Receptors in lateral hypothalamic area involved in insular cortex sympathetic responses

KENNETH S. BUTCHER1 AND DAVID F. CECHETTO1,2
Departments of 1Physiology and 2Anatomy and Cell Biology,
University of Western Ontario, London, Ontario, Canada N6A 5C1

Butcher, Kenneth S., and David F. Cechetto. Receptors in lateral hypothalamic area involved in insular cortex sympathetic responses. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H689–H696, 1998.—Previous evidence has shown that sympathetic nerve responses to insular cortical (IC) stimulation are mediated by synapses within the lateral hypothalamic area (LHA) and ventrolateral medulla. The present study determined the receptor(s) involved at the synapse in the LHA associated with stimulation-evoked IC sympathetic responses. Twenty-seven male Wistar rats were instrumented for renal nerve activity, arterial pressure, and heart rate recording. The right IC was stimulated with a bipolar electrode (200–1,000 µA, 2 ms, 0.8 Hz) resulting in sympathetic nerve responses. Antagonists were then pressure injected into the ipsilateral LHA (300–500 nl). Kynurenicate (250 mM) injections resulted in 51 ± 8% (range 0–100%) block of IC-stimulated sympathetic nerve responses. Similarly, the N-methyl-D-aspartate (NMDA)-receptor antagonist dl-2-amino-5-phosphonopentanoic acid (200 µM) resulted in an inhibition (82 ± 8%; range 51–100%) of IC-stimulated sympathetic responses. Injection of the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (200 µM) had no effect on IC sympathetic responses. Injection of antagonists to GABA, acetylcholine, and adrenergic receptors was also without effect. No antagonist injections had any effects on baseline sympathetic nerve discharge, arterial pressure, or heart rate. These results suggest that the IC autonomic efferents projecting to the LHA utilize NMDA glutamatergic receptors.

 METHODS

Animals. Twenty-seven male Wistar rats (250–350 g) between 15 and 20 wk old were used in the experiments. Food and tap water were provided ad libitum, except that food was removed ∼12 h before surgery.

General surgical methods. Rats were initially anesthetized with a mixture of α-chloralose (30 mg/kg ip) and urethan (0.75 g/kg ip). During the experiment, anesthesia was maintained with urethan supplements (0.75 g/kg iv). Supplements were given on the first sign of withdrawal to pinching of the foot. Core temperature was maintained at 37°C with the use of a rectal thermometer, temperature controller, and heating pad. The right femoral artery was cannulated with PE-50 tubing filled with heparinized saline. Pulsatile arterial pressure was measured with a Statham P23 ID transducer and continuously monitored on a Grass model 7E polygraph. Mean arterial pressure (MAP) was recorded on the polygraph by filtering the pulse pressure with a 0.5-Hz high-frequency filter. Heart rate (HR) was determined with a Grass 7P44 tachograph and monitored on the polygraph. The right femoral vein was catheterized with PE-10 tubing for the administration of drugs and anesthetic.

The animal was tracheotomized and placed in a Kopf stereotaxic apparatus. The right kidney was exposed using a retroperitoneal approach. With the aid of a Zeiss operating stereomicroscope, the renal nerve branches were isolated from the surrounding tissue and a loose ligature was placed around one of them. A bipolar stainless steel electrode was used to record nerve activity. The electrode was secured to the nerve using dental impression material (Perfourn, Miles Laboratories). The nerve was tested for a reflex decrease in sympathetic activity by activating baroreceptors with an infusion of the α-receptor agonist phenylephrine (40–60 µg/kg iv). The multiunit nerve activity was first amplified and filtered (from 100 Hz to 3 kHz) with a Grass model P15 preamplifier. The signal was further amplified (Neurolog NL100) and then fed into the oscilloscope and an audio monitor (model AM8C, Grass). The rectified and integrated
nerve activity was continuously monitored on a polygraph (model 7P10, Grass). Signals were also discriminated, using a window discriminator, and fed into a microcomputer for compilation of peristimulus time histograms in response to IC stimulation. Background noise levels were determined by the infusion of hexamethonium (2 mg/kg iv) at the conclusion of the experiment period.

Electrical stimulation. A steel bipolar electrode (tip separation 0.5 mm; SNEX 100, David Kopf Instruments) was lowered into the IC. Peristimulus time histograms were evoked by stimulation of the IC (200–1,000 µA, 2 ms, 0.8 Hz) for 125 s.

