Calcium/calmodulin-dependent kinase II mediates critical components of the hypoxic ventilatory response within the nucleus of the solitary tract in adult rats

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Reeves, Stephen R., Edwin S. Carter, Shang Z. Guo, and David Gozal. Calcium/calmodulin-dependent kinase II mediates critical components of the hypoxic ventilatory response within the nucleus of the solitary tract in adult rats. Am J Physiol Regul Integr Comp Physiol 289: R871–R876, 2005. First published May 12, 2005; doi:10.1152/ajpregu.00249.2005.—Calcium/calmodulin-dependent kinase II (CaMKII) is an ubiquitous second messenger that is highly expressed in neurons, where it has been implicated in some of the pathways regulating neuronal discharge as well as N-methyl-D-aspartate receptor-mediated synaptic plasticity. The full expression of the mammalian hypoxic ventilatory response (HVR) requires intact central relays within the nucleus of the solitary tract (NTS), and neural transmission of hypoxicafferent input is mediated by glutamatergic receptor activity, primarily through N-methyl-D-aspartate receptors. To examine the functional role of CaMKII in HVR, KN-93, a highly selective antagonist of CaMKII, was microinjected in the NTS via bilaterally placed osmotic pumps in freely behaving adult male Sprague-Dawley rats for 3 days. Vehicle-loaded osmotic pumps were surgically placed in control animals, and adequate placement of cannulas was ascertained for all animals. HVR was measured using whole body plethysmography during exposure to 10% O2-balance N2 for 20 min. Compared with control rats, KN-93 administration elicited marked attenuations of peak HVR (pHVR) but did not modify normoxic minute ventilation. Differences in pHVR were primarily attributable to diminished respiratory frequency recruitments during pHVR without significant differences in tidal volume. These findings indicate that CaMKII activation in the NTS mediates respiratory frequency components of the ventilatory response to acute hypoxia; however, CaMKII activity does not appear to underlie components of normoxic ventilation.

Acute exposure of conscious rats to mild hypoxia elicits a characteristic ventilatory pattern termed the hypoxic ventilatory response (HVR). This biphasic response is initially mediated by activation of peripheral chemoreceptors, which produces marked elevation in minute ventilation (VE), peak HVR (pHVR), followed by a subsequent decrease in ventilation to levels that are lower than the peak early ventilatory increase (36). This latter decrease in ventilatory output has been termed hypoxic ventilatory decline (HVD) and appears to be the result of complex interactions between excitatory and inhibitory influences on peripheral chemoreceptors, central respiratory neurons, metabolic pathways, and signaling molecules such as adenosine, nitric oxide, and peptide-based receptors (14, 16, 17, 22, 31, 43, 44, 47).

The early ventilatory response to hypoxia requires intact central relays within the nucleus of the solitary tract (NTS), and neural transmission of hypoxic afferent input is critically dependent on glutamatergic signaling, particularly the activity of N-methyl-D-aspartate (NMDA) receptors in this critical brain stem region (NTS) (25, 30, 33, 40).

Calcium/calmodulin-dependent kinase II (CaMKII) is an ubiquitously expressed protein kinase that is involved in a multitude of signal transduction pathways and mediates many diverse physiological processes in response to increases in intracellular Ca2+. CaMKII is particularly abundant in neurons, in which it modulates gene expression, synthesis, and release of neurotransmitters and influences the properties of ion channels such as NMDA receptors (reviewed in Ref. 6). In vitro studies showed that CaMKII acts on the NMDA receptor complex primarily through association and phosphorylation of the NR2B receptor subunit (29, 41), although association with the NR1 (24) and NR2A (11) subunits has also been reported. Furthermore, CaMKII can regulate the trafficking of the NMDA receptor to the synapse by modulating the association of synaptic associated protein 97 and the NR2A subunit, thereby providing an alternate model of NMDA receptor modulation (13). The involvement of CaMKII in synaptic plasticity has been extensively explored in models of learning and memory, hippocampal long-term potentiation (LTP), and C-fiber-evoked potential LTP (10, 48). However, the role of CaMKII in ventilatory control, particularly in HVR, has not been systematically investigated. On the basis of evidence that CaMKII is critical in signaling pathways of neural events that are critically dependent on NMDA receptor activity, we hypothesized that CaMKII may also be implicated in HVR at the NTS level.

