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Activation of locus coeruleus neurons by nucleus paragigantocellularis or noxious sensory stimulation is mediated by intracoerulear excitatory amino acid neurotransmission

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The nucleus paragigantocellularis (PGi), located in the rostral ventrolateral medulla, is one of two major afferents to the nucleus locus coeruleus (LC). Electrical stimulation of PGi exerts a robust, predominantly excitatory influence on LC neurons that is blocked by intracerebroventricular (i.c.v.) administration of the broad spectrum excitatory amino acid (EAA) antagonists kynurenic acid (KYN) or γ-D-glutamylglycine (DGG), but not by the selective *N*-methyl-D-aspartate (NMDA) receptor antagonist 2-amino-7-phosphonoheptanoate (AP7). I.c.v. injection of KYN or DGG also blocked activation of LC neurons evoked by noxious somatosensory stimuli. These results indicate that activation of LC neurons by PGi and noxious stimuli may be mediated by an EAA acting at a non-NMDA receptor in LC. In the present study, microiontophoretic techniques were used to determine the sensitivity of LC neurons in vivo to the selective EAA receptor agonists kainate (KA), NMDA and quisqualate (QUIS). Microinfusion and microiontophoresis were also used to determine whether direct application of KYN, the preferential non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3 dione (CNQX) or the selective NMDA receptor antagonist 2-amino-5-phosphonovalerate (AP5) onto LC neurons blocked excitation elicited by stimulation of PGi or the sciatic nerve. The results demonstrated that individual LC neurons were robustly activated by direct application of KA, NMDA and QUIS. Iontophoretically applied KYN reduced or completely antagonized responses evoked by all 3 agonists. In contrast, iontophoretically applied AP5 strongly attenuated NMDA-evoked excitation, while KA-and QUIS-evoked responses were not affected by this agent. Furthermore, direct application of KYN or the specific non-NMDA receptor antagonist, CNQX, onto LC neurons substantially attenuated or completely blocked synaptic activation produced by PGi or sciatic nerve stimulation in nearly every LC neuron tested. Microinfusion of the selective NMDA receptor antagonist AP5 had no effect on sciatic nerve-evoked responses. These results confirm our hypothesis that activation of LC neurons from PGi is mediated by an EAA operating primarily at a non-NMDA receptor subtype on LC neurons. Furthermore, these findings provide additional support for the hypothesis that this pathway mediates at least some sensory-evoked responses of LC neurons.

INTRODUCTION

Recent retrograde tracing studies have identified the nucleus paragigantocellularis (PGi) in the rostral ventrolateral medulla as a major afferent to the nucleus locus coeruleus (LC)[13,15]. The strong projection from the PGi to the LC has been confirmed with retrograde and anterograde tracing using a variety of tracers[4,37,47], and this pathway has also been confirmed with antidromic activation[30]. Electrical activation of PGi produces predominant excitation of LC neurons[28,29]; approximately 73% of LC neurons were synaptically activated by low intensity, single-pulse electrical stimulation of PGi.

PGi neurons stain for markers of several neurotransmitters and neuropeptides which have been reported to excite LC neurons (for review, see ref. 15). Recently, we demonstrated[29] that PGi-evoked excitation of LC neurons is completely blocked after intracerebroventricular (i.c.v.) administration of the broad spectrum excitatory amino acid (EAA) antagonists kynurenic acid (KYN) or γ-D-glutamylglycine (DGG)[22,23,61]. In addition, results from our laboratory[29], recently confirmed by others[27,39,49,54], showed that i.c.v. KYN injection also blocked activation of LC neurons evoked by a paw-pinch or by electrical activation of the sciatic nerve directly or via rear footpad stimulation (FS). However, i.c.v. administration of the selective

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N-methyl-D-aspartate (NMDA) receptor antagonist 2-amino-7-phosphonoheptanoate (AP7)[31] did not affect PGi-evoked excitation, and only modestly reduced FS-evoked activation of LC neurons[29]. Taken together, these results indicate that activation of LC from PGi may be mediated by an EAA acting primarily at a non-NMDA receptor, and suggest that this pathway mediates the characteristic excitation of LC neurons evoked by noxious somatosensory stimuli.

While these pharmacologic results indicate that EAA projections from PGi may activate LC, the sensitivity of LC neurons in vivo to kainate (KA), NMDA and quisqualate (QUIS) receptor agonists, and the ability of KYN to block responses evoked by these agonists, has not been determined. Furthermore, our previous studies with i.c.v. injection of KYN and DGG did not allow identification of the site(s) at which EAA neurotransmission occurs to mediate activation of LC neurons from PGi or noxious sensory stimulation[29].

The present experiments were performed to resolve these issues. The results demonstrated that LC neurons are robustly activated by microiontophoretic application of KA, NMDA, or QUIS. KYN attenuated responses evoked by all 3 agonists, but most potently antagonized responses evoked by KA, while application of the selective NMDA receptor antagonist 2-amino-5-phosphonovalerate (AP5) attenuated NMDA-evoked responses, but not QUIS- or KA-evoked responses. Direct application of KYN or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) by microiontophoresis or microinfusion substantially reduced or completely blocked synaptic activation of LC neurons elicited from either PGi or FS. In contrast, microinfusion of NMDA receptor antagonists did not affect sensory-evoked excitation of LC neurons. These results confirm our previous hypothesis[12,29] that activation of LC neurons from PGi or FS is mediated by an EAA operating primarily at a non-NMDA receptor, and demonstrate that this transmission occurs within LC. Taken together with other recent findings, these results support the hypothesis that the projection from PGi to LC mediates at least some sensory-evoked responses of LC neurons. Preliminary reports of this work have appeared elsewhere[12,15].

MATERIALS AND METHODS

Twenty-seven male Sprague–Dawley rats (Zivic-Miller; 275–350 g) were used in these experiments. Animals were initially anesthetized with 400 mg/kg chloral hydrate, i.p.; additional injections of 10 mg chloral hydrate every 30 min maintained a deep and constant level of anesthesia as determined by lack of movement to a strong tail pinch. A tracheal cannula was inserted (tracheal ventilation occurred by spontaneous respiration) and animals were placed in a stereotaxic instrument with the incisor bar lowered to angle the skull approximately 24 degrees from the horizontal plane (bregma 3 mm below lambda). Body temperature was maintained at 36–37°C with a thermistor-controlled heating pad.

