

Localization of Ventral Brainstem CO₂ Chemosensors by Kainic Acid Neurotoxic Lesion

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I. Introduction

Physiological evidence indicates that neurons in the caudal ventrolateral medullary surface (cVMS) of rats and cats have an important role in the central regulation of both the cardiovascular and respiratory systems (1-5). Electrical stimulation (6,7) and microinjections of excitatory amino acids (EAA) (8-10) at the ventral medullary surface (VMS) elevate blood pressure and increase respiration. Gamma-amino butyric acid (GABA), EAA antagonists, and bilateral electrolytic and chemical lesions of the VMS decrease blood pressure and respiration (11-18). Immunocytochemical expression of *c-fos*, a marker for increased neuronal activity, has been used to characterize a population of carbon dioxide (CO₂)-sensitive neurons at the cVMS after 1 h of hypercapnia (19). These studies indicate that the VMS contains chemosensitive structures that are sensitive to pH and CO₂ changes. They also suggest that EAA play an important role in the central processing of cardiorespiratory afferent information. Afferent activity of these and other intracranial chemosensors at or near the VMS appear to be the dominant source of chemoreceptor drive to the respiratory controller (20-22). However, several workers have suggested that

central respiratory chemosensitivity is at least partially provided by the deeper-lying respiratory centers (23,24) and that a localized and circumscribed region of central chemosensitivity other than the respiratory centers themselves may not be necessary (25,26).

Several techniques have been utilized to describe the morphological substrates subserving respiratory chemosensitivity at the ventral medulla. Schläfke and Loeschcke (27) thermally lesioned the intermediate chemosensitive zone (area S) (Fig. 1) and induced respiratory arrest in peripheral chemoreceptor-denervated cats in which the contralateral area S was coagulated. They proposed that central chemosensitivity was lost by this procedure. In a later study, Schläfke et al. (12) coagulated the chemosensitive apparatus that was believed to be operational at area S. They noted that there was diminished or no response to acidity or hypercapnia when the central chemosensory apparatus at area S was compromised. Histological examination of the coagulated tissue

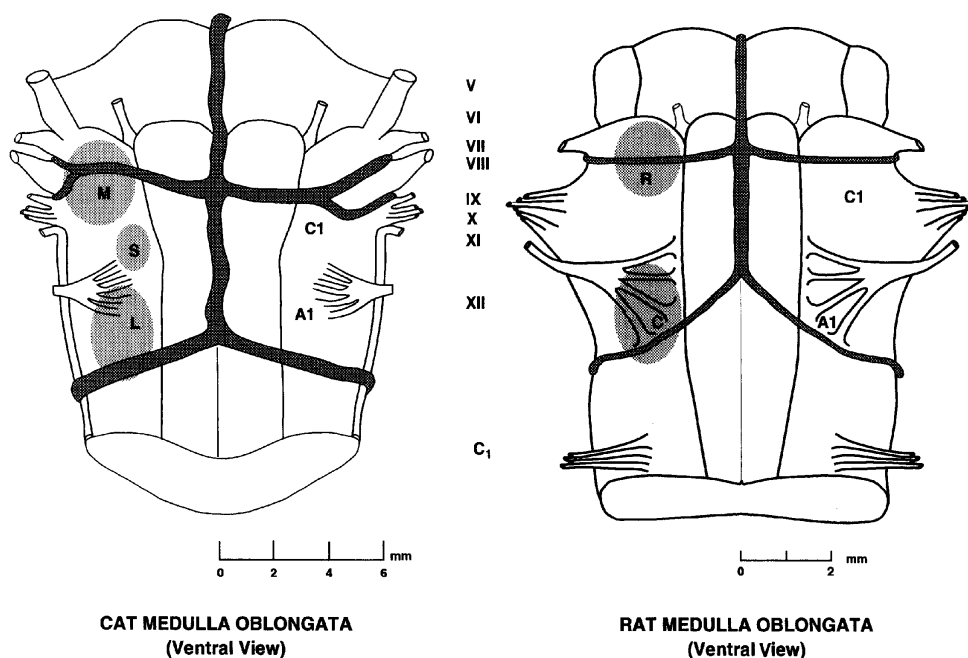


Figure 1 Ventral surface of the medulla oblongata of the cat (left) and the rat (right). In the cat, the three respiratory chemosensitive areas, "M" (Mitchell et al.) (2), "S," and "L" (Schläfke et al.) (27) are indicated on the left half of the diagram and represent the rostral, intermediate and caudal areas, respectively. In the rat, the two chemosensitive zones (Mittra et al.) (49), "R" and "C," are indicated on the left half of the diagram and represent the rostral and caudal chemosensitive zones, respectively. In both right hemimedullas, the symbols C1 and A1 represent the vasopressor and vasodepressor areas, respectively. V-XII = cranial nerves.

revealed destruction of marginal glia, superficial neurons, small blood vessels, capillaries, arcuate fibers, and other superficial fibers (12).

