, shown as Fig. 4, bilateral microinjection of 2.5 nmol of L-glutamate produced the expected 'transient' apnea. This dose was then repeated after 0.62 nmol of CPP and the L-glutamate effect was found to be still present. In the same animal, L-glutamate was again microinjected after prior treatment with a 9-fold higher dose of CPP (5.6 nmol), and apnea still occurred (not shown). Indeed, the duration of apnea was increased by the CPP treatment (Fig. 4). Data from four animals can be summarized as follows: CPP had no significant effect on the incidence of L-glutamate-induced apnea; apnea occurred in four of four animals tested. Furthermore, CPP did not shorten the duration of L-glutamate-induced apnea; if anything, the duration of apnea appeared to be prolonged (38 ± 6 s versus 28 ± 5 s). In terms of the effects of CPP per se, CPP produced a significant reduction in V_t (a decrease to 17 ± 3 ml ($P < 0.05$) from a baseline of 20 ± 4 ml) and an increase in f (an increase to 24 ± 4 breaths/min ($P < 0.05$) from a baseline of 20 ± 4 breaths/min), thus confirming our previous findings with this NMDA antagonist microinjected into the caudal subretrofacial area (Abrahams et al., 1991).

3.4. Effects of bilateral microinjections of L-glutamate into the caudal subretrofacial area on respiratory function before and after blockade of non-NMDA receptors with CNQX

Since L-glutamate did not appear to be producing apnea after microinjection into the caudal subretrofacial area via NMDA receptor activation, we next turned our attention to the non-NMDA receptor. For this purpose we found a dose of the non-NMDA receptor antagonist drug, CNQX, that would block an effect of the non-NMDA receptor agonist, AMPA, that was equivalent to the effect noted with L-glutamate microinjected into the caudal subretrofacial area (Fig. 5). As shown, AMPA was first microinjected bilaterally into the caudal subretrofacial area in a dose of 2.5 pmol and evoked a period of apnea similar to that seen with L-glutamate (Fig. 1). After obtaining control responses to AMPA, CNQX (125 pmol) was microinjected bilaterally into the same site in the caudal sub-

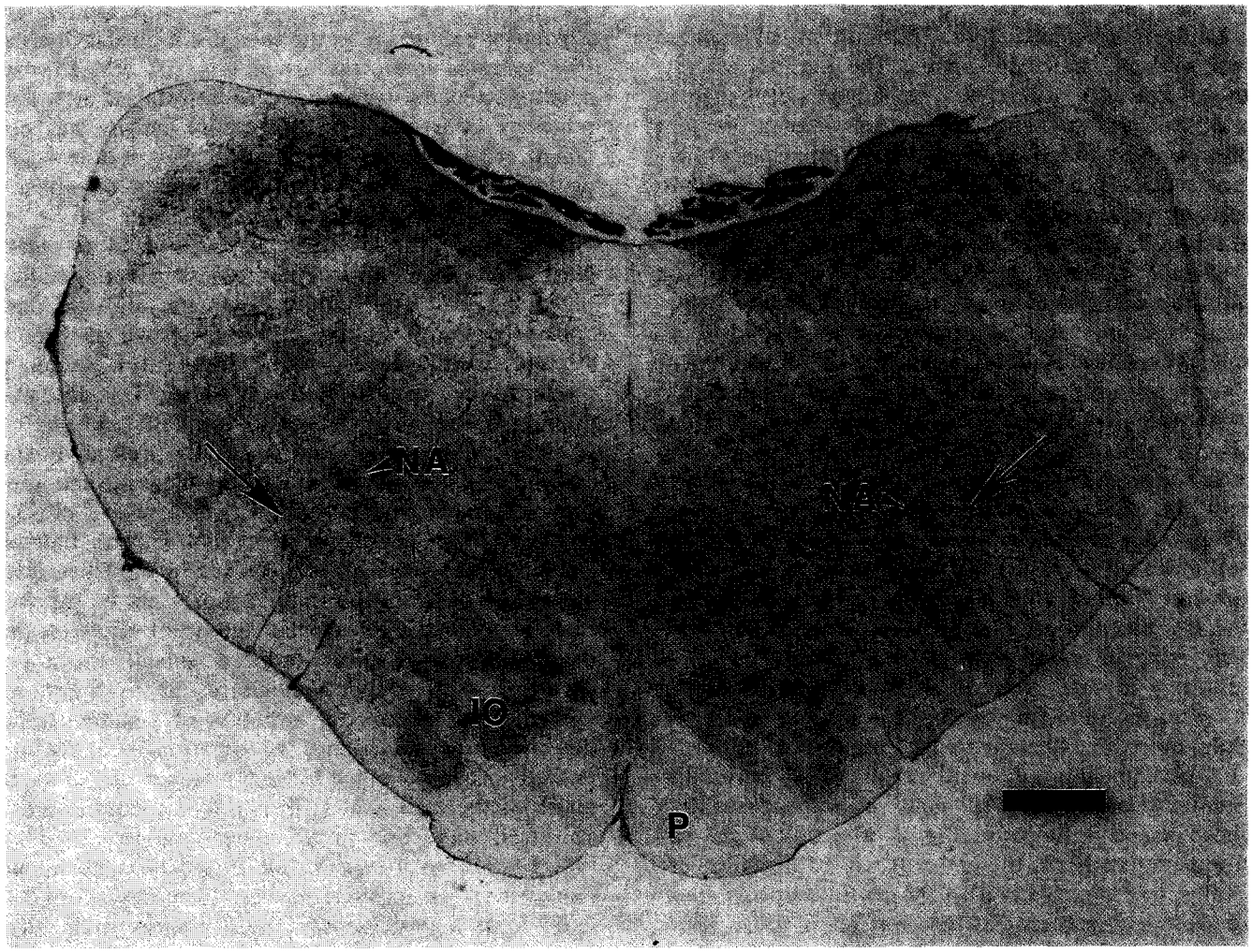

Fig. 2. Photomicrograph of the cat medulla where bilateral microinjection of 2.5 nmol of L-glutamate produces transient apnea. The tip of the micropipette as indicated by the presence of the green dye in the injectate is indicated by the large arrow. Abbreviations: NA, nucleus ambiguus (retrofacial position); IO, inferior olive; P, pyramids. Bar = 1 mm.

retrofacial area. Three minutes later, the same dose of AMPA microinjected into the caudal subretrofacial area failed to produce apnea. This experimental protocol was performed in three additional animals and CNQX was found to consistently block the apnea response of AMPA in all cases.

Next, the dose of CNQX that was to block AMPA was tested for its effectiveness in counteracting the respiratory effects of L-glutamate, and these data appear in Fig. 6. In the example shown as Fig. 6, bilateral microinjection of 2.5 nmol of L-glutamate produced apnea. This dose was then repeated 3 min after 125 pmol of CNQX and apnea still occurred. Data from four animals can be summarized as follows: CNQX had no effect on the incidence of L-glutamate-induced apnea; apnea lasting an average 49 ± 7 s occurred in all four animals. In terms of the effects of CNQX per se, CNQX alone produced no significant changes in V_t and f when microinjected into the caudal subretrofacial area. This confirms our previous findings with this non-NMDA receptor antagonist (Abrahams et al., 1991).

3.5. Effects of unilateral microinjection of L-glutamate into the caudal subretrofacial area on respiratory function before and after the combination of CPP and CNQX

As shown in the previous two sections, neither CPP nor CNQX alone, in doses adequate to block the apneic effects of NMDA and AMPA, respectively, prevented L-glutamate from producing apnea when microinjected into the caudal subretrofacial area. We next studied L-glutamate administered before and 3 min after a combination of CPP and CNQX. In this series of experiments microinjections were unilateral only. This was because bilateral microinjection of CPP

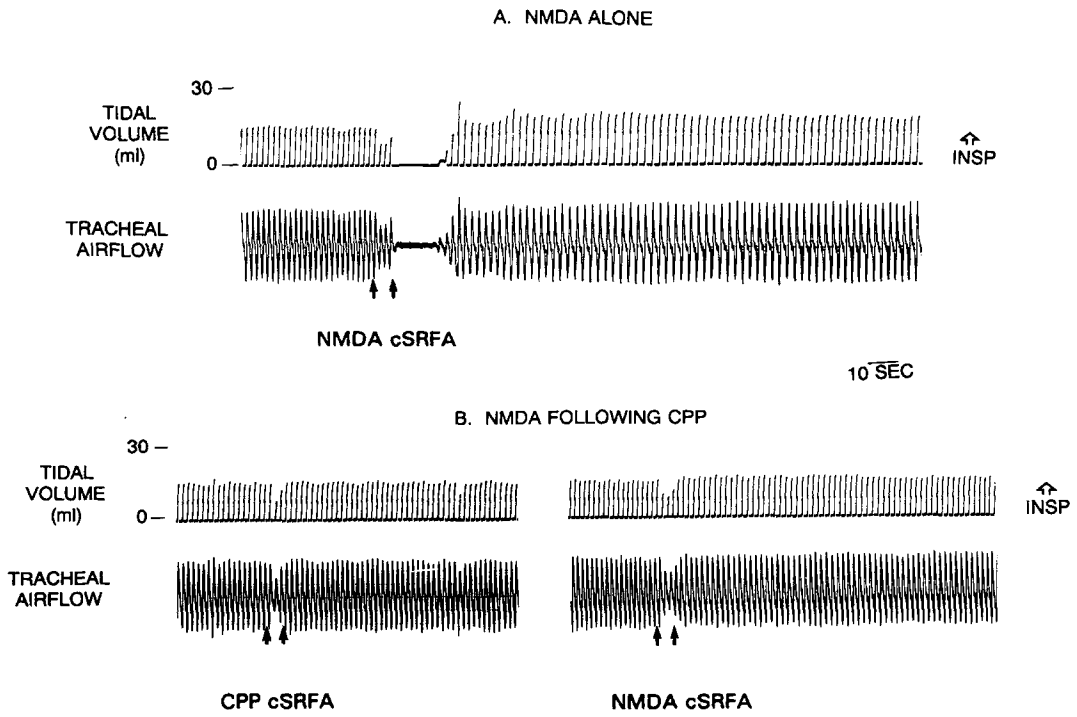

Fig. 3. Experiment showing respiratory effects of *N*-methyl-D-aspartate (NMDA) (37.5 pmol) microinjected bilaterally (as indicated by the arrows) into the caudal subretrofacial area (cSRFA) before and after CPP (0.62 nmol).