Stimulation electrode implantation

The neck tissue at the caudal skull margin was reflected and the occipital bone over the caudal cerebellum was partially removed to reveal the caudal apex of the IVth ventricle. Placement of stimulation electrodes in PGi was accomplished using electrophysiological landmarks as previously described[29,30]. Stimulation electrodes consisted of a pair of twisted 150-μm- or 250-μm-diameter stainless steel wires, insulated except for bluntly cut tips. Two 26-gauge needles were placed subcutaneously in the medial contralateral rear foot for electrical stimulation of the sciatic nerve[14]. Electrical stimuli were bipolar pulses (0.5-ms duration) from a square wave stimulator (Grass S88) and a constant current stimulus isolation unit (Grass PSIU6). Logic pulses synchronized with PGi or footpad stimulation (FS) were led to a computer for on-line peri-stimulus time histogram (PSTH) generation.

LC recordings

A 3-mm-diameter hole was drilled in the skull above LC (4.7 mm caudal to lambda and 1.2 mm lateral to midline), and the dura was reflected. Extracellular recordings from individual neurons were obtained with glass micropipettes (2–4 μm tip diameter, 10–20 MΩ impedance) filled with 2% Pontamine sky blue dye in 0.5 M sodium acetate. Recording micropipettes were glued adjacent to infusion or iontophoretic arrangements as described below. Recordings were obtained and processed by standard electrophysiological methods and LC neurons were tentatively identified by their characteristic electrophysiologic properties (Fig. 2), as previously described[29]. Baseline spontaneous discharge was monitored for 1.5–3 min and collected on-line by computer. Neuronal responses to single-pulse PGi stimulation were examined and threshold for synaptic activation (driving on approximately half the stimuli) was determined. PSTHs of LC responses to PGi or FS were generated for 50 consecutive stimulus trials presented at 0.5/s, and 1.5–2×threshold (up to 1.2 mA).

Microiontophoresis

A 7-barrel iontophoretic micropipette (10–20 μm tip diameter) was glued adjacent to a single-barrel recording micropipette with the recording micropipette tip extending 12–35 μm beyond the tip of the 7-barrel array. As described previously[6,21], these multibarrel iontophoretic pipettes yielded large amplitude, stable extracellular recordings from individual LC neurons (Fig. 2). The center and one outer barrel of the iontophoretic pipette were filled with 4 M NaCl for automatic current balancing and control current ejections. The other 5 barrels were filled with the following iontophoretic solutions (in distilled water): KA (10 mM, pH 8.2), NMDA (10 mM, pH 8.3), QUIS (5 mM, pH 8.3), AP5 (20 mM, pH 8.1) and KYN (100 mM, pH 8.1). Agents were iontophoretically ejected with currents of −1 to −200 nA, and retained with currents of +1 to +20 nA. To determine the effects of antagonists on PGi- or FS-evoked responses, continuous iontophoretic application of a desired antagonist was initiated 1.5 min before, and continued throughout, accumulation of PSTHs.

Local microinfusion

A 23 gauge guide cannula was glued adjacent to a recording micropipette, placing the tip of cannula approximately 3.0 mm above the tip of the pipette. KYN was infused through a 33 gauge needle, connected with polyethylene tubing to a 1 μl Hamilton syringe. Insertion of the injection needle into the guide cannula placed the tip of the injection needle 250–900 μm from the tip of the recording pipette. KYN was freshly prepared as a 0.1 M solution in 0.9% saline (pH 7.4), and infused in 50–200 nl volumes. The specific non-NMDA receptor antagonist CNQX and the selective NMDA receptor antagonist AP5 were dissolved in artificial cerebrospinal fluid at a concentration of 8 μM and were injected by pneumatic pressure (Picospritzer, General Valve, Inc.) from a calibrated glass capillary pipettes (Fisher Scientific; 35–50 μm tip diameter). The injection pipette was

glued adjacent to the recording micropipette which extended 125–150 μm beyond the injection tip as described previously[53]. Volumes injected were measured by monitoring the movement of the liquid column in the ejection pipette with an accuracy of ± 15 nl[53]. The pH of all solutions ranged from 7.4 to 7.8.

Histology

Micropipette penetrations were marked by iontophoretic ejection of dye from recording pipettes (-7 μA, 50% duty cycle for 10 min). At the end of recording sessions, $+30$ μA was passed through the stimulation electrode for 1 min to deposit iron at electrode tips. A 200–400 nl bolus of neutral red was injected through the inner cannula to confirm the LC microinfusion site. Animals were then deeply anesthetized and perfused with 10% formalin in 0.1 M phosphate buffer containing 5% potassium ferrocyanide, producing a Prussian blue reaction at iron deposits. All stimulation, recording, and infusion sites were histologically localized in 50-μm-thick tissue sections (Fig. 1).

Data analysis

Interspike interval histograms, computer-ratemeter records and chart recordings were used to monitor spontaneous discharge. To quantitate the effects of iontophoretically applied EAA agonists/antagonists, the mean discharge rate between drug ejection pulses was used as a control rate. Drug-induced changes in discharge rate were calculated by dividing the mean discharge rate of agonist-evoked responses by the mean control discharge rate to obtain the percent of control discharge rate. Statistical comparisons of absolute spontaneous firing rates were performed with 2-tailed Student's t-test. Other comparisons (i.e. percentage data) were performed with the non-parametric Wilcoxon signed rank test.

To quantitate PGi- or FS-evoked responses, individual PSTHs were analyzed by computer to determine excitatory and inhibitory epochs, as previously described[10,29]. A baseline period was defined as the 500 ms epoch preceding stimulation, and the mean and standard deviation of counts per baseline bin were determined. The onset of significant excitation was defined as the first of 5 consecutive bins (8 ms bin width) whose mean value exceeded mean baseline activity by two standard deviations, and response offset was determined as the time at which activity had returned to be consistently within 2 standard deviations of baseline. The following equation was used to normalize PSTHs for differing baseline activity and to calculate the magnitude of excitatory responses (Excitation R_{mag}):

$$\text{Excitation } R_{mag} = (\text{counts in excitatory epoch}) - (\text{mean counts per baseline bin} \times \text{number of bins in excitatory epoch}).$$

Inhibition was defined as an epoch of at least 15 bins in which the mean count per bin was less than 35% of that during baseline, and the magnitude of inhibition (Inhibition R_{mag}) was calculated as follows:

$$\text{Inhibition } R_{mag} = (\text{counts in inhibitory epoch}) - (\text{mean counts per baseline bin} \times \text{number of bins in inhibitory epoch}).$$