Berndt et al. (28) applied procaine and toxic concentrations of potassium (K^+) ions to the VMS of cats while stimulating the cVMS and the respiratory centers in peripheral chemoreceptor-denervated animals. Electrical stimulation of the cVMS caused increased ventilation, which was blocked, causing apnea following topical application of procaine to the cVMS. Electrical stimulation of the ventral respiratory group (VRG), however, reversed the procaine blockade, causing an inspiratory response. These studies indicated that the respiratory centers were still intact and functional despite the procaine-induced apnea. As with procaine, toxic concentrations of K^+ topically applied to the VMS induced apnea that was initially overridden by electrical stimulation of the VRG. However, within a few minutes, total apnea that was not overridden by electrical stimulation of the VRG ensued, indicating that K^+ had diffused to the respiratory centers and rendered them dysfunctional. These experiments demonstrated that the integrity of the VMS structures was essential to driving the respiratory centers in the absence of peripheral chemoreceptor afferents (28). Because these procedures destroyed or blocked cell bodies as well as fibers in transit, these studies failed to discriminate between surface neurons at chemosensitive sites and fibers of passage originating from other central or peripheral nervous structures. A better method had to be found.

The intraneuronal events triggered by EAA receptor stimulation have developed considerable interest over the past decade. Not only are EAA major excitatory neurotransmitters in mammalian brain but they also produce neuronal damage when present in excessive amounts. In 1978, J. T. Coyle (29), utilizing the restricted glutamate (Glu) analog kainic acid (KA), described a superior lesioning technique that destroyed cell bodies of neurons but not fibers of passage. Toxic doses of EAA are believed to destroy cell bodies of neurons but not fibers of passage (30,31). Indeed, this property provides an exquisite lesioning tool that allows for a finer localization, definition, and separation of the structures under investigation. Depending on the duration of the EAA treatment, neuronal death can be produced either by a rapid sodium- and chloride-dependent osmotic lysis (32,33) or by a more protracted calcium (Ca^{2+})-dependent process (33-35). Destabilization of ionized cytosolic calcium homeostasis and the biochemical events that are modulated by intracellular calcium (Ca_i) may also play a pivotal role in excessive Glu receptor stimulation and subsequent neuronal demise (35-38). By chemical lesioning with KA, the neuronal substrates subserving cVMS responsiveness to inspired CO_2 and topically applied acetylcholine (ACh) can be identified. We therefore applied toxic doses of KA to the VMS of rats before and after testing for the ventilatory and neuronal responses to inspired CO_2 and ACh.

In spontaneously breathing animals ($n = 8$) anesthetized with chloralose-urethane, we tested superficial neurons in the caudal chemosensitive area (area L)