and CNQX at the caudal subretrofacial area leads to prolonged apnea, and an example of this is shown in Fig. 7. The apnea that occurs is of gradual onset and is characterized by a steady deterioration of the tidal volume. The CPP/CNQX 'cocktail' contained 5.6 nmol of CPP and 125 pmol of CNQX, the same dosage used previously. A representative experiment using unilateral microinjection is shown as Fig. 8. In this example unilateral microinjection of 50 nl (2.5 nmol) of L-glutamate produced a transient apnea. In this same animal, L-glutamate was again microinjected 3 min after prior treatment with the CPP/CNQX 'cocktail'

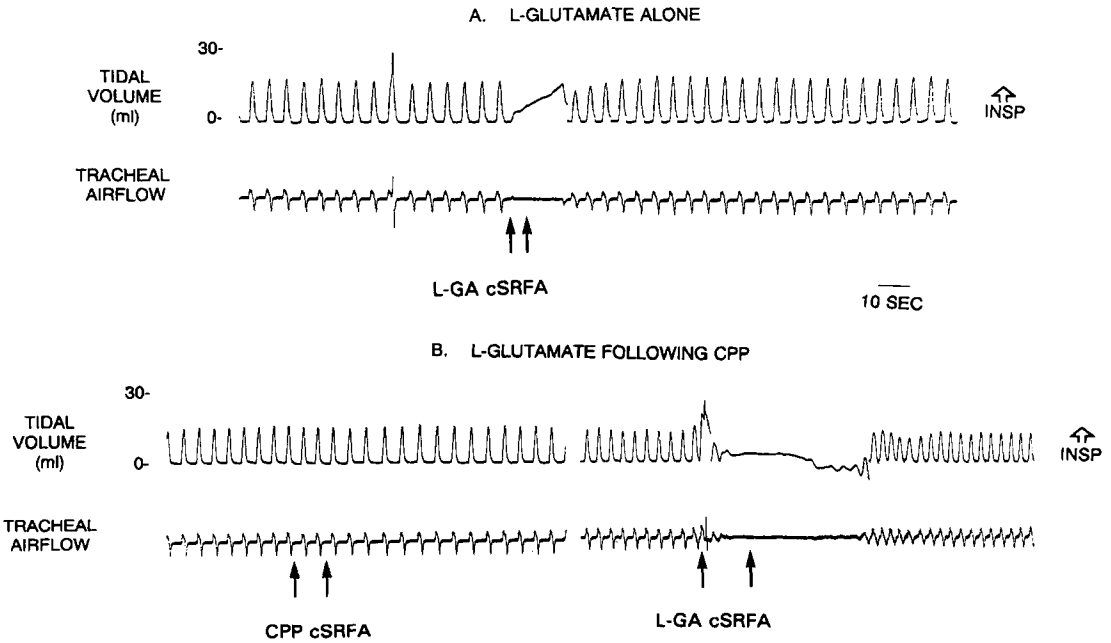

Fig. 4. Experiment showing respiratory effects of L-glutamate (L-GA) (2.5 nmol) microinjected bilaterally (as indicated by the arrows) into the caudal subretrofacial area (cSRFA) before and after CPP (0.62 nmol).

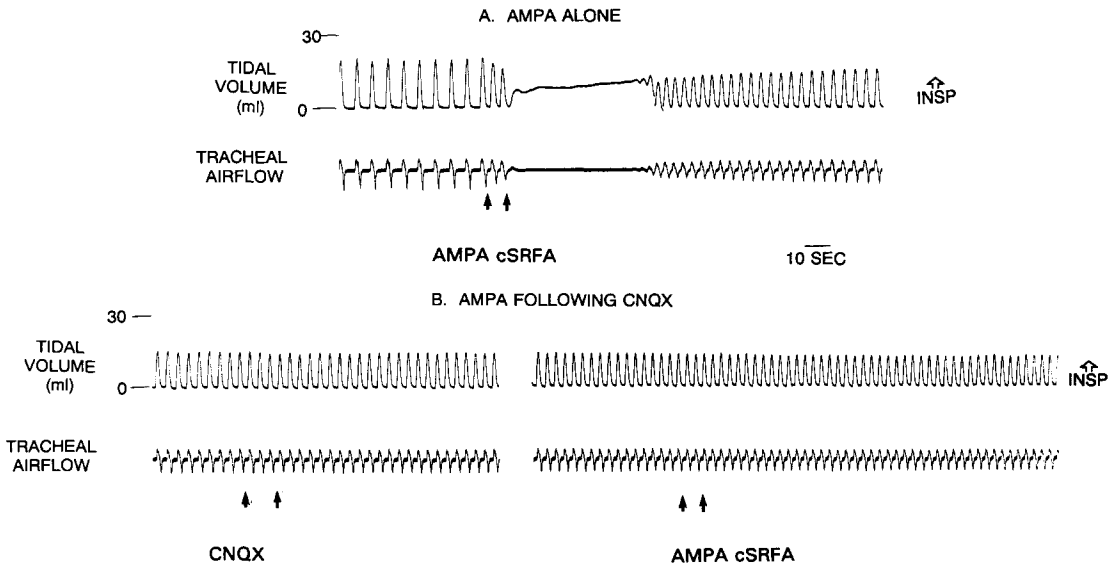

Fig. 5. Experiment showing respiratory effects of AMPA (2.5 pmol) microinjected bilaterally (as indicated by the arrows) into the caudal subretrofacial area (cSRFA) before and after CNQX (125 pmol).

and apnea still occurred. Data obtained from three experiments (including the experiment depicted in Fig. 8) appear in Table 1 and indicate that the CPP/CNQX 'cocktail' had no effect on the incidence of L-glutamate-induced apnea. As noted, one of three animals exhibited a brief apnea immediately following unilateral microinjection of CPP/CNQX cocktail but no animal showed any significant change from baseline respiratory activity 3 min later.

The lack of blockade of L-glutamate-induced transient apnea might be misconstrued as being due to the presence of an excess amount of L-glutamate relative to the CPP/CNQX 'cocktail' even though our L-glutamate response matched the responses elicited by NMDA (Fig. 3) and AMPA (Fig. 5), and was blocked by CPP and CNQX, respectively (Figs. 3 and 5). To address this issue, one experiment was performed where the interaction of one-tenth the usual dose of

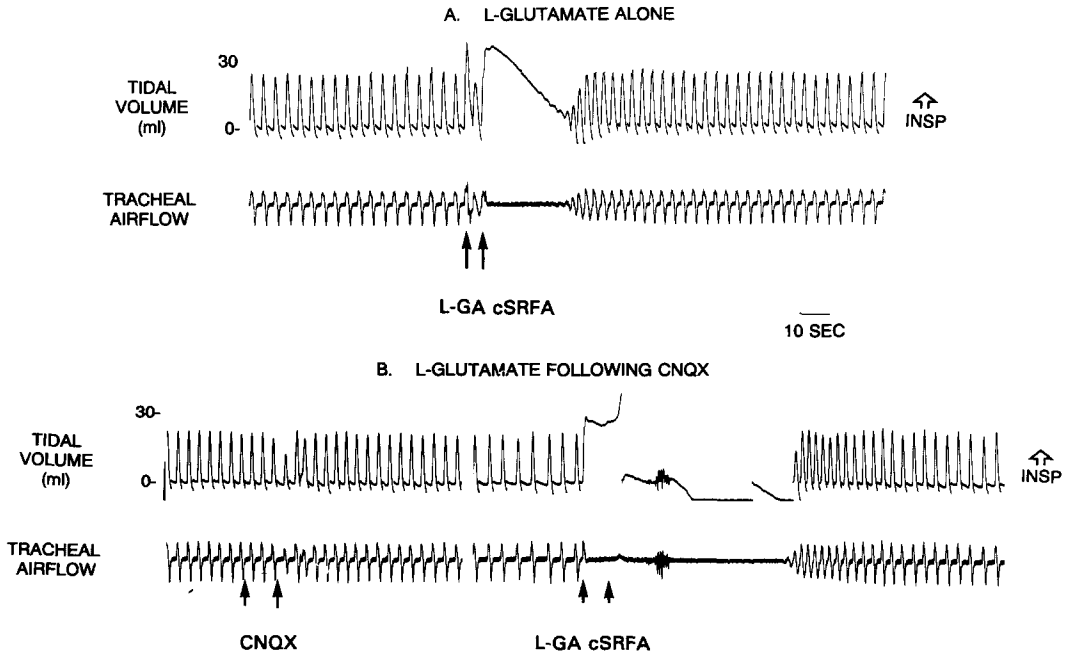

Fig. 6. Experiment showing respiratory effect of L-glutamate (L-GA) (2.5 nmol) microinjected bilaterally (as indicated by the arrows) into the caudal subretrofacial area (cSRFA) before and after CNQX (125 pmol).