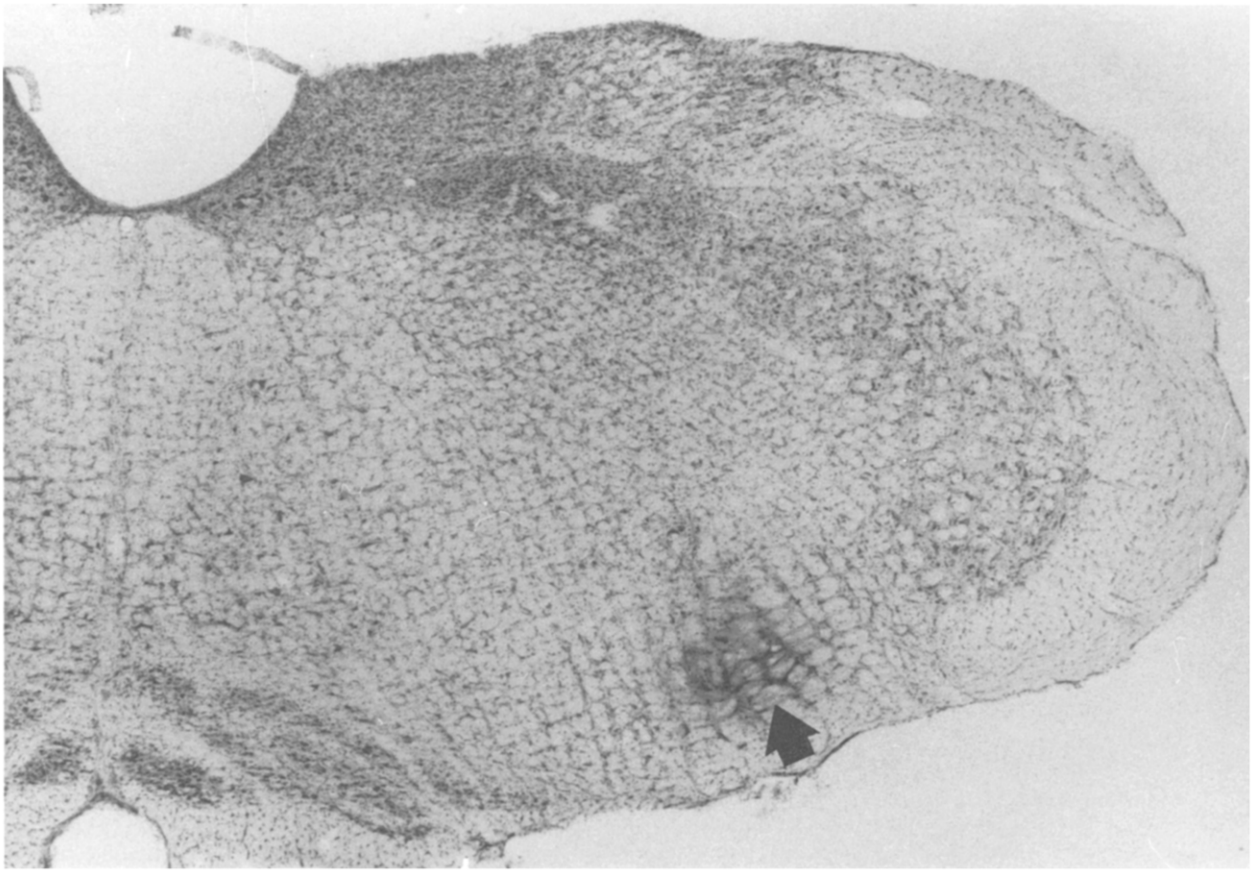

Fig. 1. Photomicrograph of a 50-μm-thick coronal section taken through the rostral medulla (Neutral red stain). Prussian blue mark (dark area in the ventrolateral medulla, at arrow) reveals stimulation site in PGi. Dorsal is at top, midline is to the left.

RESULTS

Effects of iontophoretically applied EAAs and EAA antagonists on LC discharge

Most LC neurons recorded with 7-barrel pipettes containing EAAs exhibited unusually high spontaneous discharge, ranging from 1.6 to 14 spikes/s, even with retaining currents of up to 20 nA. The mean spontaneous discharge rate (5.9 ± 0.8 spikes/s, $n = 22$) of LC neurons recorded with iontophoretic pipettes containing EAAs was significantly greater than that of LC neurons recorded with single barrel pipettes ($n = 12$, mean rate = 1.70 ± 0.24 spikes/s; $P < 0.001$, t-test). The mean spontaneous discharge of LC neurons recorded with EAA-containing iontophoretic pipettes was also substantially higher than that of LC neurons recorded with double barrel, KYN infusion/recording electrode assemblies (1.6 ± 0.7, $n = 5$; $P < 0.02$) or 7-barrel iontophoretic/recording assemblies containing GABA and GABA antagonists (2.2 ± 1.1, $n = 10$; $P < 0.005$). Thus, as previously described[6], the elevated spontaneous discharge rates of LC neurons recorded with EAA-containing iontophoretic pipettes is probably not due to tissue damage produced by the composite recording/iontophoretic pipettes, but instead, to leakage of EAAs from these pipettes.

As summarized in Table I and Fig. 3, LC neurons were potently activated by iontophoretic application of all 3 of the receptor subtype agonists tested. The response of LC neurons to QUIS was significantly greater than that for KA or NMDA ($P < 0.05$, Wilcoxon test). Furthermore, robust excitation of LC discharge with QUIS was obtained with consistently lower ejection currents than with KA or NMDA even though the concentration of QUIS used was one-half that of KA or NMDA. Current ejection through an adjacent sodium chloride barrel at intensities equal to or greater than those for the 3 agonists tested did not significantly affect LC discharge rate ($n = 8$, $P > 0.75$).

As shown in Fig. 3, continuous iontophoretic application of KYN significantly attenuated the response of LC neurons to all 3 agonists (Table I). KYN attenuated KA-evoked excitation of LC discharge to a greater extent than NMDA- or QUIS-evoked excitations ($P < 0.05$, Wilcoxon test). Although KYN consistently atten-

TABLE I

Effects of EAA agonists and antagonists on the spontaneous discharge rate of LC neurons

Effects of iontophoretically applied excitatory amino acid agonists and antagonist on the spontaneous discharge rate of LC neurons. Values are mean percent of control response ± standard error of the mean (S.E.M.).

	Agonist alone		*Agonist + KYN*		*Agonist + AP5*	
	n	*Mean*	*n*	*Mean*	*n*	*Mean*
KA	13	265.8 ± 34.3 ***	8	23.1 ± 5.5 **	2	90.2 ± 2.1
NMDA	15	278.4 ± 46.1 ***	7	45.8 ± 9.1 *	4	19.1 ± 8.4 **
QUIS	14	380.2 ± 49.9 ***	9	51.6 ± 10.5 *	4	112.1 ± 28.2

* $P < 0.04$, ** $P < 0.02$, *** $P < 0.001$. Paired t-tests were used to analyze differences between mean baseline and mean agonist-evoked discharge rate (column 1, Agonist Alone); Wilcoxon tests were used to compare the percent of control discharge rate during agonist application to that during agonist + antagonist application (columns 2 and 3).