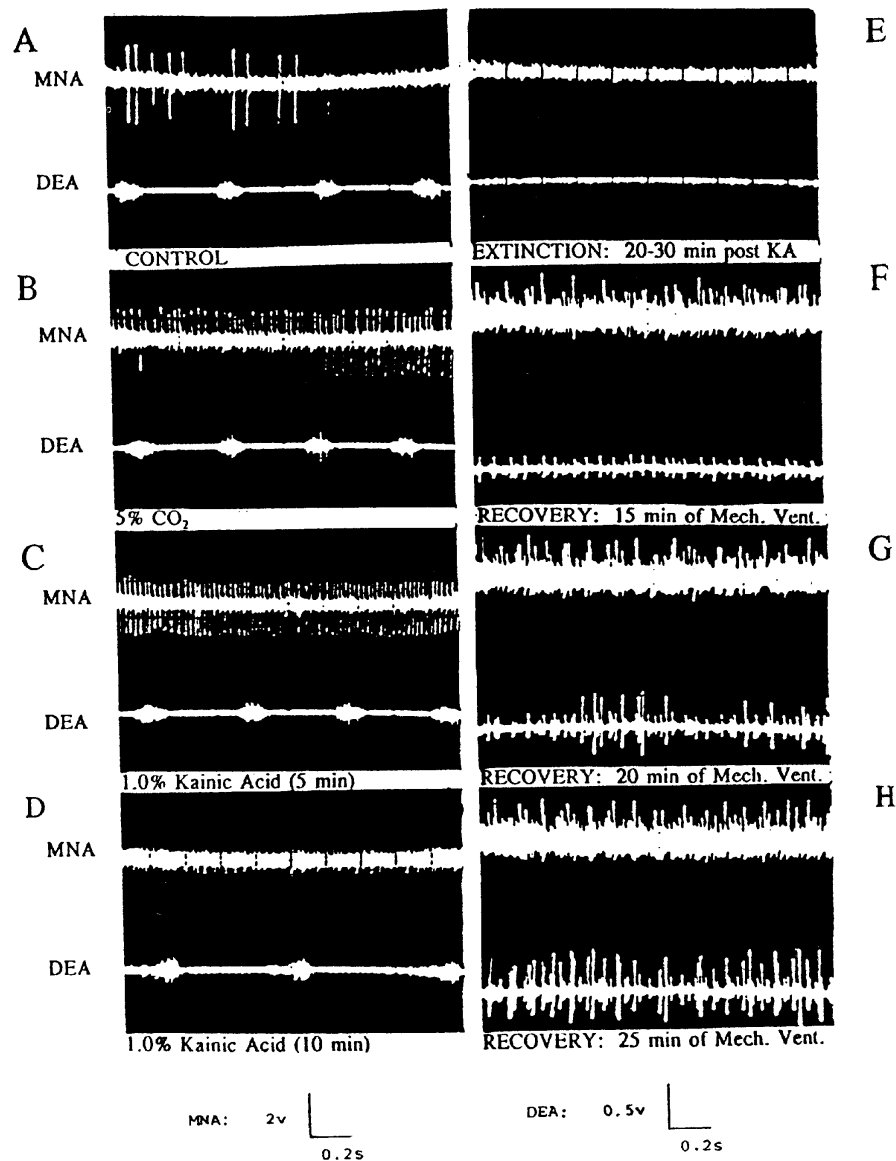


Figure 2 Nonphasic CO₂-sensitive unit discharge during the topical application of excitatory amino acids to the ventral medullary surface. Upper tracings: medullary neuronal activity (MNA). Lower tracings: diaphragmatic electromyographic activity (DEA). (A) control (MNA = 19 Hz; DEA = 48 bpm); (B) 5% PICO₂ (MNA = 27.9 Hz; DEA = 63 bpm); (C) 5 min post 1% kainic acid (KA) (MNA = 34.8 Hz; DEA = 53 bpm); (D) 10 min post 1% KA; (E) extinction (20 to 30 min post 1% KA), i.e., apnea and neuronal silence; (F) recovery: mechanical ventilation, 15 min postextinction; (G and H) recovery at 20 and 25 min postextinction, respectively (bpm = bursts per minute).

for their responsiveness to increased inspired CO₂ and topically applied ACh, while recording medullary neuronal activity (MNA) extracellularly and diaphragmatic electromyographic activity (DEA). Increased inspired CO₂ and ACh transiently increased MNA as well as DEA (Fig. 2A to E). Following topical application of 1% KA, there was a marked increase in MNA and DEA within 2 to 5 min. Ten to 15 min later, there were decrements in both MNA and DEA, with the animals demonstrating periodic gasping activity. At approximately 15 to 20 min after KA application, MNA subsided to extinction and DEA ceased; the animal expired. Thus KA, an excitotoxic amino acid, caused marked and prolonged increases in MNA and DEA, followed by depression to extinction and respiratory arrest.

The chemosensitive response to CO₂ and ACh must have originated from neurons at the surface and not from fibers of passage. Therefore, KA may have destroyed superficial chemosensitive VMS cell bodies, and the integrity of these chemosensitive VMS neurons in the anesthetized rat appears essential to the maintenance of the central drive of respiration (3,39-44). These results support studies of Berndt et al. (28) and Schläfke et al. (12).