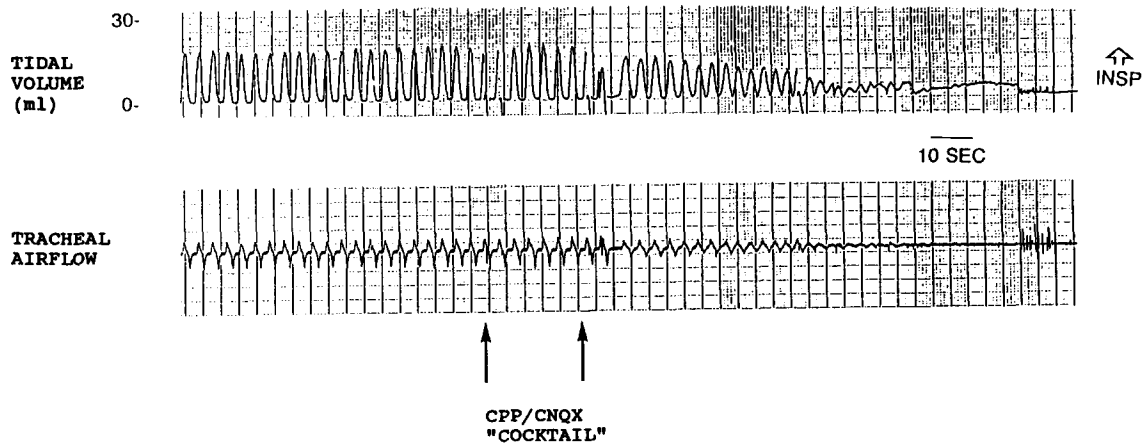

Fig. 7. Experiment showing respiratory effects of a combination of CPP (5.6 nmol) and CNQX (125 pmol) microinjected bilaterally (as indicated by the arrows) into the caudal subretrofacial area.

L-glutamate with CPP/CNQX 'cocktail' was examined. The result is shown in Fig. 9 and indicates that this small dose of L-glutamate produced transient reductions in V_t and f, but no transient apnea. This response, which was much less robust than seen with 2.5 nmol of L-glutamate, was also not counteracted by the CPP/CNQX 'cocktail' (Fig. 9).

3.6. Effects of unilateral microinjection of L-glutamate into the caudal subretrofacial area on respiratory function before and after kynurenic acid

Next, we investigated whether the broad-spectrum excitatory amino acid antagonist, kynurenic acid, would block the respiratory effects of L-glutamate microinjected at the caudal subretrofacial area. This series of experiments was performed unilaterally as bilateral microinjection of kynurenic acid at the caudal subretrofacial area produced a prolonged apnea similar to CPP/CNQX 'cocktail' (Abrahams et al., 1991). The dose of kynurenic acid used (31.2 nmol) is 2.5 times the dose used successfully by Gebber et al. (1989) in their microinjection studies in the cat. The data from these experiments appear in Table 2. As indicated, unilateral microinjection of 50 nl (2.5 nmol) L-glutamate produced apnea. This dose was then repeated 3 min after prior treatment with 31.2 nmol of kynurenic acid into the caudal subretrofacial area and produced apnea. Unilateral microinjection of kynurenic acid into the caudal subretrofacial area exerted no discernible effect on respiratory function (Table 2).

3.7. Studies of unilateral microinjection of the metabotropic receptor agonist drug L-trans-ACPD into the caudal subretrofacial area on respiratory function

Since drugs known to antagonize L-glutamate on ionotropic receptors were ineffective in blocking L-glutamate at the caudal subretrofacial area, we considered the possibility that L-glutamate could be eliciting its respiratory depressant effect through activation of a metabotropic receptor. To test this possibility we found

Table 1
Effects of unilateral microinjection of L-glutamate before and after the combination of CPP and CNQX into the caudal subretrofacial area on respiratory activity, mean arterial blood pressure and heart rate [a]

Experimental conditions	V_t (ml)	f (breaths/min)	Incidence of apnea	Duration of apnea (s)	Mean blood pressure (mm Hg)	Heart rate (beats/min)
(A) L-Glutamate alone						
Initial values	24 ± 4	13 ± 3	NA	NA	117 ± 10	190 ± 17
Maximal change following L-GA	Apnea	Apnea	3/3	16 ± 3	+13 ± 23	+2 ± 11
(B) L-Glutamate following CCP/CNQX 'cocktail'						
Initial values	23 ± 4	15 ± 3	NA	NA	118 ± 5	210 ± 9
Effect of CPP/CNQX 'cocktail'	23 ± 5	15 ± 4	1/3	20	120 ± 6	199 ± 5
Maximal change following L-GA	Apnea	Apnea	3/3	23 ± 6	−39 ± 11	−20 ± 11

[a] Values are means ± S.E.M. of three animals studied. NA = not applicable.

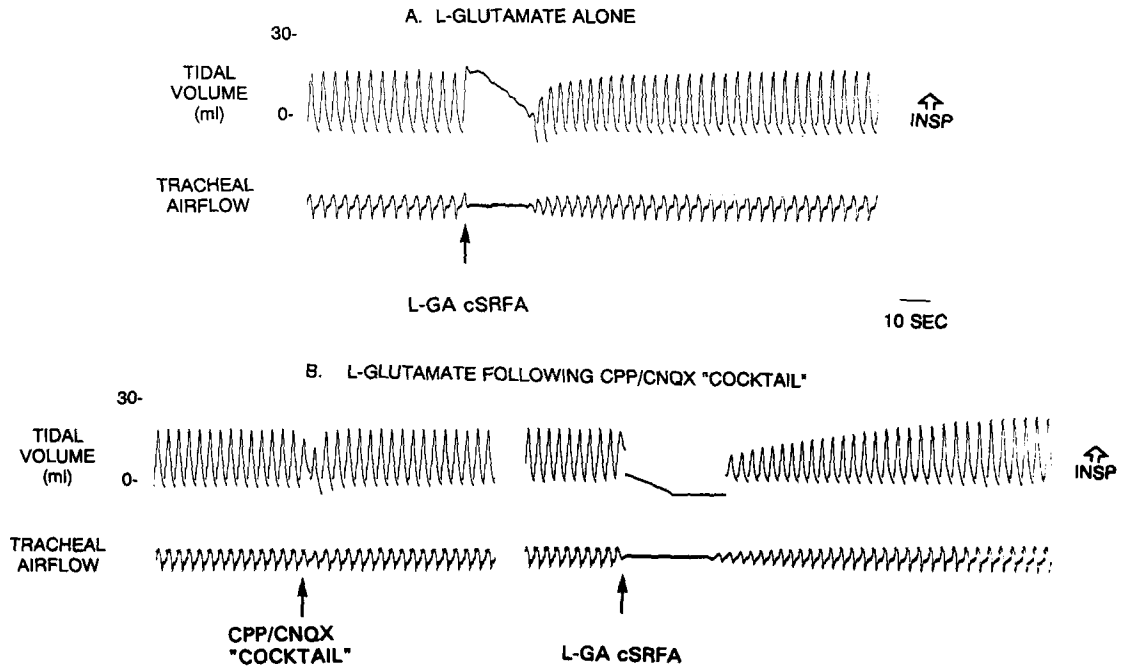

Fig. 8. Experiment showing respiratory effects of L-glutamate (L-GA) (2.5 nmol) microinjected unilaterally (as indicated by the arrow) into the caudal subretrofacial area (cSRFA) before and after the combination of CPP (5.6 nmol) and CNQX (125 pmol) microinjected unilaterally into the same site.

a site in the ventrolateral medulla where L-*trans*-ACPD elicited an effect. This site was the caudal ventrolateral medulla, and microinjection of L-*trans*-ACPD into this site in a dose of 200 pmol produced marked decreases in arterial blood pressure and heart rate (Fig. 10). This effect was observed in five of five animals tested (McManigle et al., 1992). We next tested the dose of 200 pmol of L-*trans*-ACPD at the caudal subretrofacial

