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INHIBITION OF NORADRENERGIC LOCUS COERULEUS NEURONS BY C1 ADRENERGIC CELLS IN THE ROSTRAL VENTRAL MEDULLA

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Abstract—Recent anatomical and physiological experiments indicate that the nucleus locus coeruleus receives a predominant excitatory amino acid input, as well as a substantial inhibitory input, from the nucleus paragigantocellularis in the ventrolateral medulla. To determine whether C1 adrenergic neurons are involved in the inhibitory projection, the effects of the alpha-2 adrenoceptor antagonist, idazoxan, on inhibitory responses of locus coeruleus neurons to paragigantocellularis stimulation were characterized in the rat. Intravenous administration of idazoxan (0.2–1 mg/kg) attenuated paragigantocellularis-evoked inhibition, and often revealed an underlying weak excitation. Intraventricular administration of kynurenate, an excitatory amino acid antagonist, eliminated excitation from paragigantocellularis and disclosed an underlying inhibitory response in many locus coeruleus neurons that were previously excited by paragigantocellularis stimulation. These results revealed that about 90% of locus coeruleus neurons receive inhibition from the paragigantocellularis. Intravenous idazoxan significantly reduced such paragigantocellularis-evoked inibition, completely blocking this response in three of eight locus coeruleus cells tested. Idazoxan was much more potent when locally infused into the locus coeruleus. Local infusion of idazoxan (0.1–2.5 ng) into locus coeruleus produced a dose-dependent decrease of paragigantocellularis-evoked inhibition and completely blocked the inhibition in 10/33 locus coeruleus neurons, indicating that the site of idazoxan action was in the locus coeruleus.

These results extend our previous anatomical studies of adrenergic input to locus coeruleus, and indicate that C1 adrenergic neurons in the paragigantocellularis provide a direct inhibitory input to the great majority of locus coeruleus noradrenergic neurons. In addition, this is the first report of a neuronal response to activation of C1 adrenergic cells indicating that these neurons are strongly inhibitory when acting at alpha-2 receptors *in vivo*.

Recent anatomic and physiologic investigations demonstrated that one of the major afferents to the brain nucleus locus coeruleus (LC) is the nucleus paragigantocellularis (PGi)[5] in the ventrolateral rostral medulla.[13,15] This projection has been observed with a variety of anatomic techniques, including retrograde transport of wheat germ agglutinin-conjugated horseradish peroxidase (WGA–HRP),[13] rhodamine-labeled latex microspheres,[35] Fluoro-Gold[10,57] and cholera toxin- or WGA–HRP-coupled colloidal gold particles.[25,58] In addition, this pathway has been confirmed with anterograde transport of WGA–HRP[13] and *Phaseolus vulgaris* leuco-aglutinin.[35,74] Antidromic stimulation experiments have also confirmed projections from PGi to LC,[29] and revealed that there are at least two physiologically distinct populations of PGi neurons afferent to LC.

Additional studies found that electrical stimulation of PGi yielded two effects on LC discharge: a strong excitatory influence (73% of LC cells) and a pure inhibition (16% of LC cells).[27] The PGi excitatory input to LC appears to be mediated by an excitatory amino acid (EAA) pathway, as the excitatory LC response was blocked by the EAA antagonists kynurenic acid and gamma-D-glutamylglycine.[30] Interestingly, such blockade of excitation unmasked an underlying inhibitory response in many LC neurons tested, indicating that inhibitory control of LC from the PGi may be a prevalent influence of this major afferent as well.

The inhibition of LC from PGi has not been pharmacologically characterized. Among the putative neurotransmitters that may mediate PGi's inhibitory influence on LC, adrenaline is a strong candidate for several reasons. (i) Anatomical studies[10,56,57] demonstrated that the adrenergic innervation of LC[40,41] is largely provided by the C1 adrenergic cell

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Abbreviations: ACSF, artificial cerebrospinal fluid; EAA, excitatory amino acid; LC, locus coeruleus; i.c.v., intracerebroventricular; PGi, paragigantocellularis; PNMT, phenylethanolamine-*N*-methyltransferase; PNMT-IR, PNMT-immunoreactive; PSTH, peristimulus time histogram; R_{mag}, response magnitude; TH, tyrosine hydroxylase; WGA–HRP, wheatgerm agglutinin-conjugated horseradish peroxidase.

group located in the PGi.[6,41,43,62] (ii) Alpha-2 adrenoceptors are dense in LC[22,81] and adrenaline strongly inhibits LC discharge.[19,20,77] Conversely, alpha-2 receptor antagonists block the hyperpolarization of LC induced by adrenaline, noradrenaline, or the selective alpha-2 receptor agonist clonidine.[3,77] (iii) Inhibitors of phenylethanolamine-*N*-methyltransferase (PNMT), the specific biosynthetic enzyme for adrenaline, increase tyrosine hydroxylase (TH) activity in brainstem.[66] Similarly, electrolytic lesion of adrenergic fibers of the medullary bundle increases TH activity in the LC.[8] (iv) Recent ultrastructural studies demonstrate synapses between PNMT-immunoreactive (PNMT-IR) terminals and TH-IR somata and dendrites in LC.[42,49]

Using electrophysiologic and pharmacologic approaches, the present study tested the hypothesis that the inhibition of LC from PGi is mediated by adrenergic projections. The effects of the alpha-2 antagonist, idazoxan, on the inhibitory response of LC neurons to PGi activation were characterized. The results have been published in part in abstract form.[7]

EXPERIMENTAL PROCEDURES

Forty male Sprague–Dawley rats (Hilltop Labs or Taconic Farms, 275–375 g) were used in these experiments. Animals were anesthetized with chloral hydrate at an initial dose of 400 mg/kg, intraperitoneally, and additional injections (100 mg/kg) were administered throughout experiments as needed. Animals were intubated with a tracheal cannula and placed in a stereotaxic frame with the incisor bar lowered to place the skull approximately 24° from the horizontal plane (bregma 3 mm below lambda). Core temperature was maintained at 36–38°C with a feedback-controled heating pad.

