AMPAR receptor activation of area postrema neurons

Meredith Hay and Kathy A. Lindsley
Dalton Cardiovascular Research Center, Department of Veterinary Biomedical Sciences, University of Missouri, Columbia, Missouri 65251

AMPAR receptor activation of area postrema neurons. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R586–R590, 1999.—This study reports on the effects of activation of ionotropic glutamate receptors on area postrema neuron cytosolic calcium concentration ([Ca\(^{2+}\)]. In 140 of 242 area postrema neurons isolated from postnatal rats, application of 100 µM L-glutamate (L-Glu) resulted in a significant increase in [Ca\(^{2+}\)]. The remaining neurons were unaffected. The effects of L-Glu on area postrema [Ca\(^{2+}\)] were dose dependent, with a threshold of response near 1.0 µM and maximal response near 100 µM. To determine if the response of L-Glu in area postrema neurons was due to activation of ionotropic glutamate receptors, the effects of the broad-spectrum ionotropic glutamate receptor antagonist kynurinic acid (Kyn) was determined. Application of 1.0 mM Kyn resulted in a 62.6 ± 4% inhibition of the L-Glu-evoked response. Application of the selective N-methyl-D-aspartate (NMDA) antagonist 2-amino-5-phosphonopentanoic acid had no effect on the response of area postrema neurons to 100 µM L-Glu. In contrast, application of the selective D,L-α-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA)/kainate receptor antagonist 6,7-dinitroquinoxaline (DNQX) effectively blocked the 100 µM L-Glu response. Application of (±)-AMPA mimicked the effects observed with L-Glu and was selectively blocked by DNQX. These results suggest that L-Glu activation of area postrema neurons involves activation of AMPA receptors but not NMDA receptors.

circumventricular organs; glutamate receptors; fura 2; cell culture

THE AREA POSTREMA is a circumventricular organ localized in the hindbrain. As a circumventricular organ, the area postrema is devoid of a blood-brain barrier and is subject to modulation by both neural and humoral factors. Substantial evidence has been accumulated suggesting that circulating vasoactive peptides such as ANG II and arginine vasopressin act at the area postrema to modulate sympathetic activity (1, 7–9, 25). In addition, area postrema neurons are known to receive vagal afferent projections that are putatively glutamatergic (20, 21, 24) and have been found to be activated by L-glutamate (L-Glu) (3, 10, 17, 18, 23). However, there is little information on which L-Glu receptors are involved in the activation of area postrema neurons.

Glutamate receptors can be grouped into two major categories: ionotropic receptors and G protein-linked metabotropic receptors. The ionotropic receptors are further subdivided into N-methyl-D-aspartate (NMDA)-sensitive receptors, kainic acid-sensitive receptors, and D,L-α-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA)-sensitive receptors. In situ hybridization studies have identified a number of ionotropic glutamate receptor splice variants in rabbit area postrema neurons (17). Blockade of area postrema glutamate receptors with L-glutamic acid diethyl ester (GDEE) inhibits the cardiovascular responses to microinjections of ANG II, suggesting that endogenous glutamate may have a tonic influence on area postrema activation. These results support the hypothesis that L-Glu may either directly activate area postrema neurons or may regulate the degree of peptide activation of these cells. Identification of the L-Glu receptors expressed on area postrema neurons is important for the understanding of how these cells integrate both neuronal and humoral information. In the present study, the subtypes of ionotropic glutamate receptors involved in L-Glu-induced increases in cytosolic calcium concentration ([Ca\(^{2+}\)] in area postrema neurons were determined.

