Prokineticin 2 Modulates the Excitability of Area Postrema Neurons in vitro in the Rat.

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Running head: PK2 influences AP neurons

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**Abstract**

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 regulation of neuronal excitability by PK2 on 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 1µM PK2 reversibly influenced the excitability of the majority of dissociated AP cells tested, producing both depolarizations (38%) and hyperpolarizations (28%) in a concentration-dependent manner. Slow voltage ramps and ion substitution experiments revealed a PK2-induced Cl⁻ current was responsible for membrane depolarization, while hyperpolarizations were the result of inhibition of a non-selective 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 out of 10 AP neurons depolarized by PK2 were enkephalin-expressing cells, indicating 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.

**Introduction**

Many autonomic processes follow a 24 hour circadian cycle important for maintaining physiological homeostasis. The master pacemaker that governs circadian rhythms resides in the suprachiasmatic nucleus (SCN) of the hypothalamus, whose neural activity and clock gene expression
follow a diurnal cycle (29). Prokineticin 2 (PK2) is a recently described secretory peptide found in the gastrointestinal tract and brain that regulates circadian rhythms in the central nervous system (3; 22; 24). Transcript for this peptide follows a circadian expression profile in the SCN, being maximal during the daytime, and delivery of PK2 into the brain during the night when endogenous levels are minimal suppresses feeding behaviour and locomotor activity (3; 31). Furthermore, animals with mutations in PK2 signalling show disrupted circadian behaviour and homeostatic function, indicating PK2 is essential for maintaining robust circadian rhythms in key physiological processes (15; 16; 23; 35). The G-protein-coupled prokineticin receptor 2 is the main receptor expressed in the central nervous system, with expression in many hypothalamic and medullary nuclei important in regulating circadian autonomic processes, and suggests these structures likely play pivotal roles in the integration of circadian PK2 signalling (4; 25; 26; 40). In light of these findings, previous research from our laboratory has demonstrated an excitatory role for PK2 on neurons of the paraventricular nucleus of the hypothalamus and subfornical organ, thus providing a link between oscillating PK2 expression in the SCN and neuronal firing patterns that contribute to controlling autonomic function (6; 8; 48).

The area postrema (AP) is a sensory circumventricular organ in the medulla, lacks a blood brain barrier, and therefore has the unique ability to monitor both blood-borne and neural signals regarding the status of the body (27; 45). The AP expresses receptors for, and responds to many hormones controlling metabolic, immune, and cardiovascular function (5; 34). Homeostatic signals detected by the AP are presumably relayed to medullary autonomic control centres in the brainstem via neuropeptide and neurotransmitter release at nerve terminals of efferent projection sites, such as major axonal projections to the nucleus tractus solitarius and lateral parabrachial nucleus (39; 43). The AP therefore represents a conduit through which neural and humoral signals can reach brain nuclei situated behind the blood brain barrier. Importantly, the regulation of feeding and metabolism is dependent on the time of day, and many circulating satiety hormones show a diurnal variation in their ability to limit food
intake (19; 20). Moreover, the daily fluctuation in cardiovascular activity is also tightly regulated by the circadian control of hormone secretion (38). Owing to the fact that prokineticin receptor 2 mRNA is highly expressed in the AP (4), we have investigated a potential role for modulation of neuronal excitability by PK2 in the AP.

