Brief Communication

Prokineticin 2 Modulates the Excitability of Subfornical Organ Neurons

G. Trevor Cottrell,1 Qun-Yong Zhou,2 and Alastair V. Ferguson1
1Department of Physiology, Queen’s University, Kingston, Ontario, Canada K7L 3N6, and 2Department of Pharmacology, University of California, Irvine, California 92697

The recent discovery of prokineticin 2 (PK2) expression in the suprachiasmatic nucleus and its receptors in critical autonomic control centers of the brain, including the subfornical organ (SFO), suggests the intriguing possibility that PK2 regulates the excitability of SFO neurons and thus influences autonomic function. Using current-clamp techniques to record from dissociated SFO neurons, we examined the effects of PK2 on the excitability of these cells. PK2 (20 nM) induced depolarizations in 40% of SFO neurons (n = 45; mean, 7.5 ± 1.7 mV), an effect that was reversible, PK2-specific, and concentration dependent. The depolarization was accompanied by an increase in action potential frequency from 0.4 ± 0.1 to 1.4 ± 0.5 Hz in responding cells (n = 10). This excitatory effect appears to be, in part, attributable to a PK2-induced decrease in the delayed rectifier potassium current (I_K). In 10 SFO neurons recorded using perforated patch voltage-clamp techniques, six demonstrated a reversible decrease in I_K (mean decrease, 26.7 ± 6.4%) in response to 20 nM PK2, whereas artificial CSF alone was without an effect on these currents. These data are the first to show excitatory effects of PK2 on neurons and, in addition, demonstrate that this peptide modulates voltage-activated K⁺ channels. The activation of SFO neurons by PK2 illustrates a mechanism through which this peptide may exert circadian control of autonomic functions.

Key words: autonomic; circadian; circumventricular; neuropeptide; patch clamp; potassium [K]

Introduction

Prokineticin 2 (PK2) is a novel 81 amino acid peptide with a complex folding structure including five disulphide linkages. It is a mammalian homolog to the mamba snake venom protein mamba intestinal toxin 1 and the frog protein Bv8, and has been demonstrated to be a paracrine mediator of gastrointestinal smooth muscle contractility (Li et al., 2001). Prokineticin 1 (PK1), a homolog of PK2, is also identical to endocrine gland-derived vascular endothelial growth factor, a potent angiogenic mitogen in secretory organs (LeCouter et al., 2001; Masuda et al., 2002).

PK2 is expressed throughout the mammalian organism; however, in the brain, its expression is specifically concentrated in the suprachiasmatic nucleus (SCN) (Cheng et al., 2002). PK2 is rhythmically expressed in the SCN, transcriptionally regulated by clock control genes, and has the capacity to strongly suppress nocturnal locomotor activity. PK2 receptors are located in the SCN as well as numerous autonomic control centers in the brain such as the paraventricular nucleus (PVN) and the subfornical organ (SFO) (Cheng et al., 2002). PK2 is one of the first neuropeptides that appears to directly convey circadian rhythm signaling to other CNS structures and, therefore, it may be a critical output molecule regulating biological rhythms.

The SFO is a circumventricular organ structure that has essential actions in the integrative regulation of cardiovascular, immune, feeding, and reproductive function (McKinley et al., 1998; Cottrell and Ferguson, 2004). It is ideally situated in the forebrain to be exposed to both blood-borne signals and those circulating within the CNS. The role of the SFO in the regulation of cardiovascular function has been well characterized. It is sensitive to stimulation by angiotensin II (ANG) and vasopressin and is intrinsically osmosensitive. The SFO influences cardiovascular function through projections to the hypothalamus and other autonomic control centers (Lind et al., 1982; Ferguson and Renaud, 1986).

The integrative action of the SFO makes it an ideal regulatory target for signaling molecules designed to influence autonomic function. As a result, it could play a significant role in PK2 signaling from either the periphery or the CNS. The purpose of these experiments was to determine whether the exposure of SFO neurons to PK2 resulted in increased neuronal excitability. We demonstrated that PK2 is capable of depolarizing SFO neurons and increasing their firing frequency. This effect was at least partially mediated by an inhibition of the delayed rectifier K⁺ current but not the transient outward K⁺ current.