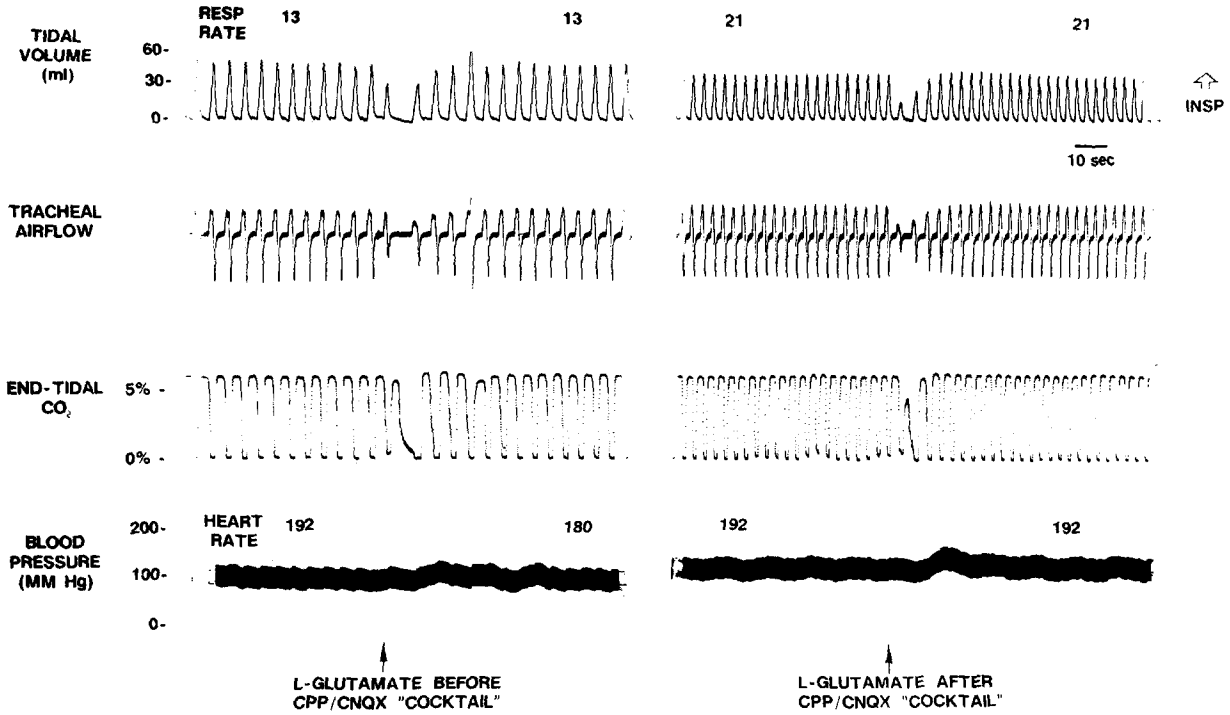

Fig. 9. Experiment showing cardiorespiratory effects of L-glutamate (0.25 nmol) microinjected unilaterally (as indicated by the arrow) into the caudal subretrofacial area before and after the combination of CPP (5.6 nmol) and CNQX (125 pmol) microinjected unilaterally into the same site.

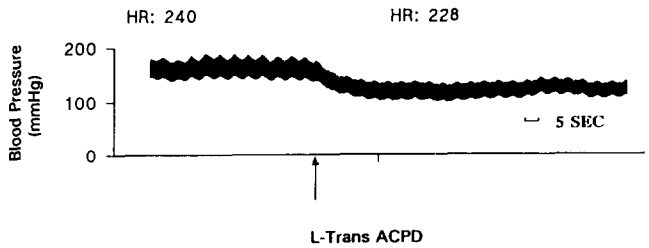

Fig. 10. Experiment showing blood pressure and heart rate effects of L-*trans*-ACPD (200 pmol) microinjected unilaterally (as indicated by the arrow) into the caudal ventrolateral medulla.

area and did not observe any effect of this metabotropic receptor agonist on respiratory activity. An experiment depicting the absence of an effect of L-*trans*-ACPD at the caudal subretrofacial area appears as Fig. 11. This lack of effect was noted in three of three animals tested.

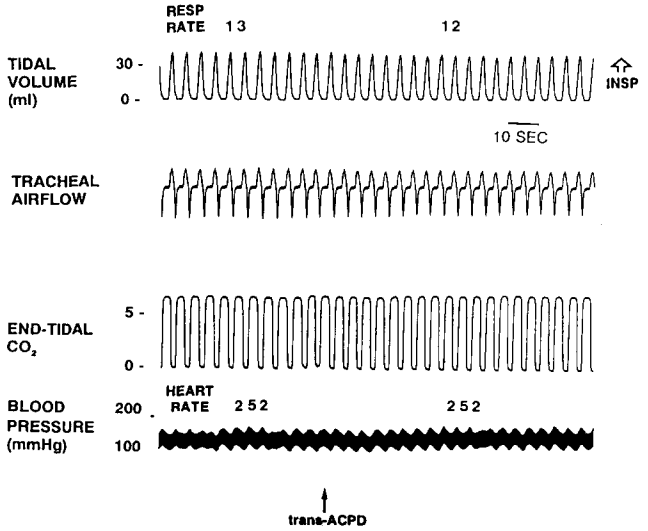

Fig. 11. Experiment showing the lack of any cardiorespiratory effects of L-*trans*-ACPD (200 pmol) microinjected unilaterally (as indicated by the arrow) into the caudal subretrofacial area.

3.8. Effects of L-pyrrolidine-2,4-dicarboxylate

L-Pyrrolidine-2,4-dicarboxylate (L-*trans*-2,4-PDC) was discovered in 1991 by Bridges and colleagues, and was documented to inhibit the high-affinity transport of [^{3}H]L-glutamate into synaptosomes (Bridges et al., 1991). To determine the dose of L-*trans*-2,4-PDC to study, we first performed a dose-ranging study of this agent using doses of 165, 412, 500 and 1250 pmol. These doses were microinjected bilaterally into the caudal subretrofacial area and the data of the five animals evaluated were as follows: the lowest dose exerted little effect on cardiorespiratory activity. The next highest dose, i.e., 412 pmol, produced a 15 s period of apnea. With 500 pmol, apnea of 23 and 25 s duration occurred, and mean blood pressure and heart rate were little affected. The highest dose tested, i.e., 1250 pmol, produced apnea of 25 s, but changes in mean blood pressure and heart rate appeared to be quite pronounced. Because of a less intensive effect of 500 pmol (i.e., 0.5 nmol) L-*trans*-2,4-PDC on cardiovascular function, we used this dose for subsequent studies.

L-*trans*-2,4-PDC was microinjected both unilaterally ($n = 9$), and bilaterally ($n = 10$) into the caudal subretrofacial area while monitoring V_t, f, arterial blood pressure, and heart rate. Data obtained are tabulated in Table 3 and indicate that L-*trans*-2,4-PDC consistently produces a brief period of apnea. The duration of apnea was similar regardless of whether unilateral or bilateral microinjections were given. In terms of V_t, a significant decrease was noted with unilateral microinjection, whereas a significant increase in V_t was observed with bilateral microinjection. No significant changes in either f, mean arterial blood pressure or heart rate were noted. An experiment illustrating apnea induced by unilateral microinjection of L-*trans*-2,4-PDC appears as Fig. 12. As can be noted, apnea

Table 2
Effects of unilateral microinjection of L-glutamate into the caudal subretrofacial area on respiratory activity, mean arterial blood pressure and heart rate before and after kynurenic acid [a]

Experimental conditions	V_t (ml)	f (breaths/min)	Incidence of apnea	Duration of apnea (s)	Mean blood pressure (mm Hg)	Heart rate (beats/min)
(A) L-Glutamate alone						
Initial values	23 ± 4	13 ± 1	NA	NA	134 ± 25	208 ± 23
Maximal change following L-GA	Apnea	Apnea	3/3	19 ± 4	+19 ± 20	−46 ± 30
(B) L-Glutamate following kynurenic acid						
Initial values	20 ± 2	14 ± 1	NA	NA	111 ± 17	208 ± 11
Effect of kynurenic acid	19 ± 2	14 ± 1	0/3	NA	105 ± 14	208 ± 11
Maximal change following L-GA	Apnea	Apnea	3/3	23 ± 3	+32 ± 35	−2 ± 11

[a] Values are means ± S.E.M. of three animals studied. NA = not applicable.

Table 3
Effects of microinjection of 0.5 nmol of L-*trans*-2,4-PDC into the caudal subretrofacial area on respiratory activity, mean arterial pressure and heart rate [a]

Experimental conditions	V_t (ml)	f (breaths/min)	Incidence of apnea	Duration of apnea (s)	Mean blood pressure (mm Hg)	Heart rate (beats/min)
Unilateral microinjection						
Initial values	29 ± 2	14 ± 1	NA	NA	110 ± 8	183 ± 10
Maximal change following L-*trans*-2,4-PDE	−4.0 ± 1 [b]	−1 ± 1	9/9	19 ± 1	−3 ± 10	+1 ± 4
Bilateral microinjection						
Initial values	28 ± 2	18 ± 2	NA	NA	118 ± 6	188 ± 6
Maximal change following L-*trans*-2,4-PDC	+4 ± 1 [b]	0 ± 0	10/10	20 ± 2	−10 ± 6	−4 ± 4

[a] Values are means ± S.E.M. of three animals studied. NA = not applicable. [b] $P < 0.05$ using the Student's t-test for paired data.

occurred immediately after microinjection of L-*trans*-2,4 PDC into the caudal subretrofacial area.