Microinjections. Injections into the LHA were made with the use of a glass micropipette (tip diameter ~20 µm) filled with a receptor antagonist or vehicle solution. All antagonists were dissolved in 0.9% NaCl, and the solution was titrated to pH 7.4 before it was used for injection. The NaCl solution was also used in control injections. The pipette was stereotaxically placed in the LHA, and solutions were pressure injected (300–500 nl). We have previously shown (11, 28) that this volume is adequate for covering the region of the LHA mediating insular responses, without extensive spread to surrounding structures. DL-2-Amino-5-phosphonopentanoic acid (AP-5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris Neuroamin) were dissolved in NaCl to a concentration of 200 µM. Kynurenate (Sigma) was injected at a concentration of 250 mM. Bicuculline, atropine, phentolamine, and propranolol (Sigma) were all injected at a concentration of 0.1 µM. These antagonist concentrations have previously been shown to be effective in blocking their respective receptors (14, 16, 21, 30, 31, 38).

Histological procedures. At the conclusion of the experiment, rats were deeply anesthetized with a bolus injection of urethane (1.5 g/kg ip). All animals were then perfused transcardially with 0.9% saline followed by 4% formaldehyde. The brains were removed, sectioned (50 µm) with a freezing microtome, and stained with thionin. The sections were examined with a microscope (Leitz Diaplan), and all pipette tracks were drawn using a camera lucida drawing attachment.

Data analysis. Peristimulus time histograms were generated and analyzed with the use of a microcomputer and an integrated program for electrophysiological experiments. The program was used to calculate a mean level of baseline activity before the stimulus artifact. After the stimulus artifact, an excitatory sympathetic response was identified in which activity was calculated to be one standard deviation above baseline activity for five consecutive bins (1 bin = 2 ms). The absolute response was determined by subtracting the baseline activity from the total number of spikes during the responsive period. Statistical comparisons were made using ANOVA followed by Dunnett’s test for significance of all versus control. For all tests, a P value of 0.05 or less was considered to indicate significance. Values in the results are expressed as means ± SE.

RESULTS

Sympathetic responses to stimulation of IC. Sympathetic nerve responses were elicited by stimulation of sites within the posterior IC (Fig. 1). These sites were primarily located in the agranular and dysgranular regions of the posterior IC, as previously described (12). Peristimulus time histograms generated during stimulation of sites within the IC demonstrated an initial increase, followed by a decrease, in renal nerve activity (see e.g., Fig. 2A). Latencies to the onset of the excitatory and inhibitory phases of the renal nerve response were ~90 and 130 ms, respectively. The duration of the excitatory response was ~60 ms (Fig. 2A). These latencies and durations are similar to those reported previously in our laboratory (11, 12).

Effect of glutamate antagonist injection. Injection of kynurenate, a blocker of all ionotropic glutamate receptors, including NMDA and non-NMDA, into the tuberal and posterior LHA resulted in a significant attenuation of the sympathetic nerve response to IC stimulation (51 ± 8%, range 0–100%, n = 17) (Fig. 2). Recovery of the response occurred ~45 min after injection, at which time the IC sympathetic responses were not significantly different from initial values. Not all kynurenate injections into the LHA resulted in an effective block (i.e., >50%) of the IC sympathetic responses (Figs. 2 and 3). Injections not directly centered in the tuberal and posterior LHA resulted in <50% inhibition of the response to IC stimulation (Figs. 2 and 3). The most effective sites were the most ventral portions of the tuberal and posterior LHA, lateral to the fornix (Fig. 2).

Injection of the NMDA-receptor antagonist AP-5 also resulted in a significant attenuation of the sympathetic nerve response to IC stimulation (Fig. 4). This dose of AP-5 in general yielded a consistently effective block of the evoked response (82 ± 8%, range 51–100%, n = 7). The effective injections were primarily located within the tuberal and posterior LHA (Fig. 3).

Injections of the non-NMDA-receptor antagonist CNQX had no effect on the sympathetic nerve response to IC stimulation, despite the fact that the majority of sites were located in the posterior and tuberal LHA.
Similarly, saline control injections into the LHA did not affect the sympathetic nerve response to stimulation of the IC.

The examples in Fig. 5 indicate that injection of either kynurenate or AP-5 into the LHA had no consistent effect on baseline sympathetic renal nerve discharge. When all of the kynurenate and AP-5 injections are considered, the average change in baseline sympathetic renal nerve discharge is $0.7 \pm 4.5\%$. Similarly, there was no change in arterial pressure or HR following injection of these antagonists into the LHA (Fig. 5). The average changes were $0.4 \pm 1$ mmHg and $8 \pm 6$ beats/min, respectively.

**Effect of injection of other antagonists.** Bicuculline (GABA-receptor antagonist), atropine (muscarinic cholinergic receptor blocker), phentolamine (both $\alpha_1$- and $\alpha_2$-adrenoceptor antagonist), and propranolol (both $\beta_1$- and $\beta_2$-adrenoceptor antagonist) injections into the LHA had no effect on the IC sympathetic response. In addition, these antagonists had no observable effect on baseline renal sympathetic nerve discharge, arterial pressure, or HR (data not shown).