To further examine whether CaMKII plays a role in HVR, we employed targeted pharmacological inhibition of CaMKII in the NTS using a highly selective CaMKII inhibitor, KN-93, and assessed ventilatory responses during normoxia and acute hypoxia in freely behaving adult rats.

Methods

Adult Sprague-Dawley male rats were purchased from Charles River and used for all experiments (weight: 275–300 g). The experimental protocols were approved by our Institutional Animal Use and
Care Committee and are in close agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

CaMKII inhibition. Pharmacological inhibition of CaMKII was achieved through local administration of KN-93 (100 μM, Calbiochem), whereas controls received PBS vehicle, via surgically implanted Alzet microosmotic pumps, which deliver pharmacological solutions for 7 days at the constant rate of 0.25 μl/h. Dosage was selected based on a series of preliminary experiments designed to ascertain a dose-response curve to KN-93 and encompassing a range from 10 to 1,000 μM (Fig. 1). The infusion pumps were assembled and filled with either vehicle or KN-93 and incubated at 37°C overnight in PBS before the surgery. Adult male rats were then anesthetized with pentobarbital sodium (Nembutal, 50 mg/kg ip), and their scalps were shaved and cleaned. The animals were then placed into a stereotaxic apparatus, and a 2.5-cm midline incision was made beginning slightly behind the eyes and extending to the first cervical vertebrae. The base of the skull was dissected, the brain stem was exposed, and the obex was visualized. Cannulas were placed bilaterally in the region corresponding to the caudal NTS. The infusion systems were then secured to the base of the skull with physiologically inert cement. Each of the two pumps was then placed in the ipsilateral subcutaneous space of the rat shoulder, at which point in time the scalp wound was sutured; rats were then allowed to recover for 3 days before physiological recordings were initiated.

Verification of injection sites in NTS. A fluorescent labeling strategy was used to verify the location and estimate the extent of KN-93 injections in the dorsocaudal brain stem, including the NTS. On completion of physiological experiments, animals were anesthetized, as above, and the tracer TMR-D (7%, 3,000 molecular wt; catalog number D-3308, Molecular Probes, Eugene, OR) was loaded into osmotic pumps and connected to the implanted cannulas as delineated above. Three days after the surgery, animals were euthanized with an overdose of pentobarbital sodium (100 mg/kg), perfused through the heart with 0.9% saline (300 ml) and phosphate-buffered (pH 7.4, 600 ml) 10% formalin; brain stems were then removed and stored in 15% sucrose formalin overnight, after which they were sectioned transversely at 50 μm using a cryostat on the second day. All tissues were then dehydrated through a graded series of ethanol rinses (70%, 2 min; 90%, 2 min; and two times 100%, 1.5 min each). Finally, the tissue was mounted and cover-slipped in Cytoseal XYL. We examined slides using conventional epifluorescence microscopy; a representative section is shown in Fig. 2.

Ventilatory recordings. Respiratory measures were continuously acquired in the freely behaving animals via the barometric method (Buxco Electronics, Sharon, CT) (1, 34) 3 days after surgery to allow for recovery from surgical instrumentation and to ensure that the drug concentration at the targeted sites would reach a stable optimal plateau. To minimize the long-term effect of signal drift due to temperature and pressure changes outside the chamber, a reference chamber of equal size in which temperature was measured using a T-type thermocouple was used. In addition, a correction factor was incorporated into the software routine to account for inspiratory and expiratory barometric asymmetries (9). Environmental temperature was maintained slightly below the thermoneutral range (24–26°C). At least 60 min before the start of each protocol, animals were allowed to acclimate to the chamber, in which humidified air (70–90% relative humidity) was passed through at a rate of 5 l/min, by use of a precision flow pump-reservoir system. Pressure changes in the chamber due to

Fig. 1. Dose-dependent attenuation of the hypoxic ventilatory response (HVR), expressed as %change from baseline of minute ventilation (V̇E), following KN-93 administered within the dorsocaudal brain stem.