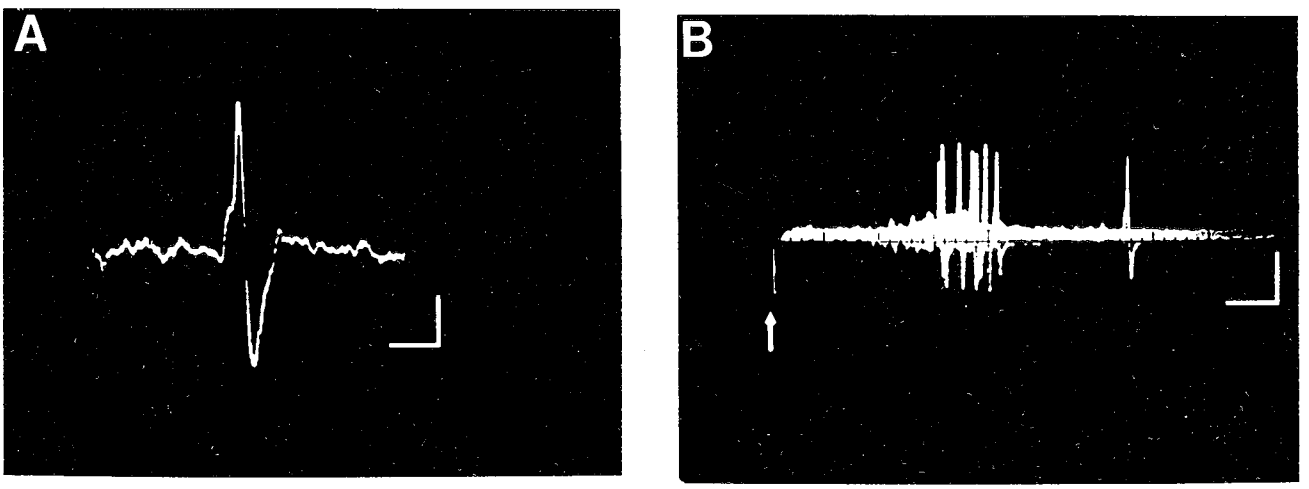

Fig. 2. A: oscilloscope photograph (filtered recording) of a typical action potential of an LC neuron recorded from the composite multibarrel glass micropipette used for extracellular recording and microiontophoresis in the present studies. Note, pronounced initial segment and broad waveform characteristic of LC action potentials recorded extracellularly, and large signal-to-noise discrimination. Calibration: vertical bar = 1.0 mV, horizontal bar = 2 ms. B: oscilloscope photograph (filtered recording) taken during electrical footpad stimulation (FS). FS (shown at arrow) activates this LC neurons at onset latencies ranging from 15 to 21 ms. Record contains 12 superimposed sweeps (0.5 stimuli/s). Calibration: vertical bar = 2.0 mV, horizontal bar = 5 ms.

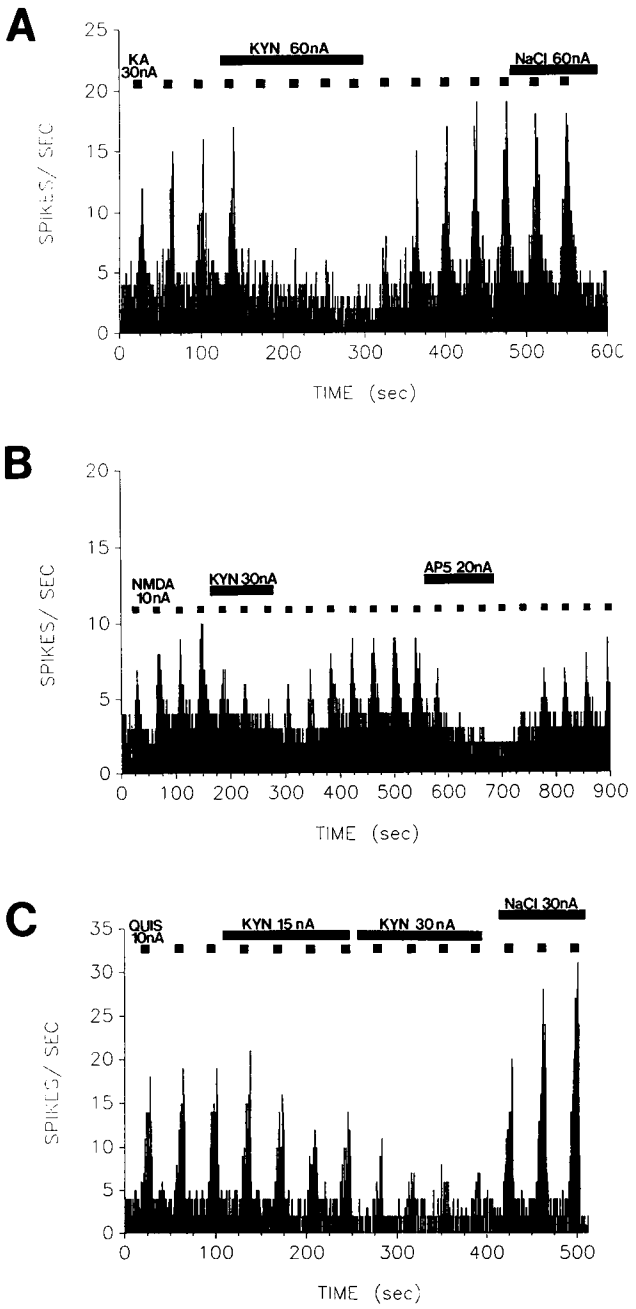

Fig. 3. A: computer ratemeter record showing excitation of an LC neuron by iontophoretic pulses of kainate (KA, 30 nA; applied at squares). Application of 60 nA kynurenate (KYN; solid bar at upper left) completely attenuated KA-evoked excitation whereas a similar current through an adjacent NaCl barrel (solid bar at upper right) had no effect on KA-evoked excitation. B: excitation of an LC neuron evoked by iontophoretically applied *N*-methyl-D-aspartate (NMDA, 10 nA; at squares) was attenuated by application of KYN (30 nA, solid bar at upper left). NMDA-evoked excitation was completely blocked during application of AP5 (20 nA, solid bar at upper right). C: ratemeter records showing excitation of an LC neuron elicited by iontophoretically applied quisqualate (QUIS, 10 nA; applied at squares). This excitation was attenuated, but not completely blocked, by KYN (15 and 30 nA, applied at upper solid bars). In contrast, similar current through an adjacent NaCl barrel (applied at upper solid bar, far right) did not affect QUIS-evoked excitation.

uated KA- or NMDA-evoked excitation in every cell tested, the ability of KYN to block QUIS-evoked excitation varied considerably from cell to cell, with some cells remaining responsive to QUIS in the presence of KYN while responses to KA or NMDA were blocked.