**DISCUSSION**

Effect of glutamate antagonist injection into LHA on IC sympathetic responses. Electrical stimulation of the IC in the present experiments resulted in renal sympathetic nerve responses consistent with those observed previously (11, 12). The nonspecific excitatory amino acid-receptor antagonist kynurenate effectively blocked the increase in renal sympathetic nerve response to stimulation of the IC. The most effective injection sites were in the most ventral portions of the tuberal and posterior ipsilateral LHA, lateral to the fornix. This is consistent with an anterograde tracing study (41), which showed that the IC projects heavily to this portion of the ipsilateral LHA. A previous investigation (11) in this laboratory demonstrated that injection of the nonspecific presynaptic inhibitor cobaltous chloride into the LHA also attenuated the IC sympathetic response. In that study, effective sites were also found to be in the most ventral portions of the LHA, lateral to the fornix. Unlike the present study, however, effective injections were limited to the most posterior portions of
the LHA and did not include the more rostral tuberal LHA (11). This is likely a reflection of the distribution of IC efferent synapses within the IC. In both this study and the cobaltous chloride experiments (11), inhibition of the response was rarely complete. This suggests that the connections between IC autonomic efferents and LHA neurons may be relatively diffusely distributed within this region. This postulation is supported by the anterograde labeling of IC autonomic efferents, which shows diffuse projections throughout the ipsilateral tuberal and posterior LHA (41).

The LHA has also been shown (28) to contain an obligatory synapse for cardiac chronotropic sites within the IC. Phasic microstimulation of these sites, linked to the R wave of the cardiac cycle, results in tachycardia or bradycardia without any concomitant effects on blood pressure (27). It is not known whether these responses represent distinct sites within the IC or are a specific result of the phasic stimulus. Kynurenate injections into the LHA attenuated the cardiac response to phasic microstimulation of the IC (28). As in the present study, injections into the ventral and lateral portions of the tuberal LHA were effective in inhibiting the response to stimulation of the IC.

An effort was made in the present investigation to identify the receptor subtype(s) mediating IC sympathetic responses within the LHA. At least five distinct glutamate receptors have been identified to date (23). These have been termed the NMDA, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid, metabotropic, kainate, and 2-amino-4-phosphonobutanoic acid receptors (23). Injection of the relatively specific NMDA glutamate receptor AP-5 was extremely effective in inhibiting the sympathetic nerve response to stimulation of the IC. The effective sites were very similar to those for kynurenate. Unlike AP-5, the non-NMDA glutamate receptor antagonist CNQX had no effect on the sympathetic nerve response to IC stimulation. These results indicate that the IC autonomic efferents exert their autonomic effects via an NMDA glutamatergic synapse in the LHA.

Recently, it has been shown (7, 37) that the effects of both IC and LHA stimulation are mediated by a glutamatergic synapse in the VLM. Unlike that in the LHA, however, the synapse in the VLM is non-NMDA mediated (7). The function of these two different populations of glutamate receptors in a single pathway mediating the sympathetic effects of IC stimulation is unclear.

Somatosensory input to the ventrobasal thalamus has also been shown (32) to involve both NMDA and non-NMDA receptors. Non-NMDA receptors mediate short-latency somatosensory responses, whereas NMDA receptor effects are manifested only in response to maintained sensory stimulation. This suggests that NMDA and non-NMDA receptors are each suited to a particular type of presynaptic input. Similarly, in the spinal cord, monosynaptic excitation of Renshaw cells is mediated by non-NMDA receptors, whereas NMDA receptors are responsible for polysynaptic activation (15). It has been postulated (33) that non-NMDA receptors mediate fast synaptic transmission, whereas NMDA receptors are responsible for slower potentials with longer time courses. This may be related to the two different populations of glutamate receptors in the LHA and VLM.

The IC, LHA, and VLM form an anatomic axis whereby the IC can affect the sympathetic nervous tone (Fig. 6). It appears that the IC projection to the LHA is mediated by the slower NMDA synaptic mechanism. The LHA, in contrast, appears to influence the VLM via a fast-acting non-NMDA receptor. This may be related to the overall functions of the IC and LHA. It has been proposed (3) that the IC sets the autonomic tone appropriate to the visceral and limbic stimuli it receives. The LHA receives projections from several other limbic nuclei, including the infralimbic cortex, septal nuclei, central nucleus of the amygdala, and the bed nucleus of the stria terminalis (20). In addition, the LHA has direct connections with brain stem autonomic nuclei, including the VLM (1). The LHA has therefore been proposed (2) to be a site capable of integrating the autonomic aspects of emotions. The synaptic potential of the NMDA receptor has been shown (25) to vary with the potential of the cell membrane because of a voltage-dependent blockade by Mg$^{2+}$. Thus the NMDA receptor
may be ideal for the integration of tonic or sustained signals from the IC with those from other limbic nuclei. The LHA has also been shown (4, 37, 40) to have profound effects on ongoing sympathetic tone. The LHA contains neurons whose activity is synchronized to the 2- to 6-Hz component of sympathetic nerve discharge (4). This relationship to the sympathetic nervous system appears to be mediated by the rostral VLM (4, 7, 37). The faster non-NMDA receptor mediating LHA responses in the VLM appears to be most appropriate for this type of direct synaptic transmission (Fig. 6) (7).

