Prokineticin 2 modulates the excitability of area postrema neurons in vitro in the rat

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Ingves MV, Ferguson AV. Prokineticin 2 modulates the excitability of area postrema neurons in vitro in the rat. Am J Physiol Regul Integr Comp Physiol 298: R617–R626, 2010. First published January 6, 2010; doi:10.1152/ajpregu.00620.2009.—Despite recent evidence describing prokineticin 2 (PK2)-producing neurons and receptors in the dorsomedial medulla, little is known regarding the potential mechanisms by which this circadian neuropeptide acts in the medulla to influence autonomic function. Using whole cell electrophysiology, we have investigated a potential role for PK2 in the regulation of excitability in neurons of the area postrema (AP), a medullary structure known to influence autonomic processes in the central nervous system. In current-clamp recordings, focal application of 1 μM PK2 reversibly influenced the excitability of the majority of dissociated AP cells tested, producing depolarizations (38%) and hyperpolarizations (28%) in a concentration-dependent manner. Slow voltage ramps and ion-substitution experiments revealed that a PK2-induced Cl⁻ current was responsible for membrane depolarization, whereas hyperpolarizations were the result of inhibition of a nonselective cation current. In contrast to these differential effects on membrane potential, nearly all neurons that displayed spontaneous activity responded to PK2 with a decrease in spike frequency. These observations are in accordance with voltage-clamp experiments showing that PK2 caused a leftward shift in Na⁺ channel activation and inactivation gating. Lastly, using post hoc single-cell RT-PCR technology, we have shown that 7 of 10 enkephalin-expressing AP neurons were depolarized by PK2 indicating that PK2 may have specific inhibitory actions on this population of neurons in the AP to reduce their sensitivity to homeostatic signals. These data suggest that the level of AP neuronal excitability may be regulated by PK2, ultimately affecting AP autonomic control.

MATERIALS AND METHODS

Cell culture. All animal protocols were in accordance with guidelines of the Canadian Council on Animal Care and were approved by the Queen’s University Animal Care Committee. Male Sprague-Dawley rats (100–200 g body wt; Charles River) were decapitated, and the brain stem was quickly removed and placed in cold (1–4°C) artificial cerebrospinal fluid (aCSF) containing (in mM) 124 NaCl, 2.5 KCl, 1.3 MgSO₄, 1.24 KH₂PO₄, 20 NaHCO₃, 2.27 CaCl₂, and 10 glucose. The brain stem was mounted on a stage, and 300-μm coronal slices containing the AP were cut using a Vibratome (Leica, Nussloch, Germany) and placed in Hibernate medium (Brain Bits, Springfield, IL) supplemented with 0.5 ml of 1× B27 (GIBCO, Invitrogen, Burlington, ON, Canada). The AP was microdissected from brain stem slices, which were visualized using light microscopy, cut with care taken to ensure that no contaminating tissue was included, and incubated in Hibernate medium containing papain (2 mg/ml; Worthington, Lakewood, NJ) at 30°C for 30 min. After incubation, AP tissue was washed and triturated in Hibernate-B27 medium, and dissociated cells were centrifuged at 500 rpm for 8 min. The super-

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natant was removed, and the pellet was resuspended in Neurobasal A-B27 medium (Invitrogen) supplemented with 5 mM glucose, 100 U/ml penicillin-streptomycin, and 0.5 mM l-glutamine (Invitrogen). Dissociated cells were plated on 35-mm uncoated glass-bottom culture dishes (MatTek, Ashland, MA) at a low density (~10 cells/mm²) to ensure that synaptic contacts did not form and then incubated at 37°C in 5% CO₂. Electrophysiological experiments were performed on neurons maintained in culture for 1–5 days, during which time some small process developed, none of which appeared (by visual inspection using a patch-clamp microscope) to make contact with other cells.

**Electrophysiology.** Whole cell recordings from dissociated AP neurons were made using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Palo Alto, CA). Data were collected using Signal (voltage-clamp recordings) and Spike2 (current-clamp recordings) software packages (Cambridge Electronics Design, Cambridge, UK). Signals were filtered at 2 kHz and digitized at 5 kHz using a Micro 1401 MKII interface (Cambridge Electronics Design). Voltage measurements were corrected for liquid junction potential. Unless otherwise noted, recordings were obtained using extracellular solution containing (in mM) 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, and 5 glucose (with pH adjusted to 7.2 with NaOH). Patch electrodes were made from borosilicate glass (World Precision Instruments, Sarasota, FL) on a Flaming Brown micropipette puller (P87, Sutter Instrument, Novato, CA) and heat polished; resistance of the electrodes was 3–6 MΩ.