Materials and Methods

Chemicals. Unless otherwise indicated, all chemical reagents were from Sigma (St. Louis, MO). PK2 was produced using recombinant techniques described previously (Li et al., 2001) and was used at a standard concentration of 20 nM for all experiments unless otherwise indicated. This...
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concentration corresponds to the top range of the dose–response for PK2 effects in gastrointestinal smooth muscle and is similar to that used to induce calcium transients in neurons (Li et al., 2001; Negri et al., 2002). The PK2 antagonist A1MPK1 was produced using recombinant techniques described previously (Bullock et al., 2004). A1MPK1 is an effective inhibitor of PK2 action when at a 40-fold greater concentration than PK2. Nystatin stock solution (100 g/ml in dimethylsulfoxide) was made fresh each day and stored at 4°C until use in experiments when it was then suspended in pipette solution (see Electrophysiology) at a final concentration ranging from 100 to 250 μg/ml. DMSO content in the internal solution was never >0.03%. Tetrodotoxin (TTX; Alomone Labs, Jerusalem, Israel) was stored as a stock solution in 1 ml aliquots and made fresh daily by suspension in artificial CSF (aCSF) to a final concentration of 100 nM.

Subfornical organ neuron dissociation. Experiments with animals were approved by the Queen’s University Animal Care Committee. The SFO dissociation procedure was performed as described previously (Ferguson et al., 1997). Male Sprague Dawley rats (125–175 gm) were decapitated, and the brains were removed and immediately immersed in ice-cold Ca²⁺- and Mg²⁺-free HBSS (Invitrogen, Grand Island, NY) supplemented with 0.09 mM sucrose. A tissue block containing the hippocampal commissure and SFO was dissected free from the brain, and the SFO was separated from the surrounding tissue. The isolated SFO was immersed in 1 mg/ml Trypsin and incubated at 37°C in 5%CO₂, 95%O₂ for 30 min. Cells were then suspended in ice-cold Ca²⁺- containing HBSS (Invitrogen) supplemented with 4 μg/ml BSA, triturated through a 20 gauge needle using a 1 ml syringe, and centrifuged at 900 × g for 5 min. The supernatant was then removed, cells were resuspended, and a second 5 min centrifugation was performed. Cells were resuspended, plated in culture dishes (Corning, Corning, NY) with a Neurobasal A Medium (Invitrogen), and incubated for a minimum of 24 hr before electrophysiology experiments.

Electrophysiology. Whole-cell patch-clamp recordings were performed as reported previously (Washburn et al., 1999; Desson and Ferguson, 2003). Electrodes were fabricated from 1.2 mm filament glass (A–M Systems, Carlsborg, WA) on a Sutter Instruments (Novato, CA) puller and were filled with the following (in mM): 130 potassium gluconate, 10 HEPES, 10 EGTA, 1 MgCl₂, 4 Na₂ATP, and 0.1 GTP (290–300 mOsm, pH 7.2). For perforated patch experiments, 10 mM KCl was added to the internal solution. The cells were bathed in an external solution (aCSF) containing (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 10 HEPES, 10 glucose, and 2 CaCl₂ (290–300 mOsm, pH 7.2).

Whole-cell recordings were obtained from SFO cells identified as neurons on the basis of the presence of voltage-gated Na⁺ currents under voltage-clamp conditions, and at least 80 mV action potentials induced at a holding potential below −55 mV in current-clamp recordings. Signals were amplified using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) filtered with an 8-pole Bessel filter at 1 kHz and digitized using a Cambridge Electronics Design micro1401 interface (Cambridge Electronics Design, Cambridge, UK) at 8 kHz. Data were collected using Signal (voltage clamp) or Spike 2 (current clamp) software packages (Cambridge Electronics Design).

To minimize current rundown during voltage-clamp experiments, perforated patch recordings were performed with electrodes back-filled with Nystatin (see Reagents). After making cell contact with the electrode, light suction was applied to achieve a high resistance seal (≈2–8 GΩ). Seal quality and cell access were monitored using −10 mV pulses from a holding potential of −65 mV. Once series resistance was below 30 MΩ, outward currents were monitored over time until cell access plateaued (≈15 min). Sudden changes in series resistance were monitored between recordings, and electrode suction was applied at the end of experiments to ensure that the perforated patch configuration was patent throughout the experiments. All voltage-clamp experiments were performed in the presence of 100 nM TTX.