II. Cardiorespiratory Effects on VMS Cardiorespiratory Control

The VMS has both respiratory and cardiovascular control elements (1,5,45,46) and is thought to perform an important integrative function in the central regulation of the respiratory and cardiovascular systems (4). Vasopressor and vasodepressor regions have been identified within the VMS (10,16,17,47,48) and on the VMS (49-52). It has also been recognized that EAA play a significant role in the modulation of both respiratory and cardiovascular elements within and upon the ventrolateral medulla (VLM) (14,18,44,53-56). We therefore attempted to determine whether the KA-induced demise of CO₂-sensitive neurons (and elimination of CO₂ chemosensitivity) would result in dysfunction of the respiratory reflex apparatus or disruption of cardiovascular elements within the cVMS.

In these investigations, we utilized the neurotoxin KA to discriminate between cardiovascular and respiratory structures in the VMS. In spontaneously breathing animals anesthetized with chloralose-urethane, neurons in the caudal chemosensitive area (area L) were tested for their responsiveness to physiological and pharmacological stimuli before and after the topical application of 1% (47 mM) KA to the cVMS. As in previous experiments, 1% KA at first increased ventilation, then induced apnea within 10 to 20 min. It is possible that KA destroys only cell bodies (29); however, it is uncertain (a) whether KA causes cell death or merely metabolically depletes the cell or (b) whether fibers of passage are affected by toxic doses of EAA.

In an attempt to determine which of the two systems, respiratory or cardiovascular, was compromised in the KA-induced apneic demise of the animal, we instituted artificial ventilation with a Narco small-animal ventilator for up to 6 h immediately following the induction of the apnea by the neurotoxin KA. We speculated that the cVMS neurons in these studies may have been depolarized to the point where they were refractory to incoming stimuli, thus leading to the apnea induced by KA. After 5 to 10 min of mechanical ventilation, MNA and DEA recovered. MNA initially appeared as low-amplitude spikes that increased in frequency over the next few minutes. After 5 to 10 min, MNA increased to a maximal frequency of 84.14 ± 10.19 Hz (Fig. 2F to H). The MNA during this period of recovery is reminiscent of evoked and spontaneous epileptiform bursting described in hippocampal slice preparations (57–60) and in *in vivo* models of epilepsy (61–63). KA may induce activation of *N*-methyl-D-aspartate (NMDA) receptors (one of the three classic Glu receptors) which have been implicated in the generation of evoked and spontaneous burst generation (64–66).

In toxic doses, KA can directly depolarize postsynaptic elements, mobilize other EAA, induce depolarization-modulated release of the Mg^{2+} block on the NMDA channel, and markedly increase intracellular Ca^{2+} accumulation (65–72). One might assume that the KA-induced respiratory failure might in some way be associated with the toxic effects of Ca^{2+} on neurons at caudal ventral brainstem chemosensitive sites, but this must be proven. Failure of these chemosensory neurons to communicate with or modulate the activity of the central respiratory integrating centers would result in apnea. This would imply that these circumscribed superficial neuronal elements are necessary to the central drive of respiration.

During the recovery period, DEA initially demonstrated low-frequency, low-amplitude random spikes that increased in frequency and amplitude over the next 60 min to a generalized tonic state. Thereafter, DEA diminished in amplitude and frequency to the point of extinction within 3 to 4 h. The DEA indicated that the burstlike (phasic) activity reflective of phrenic nerve or diaphragmatic electromyographic activity never resumed. This result further indicates a disruption of the organization of respiratory motor output coordination and a loss of communication between the respiratory centers, *per se*, and the phrenic nucleus. We therefore postulate that KA renders the superficial CO_2 -sensitive neuronal pool at the caudal VMS inoperable and that, in spite of the continued efficacious function of the respiratory centers and phrenic nucleus, the animal appears to undergo irreversible apnea.

After 3 to 4 h, and even though DEA had achieved extinction, MNA continued unabated until the animal expired or was sacrificed. Attempts to wean the animals from the respirator were unsuccessful, demonstrating a rapid decline in MNA, DEA, or both within 1 to 2 min. The animals expired if they were not immediately returned to the ventilator. Thus, testing for CO_2 respon-

siveness under these conditions has not been possible, since MNA, DEA, and ventilation could not be sustained independent of assisted breathing.

Assuming that there are cardiovascular control elements located at or near the VMS (50,73) as well as 1 to 2 mm deep (the A1 area) (10,48,74,75), it was noteworthy that superficial respiratory components were disabled by KA, yet the animal could be maintained by artificial respiration for several hours after the induction of apnea. This result implies that these cardiovascular elements were either intact or, if compromised, of insufficient influence on the putative vasomotor centers to deprive the animal of sympathetic vasomotor drive. This led to the examination of the effects of KA on the cardiovascular system.