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 (100-200g, Charles River, QC) were decapitated, the brainstem quickly removed, and placed in cold artificial cerebrospinal fluid (1-4°C) containing (in mM): 124 NaCl, 2.5 KCl, 1.3 MgSO4, 1.24 KH2PO4, 20 NaHCO3, 2.27 CaCl2, and 10 glucose. The brainstem was mounted on a stage and 300µm coronal slices containing the AP were cut using a vibratome (Leica, Nussloch, Germany) and placed in Hibernate media (Brain Bits, Springfield, IL) supplemented with 0.5ml 1 x B27 (Gibco, Invitrogen, Burlington, ON). AP was microdissected from brainstem slices (visualized using light microscopy and cuts were conservative to ensure no contaminating tissue) and incubated in Hibernate media containing 2mg/ml of papain (Worthington, Lakewood, NJ) at 30°C for 30 min. Following incubation, AP tissue was washed and triturated in Hibernate/B27 media and dissociated cells were centrifuged at 500 x g for 8 min. The supernatant was removed and the pellet resuspended in Neurobasal A/B27 media (Invitrogen) supplemented with 5mM glucose, 100U/ml penicillin/streptomycin, and 0.5mM L-glutamine (Invitrogen). Dissociated cells were plated on 35mm uncoated glass bottom culture dishes (MatTek, Ashland, MA) at a low density (~10 cells/mm²) to ensure synaptic contacts did not form, and 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 were developed, none of which were seen (visual inspection using 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) and Spike2 (current-clamp) software packages (Cambridge Electronics Design, Cambridge, United Kingdom). Signals were filtered at 2kHz and digitized at 5kHz using a Micro 1401 MKII interface (Cambridge Electronics Design). Voltage measurements were corrected for liquid junction potential. Unless otherwise noted, recordings were obtained using external recording solution containing (in mM): 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, and 5 glucose, pH 7.2 (adjusted with NaOH). Patch electrodes were made from borosilicate glass (World Precision Instruments, Sarasota, FL) on a Flaming Brown micropipette puller (P87, Sutter Instrument Co, Novato, CA), heat polished, and had resistances of 3-6MΩ. Unless noted, electrodes were filled with intracellular recording solution that contained (in mM): 130 K-gluconate, 10 KCl, 1 MgCl2, 0.1 CaCl2, 10 HEPES, 10 EGTA, 2 NaATP, pH 7.2 (adjusted with KOH).

AP cells were perfused with external recording solution (37°C) using a gravity fed perfusion system at a rate of 1-2ml/min. Cells in whole-cell recording configuration were defined as neurons by the presence of voltage-gated Na+ currents in voltage-clamp and >60mV action potentials in current-clamp. PK2 was filled into puffer pipettes for pneumatic application (10s, 3-8psi) using a Multichannel Picospritzer (General Valve Corporation, Fairfield, NJ) under visual guidance approximately 10-50µm from the neuron, once a stable baseline membrane potential of at least 100s was achieved. Changes in membrane potential were calculated from the maximal difference between the average membrane potential in 50s segments prior to and following peptide application. AP neurons were considered responsive if this difference was at least 3 standard deviations of the mean baseline membrane potential and the cell showed recovery towards baseline. Changes in action potential frequency were
assessed by comparing the difference between the mean action potential frequency 100s immediately prior to and following PK2 application. Comparison of action potential height was analyzed in neurons that did not respond with a change in membrane potential by comparing the difference in mean action potential amplitude during the control segment of the recording and the segment containing the peak effect on action potential height.

A voltage ramp protocol (12.5mV/s) was used to assess the effects of bath-applied PK2 on whole-cell currents. AP neurons were clamped at -75mV and ramp currents were determined from an average of 3 ramps between -100 and -20mV before (control) and following 3 min PK2 application. During the experiment the access resistance did not vary by more than 25%. The current-voltage relationship was plotted and difference current (PK2-induced current) calculated by subtracting the control current from the current obtained after peptide treatment. Linear regression analysis was used to determine conductance (slope of the PK2-induced current) and reversal potential. Voltage ramps performed under high internal Cl⁻ utilized an intracellular solution similar to above, but substituted KCl for K-glucuronate.