Statistical analysis. In current-clamp recordings, cells were classified as responders if they showed sustained changes (>60 sec) in membrane potential (Em) of >5 mV in the 1–3 min period after the initiation of PK2 perfusion. This measure was made relative to the 60 sec period immediately before treatment. The number of responding cells in the PK2 treatment group was compared with control aCSF treatment using a χ² test. Changes in membrane potential and action potential frequency were compared between control and treatment groups using a Student’s t test. The concentration–response curve was constructed from a sigmoidal function of the following form:

\[ Y = Y_{\text{min}} + \frac{(Y_{\text{max}} - Y_{\text{min}})}{1 + 10^{(\log IC_{50} - \log C)}} \]

In voltage clamp, cells were distinguished as responders on the basis of a 10% decrease in current followed by at least a partial recovery toward baseline after an aCSF wash. Responders were compared between control and treatment groups using a χ² test to assess a treatment effect. Peak current change was compared between control and treatment groups using a Student’s t test. Current values in all I–V plots were normalized to the peak current measured during baseline recording. Normalized current change at each voltage step was compared between baseline and treatment conditions using a paired t test. All values are plotted as means ± SEM with p values set at 0.05.

Results

PK2 depolarizes subfornical organ neurons

The effects of 20 nM PK2 treatment on membrane potential were assessed during continuous current-clamp recording. In 45 cells recorded, there was a depolarizing effect of PK2 on 18 cells (40%) with this effect being reversible in 85% of these responding cells (Fig. 1A–C). In 14 controls treated with an aCSF wash, only three had a “response” on the basis of the imposed criteria for depolarization (see Materials and Methods); however, none of these cells had any recovery toward baseline after a second aCSF wash. χ² comparison of the control and treatment groups shows that the number of responders in the PK2 group was significantly

Figure 1. PK2 depolarizes SFO neurons and increases action potential firing frequency. A–C, Current-clamp recordings demonstrating the effect of 20 nM PK2 on whole-cell membrane potential. The time period of PK2 application is depicted by the gray bars. All horizontal scale bars represent 60 sec. D, Mean change in membrane potential measured relative to baseline during treatment with either aCSF (white bar; n = 14), 20 nM PK2 (hatched bar; n = 45), or 1 nM PK2 in combination with 40 nM A1MPK1 (gray bar; n = 9). The black bar represents the mean depolarization of those SFO neurons classified as responders (PK2-res; n = 18). Asterisks indicate a significant difference compared with aCSF treatment (p < 0.05). E, PK2 increased the firing frequency of 7 of 10 cells that demonstrated a reversible PK2 effect on membrane potential. Con (white bar), Firing frequency recorded in the last minute of baseline recording; PK2 (hatched bar), firing frequency recorded 2–3 min after 20 nM PK2 treatment; Rec, firing frequency recorded in the last minute of the washout period. Asterisks indicate a significant effect compared with the baseline measure.
greater than that expected under control conditions, thus implying that there is a distinct population of SFO cells capable of responding to PK2.

The mean depolarization of all SFO cells (n = 45) in response to 20 nM PK2 was 7.5 ± 1.7 mV, a value significantly greater than that of the control group (n = 14; 2.8 ± 1.1 mV) (Fig. 1D). The mean depolarization of cells identified as responders was 19.0 ± 2.2 mV (n = 18) (Fig. 1D). To ensure that the PK2 effects observed were the direct result of PK2 action and not that of a contaminant, the PK2 receptor antagonist A1MPK1 was used. Perfusion of SFO neurons with 40 nM A1MPK1 5 min before perfusion with 1 nM PK2 prevented any depolarization (Fig. 1D; see also Fig. 2 for 1 nM PK2 effect). In the 10 cells demonstrating a reversible PK2 effect, there was an increase in the action potential firing frequency in seven of the cells with the mean firing frequency being almost fourfold greater after PK2 treatment when compared with baseline values (control, 0.4 ± 0.1 vs PK2, 1.4 ± 0.5 Hz; n = 10; p < 0.05) (Fig. 1E). Changes in membrane potential were concentration dependent, with peak depolarization occurring at 1 nM PK2, no response being observed at 1 fM, and an EC50 of 63 pm (Fig. 2).

**PK2 inhibits delayed rectifier K+ current (IA)**

Previous research has demonstrated a pattern of hyperexcitability of SFO neurons induced by external ligands, with this effect being mediated in part by the inhibition of outward K+ currents (Ferguson and Li, 1996; Washburn et al., 1999). Based on this, and a decreased afterhyperpolarization amplitude and duration following action potentials in some of the responding cells, we examined the effects of PK2 on delayed rectifier (I_A) and transient outward (I_O) K+ currents.