Animal care was in accordance with the Public Health Service policy on the care and use of laboratory animals, and an assurance is on file with the Office for Protection from Research Risks.

Stimulation electrode implantation

The tissue and muscles of the neck at the caudal dorsal skull margin were reflected and the occipital bone over the caudal cerebellum was removed in part. The underlying dura was reflected to reveal the caudal apex of the IVth ventricle. Extracellular unit recordings from a glass micropipette were used to localize the exact coordinates for PGi stimulation electrode implantation, as previously described.[26,30,75] In brief, a micropipette was centered at the coordinates for the PGi and lowered through the cerebellum into the medulla. Typically, a group of neurons exhibiting prominent discharge with respiration was encountered within 1.5 mm of the ventral brain surface. The depth of the ventral brain surface (revealed by a sharp increase in noise in unfiltered pipette records) was noted. The pipette was removed and a stimulation electrode was implanted at the same coordinates, except for being 300–700 μm above the ventral brain surface (typically 6.5–7.5 mm ventral to the cerebellar surface). This procedure resulted in the consistent placement of stimulation electrodes within PGi (Fig. 1). Stimulation electrodes consisted of a pair of twisted 250-μm-diameter (20 animals) or 125-μm-diameter (20 animals) wires, insulated except for bluntly cut tips. Electrical stimuli were bipolar pulses (0.5 ms duration) from a constant-current stimulus isolation unit (Grass PSIU6) triggered by a square-wave stimulator (Grass S48). Logic pulses synchronized with brain stimuli were led to a computer for on-line peristimulus time histogram (PSTH) generation.

Locus coeruleus recording

Standard electrophysiologic procedures were used to record impulses extracellularly from individual LC neurons with glass micropipettes filled with a Pontamine Sky Blue dye solution (3–4 μm tip diameter, 10–20 MΩ impedance).[14,30] Digital pulses for action potentials were led to a computer and chart recorder for on-line data collection.

Micropipettes were stereotaxically advanced into LC ipsilateral to the PGi stimulation site, and LC neurons were tentatively localized by electrophysiologic characteristics, as described previously.[14,21,30] After stable recording from an individual LC neuron was obtained, a PSTH of the response to PGi stimulation was generated for 50 consecutive stimuli presented at 0.5 Hz and 300–700 μA.

Pharmacology

Intravenous (i.v.) injections were made into a jugular vein with idazoxan (0.5 or 1 mg/ml) dissolved in 0.9% saline. For local microinfusions, idazoxan [50 nl of a 200, 40 or 8 μM solution in 0.9% saline or artificial cerebrospinal fluid (ACSF), pH 7.4, over 30–60 s] was infused into the LC from a calibrated glass pipette (40–70 μm tip diameter) glued immediately adjacent to the recording pipette but recessed 100–200 μm. Microscopic measurement of the liquid column relative to the calibration grid on the injection pipette assured accurate injection of volumes as small as 6 nl.

Kynurenic acid (0.1 M, pH 6.5-8) was dissolved in 0.1 M phosphate buffer daily, and injected intracerebroventricularly (i.c.v.) as follows. A 23-gauge guide cannula was implanted into a lateral ventricle (1.0 mm caudal to bregma, 1.5 mm lateral to the midline, 4.0 mm ventral to the skull surface) and secured to skull screws with dental cement. Kynurenic acid was injected through a 30-gauge cannula (5 μl over 1 min) which extended beyond the guide cannula by approximately 1 mm.

Histology

Recording micropipette penetrations were marked by iontophoretic ejection of Pontamine Sky Blue dye (-7 μA, 50% duty cycle for 10 min). At the end of recording sessions, $+30$ μA was passed through the stimulation electrode for 30 s to deposit iron at electrode tips. A 5-μl bolus of Neutral Red was injected through the lateral ventricular cannula to confirm proper i.c.v. injection placement. Animals were then deeply anesthetized and perfused with 10% formalin in 0.1 M phosphate buffer containing 5% potassium ferrocyanide, yielding a visible Prussian Blue reaction product at the site of the iron deposit (Fig. 1A).[45] Select brain regions were cut in 50-μm-thick sections with a freezing microtome, mounted on gelatinized glass slides, and stained with Neutral Red. All stimulation, recording and ventricular infusion sites were histologically verified from such tissue sections (Fig. 1). Data were included for neurons located in the subcoeruleus region, ventral to the LC nucleus, when pre-drug discharge characteristics matched those of LC neurons (described above). These cells were categorized separately as subcoeruleus neurons.

Data analysis

Individual PSTHs were analysed by computer to determine excitatory and inhibitory epochs, as described previously.[12,30] A baseline period was defined as the 500 ms epoch preceding stimulation, and the mean and standard deviation (S.D.) of counts per baseline bin were determined. The onset of excitation was defined as the first of five consecutive bins (8 ms bin width) whose mean value exceeded mean baseline activity by 2 S.D., and response offset was determined as the time at which activity had returned to be consistently within 2 S.D. of baseline.

Inhibition was defined as an epoch of at least 15 bins in which the mean count per bin was less than 25% of that during baseline. Response magnitude (R_{mag}) for inhibition