Experiments analyzing changes in Na⁺ channel gating used external and intracellular recording solutions that blocked all other voltage-gated currents. The external solution contained the following (in mM): 25 NaCl, 130 TEA, 1 MgCl₂, 2 CaCl₂, 1 CsCl, 1 BaCl₂, 0.3 CdCl₂, 10 HEPES, 5 glucose, pH 7.2 (adjusted with NaOH). Intracellular solution contained (in mM): 125 CsMeSO₄, 2 MgCl₂, 5.5 EGTA, 10 CsCl, 0.1 CaCl₂, 2 NaATP, pH 7.2 (adjusted with KOH). Both activation and steady-state inactivation protocols were performed in each cell before and following 3 min PK2 application. Na⁺ channel activation was tested using a voltage step protocol between -80 and -20mV in 10mV increments from -90mV. After correcting for changes in driving force, normalized conductance was plotted against test potential and points fitted with a Boltzmann function. Steady-state inactivation was assessed using 200ms prepulse steps between -110 and -20mV in 10mV increments, followed by a test pulse to -10mV. Normalized currents were
plotted against prepulse potential and points fitted with a Boltzmann function. The half maximal activation and inactivation voltages and slope factor $k$ were determined for control and PK2 treatment.

**Single Cell RT-PCR**

Whole-cell current-clamp recordings were performed using bath-applied PK2 and electrodes that had been sterilized at 200°C for at least 6h and filled with 12µl of RNase-free intracellular recording solution. After completion of the experiment and recovery toward baseline during the washout period, suction was applied to the pipette interior and the cell collected. Immediately following cytoplasm collection, the contents of the cell were expelled into a 0.5ml centrifuge tube containing DNase (1µl) and DNase buffer (1µl) (Fermentas, Burlington, ON). The tube was incubated for 30 min at 37°C, after which EDTA (10mM) was added and the tube heated at 65°C for an additional 10 min. To synthesize cDNA the following were added: dithiothreitol (26mM), dNTPs (3mM), random hexamer primers (3µM), MgCl$_2$ (4mM), RNase inhibitor (20U), and Superscript II reverse transcriptase (100U) (all from Invitrogen). The cDNA synthesis reaction was incubated overnight at 37°C and cDNA 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 Qiagen Multiplex Kit (Qiagen, Mississauga, ON). The first amplification step consisted of a multiplex reaction in 100µl volume with the synthesized cDNA and ‘outside’ primers (0.2µM 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 30s at 94°C, 90s at 60°C, and 90s at 72°C. In the second nested reaction, ‘inside’ primers were used in individual 50µl reactions for each gene of interest using 2µl of first round product as the template and 0.2µM of each primer. The reaction mixture was cycled 35 times using the same temperature protocol described above. Finally, PCR products were run on a 2% (w/v) agarose gel containing ethidium bromide and periodically sequenced to confirm their identity (Robarts Institute, London, ON).
Chemicals and Peptides

All chemicals used to make solutions were purchased from Sigma (Oakville, ON). 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. Following the development of a stable control baseline membrane potential for a minimum of 100s, PK2 was rapidly applied by pressure ejection under visual guidance for 10s in the immediate vicinity of the recorded neuron. Local application of 1µM PK2 influenced the membrane potential of 66% of AP neurons tested (n=29), producing either membrane depolarization (mean resting membrane potential 64.6 ± 2.4 - Fig 1A) or hyperpolarization (mean resting membrane potential 60.4 ± 1.4 - 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, unpaired t-test, p<0.001), zero of which met imposed criteria to be considered responsive. The remaining AP neurons tested did not respond to PK2 with significant changes in membrane potential (mean resting membrane potential 60.9 ± 1.3 - Fig 1C). The mean change in membrane potential for each group is summarized in Fig 1D.