Voltage-clamp recordings using perforated patch techniques were used to isolate I_A from the I_K by administering 10 mM voltage steps between −80 and 20 mV from a holding potential of −40 mV. Current traces represent baseline (Con), 20 nM PK2 treatment (PK2), and PK2 washout (Rec) conditions in the same neuron. B, I–V plot of normalized steady-state current, averaged across experiments (n = 6) and plotted at each voltage step for the control period (filled circles) during 20 nM PK2 treatment (open squares) and after PK2 washout (filled triangles). The PK2 treatment effect was first observed at the 0 mV step. Asterisks indicate a significant difference from control (p < 0.05). Inset, Overlaid current traces from pulses to 20 mV during control, PK2, and recovery periods. C, Mean change in current measured at the 20 mV pulse for an aCSF wash (white bars; n = 7) and 20 nM PK2 treatment (hatched bars; n = 6). The mean PK2 current change was derived from neurons defined as responders. Asterisks indicate a significant difference from the aCSF treatment (p < 0.05).

Voltage-clamp recordings using perforated patch techniques were used to isolate I_A from the I_K by administering 10 mM voltage steps between −80 and 20 mV from a holding potential of −40 mV, a protocol which inactivates I_A and thus permits direct assessment of I_K (Washburn et al., 1999). In 10 cells recorded, six demonstrated a decrease in I_A current, which was reversible after washout at a CSF (Fig. 3A). The number of responding cells in the PK2 treatment group was significantly greater than a control aCSF wash (Con), where no cells (0 of 7) demonstrated a response on the basis of the criteria established (see Materials and Methods).

Comparison of I–V relationships shows a significant decrease in I_A at the 0 mV step, with the current difference increasing progressively up to the 20 mV step after 20 nM PK2 treatment (Fig. 3B). The mean change in current at the 20 mV step was significantly greater in the PK2-treated cells labeled responders (26 ± 6% decrease) when compared with control aCSF perfusion for the same time period (10 ± 12% increase; p < 0.05) (Fig. 3C). There were no differences in mean I_A change between the control-treated cells and the PK2-treated cells identified as non-responders (19 ± 13% increase; data not shown).

**PK2 has no effect on transient outward K+ current (I_O)**

I_O was pharmacologically isolated from sustained outward currents in SFO neurons using aCSF containing 10 mM TEA. Voltage-clamp protocols incorporated 500 msec, 10 mM steps between −100 and 20 mV from a holding potential of −100 mV. These steps resulted in rapidly activating, rapidly inactivating currents typical of I_O (Fig. 4A). PK2 had no effect on this isolated I_A current, with no significant change in normalized current being recorded at any voltage step after treatment (Fig. 4B). This effect was identical to a control aCSF perfusion for a similar time period.
period (Fig. 4C), with the mean change in current at the 20 mV step being 9.0 ± 12% \((n = 5)\), as compared with 4.7 ± 10% in the PK2 treatment group \((n = 7)\).

Discussion

These data are the first to demonstrate an effect of PK2 on the excitability of CNS neurons. PK2 depolarized 40% of the SFO neurons tested and increased the action potential firing frequency in the majority of these cells. It is also the first time PK2 has been demonstrated to have an effect on voltage-gated \(K^+\) \((K_v)\) channels. This observation could account for the increase in action potential firing frequency in PK2-treated neurons, although not necessarily for the membrane depolarizations observed at hyperpolarizing holding potentials, thus suggesting that PK2 influences other membrane properties. These findings demonstrate a neuromodulatory role for PK2 and identify the SFO as a potential site for circadian regulation of autonomic function.

PK2 is expressed both centrally and in the periphery. In the periphery, its expression is focused in gastrointestinal tissues at levels significantly lower than PK1. In this region, PK2 has a paracrine effect on the stimulation of gastrointestinal smooth muscle contraction \(\left(\text{Li et al., 2001}\right)\). Centrally, PK2 expression is focused in the SCN; however, the median preoptic area, nucleus accumbens, and islands of Calleja also contain PK2 mRNA signal \(\left(\text{Cheng et al., 2002}\right)\). Two studies have examined the effects of the PK2 orthologue, Bv8, on neuronal function and determined that it increases nociceptive sensitization of the tail and paw in rats, increases intracellular calcium in small diameter dorsal root ganglion neurons in culture \(\left(\text{Negri et al., 2002}\right)\), and supports neuronal survival \(\left(\text{Melchiorri et al., 2001}\right)\). Only the original work by Cheng et al. \(\left(2002\right)\) examined PK2 action in the brain.