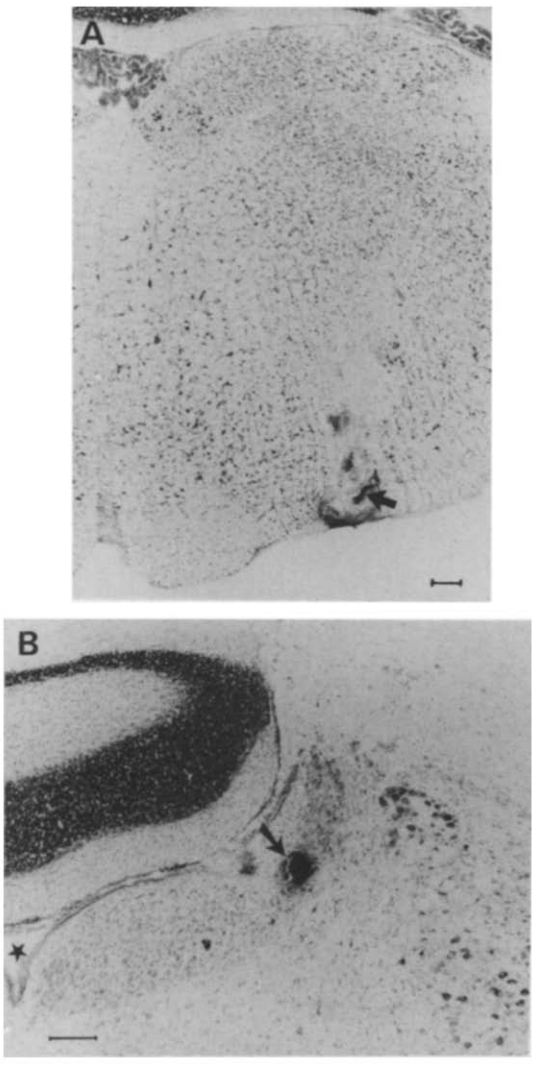

Fig. 1. Histological verification of stimulation and recording sites. (A) Photomicrograph of a 40-μm-thick coronal section (Neutral Red stain) through the rostral medulla of an experimental rat brain. Prussian Blue spot (dark area in ventral medulla, at arrow) reveals the location of stimulation electrode tips in PGi. (B) Photomicrograph of a 40-μm-thick coronal section (Neutral Red stain) through the LC. Iontophoretic ejection of Pontamine Sky Blue (at arrow) marks the recording location in ventral LC. Star indicates the IVth ventricle. For A and B, dorsal at top, midline at the left. Scale bars = 200 μm (A, B).

was normalized for differences in spontaneous firing (baseline) according to the following equation: Inhibition R_{mag} = (mean counts per baseline bin × number of bins in inhibitory epoch) − (counts in inhibitory epoch). Data were analysed by t-tests (paired or unpaired, as indicated) using raw values. A significance criterion of $P < 0.05$ was used.

RESULTS

Evoked responses

Single-pulse stimulation (0.5/s) of PGi produced a dual effect in LC. (i) One-hundred and three of 119 LC neurons, and each of two subcoeruleus neurons, exhibited a phasic, initially excitatory response (Fig. 2A) with a mean onset latency of 14.1 ± 1.0 ms and a mean duration of 28.3 ± 1.2 ms. This excitation was followed by postactivation inhibition, characteristic of LC neurons when activated by any of a variety of means.[2,4,21,28] (ii) Eight LC cells (7% of those tested) exhibited a phasic, pure inhibition, with a mean onset latency of 36.3 ± 8.4 ms and a mean duration of 352.0 ± 76.1 ms (Fig. 3A,C). The remainder of the cells had mixed, non-significant effects. (In one cell, an inhibitory response occurred at a relatively long latency of 112 ms, following a small but non-significant excitation. As this excitation may have interacted with a purely inhibitory response, this cell was omitted from the above calculation of inhibition latency and duration.)

Attenuation of purely inhibitory responses by systemic idazoxan

The effect of the alpha-2 receptor antagonist, idazoxan, was tested on purely inhibitory responses to PGi stimulation (475 μA). Intravenous injection of idazoxan (0.1–1 mg/kg) reduced such inhibition in three of five LC neurons and in one subcoeruleus neuron examined by an average of 60 ± 7% (Fig. 3A, B; $P = 0.02$, paired t-test, $n = 6$). The inhibition

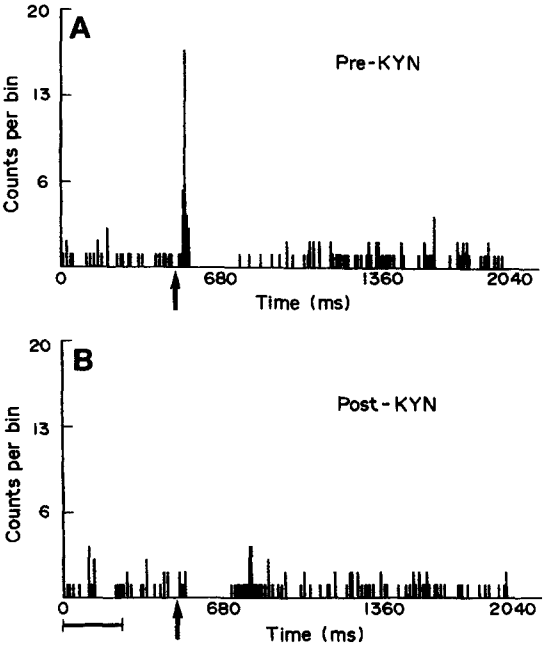

Fig. 2. Blockade of PGi-induced excitation of an LC neuron by kynurenic acid (KYN). (A) Typical PSTH generated during 0.5 Hz electrical stimulation of PGi (at arrow, intensity 700 μA). Such stimulation yielded short-latency excitation followed by a longer-lasting inhibition of impulse activity, corresponding to the characteristic postactivation inhibition of LC neurons.[2,21,28] (B) Similar PSTH for the same cell and stimulation, but 10 min after 0.5 μmol kynurenic acid (i.c.v.). Note that kynurenic acid completely blocked PGi-induced excitation of this neuron, and disclosed a purely inhibitory response to such stimulation. PSTHs were generated for 50 consecutive stimuli. Scale bar = 250 ms.