Fig. 2. Representative example of tracer studies aiming to estimate the distribution of KN-93 from the infusion sites using tetra-methyl rhodamine dextran as the fluorescent marker. Locations of the cannulas are indicated with arrows.
the inspiratory and expiratory temperature changes were measured by use of a high-gain-differential pressure transducer (Validyne, model MP45–1). Analog signals were continuously digitized and analyzed online by a microcomputer software program (BioSystem XA, Buxco Electronics). A rejection algorithm was included in the breath-by-breath analysis routine, allowing for accurate rejection of motion-induced artifacts. All ventilatory measurements are reported as BTPS values. Tidal volume (Vt), respiratory frequency (f), VE, mean inspiratory flow (VT/TI), and inspiratory duty cycle (TI/TTOT) were computed and stored for subsequent off-line analyses.

Acute hypoxic ventilatory challenges. Hypoxic ventilatory challenges were conducted 3 days after surgical instrumentation with Alzet microosmotic pumps. Animals were weighed and placed in the barometric recording chamber. After stable baseline normoxic values were obtained for at least 30 min, rats were switched to 10% O2-balance N2, using a premixed gas mixture. The hypoxic challenge lasted for 20 min, after which room air was reintroduced in the recording chamber, and recovery was recorded for 10 min. Ventilatory measures were averaged in 1-min intervals and plotted.

Data analysis. All values are shown as means ± SE, unless indicated otherwise. Differences in data among the experimental groups were compared by two-way ANOVA for repeated measures and the Newman-Keuls test or by paired t-tests as appropriate. A P value of <0.05 was considered to achieve statistical significance.

RESULTS

Respiratory effects of KN-93 during normoxia. During normoxia, infusion of KN-93 to the dorsocaudal brain stem resulted in no significant changes in normoxic VE, Vt, f, or VT/TI compared with vehicle-treated animals (Fig. 3). However, in animals treated with KN-93, TI/TTOT was significantly reduced (Fig. 3E). Thus the effects of local administration of KN-93 in the dorsocaudal brain stem during normoxic conditions appear to be limited to respiratory timing, without significant effects on overall ventilatory output.

Fig. 3. Ventilatory measurements in adult rats treated with KN-93 or vehicle administered within the dorsocaudal brain stem via chronically implanted cannulas and osmotic pump delivery systems. KN-93 elicited significant attenuations of VE during the peak HVR (pHVR; A), which was associated with diminished respiratory frequency (C) without significant changes in tidal volume (Vt; B). No significant differences were observed in the mean inspiratory flow (Vt/TI; D); however, inspiratory duty cycle (TI/TTOT; E) was significantly decreased during normoxia following treatment with KN-93. BW, body weight.
Respiratory effects of KN-93 on the HVR. Ventilatory responses to 10% \( \text{O}_2 \) in both KN-93 and vehicle-treated animals are illustrated in Figs. 3 and 4. In the latter, \( \dot{V}_E \) increased \( \sim 150\% \) (Fig. 3A, \( P < 0.01 \)) during the early phase of HVR (i.e., pHVR) and was associated with significant increases in \( f \) (Fig. 3C, \( P < 0.01 \)) without changes in \( V_t \). Significant increases in \( V_t/T_t \) were also observed (Fig. 3D, \( P < 0.01 \)). Values for \( \dot{V}_E \) remained significantly elevated above normoxic baseline during HVD (Fig. 3A, \( P < 0.05 \)) and were also accounted for by increased \( f \) (Fig. 3C, \( P < 0.05 \)). Furthermore, \( V_t \) displayed a trend toward increasing values; yet such changes did not reach statistical significance. Values measured for \( T_t/T_T \) were not significantly different from baseline values at any time point during the hypoxic challenge.