A voltage-ramp protocol (12.5 mV/s) was used to assay the effects of bath-applied PK2 on whole cell currents. AP neurons were clamped at −75 mV, and ramp currents were determined from an average of three ramps between −100 and −20 mV before (control) and after 3 min of PK2 application. After completion of the experiment, recovery toward baseline during the washout period, suction was applied to the pipette interior, and the cell was collected. Immediately after cytoplasm collection, the contents of the cell were expelled into a 0.5-ml centrifuge tube containing DNase (1 μl) and DNase buffer (1 μl; Fermentas, Burlington, ON, Canada). The tube was incubated for 30 min at 37°C, EDTA (10 mM) was added, and the tube was heated at 65°C for an additional 10 min. For synthesis of cDNA, dithiothreitol (26 mM), dNTPs (3 mM), random hexamer primers (3 μM), MgCl₂ (4 mM), RNase inhibitor (20 U), and Superscript II reverse transcriptase (100 U; all from Invitrogen) were added. The cDNA synthesis reaction was incubated overnight at 37°C, and cDNA was stored at −80°C until PCR was performed. A two-step multiplex PCR protocol was used to detect the presence of mRNA encoding genes of interest (see Table 1 for primer sets) using reagents provided in the multiplex kit (Qiagen, Mississauga, ON, Canada). The first amplification step consisted of a multiplex reaction in 100-μl volume with the synthesized cDNA and “outside” primers (0.2 μM each) for all the genes of interest. The reaction was denatured at 95°C for 15 min and cycled 20 times through a temperature protocol consisting of 30 s at 94°C, 90 s at 60°C, and 90 s at 72°C. In the nested reaction, “inside” primers were used in individual 50-μl reactions for each gene of interest in which 2 μl of first-round product were used as the template and each primer was used at 0.2 μM. The reaction mixture was cycled 35 times using the temperature protocol described above. Finally, PCR products were run on a 2% (wt/vol) agarose gel containing ethidium bromide and periodically sequenced to confirm their identity (Robarts Institute, London, ON, Canada).

**Chemicals and peptides.** All chemicals used to make solutions were purchased from Sigma (Oakville, ON, Canada). RNase-free intracellular recording solution was made using molecular biology-grade chemicals. PK2 was generously provided by Dr. Qun-Yong Zhou (University of California at Irvine), synthesized using recombinant techniques (24), and reconstituted in external recording solution to working concentrations.

**RESULTS**

We initially used whole cell current-clamp recordings to examine the effects of focal application of PK2 on the excitability of dissociated AP neurons. Long-term stable recordings were obtained from 86 dissociated AP neurons maintained in culture for 1–5 days. After the development of a stable control baseline membrane potential for ≥100 s, PK2 was rapidly applied by pressure ejection under visual guidance for 10 s in the immediate vicinity of the recorded neuron. Local application of 1 μM PK2 influenced the membrane potential of 66%...
of AP neurons tested (n = 29), producing membrane depolarization (mean resting membrane potential \(-64.6 \pm 2.4 \text{ mV}\); Fig. 1A) or hyperpolarization (mean resting membrane potential \(-60.4 \pm 1.4 \text{ mV}\); Fig. 1B) in 38% and 28% of cells, respectively. These effects were at least partially reversible upon washout and significantly different from control cells treated with aCSF (n = 10, P < 0.001 by unpaired t-test), none of which met imposed criteria to be considered responsive. The remaining AP neurons did not respond to PK2 with significant changes in membrane potential (mean resting membrane potential \(-60.9 \pm 1.3 \text{ mV}\); Fig. 1C). The mean change in membrane potential for each group is summarized in Fig. 1D.

In many cases (14 of 16 neurons), AP neurons that displayed spontaneous activity responded to 1 \(\mu\text{M}\) PK2 with decreases in action potential frequency. The mean change in spike frequency of all cells was \(-9.2 \pm 2.5 \text{ %}\), effects that resulted in a significantly greater change in mean spike frequency than was observed in aCSF-treated controls (P < 0.05 by unpaired t-test). The resulting inhibition of spike frequency was characterized using all cells tested at this concentration.