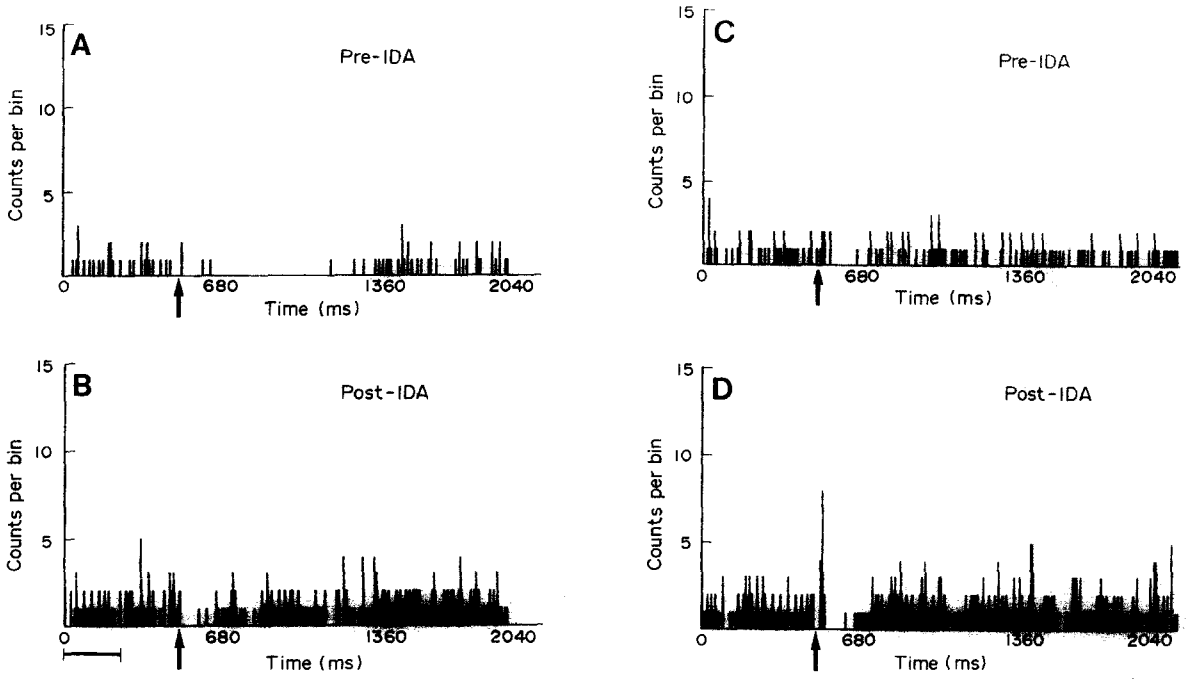

Fig. 3. Attenuation of purely inhibitory response of an LC neuron to PGi stimulation by idazoxan (IDA). (A), (C) PSTHs generated during electrical stimulation of PGi (at arrows) revealing purely inhibitory responses of two individual LC neurons. (B) PGi-evoked inhibition shown in A, pre-drug, is attenuated in the same neuron 6 min after 1 mg/kg idazoxan (i.v.). (D) Similar PSTH for the same neuron and stimulation as in C, but 5 min after 0.5 mg/kg idazoxan (i.v.), revealing underlying excitation. All PSTHs were generated for 50 consecutive stimuli, at arrows, presented at 0.5 Hz. Stimulation amplitude in A–D, 300 μA. Scale bar = 250 ms.

R_{mag} decreased by 47% following 0.1 mg/kg idazoxan ($n = 1$), by 58% with 0.5 mg/kg idazoxan ($n = 2$), and by 80% following 1 mg/kg idazoxan ($n = 1$). In the remaining two cells, idazoxan eliminated the purely inhibitory response and revealed an underlying initial excitation at doses of 0.5 or 1 mg/kg (Fig. 3C,D).

Spontaneous discharge rates of LC neurons were higher following 0.5 mg/kg idazoxan (2.8 ± 0.4 spikes/s, $n = 4$) or 1 mg/kg idazoxan (3.2 ± 0.9 spikes/s, $n = 3$) than those of LC neurons without idazoxan treatment (1.8 ± 0.3 spikes/s, $n = 9$ cells initially inhibited by PGi; $P < 0.05$ for each dose by t-tests). There was no increase in discharge after doses of 0.1 or 0.2 mg/kg.

Antagonism of excitatory amino acid transmission revealed underlying inhibition

Similar to previous reports[27,30] and as shown in Fig. 2A and B, we found that the broad spectrum EAA antagonist kynurenic acid (0.5 μmol, i.c.v.)[54] completely blocked PGi-induced excitation in all 41 LC and two subcoeruleus neurons tested ($P < 0.001$, paired t-test). Excitation from PGi was totally blocked within 3–6 min after kynurenic acid injection, and such blockade was effective for at least 45 min.

Also, as reported previously,[30] and shown in Fig. 2B, such pharmacological blockade of excitation revealed an underlying purely inhibitory response to PGi stimulation in 35 of 42 LC cells and one subcoeruleus cell; all of these neurons were originally excited by the same stimulation prior to drug administration. Including cells recorded only post-kynurenic acid, 85 of 94 LC cells and one subcoeruleus neuron examined within 30 min of i.c.v. kynurenic acid exhibited a purely inhibitory response to PGi stimulation. This inhibition occurred at an onset latency (13.5 ± 1.4 ms) similar to that of the excitatory response to PGi stimulation, but was longer in duration (273 ± 10.9 ms). However, this onset latency was substantially shorter than that for purely inhibitory responses to PGi stimulation observed before EAA antagonism (36.3 ± 8.4 ms, described above).

Kynurenic acid also significantly decreased the spontaneous discharge rate of LC neurons. For cells recorded both before and after kynurenic acid, we noted an 18% decrease in rate within 3–6 min after kynurenic acid injection (from 2.2 ± 0.3 to 1.8 ± 0.2 spikes/s; $n = 27$, $P < 0.01$, paired t-test). Overall, the spontaneous rate averaged for all LC cells recorded after kynurenic acid administration (including cells without pre-drug measures) was significantly lower than that of the total population of LC cells recorded without kynurenic acid pretreatment (1.6 ± 0.1 vs 2.3 ± 0.1 spikes/s; $n = 95$ and 121, respectively, $P < 0.001$, t-test).