Continuous iontophoretic application of the selective NMDA receptor antagonist AP5 completely blocked or strongly attenuated NMDA-evoked excitation (Fig. 3B; $n = 4$, $P < 0.02$, Wilcoxon sign test), had no significant effect on QUIS-evoked excitation ($n = 4$, $P > 0.2$, Wilcoxon sign test) and had no substantial effect on KA-evoked responses in the two cells tested.

Effects of EAA antagonists on synaptically-evoked excitation of LC neurons

Consistent with our previous studies[28,29], single-pulse stimulation (mean stimulation amplitude = 500 μA) of PGi produced phasic, short-latency discharges in 19 of 25 LC neurons examined (Figs. 4 and 5). The mean onset latency and duration of these responses were 12.0 ± 1.6 ms and 27.7 ± 3.8 ms, respectively. Purely inhibitory responses to PGi stimulation were observed in some LC neurons, as previously reported[28,29]. As a pharmacological analysis of this inhibition has been recently published[7], experiments in the present study focused on excitatory responses. LC neurons were also robustly activated by footpad stimulation (FS; 2–10 mA) with a mean onset latency of 21.0 ± 1.0 ms and a mean duration of 26.5 ± 3.0 ms.

Microiontophoresis of KYN. As illustrated for a typical cell in Fig. 4, application of KYN at currents (50–195 nA) that substantially reduced activation of LC neurons elicited by exogenously-applied EAA agonists, significantly attenuated PGi-evoked excitation ($n = 11$; $P < 0.01$, paired t-test). Iontophoretic KYN completely blocked PGi-evoked excitation in 7 of 11 LC neurons tested, reduced the magnitude of PGi-evoked excitation of one cell by 41%, and had little effect on the remaining 3 cells. Current ejection through an adjacent barrel containing sodium chloride at intensities equal to those used for application of KYN did not alter the magnitude of PGi-evoked excitation of LC neurons (Fig. 4).

Iontophoretically applied KYN significantly attenuated FS-evoked excitation of LC neurons simultaneously with the blockade of PGi-evoked excitation (Fig. 4; $n = 7$, $P < 0.001$, paired t-test). FS-evoked excitation was completely blocked in 2 of 7 cells, and the magnitude of FS-evoked excitation of the other 5 cells was reduced by a mean of $49.3 \pm 3.8\%$.

Microinfusion of KYN. As iontophoretically applied KYN may not have reached adequate concentrations to block synaptic inputs onto the dendrites of LC

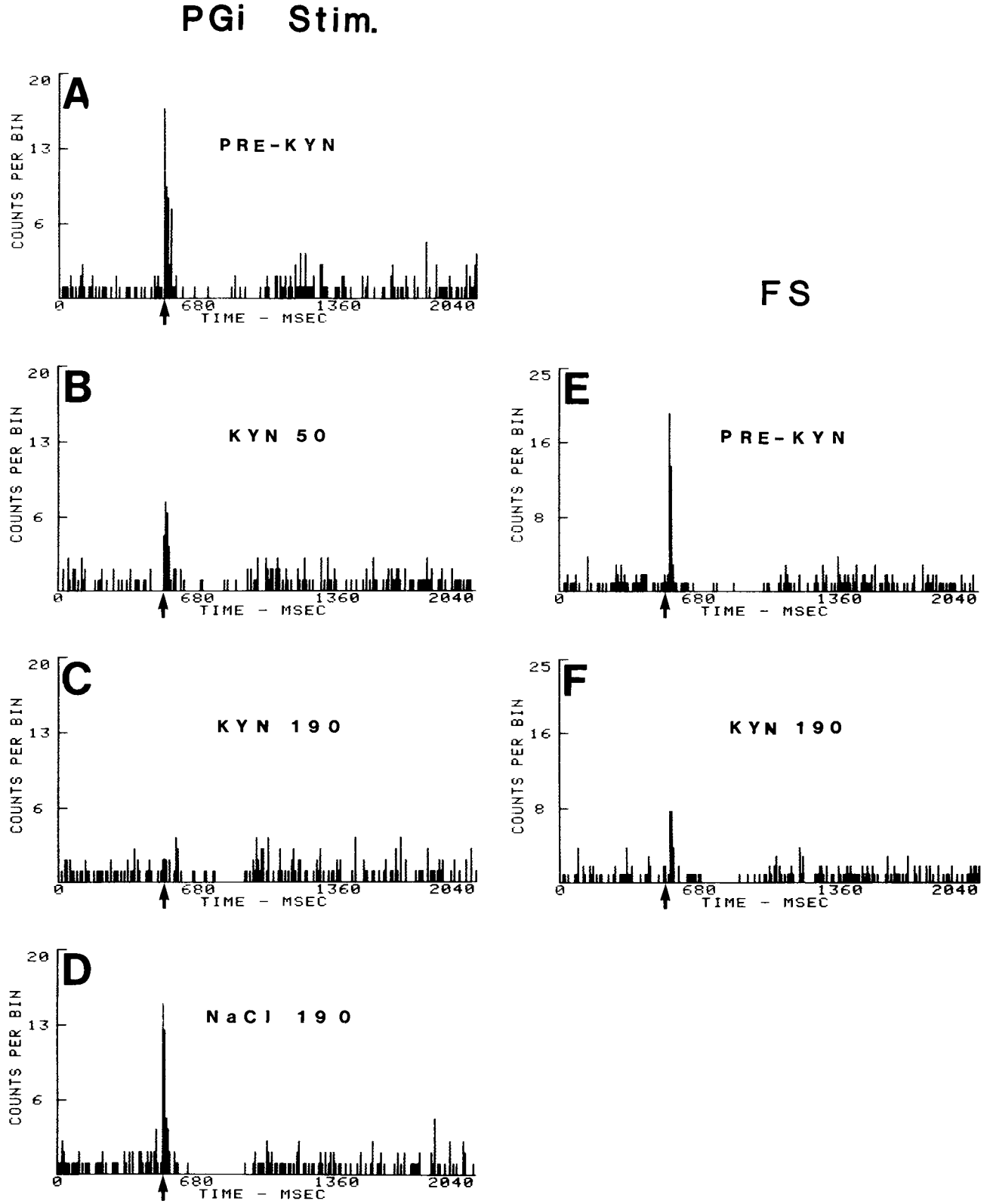

Fig. 4. Iontophoretically applied KYN simultaneously attenuates PGi- and footpad stimulation (FS)-evoked excitation of LC neurons. A: PSTH showing PGi-evoked excitation of an LC neuron. B: PGi-evoked excitation of the same cell was attenuated during continuous iontophoretic application of KYN (50 nA). C: application of KYN with a higher current (190 nA) completely blocked PGi-evoked excitation of this cell. D: continuous ejection of current through an adjacent NaCl-containing barrel did not attenuate PGi-evoked excitation of the same neuron. Stimulation amplitude in A–D = 400 μA. E: PSTH showing FS-evoked excitation of the same neuron shown in A–D. F: FS-evoked excitation was strongly attenuated during continuous iontophoretic-application of KYN (190 nA). Stimulation amplitude in (E and F) = 20 V. Stimuli (0.5/s) are at arrows in all PSTHs.