In many cases AP neurons that displayed spontaneous activity responded to 1µM PK2 treatment with decreases in action potential frequency (14 out of 16 neurons). The mean change in spike frequency of all cells tested was -71.1 ± 13.5%, a value significantly different (unpaired t-test, p<0.05) from aCSF treated control cells (n=6). Changes in action potential frequency did not always correspond
with changes in membrane potential, as there was no significant change (n=4, unpaired t-test, p>0.05) in mean spike frequency for AP neurons that depolarized although it should be emphasized that all but one of these cells showed clear decreases in spike frequency with the remaining outlier showing a large increase in spike frequency associated with the depolarization (Fig 1E). Interestingly, decreases in spike frequency were observed in all cells that either hyperpolarized (n=3) or did not show a membrane potential change (n=9), effects which resulted in a significantly greater change in mean spike frequency compared to aCSF treated controls (unpaired t-test, p<0.05). As illustrated in Fig 1A-C, the resulting inhibition of spike frequency was often profound, as 9 out of 12 neurons responded with a >94% reduction, and often took longer to show return to baseline (see Figure 1C), with full recovery normally only observed in cells where recording could be maintained for 60m following PK2 administration.

Concentrations of PK2 ranging from 1pM to 1µM produced concentration-dependent changes in membrane potential, with the highest proportion of responsive neurons and largest effects observed at 1µM (Fig 2). For experiments performed at 1pM only 2 responsive cells were observed and therefore mean responses were 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} value of 27.5pM. We analyzed both depolarizing and hyperpolarizing effects together as they both showed similar concentration response relationships. Collectively, these results show PK2 directly caused either depolarizing or hyperpolarizing effects on AP neurons, both of which were found to be concentration dependent, while effects on spike frequency were more homogenous with only inhibitory effects observed.

We next attempted to examine the ion channels influenced in AP neurons by PK2 using voltage-clamp techniques to measure currents evoked by slow voltage ramps (12.5mV/s) run from -100 to -20mV both in the presence of aCSF and following 10nM PK2 application. Out of 12 cells tested, current-voltage relationships in control conditions generated a voltage-independent current and following PK2
treatment an inward whole-cell current shift was observed in 5 cells from a holding potential of -75mV (mean -17.4 ± 7.9pA, Fig 3A). The mean change in conductance was 4.0 ± 2.7nS and the difference current that was obtained by subtracting the control current from the current obtained in PK2 elicited a mean reversal potential of -63.6 ± 3.1mV (Fig 3B). Based on 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⁻}=-64mV) and leads to membrane depolarization.

In a separate group of AP neurons, PK2 produced outward whole-cell currents (mean 7.6 ± 5.8pA, 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 ± 0.6nS and a PK2-induced current that reversed at a mean membrane potential of -34.9 ± 3.5mV (Fig 3D), suggesting that inhibition of a voltage-dependent non-selective cation current leads to membrane hyperpolarization. The proportion of responding neurons in voltage-clamp configuration closely resembles the proportion of depolarizing and hyperpolarizing responses on membrane potential (Fisher’s exact test, p>0.05).

To determine whether activation of a Cl⁻ current is responsible for membrane depolarization, slow voltage ramps were again performed using a pipette solution containing 139mM Cl⁻. Under high Cl⁻ conditions, the PK2-activated current reversal would be expected to shift towards the set E_{Cl⁻} of -2mV. 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_{Cl⁻}. Overall, the mean reversal potential of the PK2-induced depolarizing current was -24.0 ± 8.3mV (n=6), although there was considerable variability in these values ranging from the -40mV shown in Figure 4a to 0mV in other examples. These results are in accordance with the predicted depolarizing shift of the Cl⁻ reversal potential in these recording conditions. Furthermore, due to a greater Cl⁻ driving force at resting membrane potential, depolarizations (mean 17.3 ± 4.8mV, n=5) but not hyperpolarizations (mean -16.2
± 6.5mV, n=2) were significantly larger in response to 1µM PK2 (Fig 4B and C, unpaired t-test, p<0.05), indicating activation of a Cl⁻ conductance contributes to membrane depolarization.