Ventilatory measurements in animals receiving KN-93 revealed significantly attenuated \( \dot{V}_E \) during pHVR (Fig. 3, \( P < 0.05 \)) compared with controls; however, pHVR was not altogether abolished and still displayed an average of \( \sim 85\% \) increase in response to 10% \( \text{O}_2 \). This response was also mediated primarily through increases in \( f \), albeit significantly diminished compared with vehicle-treated animals (Fig. 3, \( P < 0.05 \)), without increases in \( V_t \). Measurements of \( V_t/T_t \) and \( T_t/T_T \) were not significantly different from vehicle-treated animals at any time point during the acute 10% hypoxic challenge.

**DISCUSSION**

In the present study, targeted inhibition of CaMKII activity within the dorsocaudal brain stem attenuated the early phase of HVR without significant modification of either normoxic ventilation or HVD. In addition, blockade of CaMKII activity within the dorsocaudal brain stem primarily affected the frequency response to acute hypoxia without significantly altering \( V_t \). Furthermore, reductions in inspiratory duty cycle during pHVR (\( T_t/T_T \)) reflected an augmentation of respiratory timing during normoxic respiration after CaMKII inhibition. Thus we conclude that CaMKII activity in the dorsocaudal brain stem is critical for mounting an appropriate respiratory response to acute hypoxia in adult rats.

Before we address the potential implications of our findings, some technical issues deserve comment. Our experimental approach overcomes inherent limitations of acutely instrumented preparations in which microinjections are administered. Indeed, we employed a chronically instrumented model, in which there are no behavioral constraints on the animals, while still achieving precise pharmacological manipulation in the desired region of interest within the dorsocaudal brain stem, namely, the NTS. However, notwithstanding the careful surgical instrumentation, we further proceeded with evaluation of the accuracy of our targeted approach on completion of the experimental measurements, thereby ensuring adequate placement of the cannulas. Furthermore, it is important to note that disruption of the neural tissue in the dorsocaudal brain stem can also affect ventilatory output (21). Thus strict scrutiny of the histological sections for disruption of the tissue architecture was applied and clearly does not account for any of the findings associated with CaMKII inhibition reported herein.

**Fig. 4.** Schematic diagram of a working model for glutamatergic signaling in the nucleus of the solitary tract during acute hypoxia. Upstream signal transduction involving, among other mediators activation of platelet-activating factor (PAFR) (15) leads to the release of glutamate (Glu) into the synaptic cleft. Upon binding, Glu activates the N-methyl-D-aspartate receptor (NMDAR) and allows the influx of Ca\(^{2+} \) into the cell. This will, in turn, activate many downstream signaling pathways and protein kinases, most notably protein kinase C (PKC) (18), tyrosine kinases (TK) (7), and calcium/calmodulin-dependent kinase II (CaMKII). CaMKII may then act to further modulate the signaling cascade by influencing the translocation of \( \alpha \)-amino-3-hydroxy-5-methylisoxazole-propionic acid receptors (AMPAR) and NMDAR to the postsynaptic membrane or through further signal transduction involving neuronal nitric oxide synthase (nNOS) (14, 44).
The concept that CaMKII is involved in neuronal plasticity is not novel. Indeed, the role of CaMKII as a modulator of NMDA receptor activity has been widely investigated in several nonrespiratory models, including hippocampal LTP (reviewed in Ref. 26) and spinal C-fiber-evoked field potential LTP (48). From such studies, significant insight has been gained into the mechanisms of CaMKII-NMDA receptor interaction, suggesting that on neuronal activation, CaMKII translocates to the postsynaptic density (38) and becomes directly associated with either NR2B subunits of the NMDA receptor (12, 41) or NR1 subunits (23). Interaction with NMDA receptor facilitates hyperphosphorylation of CaMKII and enhances its activity, a phenomenon that has been postulated as the “molecular memory switch” (27). Indeed, several important consequences derive from such enhancement of CaMKII activity, namely, to enable tighter binding to the NR2B subunit through interaction with a second binding domain (2), thereby allowing CaMKII to establish molecular linkages between NMDA and α,γ-3-hydroxy-5-methylisoxazole-propionic acid (AMPA) receptors, and thus recruit AMPA receptors to postsynaptic sites containing NMDA receptors (24, 28), a phenomenon that is critically implicated in LTP (19). Furthermore, phosphorylated CaMKII will, in turn, phosphorylate AMPA receptors, thereby increasing their conductance (3, 8). Beyond its role in the trafficking and phosphorylation of AMPA receptors, CaMKII has been implicated in the trafficking of NMDA receptors as well. Indeed, recent evidence indicated a novel role for CaMKII in the synaptic targeting of the NMDA receptor via modulation of the interaction of synaptic associated protein 97 and the NR2A subunit of the NMDA receptor.