The responses were normalized to the peak effect observed at each PK2 concentration, averaged, and fitted with a Hill equation to yield an \(EC_{50}\) of 27.5 \(\mu\text{M}\). We analyzed depolarizing and hyperpolarizing effects together, as they showed similar concentration-response relationships. Collectively, these results show that PK2 directly caused depolarizing or hyperpolarizing effects on AP neurons, both of which were found to be concentration-dependent, whereas effects on spike frequency were more homogenous, with only inhibitory effects observed.

We next used voltage-clamp techniques to measure currents evoked by slow voltage ramps (12.5 \(\text{mV/s}\)) from \(-100\) to \(-20\) mV in the presence of aCSF and after 10 nM PK2 application to examine the ion channels in AP neurons influenced by PK2. Of 12 cells tested, current-voltage relationships in control conditions generated a voltage-independent current, and after PK2 treatment an inward whole cell current shift was observed in 5 cells from a holding potential of \(-75 \text{ mV}\) (\(-17.4 \pm 7.9 \text{ pA}\); Fig. 3A). The mean change in conductance was 4.0 \pm 2.7 \text{ nS}, and the difference current that was obtained by subtraction of the control current from the current obtained in PK2 elicited a mean reversal potential of \(-63.6 \pm 3.1 \text{ mV}\) (Fig. 3B). On the basis of the ionic concentrations of the bath and pipette recording solutions, these findings suggest that PK2 activated a current that reversed near the calculated equilibrium potential for Cl\(^-\) (\(E_{Cl} = -64 \text{ mV}\)) and leads to membrane depolarization.

In a separate group of AP neurons, PK2 produced outward whole cell currents (mean 7.6 \pm 5.8 \text{ pA}; n = 5) that were associated with an inwardly rectifying control ramp current at hyperpolarized potentials (Fig. 3C). Peptide administration in this phenotype of cells resulted in a mean decreased conductance of 1.1 \pm 0.6 \text{ nS} and a PK2-induced current that reversed...
at a mean membrane potential of $-34.9 \pm 3.5$ mV (Fig. 3D), suggesting that inhibition of a voltage-dependent nonselective cation current leads to membrane hyperpolarization. The proportion of responding neurons in voltage-clamp configuration closely resembles the proportion of depolarizing and hyperpolarizing responses ($P > 0.05$ by Fisher’s exact test).

To determine whether activation of a $\mathrm{Cl}^-$ current is responsible for membrane depolarization, using a pipette solution containing 139 mM $\mathrm{Cl}^-$, we again performed slow voltage ramps. Under high-$\mathrm{Cl}^-$ conditions, the PK2-activated current reversal would be expected to shift toward the set $E_{\mathrm{Cl}}$ of $-2$ mV. As represented in Fig. 4A, the resultant inward current activated by PK2 treatment converged with the control ramp current at a reversal potential that showed a depolarizing shift away from $E_{\mathrm{Cl}}$. Overall, the mean reversal potential of the PK2-induced depolarizing current was $-24.0 \pm 8.3$ mV ($n = 6$), although there was considerable variability in these values ranging from $-40$ mV in Fig. 4A to $0$ mV in other examples. These results are consistent with the predicted depolarizing shift of the $\mathrm{Cl}^-$ reversal potential in these recording conditions. Furthermore, because of a greater $\mathrm{Cl}^-$ driving force at resting membrane potential, depolarizations ($17.3 \pm 4.8$ mV, $n = 5$), but not hyperpolarizations ($-16.2 \pm 6.5$ mV, $n = 2$), were significantly greater in response to $1 \mu$M PK2 (Fig. 4, B and C; $P < 0.05$ by unpaired $t$-test), indicating that activation of a $\mathrm{Cl}^-$ conductance contributes to membrane depolarization.