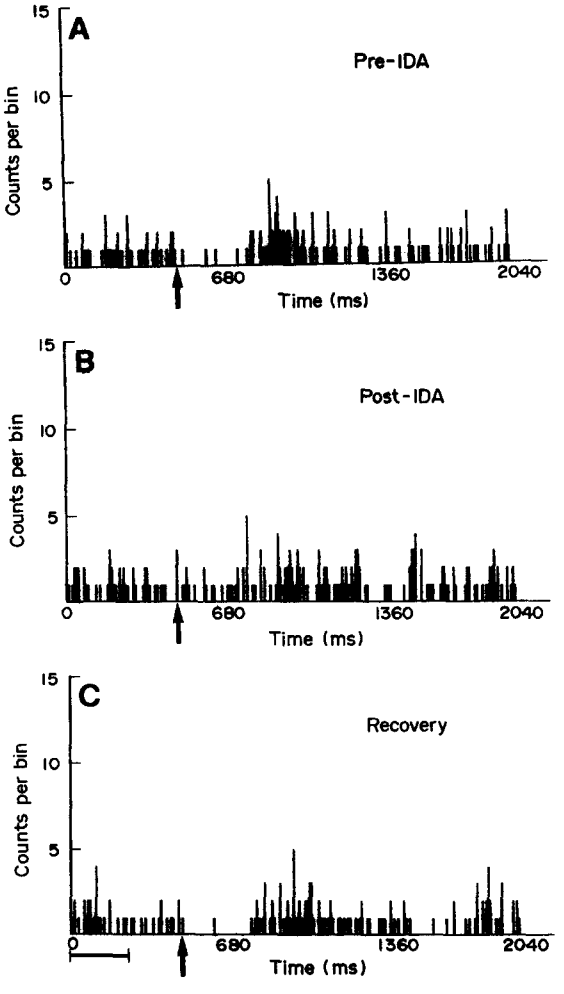

Fig. 4. Idazoxan blockade of underlying inhibition of an LC neuron evoked by PGi stimulation after EAA antagonism. (A) Typical PSTH generated during electrical stimulation of PGi (at arrow, 600 μA) after blockade of excitation by kynurenic acid (0.5 μmol, i.c.v.). Such stimulation yielded potent, short-latency inhibition. (B) Similar PSTH for the same cell and stimulation as in A, but after idazoxan administration (1 mg/kg, i.v.). Note that idazoxan blocked the PGi-induced inibition. (C) PGi-evoked inhibition of this cell recovers 50 min after idazoxan administration (an additional kynurenic acid dose was given 40 min after the first dose). All PSTHs were generated for 50 consecutive stimuli (at arrows) presented at 0.5 Hz. Scale bar = 250 ms.

Attenuation by idazoxan of inhibitory responses to paragigantocellularis stimulation after excitatory amino acid antagonism

Systemic idazoxan administration. Idazoxan (0.2–1 mg/kg, i.v.) significantly attenuated (by 35%) the inhibitory R_{mag} in LC neurons evoked by PGi stimulation after kynurenic acid pretreatment (inhibitory R_{mag} pre-idazoxan = 18.1 ± 5.0, and post-idazoxan = 11.8 ± 3.7; $n = 8$, $P < 0.03$, paired t-test). Idazoxan completely blocked such inhibition in three of eight cells tested (Fig. 4), decreased the inhibition in four of eight cells, and increased the inhibition in one cell. There was no change in the inhibition R_{mag} following 0.1 mg/kg idazoxan ($n = 2$). Idazoxan did not reveal underlying excitation in animals pretreated with kynurenic acid i.c.v.

For cells tested after kynurenic acid pretreatment, systemic idazoxan did not significantly increase the spontaneous discharge rate of LC neurons, though tendencies toward increased activity were observed: discharge increased following 0.5 mg/kg from 1.9 ± 0.1 to 2.4 ± 0.3 spikes/s ($n = 2$), and following 1 mg/kg from 1.5 ± 0.1 to 2.1 ± 0.4 spikes/s ($n = 5$). No change was observed with the lower doses of idazoxan.

Local microinfusion of idazoxan into locus coeruleus. As shown in Fig. 5 and Table 1, microinfusion of idazoxan (0.1–2.5 ng in 50 nl NaCl or ACSF) into LC in kynurenic acid-pretreated animals significantly reduced the inhibitory response of LC and subcoeruleus neurons to PGi stimulation ($n = 33$, $P < 0.001$, paired t-test). Such microinfusion completely blocked this inhibition in nine of 31 LC and one of two subcoeruleus neurons. Specifically by dose, local idazoxan decreased the PGi-evoked inhibition of LC neurons by $15 \pm 3\%$ following 0.1 ng ($n = 5$, not significantly), by $51 \pm 2\%$ following 0.5 ng ($n = 5$, $P < 0.02$, paired t-test), and by $42 \pm 3\%$ following 2.5 ng ($n = 21$, $P < 0.001$, paired t-test). Such inhibition was decreased by $52 \pm 10\%$ in two subcoeruleus neurons following microinfusion of 2.5 ng idazoxan (not significant). Analysis of variance revealed that the overall attenuation of LC and subcoeruleus neurons by idazoxan was dose-dependent ($F = 4.32$, $P < 0.05$, $n = 33$). Log probit analysis indicated that the ED_{50} for locally administered idazoxan was approximately 0.2 ng (assuming that the maximal effect is about 50% blockade of inhibition). Local idazoxan administration appeared to be effective within 60 s of infusion and recovery was typically observed within 10–15 min. The inhibition R_{mag} was not significantly changed following microinfusion of 50 nl of NaCl or ACSF into LC ($n = 9$, $P = 0.6$, paired t-test; Table 1).

Spontaneous LC discharge was significantly increased within 3 min after injection of 2.5 ng idazoxan into LC (from 1.7 ± 0.2 to 2.1 ± 0.3 spikes/s; $n = 23$, $P < 0.001$, paired t-test). Discharge rate was not significantly altered following local microinfusion of 0.5 ng idazoxan, although a tendency towards increased activity was noted (from 1.7 ± 0.1 to 2.2 ± 0.2 spikes/s; $n = 5$, $P = 0.07$, paired t-test). Spontaneous rate was not changed after microinfusion of 0.1 ng idazoxan into LC.