METHODS

Neuronal culture. Primary cultures of neurons were prepared from area postrema of 10- to 16-day-old postnatal rats. The dissociation protocol was done according to established procedures (5, 14, 16). Briefly, the rats were anesthetized with halothane, and the hindbrain and cerebellum were rapidly removed and placed in 4°C physiological buffer. A 500-µm-thick, horizontal medullary slice, including the area postrema, was obtained using a vibratome. Under a dissecting microscope, the area postrema was identified by its distinguishing shallow orange color and cut away from the surrounding tissue. The tissue was then incubated for 20 min in Earle’s balanced salt solution (Sigma) containing 5 mg/ml papain, 12 mM cysteine, 0.5 mM EDTA, and 1.5 mM CaCl\(_2\) maintained at 37°C. The tissue was then triturated in a papain-free solution with serially smaller pipettes until most of the tissue was dissociated. Dissociated cells were rinsed in DMEM (Sigma) and finally plated on poly-lysine-covered coverslips. Cells were maintained in DMEM with 8 ng/ml nerve growth factor at 37°C in a carbon dioxide incubator.

Calcium measurements. [Ca\(^{2+}\)] was assessed by ratio measurements of the calcium indicator dye fura 2, as described previously (5, 14). Cultures were loaded by addition of 4 µM of the acetoxymethyl ester of fura 2 (fura 2 AM; Molecular Probes) to the culture media and incubated at 37°C in 5% CO\(_2\). After 30 min, the cultures were rinsed once with balanced salt solution containing (in mM): 139 NaCl, 5.4 KCl, 1.8 CaCl\(_2\), 0.1 MgSO\(_4\), 0.9 NaH\(_2\)PO\(_4\), 27.75 glucose, and 10 HEPES. The cells remained in this solution at 37°C for 30–45 min before the initiation of the study. Dishes were placed on the stage of an Olympus inverted fluorescent microscope equipped with a 100-W Hg fluorescent lamp, an Olympus ×40 oil objective, a 410-nm dichroic mirror, a 520 high-pass emission filter, and a computer-controlled shutter and filter wheel with 340- and 380-nm excitation filters. With this configuration, three to four neurons were measured within a given visual field. Fluorescent images of the cells were analyzed with a computer equipped with an imaging program. Dishes were returned to a 37°C incubator for an additional 10 s before data acquisition. Calcium measurements were performed as described previously (5, 14).
obtained using a Synsys digital camera (Photometrics). Paired images were collected in <1 s and analyzed using an image processor (Perceptics). Ratio measurements were calculated each 20 s according to the method of Grynkiewicz et al. (11).

Data analysis. The values are means ± SE from cell groups. The means were compared by ANOVA followed by a Mann-Whitney test, and differences at P < 0.05 were considered statistically significant.

RESULTS

All experiments were performed on area postrema neurons maintained in culture for 7–14 days. The distinct morphology of the area postrema neurons after 7–14 days in culture, including phase-bright, 8- to 10-µm-diameter soma and 2- to 5-µm-long thin processes, were readily distinguished from the flat, large glial cells. In addition, area postrema neurons can be distinguished from glial cells through the immunocytochemical identification of neurofilament protein within neurons (Fig. 1A). Figure 1B is a pseudocolor digital image showing the temporal and spatial distribution of increased [Ca$$^{2+}$$], in response to 100 µM L-Glu. [Ca$$^{2+}$$] appeared to be distributed nonhomogeneously within the cytosol and processes. The average basal level of [Ca$$^{2+}$$] in the neurons studied was 103.6 ± 7 nM (n = 140). This basal level did not change significantly within the first 30 min of the experimental protocol. However, in experimental protocols that lasted >60 min, the basal [Ca$$^{2+}$$] increased to 139.1 ± 17 nM (n = 18).

In 140 of 242 area postrema neurons tested, application of 100 µM L-Glu resulted in a significant increase in [Ca$$^{2+}$$]. In the remaining neurons, L-Glu did not elicit any change in [Ca$$^{2+}$$]. The response to 100 µM L-Glu reached maximum within 10 s and recovered to baseline values within 2 min. The activation of area postrema neurons by L-Glu was repeatable and reversible.