The analogy between the above-described role of CaMKII and glutamate receptor-dependent activity in rostral brain regions and in the brain stem is particularly striking in the NTS. Indeed, it is now well established that within the NTS, glutamatergic pathways are not only required for normal neural transmission in HVR response (30, 32, 33, 45) but are also activated in NTS plasticity (35). Thus it is not surprising that the results of the present study are reminiscent of those previously described by Ohtake et al. (33) after intravenous injection of the NMDA receptor antagonist MK-801. Indeed, NMDA receptor antagonism resulted in marked reductions in \( \dot{V}_{E} \) during pHVR in response to an acute 10% poikilocapnic hypoxia without augmentation of normoxic \( \dot{V}_{E} \) in intact freely behaving adult rats. Furthermore, the attenuation of \( \dot{V}_{E} \) during hypoxia was accounted for by significant reductions in \( f \) without clear differences in \( V_t \). A more recent study further substantiates the role of NMDA receptors in HVR using anesthetized adult rats and systemic injections of MK-801 (42). In this study, Tarakanov et al. described remarkably similar effects following NMDA blockade during pHVR and also provided evidence that NMDA receptor activity may also play a role during HVD. However, in somewhat contradiction to such findings, we found no evidence that CaMKII activity is involved during HVD. The magnitude of \( \dot{V}_{E} \) attenuation during pHVR in the present study was not as pronounced as that described by Ohtake et al. This is not altogether surprising, given that direct blockade of the NMDA receptor will also modulate other downstream signaling pathways involved in pHVR, such as protein kinase C and platelet-activating factor (18, 39). Thus we propose a working model in which CaMKII is critically involved in the glutamatergic signaling within the NTS, where it acts in concert with other postsynaptic modulators of HVR (Fig. 4).

One additional effect of CaMKII may involve its recruitment of AMPA receptor activity (3, 8). However, although non-NMDA glutamate receptors are critically involved in cardiovascular regulation (5, 20, 37), the literature concerning their contribution to respiratory control mechanisms is relatively limited. For example, studies in both mice and cats have demonstrated marked respiratory depression in neonatal or anesthetized animals, following non-NMDA receptor blockade (4). Additionally, Vardhan et al. (45) demonstrated that microinjections of a non-NMDA receptor antagonist with the NTS of adult rats attenuated the ventilatory response to carotid body stimulation. However, in concordance with the work of Borday et al. (4), Whitney et al. (46) reported a more robust attenuation of pHVR after blockade of AMPA receptors in the developing animal compared with the adult. Interestingly, the differences in ventilatory output were also predominantly manifest as diminished \( f \) without significant differences in \( V_t \).

In summary, we conclusively demonstrate that administration of a CaMKII antagonist within the NTS during acute environmental hypoxia is associated with attenuation of critical components of the ventilatory response to hypoxia.

**REFERENCES**

CAMKII IN THE NTS MODULATES HVR