DISCUSSION

The present study confirms the EAA innervation of LC by the PGi[27,30] and provides evidence for a simultaneous, substantial inhibitory influence of PGi on LC neurons as well. Indeed, in addition to the purely inhibitory response of some LC cells to PGi stimulation, blockade of the PGi excitatory influence on LC by EAA antagonism revealed an underlying

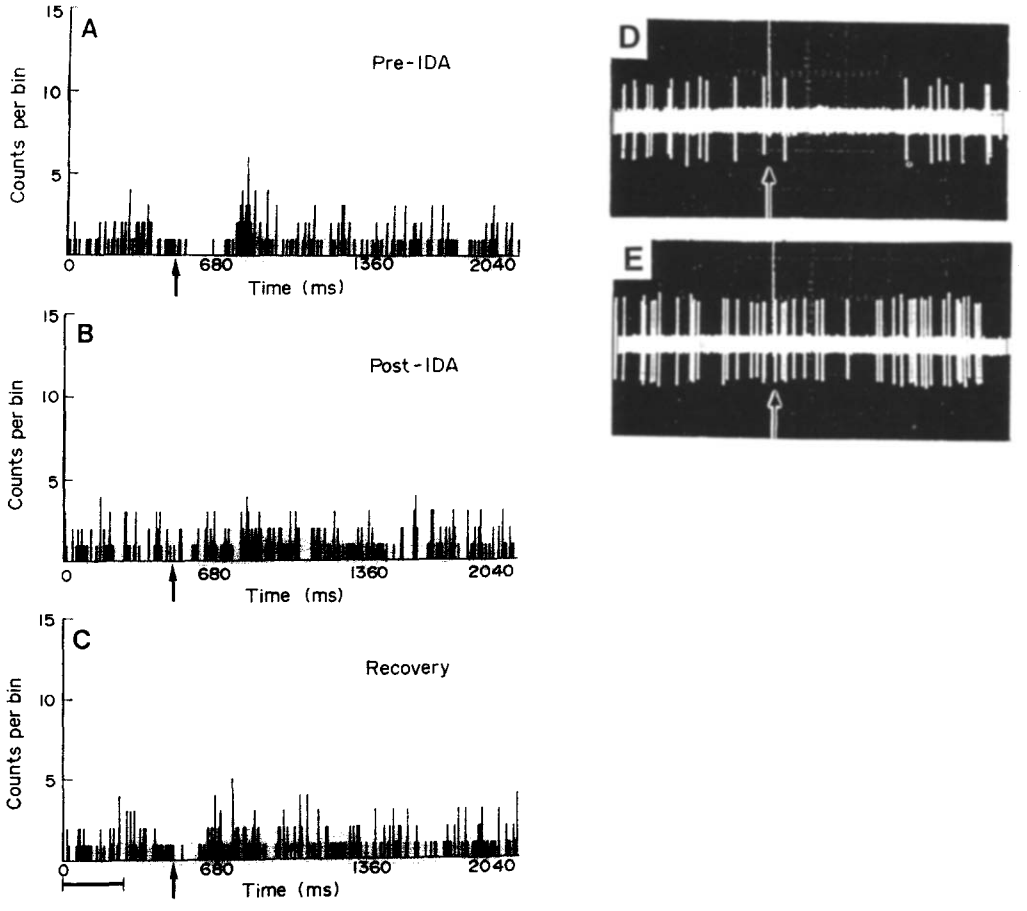

Fig. 5. Blockade of underlying inhibition of an LC neuron from PGi stimulation (10 min after EAA antagonism by 0.5 μmol kynurenic acid, i.c.v.) by idazoxan microinfusion into LC. (A) Typical PSTH showing PGi-induced inhibition of an LC neuron (after EAA antagonism). (B) Underlying inhibition from PGi shown in A, pre-drug, is blocked 3 min after microinfusion of 0.5 ng idazoxan (50 nl injection volume) into LC. (C) Underlying inhibition of LC from PGi recovers 9 min afer local idazoxan infusion. All PSTHs were generated for 50 consecutive stimuli (at arrows) presented at 0.5 Hz. (D), (E) Oscilloscope photographs of impulse activity of an LC neuron after EAA antagonism showing blockade of underlying inhibitory response to PGi stimulation by microinfusion of 0.1 ng of idazoxan (50 nl injection volume) in E, post-drug. Compare to D, pre-drug. Stimulation amplitude in A–E, 600 μA. Calibration bar = 250 ms.

inhibitory response in 90% of LC cells tested. The present findings further indicate that this inhibitory influence may be mediated in large part by the adrenergic innervation of LC from PGi. This is the first report of a physiological response to activation of C1 adrenergic neurons.

Evoked responses

We confirmed that the PGi exerts a strong excitatory influence on the LC,[27,30] with 87% of LC cells excited by PGi stimulation while 7% exhibited a purely inhibitory response. The present results for attenuation of PGi-evoked activation by kynurenate supports our previous evidence for an EAA pathway from PGi to LC.[15,27,30] The slight decrease of LC tonic discharge after kynurenate may indicate that kynurenate reduces the overall excitability of LC neurons, perhaps responsible in part for kynurenate's blockade of PGi-evoked excitation of LC neurons. However, this seems unlikely because (i) the decrease in spontaneous activity was slight while the blockade of PGi-evoked excitation by kynurenate was total, and (ii) recent studies find that a similar dose of kynurenate blocks responses to sciatic nerve activation but not activation by nitroprusside-induced hypotension.[72a]

We also confirmed our previous observation[30] that the bulk of LC cells exhibit an underlying inhibitory response after EAA antagonism, indicating that most, in fact perhaps all, LC neurons receive both excitatory and inhibitory afferents from the PGi. We found in addition that blockade of alpha-2 receptors eliminated about one-half of PGi-evoked inhibition, suggesting that non-adrenergic projections may also participate in this inhibition.