3.9. Effects of L-trans-2,4-PDC before and after blockade of NMDA receptors with CPP

Next, CPP was tested for its effectiveness in counteracting the respiratory effects of L-*trans*-2,4-PDC, and the data appear in Table 4. The dose of CPP selected was one that was previously shown to block the effects of NMDA at the caudal subretrofacial area (Fig. 3). Four animals were studied and the protocol used was as follows: (1) animals first received L-*trans*-2,4-PDC microinjected either unilaterally or bilaterally into the caudal subretrofacial area; (2) once recovery from L-*trans*-2,4-PDC occurred, CPP was microinjected either unilaterally or bilaterally into the caudal subretrofacial area; and (3) within 3–5 min after CPP was microinjected, the same dose of L-*trans*-2,4-PDC was microinjected into the caudal subretrofacial area and the response compared to the control response (i.e., response obtained with L-*trans*-2,4-PDC prior to CPP). (Note: preliminary experiments indicated that reproducible responses to L-*trans*-2,4-PDC could be obtained upon repeated microinjection into the caudal subretrofacial area of the same animal.) As can be noted from Table 4, unilateral and bilateral microinjections of L-*trans*-2,4-PDC produced apnea in each animal prior to administering CPP, and no evidence was obtained for an alteration of the response after microinjection of CPP. Indeed, in terms of the bilateral microinjection data, it appeared that the duration of apnea produced by L-*trans*-2,4-PDC was augmented by pretreatment with CPP. Finally, the effects of CPP per

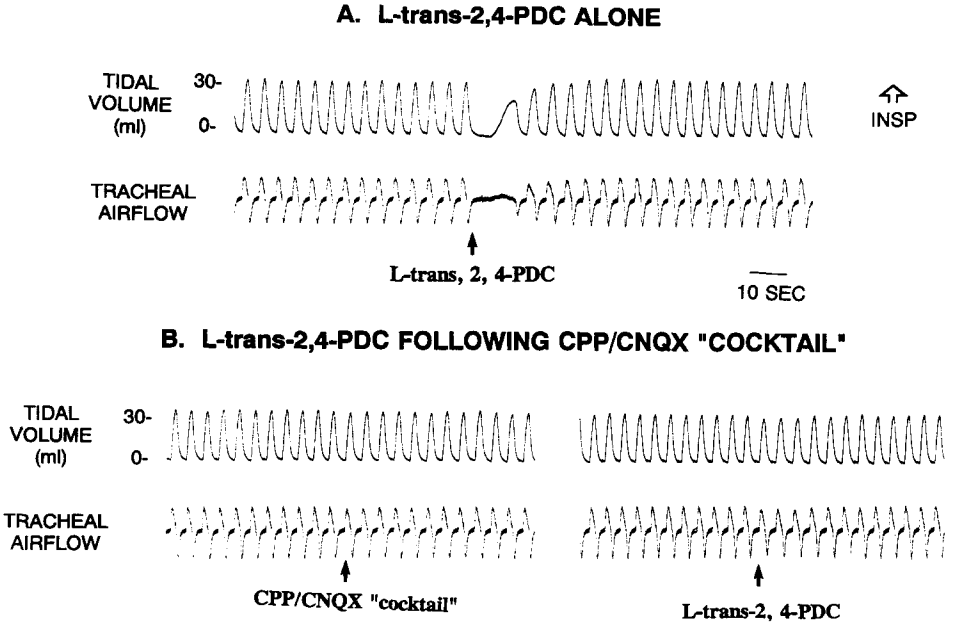

Fig. 12. Experiment showing respiratory effects of L-*trans*-2,4-PDC (0.5 nmol) microinjected unilaterally (as indicated by the arrows) into the caudal subretrofacial area before and after the combination of CPP (5.6 nmol) and CNQX (125 pmol) microinjected into the same site.

Table 4
Effects of microinjection of 0.5 nmol of L-*trans*-2,4-PDC into the caudal subretrofacial area on respiratory activity, arterial pressure and heart rate before and after CPP (5.6 nmol) [a]

Experimental conditions	V_t (ml)	f (breaths/min)	Incidence of apnea	Duration of apnea (s)	Mean blood pressure (mm Hg)	Heart rate (beats/min)
Unilateral microinjection						
(A) L-*trans*-2,4-PDC alone						
Initial values	32 ± 0	12 ± 0	NA	NA	101 ± 9	201 ± 21
Maximal change following L-*trans*-2,4-PDC	−6 ± 0	0 ± 0	2/2	20 ± 0	+2 ± 19	+6 ± 0
(B) L-*trans*-2,4-PDC following CPP						
Initial values	32 ± 0	12 ± 1	NA	NA	102 ± 4	204 ± 24
Effect of CPP	28 ± 2	14 ± 0	0/2	NA	106 ± 6	201 ± 21
Maximal change following L-*trans*-2,4-PDC	+4 ± 0	0 ± 0	2/2	18 ± 3	+30 ± 17	0 ± 6
Bilateral microinjection						
(A) L-*trans*-2,4-PDC alone						
Initial values	24 ± 1	17 ± 3	NA	NA	106 ± 6	168 ± 6
Maximal change following L-*trans*-2,4-PDC	+2 ± 0	0 ± 0	2/2	26 ± 6	+31 ± 6	+9 ± 15
(B) L-*trans*-2,4-PDC following CPP						
Initial values	22 ± 2	15 ± 3	NA	NA	85 ± 5	153 ± 3
Effect of CPP	18 ± 3	20 ± 4	0/2	NA	109 ± 11	165 ± 3
Maximal change following L-*trans*-2,4-PDC	+2 ± 1	+4 ± 2	2/2	40 ± 15	+43 ± 10	+9 ± 21

[a] Values are means ± S.E.M. of two animals (unilateral studies) and two animals (bilateral studies). NA = not applicable.

se are also tabulated in Table 4. CPP did not appear to produce any effect when it was given unilaterally. However, when CPP was given bilaterally, V_t appeared to decrease, and f appeared to increase. These are the same effects that we reported earlier for CPP (Abrahams et al., 1991).

An experiment demonstrating the lack of effectiveness of CPP in counteracting the respiratory effects of L-*trans*-2,4-PDC appears as Fig. 13. As can be seen, bilateral microinjection of 0.5 nmol of of L-*trans*-2,4-PDC produced the expected transient apnea. CPP, 5.62 nmol, was microinjected bilaterally into the caudal

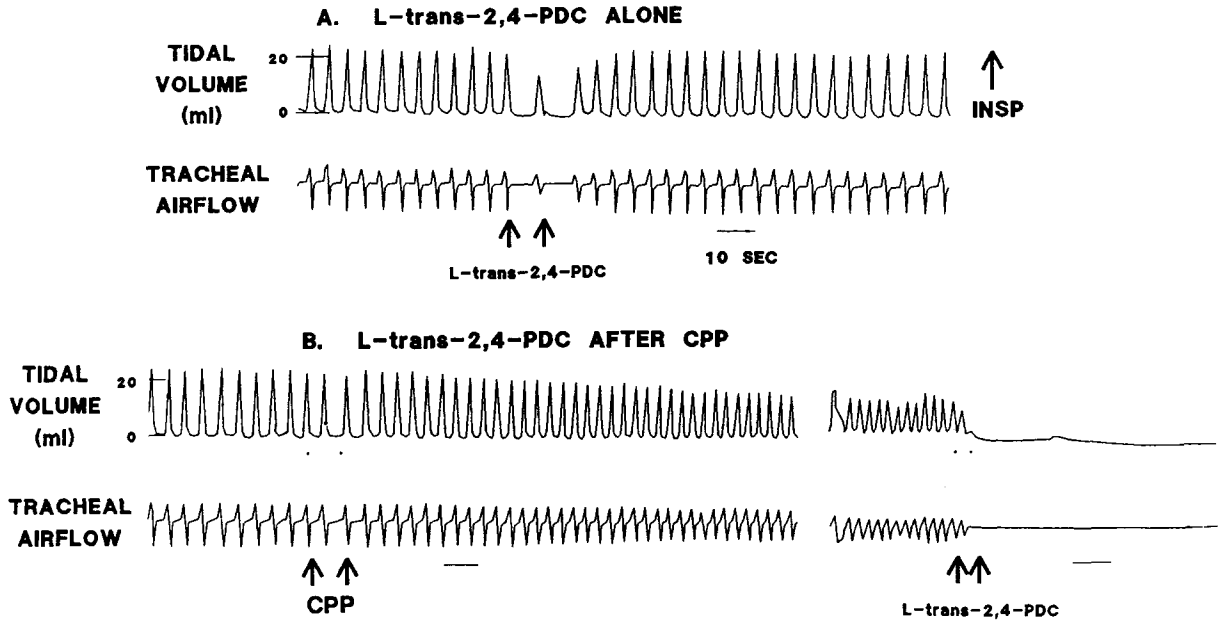

Fig. 13. Experiment showing respiratory effects of L-*trans*-2,4-PDC (0.5 nmol) microinjected bilaterally (as indicated by the arrows) into the caudal subretrofacial area before and after CPP (5.6 nmol).

Table 5
Effects of microinjection of 0.5 nmol of L-*trans*-2,4-PDC into the caudal subretrofacial area on respiratory activity mean arterial pressure and heart rate before and after CNQX (125 pmol) [a]

Experimental conditions	V_t (ml)	f (breaths/min)	Incidence of apnea	Duration of apnea (s)	Mean blood pressure (mm Hg)	Heart rate (beats/min)
Unilateral microinjection						
(A) L-*trans*-2,4-PDC alone						
Initial values	24 ± 2	14 ± 1	NA	NA	82 ± 16	180 ± 0
Maximal change following L-*trans*-2,4-PDC	+2 ± 1	+2 ± 0	2/2	22 ± 3	+9 ± 26	+4 ± 16
(B) L-*trans*-2,4-PDC following CNQX						
Initial values	22 ± 4	15 ± 1	NA	NA	98 ± 10	174 ± 18
Effect of CNQX	22 ± 4	15 ± 1	0/2	NA	94 ± 14	168 ± 18
Maximal change following L-*trans*-2,4-PDC	Duration of apnea was too long to obtain reliable values		2/2	40 ± 5	−5 ± 34	−42 ± 42
Bilateral microinjection						
(A) L-*trans*-2,4-PDC alone						
Initial values	28 ± 2	16 ± 0	NA	NA	124 ± 3	183 ± 21
Maximal change following L-*trans*-2,4-PDC	+4 [b]	0 [b]	2/2	19 ± 6	−2 ± 18	+3 ± 3
(B) L-*trans*-2,4-PDC following CNQX						
Initial values	30 ± 2	12 ± 4	NA	NA	119 ± 14	186 ± 18
Effect of CNQX	27 ± 0	14 ± 3	1/2	15	120 ± 12	192 ± 12
Maximal change following L-*trans*-2,4-PDC	Duration of apnea was too long to obtain reliable values		2/2	33 ± 4	+40 ± 10	+9 ± 3

[a] Values are means ± S.E.M. of two animals (unilateral studies) and two animals (bilateral studies). NA = not applicable. [b] Data from only one of the two animals were reliable.

subretrofacial area and produced an immediate decrease in V_t and an increase in f. (Note: this is the characteristic effect of CPP when microinjected into the caudal subretrofacial area and has been described by Abrahams et al. (1991), and CPP microinjected bilaterally at this site has not been observed to produce apnea – Abrahams et al., 1991.) L-*trans*-2,4-PDC was then repeated after CPP and the apneic effect was still present, and, indeed, intensified.