In related experiments, we therefore examined the respiratory and blood pressure responses of rats ($n = 5$) to the topical application of EAA agonists and antagonists and KA. Respiratory responses were recorded via a low-inertia Krogh spirometer and blood pressure via a Stratham strain gauge transducer. Compared with control levels, topically applied Glu-evoked dose-related increases in mean arterial blood pressure (MABP) and tidal volume (V_T) with no apparent effect on respiratory frequency (f) (Fig. 3A). There was an apparent desensitization of the Glu response, similar to that noted by Addae and Stone (76) in the response of cortical neurons to NMDA (76–79) in that subsequent applications of Glu within 5 to 10 min of the previous applications did not induce ventilatory or blood pressure responses. Those responses, however, could again be elicited after approximately 30 min. NMDA increased MABP and V_T , and decreased f (Fig. 3B).

Conversely, the NMDA antagonists (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK801), DL-2-amino-7-phosphono-heptanoic acid (AP7), and (\pm)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) decreased both MABP and V_T , and increased f (Fig. 3C). The topical application of 1% KA produced a marked but transient increase in both MABP and V_T and a decrease in f . These experiments, as well as those of Gattie et al. (52) and of Martin and Sinclair (44) demonstrated that cells in or near the VMS contain EAA receptors both of the NMDA and non-NMDA varieties. They also appear to indicate that the EAA receptors at the VMS are involved in the timing (rhythm generation) and amplitude (pattern generation) responses of the respiratory reflex apparatus located in the caudal and rostral chemosensitive zones (80–83). A more complete examination of the EAA receptor subtypes serving cardiorespiratory responses at the VMS seems warranted.

However, as in our electrophysiological experiments, topically applied KA led to decrements in V_T and f over the next 20 to 30 min, after the initially marked increase in V_T , and subsequent apnea. If the process was uninterrupted, the animal expired, but if it was placed on a mechanical ventilator, the animal could be maintained for up to 6 h. We monitored the MABP continuously throughout this procedure and noted that, after its transient increase, MABP