Although different effects of PK2 were observed on the membrane potential of different groups of AP neurons, as outlined above the majority of AP neurons treated with PK2 responded with decreases in spike frequency. These observations suggested potential additional actions of PK2 on Na⁺ channels, effects which would be in accordance with our own recent report of PK2 effects on Na⁺ channels in subfornical organ neurons (8). We therefore undertook an analysis of mean action potential height in 5 cells that demonstrated decreased spike frequency but no change in membrane potential in response to PK2, an analysis that revealed PK2 induced a significant decrease in spike amplitude (paired t-test, p<0.05) from 71.2 ± 7.5 to 52.8 ± 7.8mV (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 undertook whole-cell voltage-clamp experiments to investigate the effect of 10nM PK2 on Na⁺ channel gating. Na⁺ current activation and steady-state inactivation were studied in 6 AP neurons before and following PK2 application. Activation curves were generated by applying voltage steps between -80 and -20mV in 10mV increments from a -90mV prepulse potential, and the conductance corrected for changes in driving force and normalized (Fig 6A). After fitting the resulting activation curves with a Boltzmann function, 4 cells out of 6 showed a significant leftward shift in the half activation potential (extra sum-of-squares F-test, p<0.05) to more hyperpolarized values (control -36.5 ± 1.6mV, PK2 -41.8 ± 1.9mV), while there was no significant change in the slope factor k (control 4.5 ± 1.3mV, PK2 4.9 ± 1.7mV). To assess steady-state inactivation, AP neurons were subjected to 200ms prepulse potentials between -110 and -20mV in 10mV increments, followed by a test pulse to -10mV. A total of 6 normalized steady-state inactivation plots revealed a significant PK2-mediated hyperpolarizing shift in the half inactivation potential in 4 neurons (extra sum-of-squares F-test, p<0.05). The mean half
inactivation potential was -59.4 ± 1.0 mV in control and -68.6 ± 1.7 mV following PK2 application (Fig 6B). Again, the slope factor $k$ remained unchanged (control -6.6 ± 0.9 mV, PK2 -7.1 ± 1.5 mV). 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 the AP regulates have a circadian component to them such as feeding behaviour and cardiovascular function. A number of neurotransmitters and neuropeptides implicated in the control of feeding behaviour and cardiovascular function are expressed in the AP (for review see Price et al. (34)), and may therefore be involved in circadian autonomic output from this nucleus. We sought to identify whether specific chemical phenotypes of AP neurons are influenced by PK2 using a combination of electrophysiology and single cell RT-PCR (scRT-PCR). 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 using post hoc molecular processing techniques. Nested primer sets were designed to detect the following mRNA: enkephalin (ENK), cocaine- and amphetamine-related transcript (CART), glutamate decarboxylase 67 (GAD67), cholecystokinin (CCK), tyrosine hydroxylase (TH), and vesicular glutamate transporter 2 (VGLUT2) (Table 1) and the response to 10nM 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, 5 out of 6 ENK neurons displaying spontaneous activity responded to PK2 with decreased firing frequency (mean -99.3 ± 0.7%). We also identified 2 depolarizing ENK neurons that co-expressed GAD67; however, GAD67 expressing neurons were not homogenously influenced (n=6). GAPDH-positive cells that hyperpolarized following PK2 exposure showed no distinguishing phenotype. Because there was only a small population of cells expressing CART (n=4) and CCK (n=1), it is
difficult to make any conclusions regarding the effect of PK2 on these neurons. Lastly, there were no neurons identified to express TH.

**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, while inhibition of an inwardly rectifying non-selective 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 subfornical organ (SFO) and paraventricular nucleus of the hypothalamus identified only excitatory effects on membrane activity in these nuclei (6; 48). In contrast, in the current study we have shown PK2 produced both 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 63pM found in the paraventricular nucleus and SFO, respectively (6; 48). Similarly, other peptides which 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 indicated that inhibition of action potential frequency and amplitude were 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 in 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 signalling pathways in these nuclei.