![Image](http://ajpregu.physiology.org/)

**Fig. 1.** A: rhodamine-labeled area postrema neurons that exhibited positive immunoreactivity for neuronal filament reaction product. Arrows indicate soma of area postrema neuron (bar = 10 µM). B: pseudocolor image of cytosolic calcium distribution in a fura 2-loaded area postrema neuron after application of L-glutamate (L-Glu). Circle around center of soma indicates region from which pixel intensity was averaged to obtain the 340/380 ratio data. t, Time.
Removal of extracellular Ca\textsuperscript{2+} inhibited the responses of area postrema neurons to L-Glu (data not shown). The effects of L-Glu on area postrema [Ca\textsuperscript{2+}] were increased with increasing concentrations of L-Glu. Figure 2A illustrates the averaged effects of increasing concentrations of L-Glu in 12 neurons. The threshold dose of L-Glu was observed near 1.0 µM, and maximum response was near 100 µM. Similar responses were obtained when the different doses were applied randomly. To determine if the response of L-Glu in area postrema neurons was due to activation of ionotropic glutamate receptors, the effects of the broad-spectrum ionotropic glutamate receptor antagonist kynurenic acid (Kyn) was determined. Figure 2B illustrates the averaged effects of Kyn on the L-Glu-evoked response (n = 6). Application of 1.0 mM Kyn resulted in a 62.6 ± 4% inhibition of the L-Glu-evoked response (Fig. 2B). Application of Kyn alone had no effect on [Ca\textsuperscript{2+}].

To determine if the NMDA receptor was involved in L-Glu activation of area postrema neurons, the effect of the selective NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (L-AP-5) was determined. Figure 3A illustrates the effect of both L-AP-5 and the selective AMPA/kainate receptor antagonist 6,7-dinitroquinoxaline (DNQX) in a single neuron. In this cell, application of 50–500 µM L-AP-5 had no effect on the response of area postrema neurons to 100 µM L-Glu. Application of NMDA was also without effect on these cells (n = 5, data not shown). In contrast, application of the AMPA/kainate receptor antagonist DNQX effectively blocked the 100 µM L-Glu response, with maximum inhibition of the L-Glu response achieved with 5 µM DNQX. Figure 3B illustrates the averaged effects of L-AP-5 and DNQX on the L-Glu-evoked response. Application of 5 µM DNQX eliminated the response to L-Glu in all cells tested, whereas application of 500 µM L-AP-5 had no effect (n = 6). Application of 500 µM DNQX alone had no effect on [Ca\textsuperscript{2+}].

To determine if the response to L-Glu could be mimicked by selective activation of the AMPA receptor, the effects of (±)-AMPA was determined. Figure 4A illustrates the averaged effect of different concentrations of AMPA on area postrema neurons. In 30 of 73 neurons tested, application of AMPA resulted in responses similar to those observed with L-Glu. Increasing the concentration of AMPA from 1.0 to 100 µM resulted in a progressively greater increase in area postrema [Ca\textsuperscript{2+}]. Similar responses were obtained when
the different doses were applied randomly. In all cells tested, cells that were responsive to AMPA were also responsive to L-Glu. Figure 4B illustrates the averaged effects of DNQX on AMPA-evoked [Ca^{2+}]i in area postrema neurons. Application of 50 µM DNQX blocked the effects of 100 µM AMPA.

**DISCUSSION**

The present study used the isolated area postrema neuronal preparation to identify the receptor subtype involved in L-Glu activation of area postrema neurons in the absence of any synaptic activation by other cell types. The results from this study are the first to suggest that L-Glu activation of rat area postrema neurons involves activation of AMPA glutamate receptors. In addition, these results suggest that area postrema activation by L-Glu does not involve activation of NMDA receptors. Importantly, only 58% of the area postrema neurons tested were responsive to L-Glu. This heterogeneity of response is consistent with other studies in both the rat and rabbit brain stem slices in which 48 and 54%, respectively, were responsive to application of L-Glu (17, 23). This heterogeneity may reflect multiple populations of area postrema neurons. It is reasonable to speculate that, in vivo, these non-L-Glu-sensitive cells may represent interneurons within the area postrema or a population of cells with distinct projections to other central nuclei.