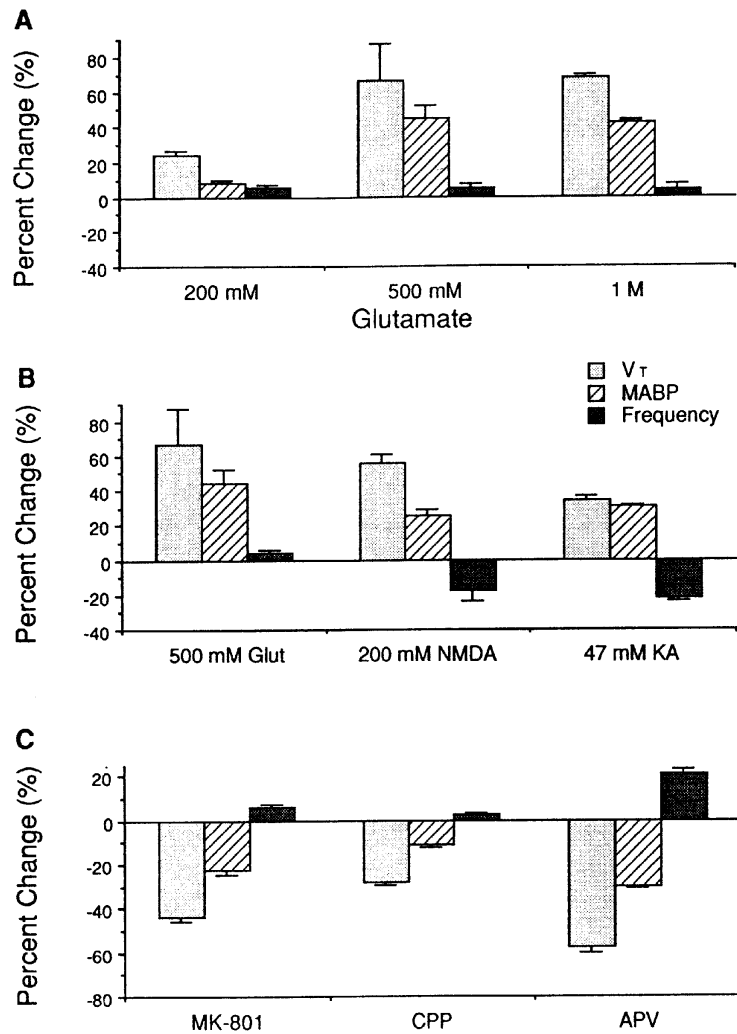


Figure 3 Effects of excitatory amino acid agonists and antagonists topically applied to the ventral medullary surface (VMS) on tidal volume (V_T), mean arterial blood pressure (MABP), and respiratory frequency (f) in rats. Mean percent changes \pm standard error of the mean (SEM). Each animal serves as its own control. (A) Dose-related increase in V_T and MABP in response to Glu; (B) effect of NMDA (500 mM Glu, 200 mM NMDA) and non-NMDA (47 mM KA) receptor stimulation; (C) effect of three specific NMDA antagonists (10 mM MK801, 400 nM CPP, and 10 mM AP7).

returned to baseline levels and remained there when the animals were maintained via mechanical ventilation. Therefore, superficial CO_2 -sensitive cells at the VMS may have some but not an overriding influence on the central cardiovascular controlling elements. A vasodepressor area (area A1) (10,84,85) whose cells contain norepinephrine has been described in the cVLM, located 1 to 1.5 mm

deep to the VMS in the rat. Microinjections of EAAs in the A1 area have yielded vasodepressor responses (74), while the topical application of EAA agonists to the VMS in the same region (area L) (44,83) induces transient increases in MABP. This implies a separation or demarcation of superficial cardiorespiratory neurons and deeper-lying purely cardiovascular neurons 1 to 1.5 mm deep in the A1 area. In consideration of the rate of diffusion of small molecules such as KA (molecular weight 214.1 g), Martin and Sinclair (44) developed a mathematical model based on the solution of the one-dimensional diffusion equation and a Gallerkin finite element model and determined that a 47 mM (1%) KA solution applied topically to the VMS for only 2 min would achieve cytotoxic levels in a few seconds at the VMS and at deeper levels within a few minutes (44,45). KA may also be cytotoxic in nanomolar concentrations (86), which are readily achieved deep within the medullary tissue within 20 to 30 min from a topical site of application. Another aspect of the rate of diffusion of substances into brain parenchyma from a subarachnoid space or cisternal locus is the access of these molecules to the interstitial (extracellular) space via the perivascular spaces and basal laminae of penetrating arteries (87,88). Rennels et al. (88), utilizing intracisternal horseradish peroxidase perfusion (HRP), described penetrant arteries and emergent veins that were enclosed by fluid-filled perivascular spaces in contiguity with the extracellular and subarachnoid spaces, which functioned as "express routes" and have been designated as paravascular spaces. They believed that the rapid paravascular influx and spread of HRP were not simply due to diffusion but depended on the pulsations of penetrating arterioles and the convective forces generated thereby (87,88). These arguments, however, do not adequately explain the differences noted in the MABP responses to topically applied KA and KA microinjected into the A1 area. They also do not address the issue of the disruption of the respiratory apparatus at the VMS leading to apnea, while the VRG and other deeper-lying medullary neuronal pools are unaffected.

The question therefore arose as to which cells at the VMS were activated and subsequently made dysfunctional by the topical application of 1% KA without apparently affecting cardiovascular control mechanisms within the cVLM. Because calcium has been implicated in the neurotoxicity of KA and EAA (36, 89,90), we investigated the subcellular localization of calcium in cVMS cells in response to the topical application of 1% KA.

III. Ultracytochemical Localization of Calcium in Rat cVLM in Response to Topically Applied KA

The mechanism of action of KA as an excitatory amino acid in the central nervous system (CNS) appears to be the mediation of the voltage-independent portion of the synaptic response to unitary synaptic activation (91) by the opening of a voltage-independent cation channel, and the modulation of synaptic activity