3.10. Effects of L-trans-2,4-PDC before and after blockade of non-NMDA receptors with CNQX

In view of the ineffectiveness of CPP in blocking the effect of L-*trans*-2,4-PDC, we next tested CNQX for its

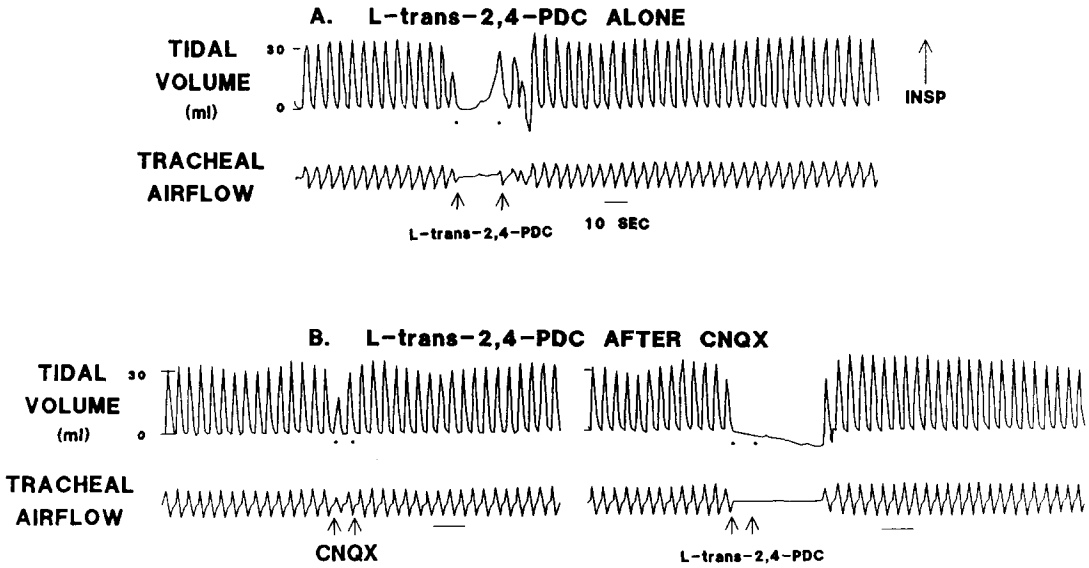

Fig. 14. Experiment showing respiratory effects of L-*trans*-2,4-PDC (0.5 nmol) microinjected bilaterally (as indicated by the arrows) into the caudal subretrofacial area before and after CNQX (125 pmol).

effectiveness in counteracting the respiratory effects of L-*trans*-2,4-PDC. The dose of CNQX selected was one that was previously shown to block the effects of the non-NMDA receptor agonist, AMPA, at the caudal subretrofacial area (Fig. 5). Four animals were studied and the protocol used was as follows: (1) animals first received L-*trans*-2,4-PDC microinjected either unilaterally or bilaterally into the caudal subretrofacial area; (2) once recovery from L-*trans*-2,4-PDC occurred, CNQX was microinjected either unilaterally or bilaterally into the caudal subretrofacial area; and (3) within 3–5 min after CNQX was microinjected, the same dose of L-*trans*-2,4-PDC was microinjected into the caudal subretrofacial area and the response compared to a control response (i.e., response obtained with L-*trans*-2,4-PDC prior to CNQX). As can be noted from Table 5, unilateral and bilateral microinjections of L-*trans*-2,4-PDC produced apnea in each animal prior to administering CNQX, and no evidence was obtained for an alteration of the response after microinjection of CNQX. Indeed, in terms of both the unilateral and bilateral microinjection data, it appeared that the duration of apnea produced by L-*trans*-2,4-PDC was augmented by pretreatment with CNQX. Finally, the effects of CNQX per se are also tabulated in Table 5. CNQX exerted very little effect when given either unilaterally or bilaterally, with the exception of one animal where bilateral microinjection did produce a brief apnea lasting 15 s.

An experiment demonstrating the lack of effectiveness of CNQX in counteracting the respiratory effects of L-*trans*-2,4-PDC appears as Fig. 14. As can be seen, bilateral microinjection of 0.5 nmol of L-*trans*-2,4-PDC produced the expected transient apnea. CNQX, 125 pmol, was microinjected bilaterally into the caudal subretrofacial area and had little effect per se. L-*trans*-2,4-PDC was then repeated after CNQX and the apneic effect was still present, and indeed, intensified.

3.11. Effects of L-trans-2,4-PDC before and after blockade of both NMDA and non-NMDA receptors with the combination of CPP and CNQX

Since neither CPP nor CNQX was effective in counteracting the transient apnea evoked by microinjecting L-*trans*-2,4-PDC into the caudal subretrofacial area, the next series of experiments were performed for the purpose of determining whether the combination of CPP and CNQX would prevent the response. Five animals were studied and the protocol used was as follows: (1) animals first received L-*trans*-2,4-PDC microinjected unilaterally into the caudal subretrofacial area; (2) once recovery from L-*trans*-2,4-PDC occurred, CPP plus CNQX were microinjected unilaterally into the same site in the cSFRA area as L-*trans*-2,4-PDC (unilateral microinjection was necessary because bilateral microinjection of CPP plus CNQX causes long-lasting apnea – Abrahams et al. (1991) and Fig. 7 of this study); and (3) within 3–5 min after CPP plus CNQX were microinjected, the same dose of L-*trans*-2,4-PDC was microinjected unilaterally into the caudal subretrofacial area and the response compared to the control response (i.e., response obtained with L-*trans*-2,4-PDC prior to CPP plus CNQX). As can be noted from Table 6, unilateral microinjections of L-*trans*-2,4-PDC did not produce transient apnea in any of the five animals tested after pretreatment with CPP plus CNQX. The effects of CPP plus CNQX per se are also tabulated in Table 6. This combination given unilaterally did produce a brief apnea in three of five animals lasting an average for 14 s. No apnea was observed in the other two animals, and no significant effects were noted on V_t, f, mean blood pressure and heart rate.

An experiment demonstrating the effectiveness of CPP plus CNQX in counteracting the respiratory effects of L-*trans*-2,4-PDC appears in Fig. 12. As can be seen, unilateral microinjection of 0.5 nmol of L-*trans*-

Table 6
Effects of unilateral microinjection of 0.5 nmol of L-*trans*-2,4-PDC into the caudal subretrofacial area on respiratory activity, mean arterial pressure and heart rate before and after CPP/CNQX 'cocktail' (i.e., CPP 5.6 nmol plus CNQX 125 pmol) [a]

Experimental conditions	V_t (ml)	f (breaths/min)	Incidence of apnea	Duration of apnea (s)	Mean blood pressure	Heart rate (beats/min)
(A) L-*trans*-2,4-PDC alone						
Initial values	30 ± 4	14 ± 2	NA	NA	125 ± 8	186 ± 13
Maximal change following L-*trans*-2,4-PDC	+3 ± 2	0 ± 1	5/5	17 ± 2	+1 ± 18	−11 ± 7
(B) L-*trans*-2,4-PDC following CPP/CNQX 'cocktail'						
Initial values	28 ± 3	15 ± 1	NA	NA	125 ± 6	185 ± 18
Effect of CPP/CNQX 'cocktail'	26 ± 3	18 ± 2	3/5	14 ± 3	126 ± 4	186 ± 14
Maximal change following L-*trans*-2,4-PDC	−6 ± 2 [b]	−1 ± 3	0/5	NA	−11 ± 2 [b]	−1 ± 3

[a] Values are means ± S.E.M. of five animals. NA = not applicable. [b] $P < 0.05$ using the Student's t-test for paired data.

2,4-PDC produced the expected transient apnea. CPP plus CNQX were microinjected into the same site and exerted no effect on breathing. L-*trans*-2,4-PDC was then microinjected after CPP plus CNQX and the apneic effect was prevented.