Although different effects of PK2 on the membrane potential of different groups of AP neurons were observed, as outlined above, the majority of AP neurons treated with PK2 responded with decreases in spike frequency. These observations sug-
ggested potential additional actions of PK2 on Na\(^+\) channels, effects that would be consistent with our own recent report of PK2 effects on Na\(^+\) channels in SFO neurons (8). We therefore undertook an analysis of mean action potential height in five cells that demonstrated decreased spike frequency but no change in membrane potential in response to PK2, an analysis that revealed a significant PK2-induced decrease in spike amplitude (\(P < 0.05\) by paired \(t\)-test) from 71.2 ± 7.5 to 52.8 ± 7.8 mV (Fig. 5A). In addition, we often observed that recovery from spike inhibition occurred at hyperpolarized membrane potentials below baseline (Fig. 5B). We therefore hypothesized that PK2 inhibits AP action potential firing through modulation of voltage-gated Na\(^+\) channels, and we undertook whole cell voltage-clamp experiments to investigate the effect of 10 nM PK2 on Na\(^+\) channel gating. Na\(^+\) current activation and steady-state inactivation were studied in six AP neurons before and after PK2 application. Activation curves were generated by application of voltage steps between −80 and −20 mV in 10-mV increments from a −90-mV prepulse potential, and the conductance was corrected for changes in driving force and normalized (Fig. 6A). After the resulting activation curves were fit with a Boltzmann function, four of six cells showed a significant leftward shift in the half-activation potential (\(P < 0.05\) by extra sum-of-squares \(F\) test) to more hyperpolarized values (−36.5 ± 1.6 and −41.8 ± 1.9 mV in control and PK2-treated cells, respectively), while there was no significant change in the slope factor \(k\) (4.5 ± 1.3 and 4.9 ± 1.7 mV in control and PK2-treated cells, respectively). To assess steady-state inactivation, we subjected AP neurons to 200-ms prepulse potentials between −110 and −20 mV in 10-mV increments followed by a test pulse to −10 mV. A total of six normalized steady-state inactivation plots revealed a significant PK2-mediated hyperpolarizing shift in the half-inactivation potential in four neurons (\(P < 0.05\) by extra sum-of-squares \(F\) test). The mean half-inactivation potential was −59.4 ± 1.0 mV in control cells and −68.6 ± 1.7 mV after PK2 application (Fig. 6B). Again, the slope factor \(k\) remained unchanged (−6.6 ± 0.9 and −7.1 ± 1.5 mV in control and PK2-treated cells, respectively). These data suggest that a shift to hyperpolarized potentials in the activation and steady-state inactivation of Na\(^+\) currents underlies the effect of PK2 on spontaneous firing in AP neurons.

Many autonomic processes regulated by the AP have a circadian component, such as feeding behavior and cardiovascular function. A number of neurotransmitters and neuropeptides implicated in the control of feeding behavior and cardiovascular function are expressed in the AP (for review see Ref. 34) and may therefore be involved in circadian autonomic output from this nucleus. Using a combination of electrophysiology and single-cell RT-PCR (scRT-PCR), we sought to
PK2 influences AP neurons

identify whether specific chemical phenotypes of AP neurons are influenced by PK2. This allowed us not only to characterize the membrane potential response of single AP neurons but, also, to identify the specific peptide mRNAs expressed by individual neurons with the use of post hoc molecular processing techniques. Nested primer sets were designed to detect enkephalin (ENK), cocaine- and amphetamine-related transcript, glutamate decarboxylase 67 (GAD67), cholecystokinin, tyrosine hydroxylase, and vesicular glutamate transporter 2 mRNAs (Table 1), and the response to 10 nM PK2 was evaluated in 30 neurons that expressed GAPDH, a “housekeeping” gene and positive control marker. ENK mRNA was expressed in 10 of these neurons, and as illustrated in Fig. 7, the vast majority of responsive ENK-positive neurons (88%) demonstrated membrane depolarization as a consequence of peptide application. Additionally, five of six ENK neurons displaying spontaneous activity responded to PK2 with decreased firing frequency (mean −99.3 ± 0.7%). We also identified two depolarizing ENK neurons that coexpressed GAD67; however, GAD67-expressing neurons were not homogeneously influenced (n = 6). GAPDH-positive cells that hyperpolarized after PK2 exposure showed no distinguishing phenotype. Because there was only a small population of cells expressing cocaine- and amphetamine-related transcript (n = 4) and cholecystokinin (n = 1), it is difficult to make any conclusions regarding the effect of PK2 on these neurons. No tyrosine hydroxylase-expressing neurons were identified.