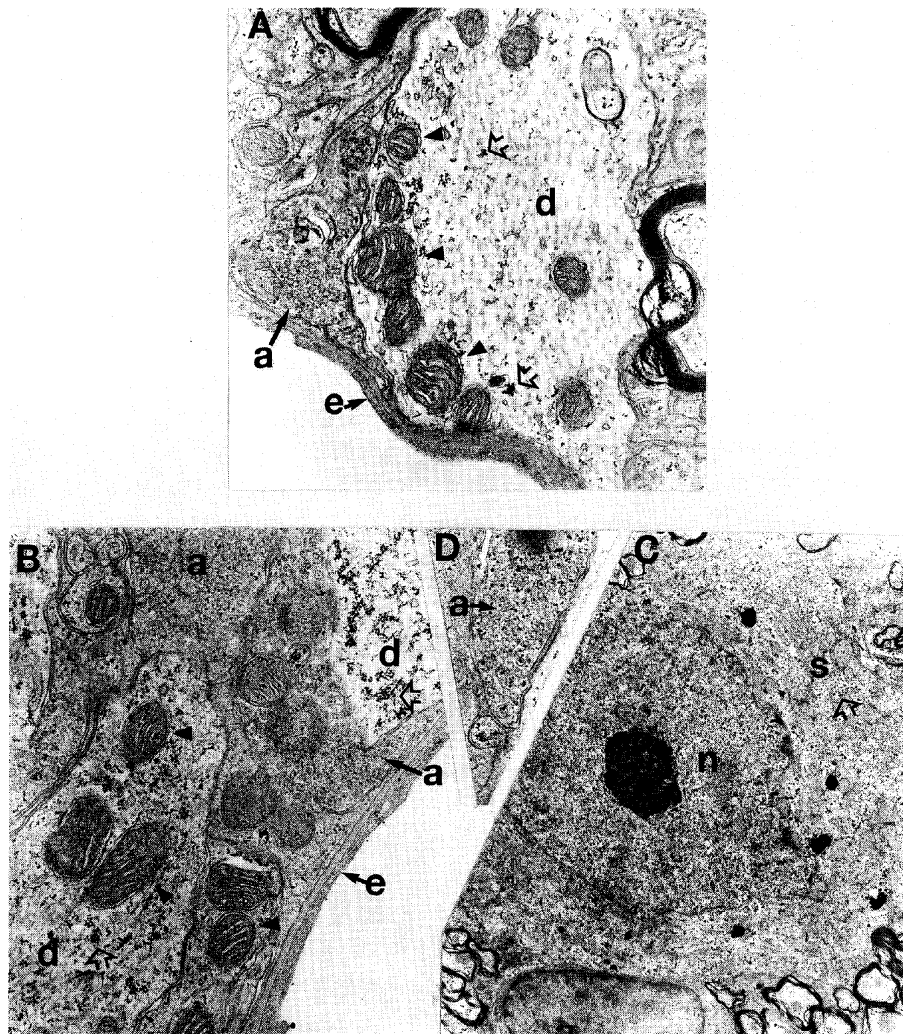


Figure 4 Control: Electron micrographs from superficial ventral brainstem at the CVLM of the normal rat processed by the oxalate-pyroantimonate method for localizing calcium. Uranyl acetate and lead citrate staining. Labels: n-nucleus, s-neuronal soma, e-endothelial wall, d-dendrite, a-axon terminal. Note fine grains or clumps of electron-dense calcium deposits (open arrows) throughout the tissue. (A) Axon terminal and dendritic process abutting blood vessel wall (neurovascular elements); numerous mitochondria (closed arrowheads) that contain variable amounts of Ca^{2+} deposit ($\times 21,000$). (B) Dendrites and axon terminals adjacent to blood vessels ($\times 28,000$). (C) Superficial neuron demonstrating fine nuclear and cytoplasmic deposition ($\times 5,200$). (D) Inset: axon terminal (a) apposed to blood vessel wall with calcium deposits in clear round vesicles ($\times 28,000$).

by calcium channels. EAA agonists depolarize cells by activation of NMDA and non-NMDA (quisqualate/AMPA, kainate) receptors that induce the influx of cationic species (Na^+ , Ca^{2+}) via voltage-sensitive and voltage-independent means (91–93). EAA neurotoxicity is thought to be mediated by the influx of Na^+ , Cl^- , and Ca^{2+} , leading to a sustained depolarization and the release of intracellular calcium (Ca_i) in amounts that overwhelm the Ca_i homeostatic apparatus and renders the neuron susceptible to swelling and the pejorative activation of lipases, proteases, and so on, by Ca^{2+} (36,93–95).