The functional consequences of the observed hyperpolarizing shift in Na\(^+\) channel voltage sensitivity in AP neurons (which in our hands have a resting membrane potential of approximately -60mV), would result in a decrease of the Na\(^+\) current mediating the action potential; an effect which 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 suggested that differential ion channel modulation by PK2 was a determinant of the effects of this peptide on membrane potential in the AP. AP cells that showed voltage-independent currents between -100 and -60mV 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 data indicate a difference in whole-cell properties between two populations of AP neurons. The observed reduction in inward
current may represent PK2-mediated inhibition of the hyperpolarization-activated cation current that is present in roughly 60% of AP neurons and possesses a similar reversal potential (-36mV) as neurons in this study (11). Similarly, the PK2 homologue, Bv8, increases pain perception by enhancing the activity of the transient receptor potential vanilloid 1 channel in dorsal root ganglion neurons, a non-selective 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 creates an ideal situation in which the responsiveness of an AP neuron to input signals, such as excitatory 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 were the result of a PK2-activated conductance that reversed at the Nernst E_{Cl⁻}. The mean reversal potential of the PK2-activated depolarizing current did not completely shift to the calculated E_{Cl⁻} (-2mV) 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 will clearly need to
identify further the functional relevance of these differential effects on different subpopulations of AP neurons.

Our scRT-PCR analysis has provided data indicating that PK2 caused 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 have been described for this circumventricular organ. The nucleus tractus solitarius (NTS) is a major cardiovascular integration centre in the medulla (36), receives direct inputs from the AP (39; 43), and represents a possible site where ENK-neurons may project. In accordance 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 regulating 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 brainstem; however, retrograde tracing from the NTS combined with ENK immunolabelling 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 AP, our scRT-PCR analysis was not able to identify the specific cell type(s) showing hyperpolarizing responses to this peptide.
While 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 either the SCN or NTS, both of which are critical autonomic control centers in which PK2 mRNA expressing neurons have been described (3; 4). While there is no evidence to support SCN neurons projecting to AP, there is an extensive literature showing NTS projections to this circumventricular organ (43), suggesting neurons in this region to be the most likely source of neurally produced PK2 that would be released from axon terminals of NTS cells terminating in the AP. A second possibility is that PK2 may access the AP as a circadian hormone in the circulation; however, neither the presence nor any circadian profile of PK2 expression has presently been described in the bloodstream, although the lack of appropriate antibodies has to date made such measurements impossible.

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 hour rhythmic expression pattern in the NTS (13; 18), a site 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. Interestingly, 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 NTS during this same time period. In addition, the circadian expression of the core clock genes are 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 which 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, suggest that the level of AP neuronal excitability may be regulated by the circadian cycle of PK2 expression; however, further experiments will be 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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**Figure Legends**

Fig 1: PK2 has direct effects on membrane potential and firing frequency in dissociated AP neurons. Representative current-clamp recordings showing AP neurons (A) depolarize (n=11), (B) hyperpolarize (n=8), or (C) show no change in membrane potential (n=10) in response to 1µM PK2 application. The majority of spontaneously active neurons showed a decrease in firing frequency. Arrows indicate time of PK2 application. Scale bars: 10mV, 20s. Bar graphs showing (D) the mean change in membrane potential and (E) mean change in spike frequency for cells tested with 1µM PK2.

Fig 2: The effects of PK2 on membrane potential are concentration dependent. Mean changes in membrane potential normalized to the maximal response observed at each concentration for depolarizations and hyperpolarizations were fitted with a Hill equation to give an EC50 of 27.5pM. Fractions indicate the proportion of neurons responding at each concentration.