DISCUSSION

In this report, we demonstrate that PK2, a circadian messenger produced in the SCN and NTS, has direct actions on the membrane properties of dissociated AP neurons. Using whole cell patch-clamp techniques, we have shown that nearly all spontaneously active AP cells respond to focal PK2 application with significant decreases in action potential frequency, an effect characterized by a leftward shift (toward hyperpolarized potentials) of voltage-dependent Na+ channel activation and inactivation gating. The predominant effect of PK2 on membrane potential was depolarization due to the activation of a voltage-independent Cl− current, whereas inhibition of an inwardly rectifying nonselective cation current led to hyperpolarization in a smaller proportion of neurons. Using scRT-PCR technology, we also identified a population of ENK-expressing neurons, the majority of which depolarized with a similar decrease in spike frequency in response to PK2, indicating the potential for specific circadian modulation of the excitability in this phenotype of AP neurons.

Earlier studies examining the effects of PK2 on forebrain neurons in the SFO and paraventricular nucleus of the hypothalamus identified only excitatory effects on membrane activity in these nuclei (6, 48). In contrast, in the present study, we have shown that PK2 produced depolarizing and hyperpolarizing actions on AP neurons, in addition to inhibitory effects on spontaneous action potential frequency. PK2 was also shown to influence AP cells in a concentration-dependent manner, with an apparent EC50 that falls between previously identified EC50 values for PK2 of 2.3 and 63 pM in the paraventricular nucleus and SFO, respectively (6, 48). Similarly, other peptides that have been shown to influence AP neuronal activity have also been reported to exert depolarizing and hyperpolarizing effects on different subpopulations of AP cells (9, 10, 46, 47). Our findings with PK2 indicate that the differential responsiveness of separate populations of AP neurons is most likely associated with the separate roles of these neurons in the regulation of integrated autonomic outputs.

The voltage-gated Na+ channel is a critical component for the initiation and upstroke of the action potential in neurons (14). Our observations indicate that inhibition of action potential frequency and amplitude is the result of PK2 shifting Na+ channel gating to more hyperpolarized potentials. Similarly, PK2 has also been shown to influence Na+ channel activity in cultured SFO neurons, although an enhancement of Na+ current was found to be responsible for increased neuronal excitability in this forebrain circumventricular organ (8). These differences between the modulatory actions of PK2 on Na+ currents in the SFO and the AP may be the result of differential...
Na⁺ channel subunit expression or intracellular PK2 signaling pathways in these nuclei.

The functional consequences of the hyperpolarizing shift in Na⁺ channel voltage sensitivity in AP neurons (which, in our hands, have a resting membrane potential of approximately −60 mV) would result in a decrease of the Na⁺ current mediating the action potential, an effect that most likely underlies the action of PK2 on decreased spike height. In addition, these findings indicate that AP cells would have a lower probability of firing an action potential in the presence of PK2, given that a greater proportion of Na⁺ channels would be in the inactivated state. Thus, at a constant resting membrane potential, fewer channels would be available to elicit a spike, and a lower spontaneous firing rate would result (17).

Whole cell recordings using slow voltage ramps suggest that differential ion channel modulation by PK2 is a determinant of the effects of this peptide on membrane potential in the AP. AP cells that showed voltage-independent currents between −100 and −60 mV responded to PK2 with depolarizing shifts in whole cell current. In contrast, cells expressing an inwardly rectifying current responded with hyperpolarizing shifts in whole cell current, and these findings indicate a difference in whole cell properties between two populations of AP neurons. The reduction in inward current may represent PK2-mediated inhibition of the hyperpolarization-activated cation current that is present in ~60% of AP neurons and has a reversal potential (−36 mV) similar to that of neurons in this study (11). Similarly, the PK2 homolog Bv8 increases pain perception by enhancing the activity of the transient receptor potential vanilloid 1 channel in dorsal root ganglion neurons, a nonselектив cation conductance that is activated by painful chemical and thermal stimuli (28, 30, 32, 44). In combination with a hyperpolarizing shift in Na⁺ channel voltage dependence, PK2-mediated membrane hyperpolarization and increased input resistance create an ideal situation in which the responsiveness of an AP neuron to input signals, such as excitatory

![Fig. 5. PK2 decreases action potential amplitude in AP neurons. A: current-clamp record showing decreased spike frequency and height in an AP neuron after application of 1 μM PK2 (left; scale bars, 10 mV and 20 s) and mean change in action potential height in response to PK2 application (right; scale bars, 20 mV and 5 ms). B: current-clamp trace showing recovery from spike inhibition at hyperpolarized membrane potentials in an AP neuron that hyperpolarized after PK2 administration, suggesting a change in Na⁺ channel voltage dependence. Scale bars, 10 mV and 50 s. Arrows indicate time of PK2 application.](fig5.png)