4. Discussion

One focus of the present study was to determine the respiratory effects of microinjection of excitatory amino acids into the caudal subretrofacial area, a brain site where we recently discovered that blockade of an endogenous excitatory amino acid results in apnea (Abrahams et al., 1991). In the present study, we microinjected three excitatory amino acids into the caudal subretrofacial area, namely, L-glutamate, NMDA and AMPA. Each of these excitatory amino acids had a similar effect on respiratory activity, and this effect consisted of apnea. This respiratory response was surprising to us in view of our earlier data indicating that antagonists of excitatory amino acids microinjected into the caudal subretrofacial area also produced apnea (Abrahams et al., 1991), and this was confirmed in the present study. The apnea, however, produced by agonist and antagonists of excitatory amino acids microinjected into the caudal subretrofacial area was not identical in time course or in character. Apnea observed with the agonist occurred immediately upon microinjection of the agent with no indication that respiratory depression was imminent (i.e., no reduction in V_t, f or prolongation of inspiratory duration or expiratory duration prior to apnea), and the duration of apnea was very short (rarely lasting more than 45 s). In contrast, apnea observed with the antagonists of excitatory amino acids (i.e., kynurenic acid and the combination of CPP and CNQX) took time to develop, and was preceded by a reduction in V_t and an increase in f. Furthermore, apnea observed with these antagonists was prolonged and recovery from apnea was rarely noted after these drugs were microinjected, with apnea persisting for the duration of the experiment (i.e., up to 1–2 h). Finally, apnea observed with agonist could occur with just unilateral microinjection while apnea observed with antagonists required bilateral microinjections.

Data obtained with NMDA and AMPA in the present study indicate that activation of NMDA and non-NMDA ionotropic receptors at the caudal subretrofacial area can evoke apnea. On the other hand, respiratory data obtained with L-*trans*-ACPD indicate that the metabotropic excitatory amino acid receptor, at least one sensitive to L-*trans*-ACPD, probably does not exist at the caudal subretrofacial area.

We considered the possibility that our unexpected findings with microinjection of excitatory amino acids into the caudal subretrofacial area produced apnea because of depolarization block of neurons at the microinjection site, thereby resulting in inactivation of these neurons. That this can occur has been described by Lipski and colleagues, using microinjections of L-glutamate and D,L-homocysteic acid (Lipski et al., 1988). However, microinjection of 5–150 nmol of each of these agonists was required in order to observe a decrease in neuronal excitability. According to these investigators, 'the amount of injected amino acids should be 5 nmol or less to minimize the establishment of concentrations that would cause local depolarizing block.' In our studies, we used amounts of excitatory amino acids below 5 nmol. For most of the experiments, 2.5 nmol of L-glutamate was used.

In terms of localizing the caudal subretrofacial area, relative to other known respiratory areas in the ventrolateral medulla, the caudal subretrofacial area appears to be caudal to the Botzinger complex and probably more lateral from the midline. Lipski and Merrill (1980) describe the rostral expiratory neurons in the ventrolateral medulla, i.e., the Botzinger complex, as being located 3.5–5.5. mm rostral to obex and 2.9–3.3 mm lateral to midline. Connelly and colleagues place the Botzinger expiratory neurons 3.55 mm rostral to obex and ventromedial to the retrofacial nucleus, whereas an area that they refer to as the pre-Botzinger area (which probably corresponds to the caudal subretrofacial area) is caudal to the retrofacial nucleus and ventrolateral to the compact division of nucleus ambiguus (Connelly et al., 1992). They regard the pre-Botzinger complex as a transition zone between expiratory-modulated Botzinger and inspiratory modulated rostral ventral respiratory neurons.

We have not examined the respiratory neuronal discharge patterns of caudal subretrofacial area neurons, but Connelly and colleagues have examined the discharge patterns of pre-Botzinger neurons in the cat (Connelly et al., 1992). The pre-Botzinger complex was characterized by a mix or neurons with inspiratory modulated (70% of the neurons), expiratory modulated (22%) and phase spanning (8%) discharge patterns. Among the phase spanning neurons, there were a relatively high percentage of pre-inspiratory neurons, and these types of neurons have been proposed by Feldman's group as key elements in respiratory rhythm generation (Connelly et al., 1992).

We are not the first to show that microinjection of excitatory amino acids into the ventrolateral medulla of anesthetized cats causes apnea. For example, McCrimmon and colleagues describe a site that is more caudal (1.5 mm rostral to obex) and medial to the caudal subretrofacial area where microinjection of excitatory amino acids produces apnea (McCrimmon et al., 1986). Bongianni and colleagues, on the other hand, describe a site that appears more rostral to the caudal subretro-

facial area where microinjection of D,L-homocysteic acid produces apnea (Bongianni et al., 1993). As shown in Fig. 8 of their paper, the critical site lies mostly ventral to the facial nucleus, a region that is clearly rostral to the caudal subretrofacial area.

In addition to the unanticipated finding that microinjection of excitatory amino acids into the caudal subretrofacial area would produce apnea, we also did not expect to find it so difficult, and in fact impossible, to block the apneic effect of microinjection of L-glutamate into the caudal subretrofacial area. Initially, we attempted to block L-glutamate with CPP, and then with CNQX, both in doses that were able to block apnea evoked by microinjection of NMDA and AMPA into the caudal subretrofacial area, respectively. Next, we attempted to block L-glutamate with a combination of CPP and CNQX, and with kynurenic acid, but with no success. From these data it appeared that L-glutamate was *not* evoking apnea at the caudal subretrofacial area by activating either a NMDA or a non-NMDA ionotropic receptor (i.e., an AMPA or kainate receptor). We also considered the possibility that L-glutamate might be affecting an L-AP4 receptor site. However, studies of Sheardown (1988) indicate that CNQX blocks the L-AP4 site as well as the non-NMDA receptor, thus suggesting a lack of involvement of the L-AP4 receptor site in mediating the respiratory effect of L-glutamate at the caudal subretrofacial area. Furthermore, we considered the possibility that L-glutamate might be exerting its respiratory effect by activating a metabotropic receptor. We investigated this possibility by studying the effect of the metabotropic receptor agonist drug, L-*trans*-ACPD. L-*trans*-ACPD microinjected into the caudal subretrofacial area in a dose that exerts a profound effect on arterial blood pressure when microinjected into the caudal ventrolateral medulla had no effect on respiration. Finally, we tested the combination of CPP and CNQX against a small dose of L-glutamate microinjected into the caudal subretrofacial area and were still unable to counteract the L-glutamate-induced respiratory depression. Taken together, our data indicate that apnea induced by L-glutamate at the caudal subretrofacial area is not mediated by a NMDA and non-NMDA ionotropic receptor (i.e., AMPA or kainate receptor), an L-AP4 receptor or a metabotropic receptor that responds to L-*trans*-ACPD.

It is difficult to understand why either the combination of CPP and CNQX or kynurenic acid does not at least partially antagonize the effect of L-glutamate since there are clearly NMDA- and AMPA-type excitatory amino acid receptors present at the caudal subretrofacial area, and L-glutamate is able to activate both of these types of receptors (Collingridge and Lester, 1989). This suggests that L-glutamate is acting specifically to activate an undefined excitatory amino acid receptor in the caudal subretrofacial area. However, L-glutamate is known to act non-specifically on all of the known excitory amino acid receptors. Hence, a tentative explanation is that L-glutamate activation of just one type of excitatory amino acid receptor (one that is yet defined) at the caudal subretrofacial area is sufficient to produce a full response on respiration.

There have been other reports wherein blockade of excitatory ionotropic receptors failed to antagonize the effect of exogenously administered glutamate on CNS neurons (Leone and Gordon, 1989; Talman, 1989; Pawloski-Dahm and Gordon, 1992). In these studies blockade of ionotropic receptors was carried out using kynurenic acid. The explanation for why kynurenic acid failed to antagonize exogenously administered glutamate at the nucleus tractus solitarius is that metabotropic L-*trans*-ACPD receptors exist in the nucleus tractus solitarius and are mediating the pharmacological effect of exogenously administered glutamate (Pawloski-Dahm and Gordon, 1992). Evidence for this is based on the finding that microinjection of L-*trans*-ACPD into a site in the nucleus tractus solitarius where exogenously administered glutamate produces kynurenic acid-insensitive cardiovascular effects mimics the cardiovascular effects of glutamate. Additionally, the cardiovascular effects evoked by L-*trans*-ACPD are unaffected by microinjection of kynurenic acid into the nucleus tractus solitarius (Pawloski-Dahm and Gordon, 1992). As mentioned above, L-*trans*-ACPD microinjected into the caudal subretrofacial area had no effect on respiratory activity. Hence, it is unlikely that the kynurenic acid-insensitive effect evoked by L-glutamate in the present study was due to L-glutamate activating a metabotropic L-*trans*-ACPD receptor.

In an attempt to understand why activation of excitatory amino acid receptors at the caudal subretrofacial area and blockade of excitatory amino acid receptors at this site both produce apnea, we recently considered the possibility that excitatory amino acids might activate GABA containing interneurons in the caudal subretrofacial area, and the GABA, in turn, would inhibit neurons involved in maintaining normal respiratory activity (McManigle et al., 1991). To test this possibility we first microinjected the $GABA_A$ receptor antagonist bicuculline into the caudal subretrofacial area and followed this microinjection with either L-glutamate, NMDA or AMPA microinjected into the same site. Under these conditions, L-glutamate, NMDA and AMPA were all observed to increase respiratory activity (McManigle et al., 1991). These preliminary data indicate that activation of excitatory amino acid receptors at the caudal subretrofacial area can stimulate breathing provided that GABA input into this area is first inhibited.