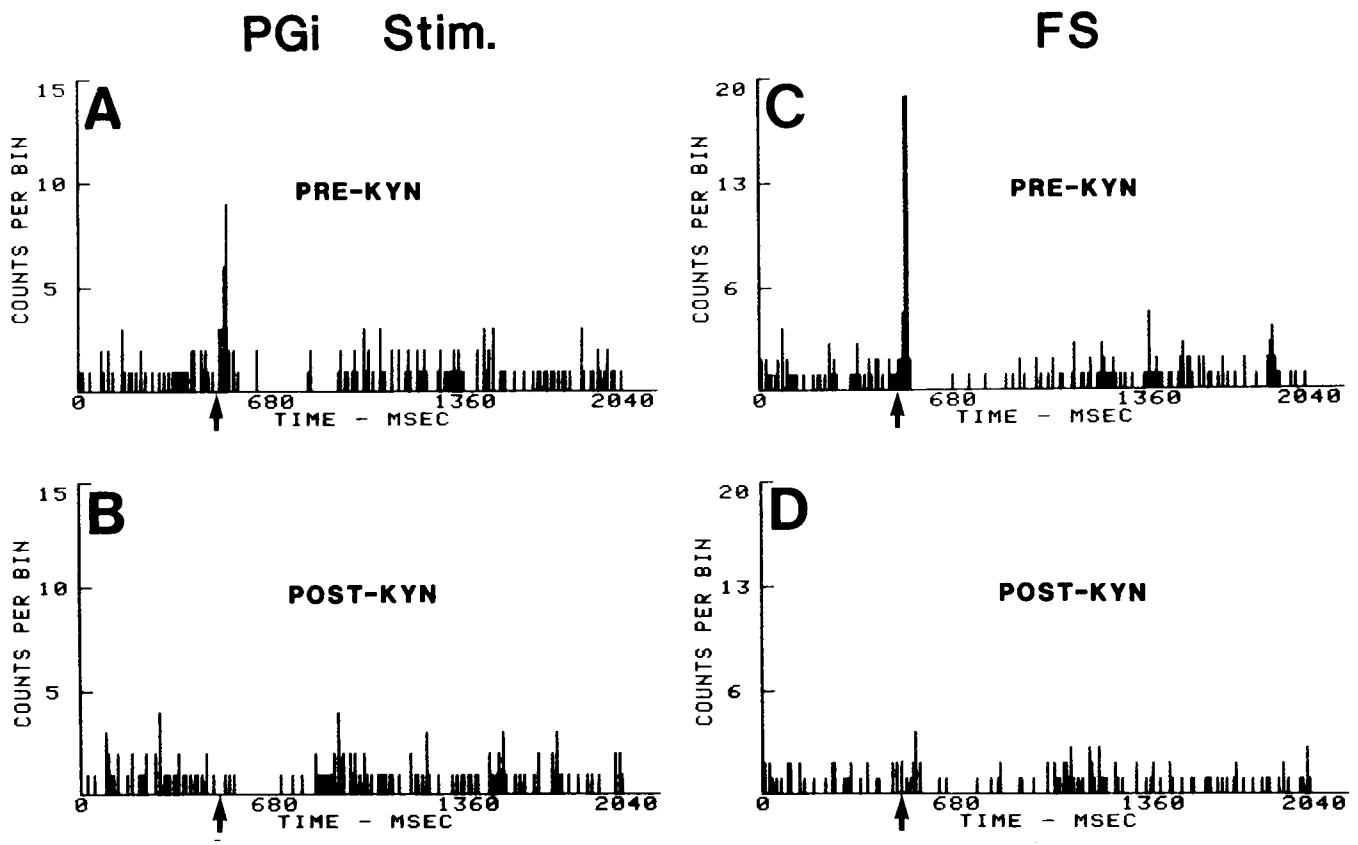

Fig. 5. Local microinfusion of KYN into LC simultaneously blocks PGi- and footpad stimulation (FS)-evoked excitation of LC neurons. A: peri-stimulus time histogram (PSTH) generated during single-pulse, electrical stimulation of PGi (at arrow). Stimulation of PGi yielded short latency, potent activation of this typical LC neuron. B: PGi-evoked excitation of the cell shown in A was completely attenuated 1 min after infusion of 0.01 μmol of KYN into LC, as shown in B post-drug. Note that blockade of excitation by KYN revealed a purely inhibitory response of this neuron to PGi stimulation. Stimulation intensity in A and B = 300 μA. C: PSTH showing FS-evoked excitation of the same LC neuron shown in A and B. D: FS-evoked excitation of this neuron was blocked 3.5 min after the same dose of KYN as in B. Stimulation intensity in panels C and D = 20 V. All PSTHs were generated for 50 consecutive stimulation trials. Stimuli (0.5/s) are at arrows in all PSTHs.

neurons, larger volumes of this agent were delivered by microinfusion directly into LC. As shown in Fig. 5, microinfusion of KYN into the LC rapidly (1–3 min) attenuated PGi-evoked excitation of LC neurons. Infusion of 0.01 μmols ($n = 3$) or 0.015 μmols ($n = 1$) of KYN completely blocked PGi-evoked excitation in all cells tested ($p = 0.008$, paired t-test). Responses returned to control values within 10–15 min after KYN infusion. KYN (0.005–0.01 μmols) infusion did not significantly change the spontaneous discharge rate of LC neurons (1.6 ± 0.4 spikes/s prior to KYN infusion compared to 1.2 ± 0.3 spikes/s after infusion; $n = 4$; $P > 0.05$).

As illustrated in Fig. 5, blockade of PGi-evoked excitation with local microinfusion of KYN revealed an underlying, purely inhibitory response to PGi stimulation in 3 of 4 LC neurons; the mean onset latency and mean duration of this inhibition were 10.7 ± 2.2 ms

TABLE II

Effects of EAA antagonists on baseline spontaneous discharge, and PGi- and FS-evoked excitation of LC neurons

Effects of locally infused (Inf.) or iontophoretically (Iont.) applied excitatory amino acid antagonists on spontaneous discharge rate and PGi- and FS-evoked excitation of LC neurons. Values are the mean percent of control (±S.E.M.) spontaneous discharge rate, and PGi- and FS-evoked excitation response magnitudes (R_{mag}, see Materials and Methods).