The inhibition of LC neurons from PGi after EAA antagonism is likely to be a direct response to input from cells in the PGi area. (i) There is only one minor afferent to the LC located caudal to PGi (in spinal lamina X[13,15]), so that activation of fibers passing

through the PGi area to terminate in LC should have a minor, if any, contribution to the observed effects. (ii) No LC neurons were activated by PGi stimulation after EAA antagonism, suggesting that the PGi-evoked inhibition in these experiments is not due to collateral-mediated postactivation inhibition among LC neurons.[2,3,21,28] However, purely inhibitory responses of LC neurons elicited in animals with EAA neurotransmission intact may well result, at least in part, from activation of collaterals of LC neurons that are excited by the PGi stimulation. (iii) The latency for inhibition observed here (mean onset 13.5 ms) is similar to the mean latency for antidromic activation from LC of a subpopulation of PGi neurons (14 ms).[29] These relatively long latencies indicate a conduction velocity of about 0.3 m/s, similar to that reported by Haselton and Guyenet[38] for electrophysiologically identified C1 adrenergic neurons antidromically driven from the rostral pons near LC.

Identity of transmitter(s) responsible for inhibition

Although the neurochemical identity of projections from PGi to LC have not been fully characterized, recent experiments reveal that the adrenergic innervation of LC is mainly provided by C1 neurons located in the PGi, where approximately 21% of neurons projecting to LC are PNMT-immunoreactive.[10,15,38,55–57] This is consistent with the finding that lesion of the medullary adrenergic bundle eliminated the bulk of PNMT-immunoreactive (PNMT-IR) fibers in the LC.[9] In addition, recent ultrastructural studies demonstrated synapses between PNMT-IR terminals and TH-IR somata and dendrites within LC.[42,49]

The possibility that adrenaline may mediate PGi-evoked inhibition of LC neurons is consistent with several pharmacological findings demonstrating that adrenaline, noradrenaline and clonidine strongly inhibit LC,[19,20,71,77,78] while alpha-2 antagonists activate LC[19] and block the hyperpolarization of LC neurons induced by the foregoing agonists.[3,26] The ability of systemic idazoxan to reduce, or in some cells completely block, PGi-evoked inhibition of LC neurons is also good evidence that this inhibition may be mediated, at least in part, by adrenergic projections from PGi. Idazoxan locally infused into LC was much more potent in antagonizing PGi-evoked inhibition (effective at 0.5 ng locally vs about 70 μg i.v.), indicating that the alpha-2 receptor sites mediating this effect are located in the LC area.

Previous studies have concluded that idazoxan interacts selectively with alpha-2 receptors in LC.[34] This is consistent with our previous finding that idazoxan was ineffective on GABA-mediated synaptic inhibition of LC[31,32] and with the fact that idazoxan has a high degree of selectivity for binding alpha-2 receptors.[18,59] However, recent studies have revealed that idazoxan interacts significantly with non-adrenergic binding sites, often denoted imidazoline-preferring receptors, which have only very low affinity for catecholamines.[17,24,46,48] While it is difficult to rule out the possible involvement of an endogenous imidazoline-like transmitter in the inhibition of LC from PGi, there are several considerations that suggest that the idazoxan-sensitive PGi-evoked inhibition of LC neurons is mediated by alpha-2 receptor mechanisms: (i) the LC is densely innervated by PNMT-IR fibers;[40,41] (ii) there is a prominent PNMT-IR projection to LC from C1 adrenergic neurons (discussed above); (iii) alpha-2 receptors are densely accumulated within LC;[22,81] and (iv) LC neurons are potently inhibited by adrenaline, noradrenaline, and other non-imidazolines that act preferentially at alpha-2 receptors.

Idazoxan increased LC basal discharge rate, in agreement with previous studies.[32,34,64] While it seems possible that the idazoxan-induced attenuation of PGi-evoked inhibition of LC may have resulted simply from the accompanying increase in spontaneous discharge, several factors indicate that this is not the case. First, R_{mag} values were calculated to normalize responses for differences in spontaneous discharge rates before vs after drug treatment or between cells (see Experimental Procedures). Thus, inhibition R_{mag} as measured herein could not be reduced simply as a result of an overall increase in basal discharge rate. Second, for many LC neurons, idazoxan decreased or blocked inhibition at doses that did not alter the cell's spontaneous rate. Third, while 0.5 ng local idazoxan attenuated PGi-evoked inhibition overall, there was no significant effect of this treatment on spontaneous rate. These factors, together with other results in the present study, indicate that the idazoxan-induced decrease of PGi-evoked inhibition in LC reflects the blockade of alpha-2 adrenoceptors in LC that mediate this

Table 1. Idazoxan attenuation of paragigantocellularis-induced inhibition in locus coeruleus

	Saline CSF	Local idazoxan			Intravenous idazoxan
Dose		0.1 ng	0.5 ng	2.5 ng	0.2–1 mg/kg
n	9 (LC)	5 (LC)	5 (LC)	23 (LC + subLC)	8 (LC)
R_{mag}/post-drug (% of R_{mag} pre-drug)	102 ± 6	85 ± 16	49 ± 10	58 ± 3	68 ± 10
t-Test	$P = 0.32$	$P = 0.37$	$P = 0.006$	$P < 0.0001$	$P = 0.034$
% Change	+2%	−15%	−51%	−42%	−32%

Effects of idazoxan on inhibitory R_{mag}s for LC or subcoeruleus neurons following PGi stimulation. Significance levels were derived from paired t-tests between the raw values for pre- and post-drug R_{mag}s.

inhibition. It is also possible that idazoxan caused a general increase in excitability of LC neurons and this may have been partly responsible for the observed attenuation of inhibition by this agent. The effectiveness of idazoxan to reduce inhibition without affecting spontaneous discharge argues against this possibility, but additional tests would be needed to definitively address this issue.