Another focus of the present study was to determine

the effect of synaptically released excitatory amino acid neurotransmitter at the caudal subretrofacial area on breathing. To make this determination we used an inhibitor of the uptake of L-glutamic acid, namely L-*trans*-2,4-PDC, and assumed that respiratory effects produced by this agent would be due to inhibition of uptake of an endogenously released excitatory amino acid, and accumulation of the excitatory amino acid at postsynaptic receptors. The uptake inhibitor was found to produce apnea, and the type of apnea noted resembled that observed with microinjection of L-glutamate, NMDA and AMPA.

L-*trans*-2,4-PDC appears to exert its pharmacological effect by inhibiting L-glutamate uptake at the synaptic cleft. L-*trans*-2,4,PDC is a stronger and more selective glutamate uptake blocker than other glutamate uptake blockers (Bridges et al., 1991; Pines et al., 1992; Sarantis et al., 1993), but does have a weak releasing effect on endogenous glutamate (Waldmeier et al., 1993). L-*trans*-2,4-PDC is transported by the glutamate uptake carrier, but with a V_{max} which is much lower than for glutamate transport (Sarantis et al., 1993). Thus, L-*trans*-2,4-PDC 'inhibits glutamate uptake both by competing for occupancy of the uptake carrier and by occupying it for a longer time than glutamate does per carrier cycle' (Sarantis et al., 1993).

In terms of determining the type of excitatory amino acid receptor(s) that mediate the transient apnea response, we were not able to counteract the transient apnea induced by L-*trans*-2,4-PDC with either CPP or CNQX alone, but the combination of CPP and CNQX was effective in blocking the response. These data suggest that both NMDA and non-NMDA ionotropic receptors mediate the effect produced by L-*trans*-2,4-PDC.

In speculating as to whether L-glutamate could be the excitatory amino acid neurotransmitter that is synaptically released at the caudal subretrofacial area, data indicate that L-glutamate is probably not the endogenously occurring excitatory amino acid neurotransmitter at the caudal subretrofacial area. L-*trans*-2,4-PDC and L-glutamate microinjected into the same site in the caudal subretrofacial area elicited nearly identical responses on respiration. However, the effect of L-*trans*-2,4-PDC was consistently blocked by administering antagonists of the NMDA and non-NMDA receptors together. In contrast, the effects of L-glutamate showed no alteration by coadministration of antagonists of the NMDA and non-NMDA receptors. These data argue against L-glutamate as being the neurotransmitter for the transient apnea evoked from the caudal subretrofacial area. If this is the case, then the question arises as to what is the excitatory amino acid neurotransmitter at the caudal subretrofacial area, and how is L-*trans*-2,4-PDC exerting its pharmacological action? The brain L-glutamate transporter has been shown not only to transport L-glutamate, but also D- and L-aspartate and cysteine sulphinate (Pines et al., 1992). On the other hand, homocysteic acid is poorly transported (Cox et al., 1977). Hence, aspartate and/or cysteine sulphinate should be considered in future studies as possible endogenously occurring excitatory amino acids at the caudal subretrofacial area.

Acknowledgements

Preliminary reports of the study appeared in Am. Rev. Respir. Dis. 143: A195, 1991, and Neurosci. Abstr. 17: Part 1, 201, 1991.

This work was supported by NIH Program Project Grant PO1 NS-28130.

Opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

The experiments herein were conducted according to the principles set forth in the Guide for The Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education, and Welfare Publication (NIH) 78-23.

The authors thank Mr. Edwin L. Gibbs, Ms. Christa Highley, and Mrs. Carolyn Greenfield for their excellent typing of the manuscript.

References

Abrahams, T.P., P.J. Hornby, D.P. Walton, A.M.T. DaSilva and R.A. Gillis, 1991, An excitatory amino acid(s) in the ventrolateral medulla is (are) required for breathing to occur in the anesthetized cat, J. Pharmacol. Exp. Ther. 259. 1388.

Benjamin, A.M. and J.N. Quastel, 1976, Cerebral uptake and exchange diffusion in vitro of L-and D-glutamate, Neurochemistry 26, 431.

Bongianni, F., M. Corda, G.A. Fontana and T. Panteleo, 1993, Excitatory and depressant respiratory responses to chemical stimulation of the rostral ventrolateral medulla in the cat, Acta. Physiol. Scand. 148, 315.

Bridges, R.J., M.S. Stanley, M.W. Anderson, C.W. Cotman and A.R. Chamberlin, 1991, Conformationally defined neurotransmitter analogues. Selective inhibition of glutamate uptake by one pyrrolidine-2,4-dicarboxylate disatereomer, J. Med. Chem. 34, 717.

Collingridge, G.L. and A.J. Lester, 1989, Excitatory amino acid receptors in the vertebrate central nervous system, Pharamcol. Rev. 40, 143.

Connelly, C.A., E.G. Dobbins and J. Feldman, 1992, Pre-Botzinger complex in cats: respiratory neuronal discharge patterns, Brain Res. 590, 337.

Cox, D.W.G., P.M. Headley and J.G. Watkins, 1977, Actions of L-and D-homocysteate in rat CNS: a correlation between low-affinity uptake and the time courses of excitation by microelectrophoretically applied L-glutamate analogues, J. Neurochem. 29, 579.

Curtis, D.R., A.W. Duggan and J.C. Borstein, 1970, The inactivation of extracellular administered amino acids in the feline spinal cord, Exp. Brain Res. 10, 447.

DiChiara, G. and G.L. Gessa (eds.), 1981, Glutamate as a Neurotransmitter (Raven Press, New York).

Gebber, G.L., S.M. Barman and M. Zviman, 1989, Sympathetic activity remains synchronized in presence of glutamate antagonist, Am. J. Physiol. 256, R722.

Gillis, R.A., D.P. Walton, J.A. Quest, I.J. Namath, P. Hamosh and K.L. Dretchen, 1988, Cardiorespiratory effects produced by activation of cholinergic muscarinic receptors on the ventral surface of the medulla, J. Pharmacol. Exp. Ther. 247, 765.

Jackson, H., E.F. Nemeth and T.N. Parks, 1985, Non-*N*-methyl-D-aspartate receptors mediating synaptic transmission in the avian cochlear nucleus: effects of kynurenic acid, dipicolinic acid and streptomycin, Neuroscience 16, 1971.

Leone, C. and F.J. Gordon, 1989, Is L-glutamate a neurotransmitter of baroreceptor information in the nucleus of the tractus solitarius?, J. Pharmacol. Exp. Ther. 250, 953.

Lipski, J. and E.G. Merrill, 1980, Electrophysiological demonstration of the projection from expiratory neurons in rostral medulla to contralateral dorsal respiratory group, Brain Res. 197, 521.

Lipski, J., M.C. Bellingham, M.J. West and P. Pilowsky, 1988, Limitations of the technique of pressure microinjection of excitatory amino acids for evoking responses from localized regions of the CNS, J. Neurosci. Methods 26, 169.

McCrimmon, D.R., J.L. Feldman and D.F. Speck, 1986, Respiratory montoneuronal activity is altered by injections of picomoles of glutamate into cat brain stem, J. Neurosci. 6, 2384.

McManigle, J.E., A.M.T. DaSilva, S. Pineo and R.A. Gillis, 1991, Blockade of GABA in the caudal-subretrofacial area of the ventrolateral medulla in the cat unmasks a respiratory stimulant effect of *N*-methyl-D-asparate, FASEB J. 5, A734.

McManigle, J.E., I.L. Berger, W.H. Panico, P. Hamosh, A.M.T. DaSilva and R.A. Gillis, 1992, Evidence for the presence of a metabotropic excitatory amino acid receptor in the ventrolateral medulla of the cat, FASEB J. 6, A1509.

Palmer, E., O.T. Monaghan and C.W. Cotman, 1989, *trans*-ACPD, a selective agonist of phosphoinositide-coupled excitatory amino acid receptor, Eur. J. Pharamacol. 166, 585.

Pawloski-Dahm, C. and F.J. Gordon, 1992, Evidence for a kynurenate-insentive glutamate receptor in nucleus tractus solitarii, Am. J. Physiol. 262, H 1611.

Pines, G., N.C. Danbolt, M. Bjoras, Y. Zhang, A. Bendahan, L. Eide, H. Koepsell, J. Storm-Mathisen, P. Seeberg and B.I. Kanner, 1992, Cloning and expression of a rat brain L-glutamate transporter, Nature *360*, 464.

Sarantis, M., L. Ballerini, B. Miller, R.A. Silver, M. Edwards and D. Attwell, 1993, Glutamate uptake from the synaptic cleft does not shape the decay of the non-NMDA component of the synaptic current, Neuron 11, 541.

Schousboe, A., 1981, Transport and metablism of glutamate and GABA in neurons and glia cells, Int. Rev. Neurobiol. 22, 1.

Sheardown, M.J., 1988, A new and specific non-NMDA receptor antagonist, FG 9065, blocks L-AP4-evoked depolarization in rat cerebral cortex, Eur. J. Pharmacol. 148, 471.

Stone, T.W. and J.H. Connick, 1985, Quinolinic acid and other kynurenines in the central nervous system, Neuroscience 15, 597.

Talman, W.T., 1989, Kynurenic acid microinjected into the nucleus tractus solitarius of rat blocks the arterial baroreflex but not responses to glutamate, Neurosci. Lett. 102, 247.

Waldmeier, P.C., P. Wicki and J.-J. Feldtrauer, 1993, Release of endogenous glutamate form rat cortical slices in presence of the glutamate uptake inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylic acid, Naunyn-Schmied. Arch. Pharmacol. 348, 478.