Fig 3: Current-voltage relationships in subpopulations of AP neurons in response to PK2 application. (A) Whole-cell current response of an AP neuron to a voltage ramp that responded to 10nM PK2 with an inward shift in current. PK2-induced inward whole-cell current shifts typically occur in AP cells possessing non-rectifying currents between -100 and -60mV and are accompanied by increased conductance. (B) The mean difference in whole-cell current between PK2 treatment period and control period plotted at 10mV intervals for cells that responded with inward current shifts (n=5). The mean difference current reversed at -64 ± 3mV. (C) Decreased conductance of an inwardly rectifying current in an AP neuron is representative of a second subpopulation of AP neurons that respond to PK2 with outward shifts in whole-cell current. (D) The mean difference current plotted in AP cells that responded with outward currents reversed at -34.9 ± 3.5mV (n=5).

Fig 4: PK2 depolarizes AP neurons through activation of a Cl⁻ carrying current. (A) Current-voltage relationship in an AP neuron under high internal Cl⁻ conditions (139mM) that responded to 10nM PK2 with an inward whole-cell current shift. The extrapolated reversal potential of the PK2-induced current (n=6) shifted towards the set E_{Cl⁻} (-2mV). (B) Current-clamp trace showing a large depolarization under high internal Cl⁻ following 1µM PK2 application, as a result of a greater driving force for Cl⁻ to leave the cell at the resting membrane potential. Arrow indicates time of PK2 application. Scale bars: 10mV, 100s. (C) Summary graph showing mean depolarizations but not hyperpolarizations were significantly larger in 139mM internal Cl⁻ in response to 1µM PK2 (* p<0.05, unpaired t-test).

Fig 5: PK2 decreases action potential amplitude in AP neurons. (A) Left panel: current-clamp record showing application of 1µM PK2 decreased spike frequency and height in an AP neuron. Scale bars: 10mV, 20s. Right panel: Mean change in action potential height in response to PK2 application. Scale bars: 20mV, 5ms. (B) Current-clamp trace showing recovery from spike inhibition occurred at hyperpolarized membrane potentials in an AP neuron that hyperpolarized following PK2 administration, suggesting a change in Na⁺ channel voltage dependence. Scale bars: 10mV, 50s. Arrows indicate time of PK2 application.
Fig 6: PK2 induces a leftward shift in Na+ channel activation and inactivation voltage dependence. (A) Upper panel: Na+ current activation from a test pulse of -40mV before and following 10nM PK2 application. Scale bars: 50pA, 1ms. Lower panel: Graph illustrating the mean normalized conductance plotted as a function of test potential in AP neurons showing a shift in activation gating induced by PK2 (n=4). Data points were fitted with a Boltzmann function. Inset shows the activation voltage step protocol. (B) Upper panel: Na+ current inactivation following a prepulse step to -60mV before and following 10nM PK2 application. Scale bars: 200pA, 1ms. Lower panel: Graph illustrating the mean normalized current plotted versus prepulse potential in AP neurons showing a shift in inactivation gating caused by PK2 (n=4). Data points were fitted with a Boltzmann function. Inset shows the inactivation voltage step protocol.

Fig 7: PK2 predominantly depolarizes ENK neurons of the AP. (A) Current-clamp recording demonstrating a 10nM PK2-induced depolarization accompanied by a decrease in spike frequency. This AP cell was identified to express ENK mRNA post hoc using scRT-PCR (inset). Bar above trace indicates the duration of PK2 application. Scale bars: 10mV, 50s. (B) Graph summarizing the frequency distribution of responses to PK2 in AP neurons expressing mRNA for ENK, CART, and GAD67.

References


The figure shows a graph plotting the mean normalized response (%) on the y-axis against the logarithm of the concentration of PK2 (M) on the x-axis. The graph includes data points marked with (2/10), (7/25), (11/22), and (19/29). The error bars indicate variability in the data.
A

B

C

Δ membrane potential (mV)

depolarize hyperpolarize

control PK2

voltage (mV)
current (nA)

12 mM Cl− 139 mM Cl−

depolarize hyperpolarize

*
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Primer sequences are listed in the 5' to 3' direction.