The fact that the LC is densely innervated by PNMT-IR fibers, most of which derive from PNMT-IR neurons in the C1 cell group (discussed above), suggests that adrenaline may be the endogenous transmitter released in LC that mediates the alpha-2 receptor response to PGi stimulation. However, recent studies indicate that adrenaline may not be used as a transmitter in some PNMT-IR neurons, and noradrenaline may be co-released or used instead.[47,68,69] As noradrenaline and adrenaline have very similar effects at alpha-2 receptors in LC,[20,77] it is difficult from our studies to definitively establish which may be the transmitter responsible for the observed effects. Nonetheless, the possibility that PGi-evoked inhibition of LC is mediated, at least in part, by adrenaline is in good agreement with previous biochemical studies showing that lesions of the medullary bundle (conveying the bulk of adrenergic innervation of the LC),[10] or inhibitors of PNMT synthesis, activate tyrosine hydroxylase in the brain stem[66] as well as in LC.[8]

While adrenergic mechanisms account for a substantial part of the PGi-evoked inhibition of LC, even high doses of idazoxan (1 mg/kg i.v. or 50 nl of 200 μM locally) did not eliminate more than about 50% of the average inhibitory R_{mag}. As idazoxan acts to block alpha-2 receptors on LC neurons with an ED_{50} of 14 μg/kg *in vivo*,[34] and blocks alpha-2-receptor-mediated synaptic potentials at 1 μM *in vitro*,[23,67] the doses used here would be expected to fully antagonize alpha-2 adrenoceptor responses of LC neurons, indicating that inhibition of LC by PGi is mediated in part by a non-alpha-2-receptor. Furthermore, as there is little or no alpha-1- or beta-adrenoceptor-mediated response to adrenaline or noradrenaline in adult LC,[2,19,20,52,77] this in turn implies that the inhibition observed is mediated in part by a non-adrenergic pathway from PGi to LC. Our anatomic studies have revealed that enkephalin- and GABA-immunoreactive cells in PGi innervate the LC,[16,25,58] and these agents (or others) may underlie the non-adrenergic component of PGi-induced inhibition of LC.

Locus coeruleus neurons receive converging excitatory and inhibitory inputs from paragigantocellularis

It should be noted that in some LC cells exhibiting pure inhibition, idazoxan revealed an underlying excitation following PGi stimulation. Thus, it seems that at least some LC cells with purely inhibitory responses also receive a modest excitatory input from PGi. This is consistent with results showing that alpha-2 agonists hyperpolarize LC neurons,[5,26,77] so that adrenergic input to LC cells may mask the influence of a co-activated excitatory input. Such underlying, weak excitation may decrease the impact of purely inhibitory responses of LC cells. Thus, this simultaneous excitation may explain why onset latencies for purely inhibitory responses (36.4 ms) were longer than for inhibition observed after EAA antagonism (14.1 ms). It seems possible, therefore, that there are few or no cells that receive only inhibitory, or only excitatory, input from PGi, but that most, and perhaps all, LC neurons are innervated by both excitatory and inhibitory afferents from this structure. It is also possible that individual PGi neurons that project to LC contain adrenaline as well as an EAA. In this regard, it is noteworthy that Forloni *et al.*[33] have demonstrated co-localization of the EAA analog *N*-acetyl-aspartyl-glutamate in many C1 neurons. Other recent studies have demonstrated that C1 neurons often also stain for glutaminase, a possible marker of neurons using glutamate as a neurotransmitter.[44] Additional experiments are necessary to determine the possible co-localization of EAAs and adrenaline in C1 cells that innervate LC.

Our results are also consistent with previous reports that idazoxan increases the excitatory responses of LC cells to peripheral sensory stimuli.[64,65] If C1 cells that innervate LC are activated by these peripheral stimuli (in parallel with the more potent EAA inputs), then idazoxan may enhance EAA-mediated sensory responses in LC in part by eliminating the inhibitory influence of co-activated adrenergic inputs. Consistent with this possibility, we have found that antagonism of the EAA-mediated excitatory response of LC neurons to sciatic nerve stimulation reveals an underlying inhibitory response to the same stimuli, much as found here for PGi stimulation (unpublished observations). Thus, in addition to augmenting sensory responses of LC cells by antagonizing noradrenergic collateral feedback inhibition as previously proposed,[64,65] enhanced sensory activation of LC following idazoxan may be due, in part, to the blockade of the presently described extrinsic inhibitory influence from PGi.

Possible functions of inhibitory C1 input to locus coeruleus

The present results show for the first time a physiologic effect of C1 activation. The function of this substantial inhibitory input to LC from PGi is at present unclear. However, previous experiments found that LC neurons were largely silent during paradoxical sleep.[11,12,39,60] The markedly reduced spontaneous and sensory-evoked discharge during paradoxidal sleep is probably the result of tonic inhibition impinging on LC from an extrinsic afferent(s).[11,12,15] It is possible that strong GABAergic input to LC from the nucleus prepositus hypoglossi may be important for such behavioral

state-dependent inhibition of LC.[32] The present results suggest that adrenergic inhibitory afferents to LC from PGi may also participate in such state-dependent regulation of LC discharge. In addition, it has been reported that stressful stimuli increase LC activity,[1,53,70,72,73,76] and other studies show that PNMT activity is increased in the LC and in the C1 area during stress.[63] Therefore, it also seems possible that the adrenergic inhibitory input to LC from PGi may help to regulate LC's response to stress (e.g., perhaps serving to limit stress-induced increases in LC activity). A similar role may exist for the parallel EAA and adrenergic projections to sympathetic preganglionic cells in the spinal cord, where an EAA is thought to mediate direct excitatory effects of PGi stimulation,[37,50,51,61] while adrenergic inputs may be predominantly inhibitory[36,79,80] and serve to modulate this effect. Further examination of such mechanisms may reveal substrates linking sympathetic cardiovascular responses with arousal and vigilance mediated by the LC system.[15]

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