![Fig. 6. PK2 induces a leftward shift in Na⁺ channel activation and inactivation voltage dependence. A: Na⁺ current activation from a test pulse of −40 mV before and after 10 nM PK2 (top; scale bars, 50 pA and 1 ms) and mean normalized conductance plotted as a function of test potential in AP neurons showing a shift in activation gating induced by PK2 (bottom; n = 4). Data points were fitted with a Boltzmann function. Inset: activation voltage-step protocol. B: Na⁺ current inactivation after a prepulse step to −60 mV before and after 10 nM PK2 (top; scale bars, 200 pA and 1 ms) and mean normalized current plotted vs. prepulse potential in AP neurons showing a shift in inactivation gating caused by PK2 (bottom; n = 4). Data points were fitted with a Boltzmann function. Inset: inactivation voltage-step protocol.](fig6.png)
activated depolarizing current did not completely shift to the internal Cl channel. The effect of Cl channel conductance in mediating PK2-induced depolarization.

In addition, a leftward shift in Na channel responsiveness of these neurons to humoral or neural signals. This shift in membrane potential of AP neurons, an effect that would decrease the excitability through modulation of Cl channel activity; more specifically, closing of Cl channels has been proposed as a mechanism of excitation in glucose-sensitive neurons of the ventromedial hypothalamic nucleus (41, 42). We have demonstrated that depolarizing shifts in whole cell current in the AP are the result of a PK2-activated conductance that reverses at the Nernst potential, i.e., the $E_{Cl}$. The mean reversal potential of the PK2-activated depolarizing current did not completely shift to the calculated $E_{Cl}$ ($-2$ mV) when AP cells were loaded with high internal Cl, indicating the possible involvement of a second ionic conductance in mediating PK2-induced depolarization. The effect of Cl channel activation on membrane potential by PK2 in vivo would depend on the resting membrane potential.

postsynaptic potentials, may result in an increased probability of firing. Therefore, PK2-mediated hyperpolarization likely maintains an optimal membrane potential necessary for Na channel function and, consequently, action potential firing.

In neurons, Cl channel activity is critical for controlling membrane excitability. Furthermore, metabolic signals, such as fatty acids and glucose, are thought to regulate membrane excitability through modulation of Cl channel activity; more specifically, closing of Cl channels has been proposed as a mechanism of excitation in glucose-sensitive neurons of the ventromedial hypothalamic nucleus (41, 42). We have demonstrated that depolarizing shifts in whole cell current in the AP are the result of a PK2-activated conductance that reverses at the Nernst $E_{Cl}$. The mean reversal potential of the PK2-activated depolarizing current did not completely shift to the calculated $E_{Cl}$ ($-2$ mV) when AP cells were loaded with high internal Cl, indicating the possible involvement of a second ionic conductance in mediating PK2-induced depolarization. The effect of Cl channel activation on membrane potential by PK2 in vivo would depend on the resting membrane potential of AP neurons and the electrochemical gradient of Cl, i.e., the $E_{Cl}$. Cl channel activation also likely shunts the membrane potential of AP neurons, an effect that would decrease the responsiveness of these neurons to humoral or neural signals. In addition, a leftward shift in Na channel gating would likely decrease firing activity and further decrease the excitability of these cells, which were depolarized by PK2. Future studies are clearly needed to identify the functional relevance of these differential effects on different subpopulations of AP neurons.