The source of PK2 that modulates the SFO is not clear. To date, there is no evidence for direct efferent SCN projections to the SFO, although it is possible that the detection sensitivity of methodologies used thus far is not sufficient to identify such interactions. Because the SFO has the unique characteristic of being a sensory circumventricular organ, it could receive PK2 signaling through a number of different pathways such as from the periphery via the blood, from another brain region such as the median preoptic area, from the SCN via diffusion through the CSF, or through direct SCN efferents that have not yet been identified.

Although the physiological implications of PK2 effects on SFO neurons are not clear, the SFO has well established roles in the regulation of cardiovascular function, body fluid balance, immune response, feeding, and reproduction; therefore, there are numerous possible outcomes for PK2 action. The early morning peak in PK2 expression that is coincident with the morning peak in cardiovascular activity \(\left(\text{Guo and Stein, 2003}\right)\) suggests that the SFO could have a regulatory role in conveying circadian rhythm signaling to other cardiovascular centers, and PK2 may be a factor in this. Future studies examining both the influence of PK2 on cardiovascular function and the SFO role in cardiovascular rhythms are warranted to clarify whether this association exists.

No previous electrophysiological experiments have assessed the effects of PK2 on neuronal excitability or ion channels. The inhibition of \(I_K\) current by PK2 reported here is novel, yet not unexpected. Washburn et al. \(\left(1999\right)\) demonstrated that vasopressin increased SFO neuron excitability in 65% of the neurons examined and inhibited both \(I_{K_1}\) and \(I_{K_A}\) currents. Similarly, Ferguson and Li \(\left(1996\right)\) demonstrated that angiotensin II inhibits \(I_{A}\) currents in SFO brain slices. In the present study, no effect of PK2 on \(I_{K}\) was found; however, the significant inhibition of \(I_{K_A}\) current likely contributed to enhanced neuronal excitability by increasing membrane potential at relatively depolarized states, prolonging action potentials, and decreasing the amplitude and duration of the afterhyperpolarization. Although some of these latter effects were observed during our current-clamp recordings, we chose to use more definitive voltage-clamp techniques to directly assess PK2 effects on \(K_1\) channels and, using such an approach, were able to identify clear specific effects on \(I_{K}\).

Voltage-clamp recordings in SFO neurons have previously characterized two populations of neurons, those that show a large degree of inactivation \(\left(I_{K_A}\right)\) and those that display a small degree of inactivation \(\left(I_{K}\right)\) \(\left(\text{Anderson et al., 2001; Desson and Ferguson, 2003}\right)\). The physiological relevance of this categorization is not clear; however, those SFO neurons projecting to the paraventricular nucleus are of the \(I_{K}\) type \(\left(\text{Anderson et al., 2001}\right)\). In the present experiments, PK2 effects were observed in both populations to an equal extent \(\left(\text{data not shown}\right)\); therefore, the PK2-sensitive SFO neurons could not be categorized as specifically projecting to the PVN. Additional work must try to identify the characteristics of the PK2-responsive SFO neurons to better determine the excitatory role that PK2 has on these cells. The observation that only 40% of the neurons tested were PK2 responsive suggests that there is a distinct population of SFO neurons that may play a specific signaling role. Similar observations have been made for ANG \(\left(65\%\right)\) responding, vasopressin \(\left(60\%\right)\) responding, and IL1-\(\beta\) \(\left(65\%\right)\) responding) actions in the SFO \(\left(\text{Ferguson et al., 1997; Washburn et al., 1999; Desson and Ferguson, 2003}\right)\), thus suggesting a heterogeneous neuronal population.

PK2 appears to fit into a class of peptides that were originally found associated with the gastrointestinal system but also have potent signaling effects in autonomic centers of the brain. These peptides include, but are not limited to, secretin, cholecystokinin, gastrin-releasing peptide, neuropeptide Y, vasointestinal peptide, bombesin, and ghrelin. The evolutionary need for the development of this diverse group of signaling peptides with multifactorial roles is not clear. However, it implies a close relationship between central and peripheral control of autonomic activity.
In conclusion, we have demonstrated that PK2 behaves as an excitatory neuropeptide, depolarizes neurons of the SFO, and inhibits delayed rectifier potassium currents. These observations are the first to demonstrate that PK2 has a neuromodulatory role in the CNS and identifies the SFO as a potential site for circadian regulation.

References