The role of L-Glu as a neurotransmitter in the area postrema has been studied both in vivo (3) and in the brain stem slice (17, 23). In the anesthetized cat, L-Glu has been reported to increase area postrema activity in the majority of cells tested (3). Likewise, in the rabbit brain stem slice, 1 mM L-Glu, quisqualate, and kainate resulted in the activation of a desensitizing inward cation current (17). It was concluded that this response was not due to activation of NMDA receptors but most likely due to activation of non-NMDA receptors. In addition, these investigators reported that rabbit area postrema neurons express multiple AMPA receptor subunits. In the rat, L-Glu has been reported to both increase and decrease area postrema activity (24). The excitatory effects of L-Glu in the rat were mimicked by application of NMDA, and it was concluded that area postrema neurons may use NMDA receptors for L-Glu neurotransmission. These studies, however, did not attempt to block the L-Glu or NMDA response with any known glutamate receptor antagonist, thus limiting any definitive conclusions about the L-Glu receptor involved. In contrast, the results from the present study in which the response to L-Glu was neither blocked by AP-5 nor mimicked by NMDA would suggest that NMDA receptors are not involved in L-Glu activation of rat area postrema neurons. Although the cause for the disparity in these two results in rat area postrema is unknown, there are a number of reasonable hypotheses for these differences. First, the results in the present study are from primary cultures of area postrema neurons. Because of enzymatic dissociation and culture conditions, these cells may not express the same complement of receptors as seen in vivo. However, in our hands, cultured area postrema neurons have been found to express nearly the same complement of receptors and ion channels that have been reported in vivo, including the angiotensin II type 1 receptor, the vasopressin 1 receptor, serotonin receptors, catecholamine receptors, AMPA receptors, and others (1, 5, 14, 16, 20). In addition, NMDA receptors have been extensively studied in other types of dissociated and cultured neurons in which expression level has been found to be similar to that in vivo (4, 22). Thus, although possible, it seems unlikely that the cultured area postrema cells are selectively not expressing NMDA receptors. Secondly, the results from the present study are from neurons taken from 12- to 14-day-old rat pups and then maintained in culture for an additional 7–14 days. These younger animals may not express the NMDA receptor.

The role of endogenous L-Glu in the regulation of area postrema activity has not yet been established. It has been found that area postrema neurons receive peripheral information from both vagal afferents and aortic nerve afferents (20, 26). Considerable evidence exists that the primary neurotransmitter in these sensory afferents is L-Glu (19, 24). Thus vagal afferents innervating the area postrema may use L-Glu to modulate not
only baseline area postrema activity but also the effects of circulating peptide and hormone activation of these cells. Evidence for this hypothesis was reported in anesthetized rats (18). In these studies, the effects of L-Glu and neuropeptide interactions within the area postrema on cardiovascular regulation were determined. Area postrema microinjection of the broad-spectrum glutamate antagonist GDEE was found to inhibit the cardiovascular responses to microinjections of ANG II. These authors concluded that endogenous glutamate may have a tonic influence on area postrema activation. The results from the present study would suggest that AMPA receptors may be responsible for the majority of L-Glu activation of rat area postrema neurons.

Perspectives

Activation of the area postrema has been shown to be involved in a number of physiological functions, including taste sensitivity, emesis, and cardiovascular regulation (2, 6, 8, 9, 25). Regarding cardiovascular control, the area postrema has been found to be activated by circulating vasoactive peptides such as ANG II and arginine vasopressin (1). Activation of area postrema neurons has been shown to have a profound effect on regulation of arterial pressure. Can. J. Physiol. Pharmacol. 65: 1596–1597, 1987.