Our scRT-PCR analysis has provided data indicating that PK2 causes membrane depolarization combined with suppression of action potential firing in the majority of ENK-expressing neurons of the AP, suggesting that PK2 has the ability to suppress neuropeptide release from this population of cells. Although ENKs are highly expressed in the AP (1, 7), neither the axonal projections nor the physiological relevance of ENK output has been described for this circumventricular organ. The NTS, a major cardiovascular integration center in the medulla (36), receives direct inputs from the AP (39, 43) and represents a possible site where ENK neurons may project. Consistent with such a proposal, ENKs have been shown to induce changes in cardiovascular function when administered into the brain ventricular system (37, 49). Direct injection of ENKs into the NTS of rats has been demonstrated to serve a pressor function by increasing mean arterial pressure that is accompanied by tachycardia, perhaps through attenuation of the baroreceptor reflex (33). Intriguingly, neurons of the AP are implicated in regulation of cardiovascular function through excitatory synaptic connections with the NTS, including interactions with barosensitive NTS neurons (2, 12). It is therefore possible that ENK-expressing neurons of the AP may project to the NTS and contribute to cardiovascular regulation in the brain stem; however, retrograde tracing from the NTS combined with ENK immunolabeling in the AP would be required to confirm such a hypothesis. Given the importance of the AP in cardiovascular regulation and the effects of ENK on blood pressure and heart rate, our description of specific actions of PK2 on ENK neurons in the AP indicates a potential mechanism for circadian modulation of cardiovascular output. In contrast to these homogenous depolarizing effects of PK2 on ENK-positive neurons in the AP, our scRT-PCR analysis was not able to identify the specific cell type(s) with hyperpolarizing responses to this peptide.

Although our studies have demonstrated effects of exogenous PK2 on the excitability of AP neurons, they do not allow us to draw any conclusions regarding the origin of endogenously active PK2 that influences PK2 receptors in the AP. PK2 of neuronal origin would most likely come from the SCN or NTS, both of which are critical autonomic control centers in the brain ventricular system (37, 49). Direct injection of ENKs into the NTS represents a possible site where ENK neurons may project. Consistent with such a proposal, ENKs have been shown to induce changes in cardiovascular function when administered into the brain ventricular system (37, 49). Direct injection of ENKs into the NTS of rats has been demonstrated to serve a pressor function by increasing mean arterial pressure that is accompanied by tachycardia, perhaps through attenuation of the baroreceptor reflex (33). Intriguingly, neurons of the AP are implicated in regulation of cardiovascular function through excitatory synaptic connections with the NTS, including interactions with barosensitive NTS neurons (2, 12). It is therefore possible that ENK-expressing neurons of the AP may project to the NTS and contribute to cardiovascular regulation in the brain stem; however, retrograde tracing from the NTS combined with ENK immunolabeling in the AP would be required to confirm such a hypothesis. Given the importance of the AP in cardiovascular regulation and the effects of ENK on blood pressure and heart rate, our description of specific actions of PK2 on ENK neurons in the AP indicates a potential mechanism for circadian modulation of cardiovascular output. In contrast to these homogenous depolarizing effects of PK2 on ENK-positive neurons in the AP, our scRT-PCR analysis was not able to identify the specific cell type(s) with hyperpolarizing responses to this peptide.

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The molecular clock genes are critical cellular components of circadian timekeeping and output from the SCN that drive rhythmic expression of PK2 in the SCN. Similar to their expression in the SCN, the clock genes also clearly show a 24-h rhythmic expression pattern in the NTS (13, 18), where mRNA for PK2 and the prokineticin receptor 2 is found (4, 31).
As mentioned above, NTS neurons represent a potential source for PK2 influencing the excitability of AP neurons. Negri et al. (31) showed that, in contrast to the large decreases in PK2 mRNA observed in the SCN during the dark phase of the circadian cycle, PK2 mRNA was, if anything, increased in the NTS during this same time period. In addition, the circadian expression of the core clock genes is altered in the NTS in a model of hypertension, suggesting that circadian regulation of cardiovascular function may occur via output from the NTS (13). Furthermore, the risk for cardiovascular incidents also follows a daily rhythm, being maximal during the early morning when blood pressure and heart rate are highest, and is coincident with peak levels of PK2 expression (3, 21). Thus the NTS represents not only a likely source of PK2 expression but, also, a potential circadian regulator of AP neuronal excitability that may translate to circadian changes in autonomic function.

**Perspectives and Significance**

We can conclude from this study that PK2 has direct effects on AP neurons to inhibit spike frequency through actions on Na\(^+\) channel voltage dependence. Additionally, the membrane potential of native AP neurons in vivo, in combination with the effects of PK2 on membrane conductance and specific ionic currents, suggests that the level of AP neuronal excitability may be regulated by the circadian cycle of PK2 expression; however, further experiments are required to determine the endogenous source and expression pattern of this neuropeptide. Therefore, PK2 may impart a circadian influence on the ability of the AP to respond to homeostatic signals, thus impacting autonomic output within the central nervous system.

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