	KYN (Iont.)		*KYN (Inf.)*		*CNQX (Inf.)*		*AP5 (Inf.)*	
	n	*Mean*	*n*	*Mean*	*n*	*Mean*	*n*	*Mean*
Spont. rate	16	69.6±5.0 ***	5	75.3±5.0	11	87.9±4.7 *	8	94.0±2.5
PGi	11	43.8±11.6 **	4	−4.4±7.7 **	–	–	–	–
FS	7	42.8±5.5 ***	7	10.2±2.9 ***	11	38.2±8.6 ***	5	100.0±4.6

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; paired t-tests.

and 349.3 ± 51.3 ms, respectively. This finding is consistent with inhibitory responses of LC neurons observed after blockade of PGi-evoked excitation with intracerebroventricular injection of KYN or DGG[7,29].

Local microinfusion of KYN (0.005–0.02 μmol) blocked FS-evoked excitation of all 7 LC neurons tested ($P < 0.001$, paired t-test). As shown in Fig. 5, the dose of KYN that blocked PGi-evoked excitation simultaneously abolished FS-evoked excitation in all 4 cells tested. Blockade of FS-evoked excitation with microinfusion of KYN revealed purely inhibitory responses to FS in 4/7 of the LC cells tested, resembling results for PGi stimulation.

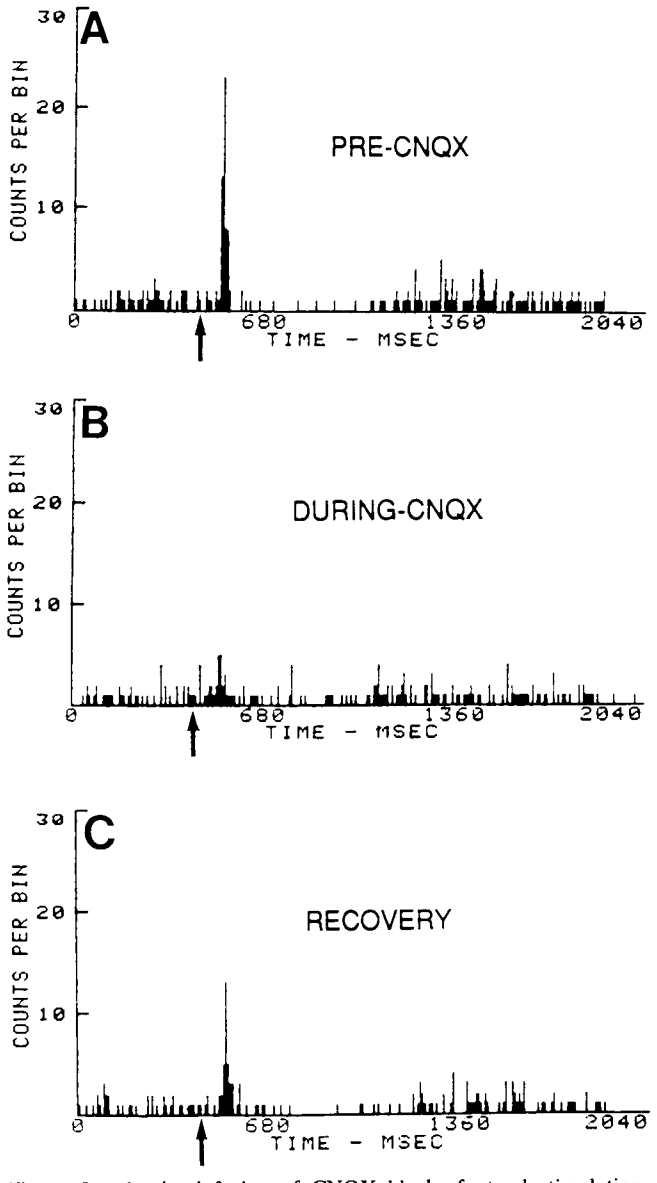

Fig. 6. Local microinfusion of CNQX blocks footpad stimulation (FS)-evoked excitation of LC neurons. A: PSTH showing FS-evoked excitation of an LC neuron. B: FS-evoked excitation of this same cell is attenuated after infusion of CNQX (2.9 pmol in 360 nl) into LC. C: FS-evoked excitation of the same neuron recovers by 4–6 min after CNQX infusion. Stimulation intensity = 90 V. Fifty consecutive stimuli (0.5/s, shown at arrows) in each PSTH.

CNQX and AP5 infusion. To further characterize the amino acid receptor subtypes mediating FS-evoked excitation of LC neurons, we examined the effect of local application of the specific amino acid receptor antagonists CNQX and AP5 (Table II and Fig. 6). Microinfusion of CNQX at a dose (0.5–5.0 pmol in 60–600 nl) previously shown to be selective for non-NMDA receptors[40], attenuated FS-evoked responses of LC neurons by a mean of 62% ($n = 11$, $P < 0.001$), completely blocking such excitation in two cells. FS-evoked responses returned to control values 3–6 min after CNQX infusion. CNQX elicited a small decrease in the spontaneous discharge rate of LC neurons (2.4 ± 0.2 spikes/s prior to CNQX infusion compared to 2.1 ± 0.3 spikes/s after infusion; $n = 11$, $P < 0.05$).

Microinfusion of the selective NMDA receptor antagonist AP5 at 50 or 100 μM (in 120–360 nl) concentrations had no effect on either the FS-evoked responses or the spontaneous discharge rate of LC neurons ($n = 4$, $P > 0.7$ for both).

DISCUSSION

The present results confirm our previous pharmacological findings that excitation of LC neurons from PGi or sciatic nerve stimulation is mediated by EAA neurotransmission, and demonstrate that these excitatory responses are mediated by activation of EAA receptors located in LC. In addition to blocking excitation from PGi, antagonism of EAA receptors in LC by intracoerulear microinfusion of KYN revealed purely inhibitory responses to PGi stimulation in LC cells that were excited prior to administration of this agent. This finding is in agreement with previous observations that blockade PGi-evoked excitation of LC neurons with KYN or DGG (i.c.v.) often revealed an underlying inhibitory response in the same neuron[7,29]. Recent anatomic and pharmacologic experiments indicated that such inhibition is at least partially mediated via direct adrenergic projections from C1 neurons in the PGi area to LC neurons[7,47].