A similar situation has recently been shown to exist within the ascending visceral sensory pathway to the IC. Visceral sensory information from the nucleus of the solitary tract is carried to the parabrachial nucleus, which in turn projects to the ventrobasal thalamus, which relays to the IC (10). It has been established (30) that the synapse within the parabrachial nucleus is mediated by NMDA receptors. Within the ventrobasal thalamus, however, synaptic transmission is mediated by non-NMDA glutamatergic receptors (5). This is consistent with the hypothesis that glutamate receptor type is related to the function of the synapse.

Effect of antagonist injection on autonomic variables. The LHA has been shown to contain sites capable of eliciting profound cardiovascular changes in response to stimulation with glutamate (1, 12, 18, 36). Kynurenate, AP-5, and CNQX all had no effects on baseline renal sympathetic nerve activity, arterial pressure, or HR. This is consistent with the findings of Oppenheimer et al. (28), who also reported no changes in basal arterial pressure or HR in response to kynurenate injection into the LHA.

Wible et al. (39) demonstrated that bicuculline injection into the posterior hypothalamus of conscious rats results in increases in arterial pressure, HR, and splanchnic sympathetic nerve activity. In that study, injections appeared to be more medial and probably affected the perifornical region of the LHA. A recent investigation (1) has shown that the most lateral portion of the LHA differs in its response to glutamate stimulation and its efferent connections from the perifornical LHA. Given the lack of response to bicuculline in the present study, the role of GABA may also differ between these two regions. A more systematic study of the entire LHA with regard to GABA and its cardiovascular effects is required. Differences between the present results and those of Wible et al. (39) may also be due to the use of conscious versus anesthetized rats.

The LHA has been shown (22, 42) to contain acetylcholinesterase-containing cells. These cells have been shown (42) to project to the basal forebrain and may be involved in cortical arousal. The LHA also contains cells that are responsive to the iontophoretic application of acetylcholine and that are sensitive to atropine (26). There is no evidence for the involvement of any of these cells in autonomic regulation, although this possibility cannot be excluded. Acetylcholine has been shown (6) to mediate increases in blood pressure and HR in the adjacent posterior hypothalamic nucleus.

The LHA has been shown (22) to contain catecholaminergic cells and fibers. In addition, α- and β-adrenergic receptors are found within the LHA (24).
The catecholamine-sensitive cells of the LHA have also been shown to affect sympathoadrenal activity in the rat. Injection of phentolamine into the lateral portion of the LHA results in an increase in circulating levels of norepinephrine after exercise in rats (34). Conversely, injection of the antagonist timolol into the same region results in increases in plasma epinephrine during exercise in rats. Catecholamines in the LHA have also been implicated in the central control of renal function (19). In addition, catecholaminergic mechanisms in the LHA are involved in the control of thirst and ingestion behavior (17). Our results suggest, however, that catecholamines are not involved in mediating autonomic responses from the IC in the LHA.

Roles for GABA, acetylcholine, and the catecholamines in the LHA with respect to autonomic regulation cannot be excluded. More systematic investigations of the cardiovascular effects of injecting agonists and antagonists to these neurotransmitters in the LHA are clearly required.

This study indicates that an NMDA glutamatergic synapse mediates the sympathetic renal nerve response to stimulation of the IC. This represents the first relay in the pathway mediating the sympathetic outflow of the IC. The nature of the neurotransmission at this synapse is different from that of the second relay, which is in the VLM. This may represent a difference in the frequency of presynaptic inputs in the two sites. This information may be important in future attempts to modulate the autonomic outflow from the IC.

Fig. 5. Effects of kynurenate (Kyn; A), AP-5 (B), and saline injection (Sal; C) into LHA on arterial pressure (AP), mean arterial pressure (MAP), heart rate (HR), and integrated renal nerve activity (IRNA). In all cases, no changes in baseline nerve activity or cardiovascular variables were observed. bpm, Beats/min.

Fig. 6. Schematic model of efferent pathways from IC to LHA and ventrolateral medulla (VLM). LHA receives efferent projections from several limbic nuclei, including infralimbic cortex (IL), central nucleus of amygdala (Ace), and bed nucleus of stria terminalis (BNST). NMDA receptor may be ideally suited to integration of these inputs with outflow from IC. Non-NMDA receptor in rostral VLM (RVLM) mediates efferent signals from LHA. IML, intermediomedial cell column.

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