EAA have also been implicated in the neurotoxicity associated with several clinical conditions [such as Huntington's chorea and hypoglycemia (96–99)], and it has been postulated that, depending on the duration of EAA treatment, neuronal death can be produced either by a rapid sodium- and chloride-dependent osmotic lysis or by a more protracted calcium-dependent process. Indeed, the KA-induced increase in Ca_i is believed to be essentially caused by influx via agonist-operated and voltage-operated Ca^{2+} channels, whereas only a small contribution is generated by release from intracellular stores (95). Because EAA neuronal activation may lead to a translocation of Ca^{2+} from the extracellular fluid (ECF) and intracellular stores, we examined the distribution of cytosolic Ca^{2+} in the cVMS of KA-stimulated neurons.

We therefore utilized the combined oxalate-pyroantimonate technique (100–103) and electron microscopy to assess the role of calcium in the presumed mortal response of the chemosensory apparatus to KA stimulation and to possibly visualize and characterize these superficial elements by their altered calcium distribution. EAA receptor activation leads to increased intracellular calcium via three potential routes: voltage-gated Ca^{2+} channels, agonist-activated channels permeable to Ca^{2+} as well as monovalent ions (e.g., the NMDA channel), and by release from intracellular stores. In these studies, pledgets containing 1% KA were topically applied to the cVMS of rats ($n = 5$). The pledgets were quickly removed after a 2-min application period and the cVMS thoroughly rinsed with mCSF, pH 7.4. At the peak of the neuronal and ventilatory response to topical KA (10 min postapplication), the animals were perfused with a 90-mM potassium-oxalate solution of 3% glutaraldehyde and 2% paraformaldehyde, postfixed with a 2% potassium pyroantimonate–1% osmium tetroxide aqueous solution, and processed for electron microscopy. In control tissues, a discrete pattern of electron-dense Ca^{2+} -pyroantimonate deposition, similar to that described by Griffiths et al. (104) in hippocampal tissue and by Garthwaite and Garthwaite (105) in cerebellar tissue, was noted overlying some neuronal nuclei, in some dendrites, occasional mitochondria, smooth endoplasmic reticulum (SER), with sparse or no deposits in the cytosol, and none in the ECF (Fig. 5A to E). The Ca^{2+} precipitate could also be visualized intraaxonally (axoplasmic) and within synaptic terminals overlying mitochondria and vesicles. In both control and experimental tissues, the Ca^{2+} -pyroantimonate reaction prod-

EAA have been implicated in the neurotoxicity associated with several clinical conditions. It has been postulated that, depending on the duration of EAA treatment, neuronal death can be produced either by rapid sodium- and chloride-dependent osmotic lysis or by a more protracted calcium-dependent process. On the other hand, destabilization of ionized cytosolic calcium homeostasis and the biomedical events that are modulated by Ca_i might play a pivotal role in excessive Glu receptor stimulation and subsequent neuronal demise. Using the combined oxalate-pyroantimonate technique, ultracytochemical localization of Ca_i was performed to assess the role of calcium in the response of the chemosensory apparatus to EAA stimulation and eventual demise. In control tissues, a discrete pattern of electron-dense Ca^{2+} -pyroantimonate deposition was noted overlying neuronal nuclei, mitochondria, and endoplasmic reticulum, with sparse deposits in the cytoplasm and none in the extracellular space. The Ca^{2+} precipitation could also be visualized intraaxonally, scattered within the myelin sheaths in bleb-like processes and within synapses overlying mitochondria and vesicles. Tissues perfused at peak MNA following KA stimulation demonstrated a dramatic increase in cytosolic calcium. The endoplasmic reticulum, however, which contained deposits of calcium in control tissues, essentially showed sparse or no calcium-pyroantimonate deposits in the KA-treated tissues. There were also notable increases in the density of precipitates overlying the nuclei and swollen mitochondria of affected neurons, within axons and swollen dendrites, and especially within synapses where vesicular deposition appeared to be maximal. The precipitate could also be detected within the extracellular compartments and within the endothelia of medullary blood vessels in this region. These results suggest that (a) KA compromises the ability of the superficial neurons to maintain the central drive to respiration; (b) KA appears to deplete the neuronal metabolic stores of superficial CO_2 -sensitive neuronal elements; (c) the cardiovascular component of the VMS seems unimpaired by the topical application of KA; and (d) EAA stimulation does indeed induce a mobilization of Ca^{2+} into the cytosol, mitochondria, and nuclei of neurons from intracellular or extracellular loci. It is postulated that increased Ca_i might be involved in the neurotoxic actions of EAA agonists at the VMS.

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