LC neurons are excited by direct application of several putative excitatory neurotransmitters contained in PGi neurons. Immunohistochemical studies demonstrate that PGi neurons stain for markers of several excitatory transmitter candidates, including acetylcholine[17,41,51], glutamate/aspartate[55], *N*-acetylaspartylglutamate[32] (NAAG), substance P[42] and corticotropin releasing factor[59]. In addition, recent experiments demonstrated that some PGi neurons that project to LC stain for glutaminase, a marker for glutamate[24]. Excitatory responses of LC neurons to PGi or FS were not consistently altered by the muscarinic receptor

antagonist scopolamine, nor by the nicotinic receptor antagonist mecamylamine, indicating that these synaptic responses do not involve a substantial cholinergic component[29]. In contrast, excitation of LC from PGi or FS was completely blocked after i.c.v. administration of the broad spectrum EAA antagonists KYN or DGG[29].

In support of a possible EAA input to LC neurons, the present experiments revealed that LC neurons in vivo are robustly activated by the preferential EAA receptor subtype agonists KA, NMDA and QUIS, consistent with results in vitro[19,45]. KYN substantially attenuated responses of LC neurons to each of these agonists, however, KA-evoked excitation was attenuated to a greater extent than NMDA- or QUIS-evoked excitation. KYN has been reported to antagonize agonist-evoked excitations on the order of NMDA > KA $\geqq$ QUIS[33], however, more recent experiments demonstrated that this agent substantially attenuates NMDA- and KA-evoked excitation, but has little or no effect on QUIS-evoked excitation[38,56]. In agreement with these latter studies, we found that KYN was much less effective in attenuating QUIS-evoked excitation than KA- or NMDA-evoked excitation. It should be noted that while KYN's differential antagonism of responses to the agonists tested were consistent and substantial for the population of LC neurons examined, these findings should be interpreted with some care due to the technical limitations in comparing relative potencies of iontophoretically applied agonists and antagonists.

A major observation of the present study was the ability of directly applied KYN or CNQX to attenuate or completely block PGi-or FS-evoked excitation of nearly every LC neuron tested. These findings further support the hypothesis[12,29] that such excitation is mediated by an EAA released by PGi terminals onto LC neurons. While the results with KYN and CNQX do not alone identify the EAA receptor subtype involved, our previous finding that PGi-evoked excitation of LC neurons was completely blocked after i.c.v. administration of KYN or DGG, but not after large i.c.v. doses of the selective NMDA receptor antagonist AP7, suggested that a non-NMDA receptor may be primarily involved.

The present in vivo results are in excellent agreement with recent experiments in vitro by Cherubini et al.[19]. Intracellular recordings demonstrated that focal electrical stimulation of the LC area in the slice preparation elicits fast depolarizing postsynaptic potentials (PSPs) in LC neurons. A bicuculline-insensitive component of these PSPs was antagonized by bath application of KYN. As this PSP was largely resistant to NMDA receptor antagonists, Cherubini et al.[19] concluded that the depolarizing PSP was mediated by an EAA operating primarily at a non-NMDA receptor. In light of the present in vivo findings, it seems possible that this KYN-sensitive PSP is mediated, at least in part, by an EAA released from PGi terminals in the LC slice.

The finding that local microinfusion or iontophoresis of KYN or CNQX, but not AP5, attenuated FS-evoked excitation is consistent with our hypothesis that the EAA pathway from PGi to LC mediates sensory evoked excitation of LC neurons acting at non-NMDA receptors[12,15,29]. This hypothesis is supported by several other findings: (i) PGi receives sensory-related inputs from the brain and spinal cord[2,60]. (ii) A large population of PGi neurons, including many of those antidromically identified as afferent to LC, are robustly activated by FS and other noxious somatosensory stimuli[16,30,52]. (iii) Microinjection of the local anesthetic lidocaine, GABA or a synaptic decoupling Mn^{2+}/Cd^{2+} solution directly into PGi blocks FS-evoked excitation of LC neurons[8,20]. This blockade was most effective with injections into medial PGi; note, however, that others[48] failed to find decreased sciatic-induced responses of LC after tissue lesions in PGi. (iv) Blockade of PGi- and FS-evoked excitations share a similar pharmacological profile, such that these responses are simultaneously blocked by KYN or DGG, but not by AP7/AP5, glutamate diethylester or cholinergic antagonists[29]. Additional experiments are needed to determine if the EAA pathway from PGi to LC mediates the excitation of LC neurons evoked by non-noxious stimuli of other modalities in unanesthetized, behaving rats[10]. In this regard, it is noteworthy that the EAA pathway from PGi to LC has been recently reported to mediate the activation of LC neurons elicited by systemically administered nicotine[18,27,58], noxious and non-noxious thermal stimulation of the tail[39], and naloxone-induced opiate withdrawal[1,49,57].

The present results, taken together with our recent pharmacological study of PGi-evoked inhibition[7], demonstrate that PGi inputs mediate a potent, EAA-induced activation of LC neurons, and also an inhibition that is at least partly mediated by epinephrine. A large percentage of LC neurons are activated by PGi stimulation (73%) and a substantial percentage of LC cells also exhibit PGi-evoked inhibition after KYN or DGG (76–83%)[7,29]. Thus, the majority of LC neurons appear to be strongly regulated by both classes of inputs. It is noteworthy that PGi appears to exert a similar, bidirectional regulatory control on spinal preganglionic sympathetic neurons mediated by an EAA pathway and an inhibitory pathway utilizing epinephrine[35,36,43]. Blockade of PGi-evoked sympathoexcitation

by intrathecally administered KYN has been reported to unmask an underlying sympathoinhibitory response to PGi stimulation[36], a result similar to the inhibition of LC neurons observed in the present study after blockade PGi-evoked excitation by KYN.

The function(s) of projections from PGi to LC have not been determined. PGi has been implicated in pain regulation and polymodal sensory integration[2,16,34,51,60]. As noted above, our recent findings suggest that the PGi to LC pathway may mediate sensory evoked activation of LC neurons. In addition, PGi plays a well documented role in autonomic regulation; activation of PGi produces a broad sympathoexcitatory response, consisting of elevated sympathetic nerve discharge, blood pressure and heart rate[36,43,44,50]. Sympathoexcitatory stimuli also activate LC neurons[25,26], and PGi's parallel control of LC and the autonomic nervous system may underlie observations that LC neurons and sympathetic nerves exhibit parallel activity profiles in certain circumstances[25,26]. Previous work suggests that the LC plays a key role in the control of attentional state or vigilance[5,9–11,15]. Based on these findings, it is reasonable to speculate that PGi may coordinately activate LC and the sympathetic nervous system to facilitate integrated cognitive and behavioral adjustments necessary to respond to imperative and/or threatening environmental events.

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