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Prokineticin 2 depolarizes paraventricular nucleus magnocellular and parvocellular neurons

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Abstract

Blind whole-cell patch-clamp techniques were used to examine the effects of prokineticin 2 (PK2) on the excitability of magnocellular (MNC), parvocellular preautonomic (PA), and parvocellular neuroendocrine (NE) neurons within the hypothalamic paraventricular nucleus (PVN) of the rat. The majority of MNC neurons (76%) depolarized in response to 10 nM PK2, effects that were eliminated in the presence of tetrodotoxin (TTX). PK2 also caused an increase in excitatory postsynaptic potential (EPSP) frequency, a finding that was confirmed by voltage clamp recordings demonstrating increases in excitatory postsynaptic current (EPSC) frequency. The depolarizing effects of PK2 on MNC neurons were also abolished by kynurenic acid (KA), supporting the conclusion that the effects of PK2 are mediated by the activation of glutamate interneurons within the hypothalamic slice. PA (68%) and NE (67%) parvocellular neurons also depolarized in response to 10 nM PK2. However, in contrast to MNC neurons, these effects were maintained in TTX, indicating that PK2 directly affects PA and NE neurons. PK2-induced depolarizations observed in PA and NE neurons were found to be concentration-related and receptor mediated, as experiments performed in the presence of A1MPK1 (a PK2 receptor antagonist) abolished the effects of PK2 on these subpopulations of neurons. The depolarizing effects of PK2 on PA and NE neurons were also shown to be abolished by PD 98059 (a mitogen activated protein kinase (MAPK) inhibitor) suggesting that PK2 depolarizes PVN parvocellular neurons through a MAPK signalling mechanism. In combination, these studies have identified separate cellular mechanisms through which PK2 influences the excitability of different subpopulations of PVN neurons.

Keywords

autonomic; circadian rhythm; electrophysiology; hypothalamus; paraventricular suprachiasmatic nucleus; prokineticin 2

Introduction

In mammals, circadian rhythms are believed to be regulated by neurons in the suprachiasmatic nucleus (SCN) of the hypothalamus. The activity of these neurons, often referred to as pacemaker cells, is tied to a 24-h cycle determined by environmental light : dark signals transmitted to the SCN via retinal-hypothalamic projections (Moore *et al.*, 2002; Scheer *et al.*, 2003). Coordinated circadian rhythms are then synchronized by outputs from the SCN to regulatory control centres involved in a variety of autonomic functions including cardiovascular, respiratory and metabolic regulation (Watts *et al.*, 1987; Buijs *et al.*, 1994;

Vrang *et al.*, 1995). The neurotransmitters utilized by SCN neurons to control such circadian outputs have been the focus of considerable recent attention with important roles suggested for both vasopressin (AVP), and vasoactive intestinal polypeptide (VIP; Buijs, 1996; Kalsbeek & Buijs, 1996; Inouye, 1996; Leak & Moore, 2001; Moore *et al.*, 2002).

Prokineticin 2 (PK2) has been identified as another potentially important circadian messenger (Cheng *et al.*, 2002). PK2, an 81-amino acid peptide, is conserved over its mammalian homologs, prokineticin 1, the frog skin protein, Bv8, and the mamba snake venom protein, MIT1 (Li *et al.*, 2001). Originally discovered as a promoter of gastrointestinal smooth muscle contractility, PK2 may also function as an angiogenic mitogen for endothelial cells derived from endocrine organs. PK2 stimulates the phosphorylation of p44/p42 mitogen activated protein kinase (MAPK) in both types of G protein-coupled receptors activated by prokineticins (Masuda *et al.*, 2002; Lin *et al.*, 2002). In the brain, PK2 expression is directly regulated within SCN by day–night cycles, with daytime levels 50 times greater than those at nighttime (Cheng *et al.*, 2002). PK2 receptors are located in the SCN as well as specific autonomic and neuroendocrine control centres in the brain including the subfornical organ (SFO) and the paraventricular nucleus (PVN; Cheng *et al.*, 2002). PK2 has also been reported to influence the excitability of neurons in one of these sites, the SFO (Cottrell *et al.*, 2004). More recently, its involvement has also been linked to a role in olfactory bulb neurogenesis (Ng *et al.*, 2005).

In addition to being a primary projection site of the SCN (Hermes & Renaud, 1993; Vrang *et al.*, 1995; Buijs, 1996; Leak & Moore, 2001; Munch *et al.*, 2002), the PVN is an essential autonomic centre controlling cardiovascular, respiratory, and feeding behaviour (Liposits, 1993; Ferguson *et al.*, 1999; Dampney *et al.*, 2002). It regulates these functions through magnocellular [MNC, project to the posterior pituitary controlling the release of oxytocin and AVP (Hatton *et al.*, 1976; Luther & Tasker, 2000)], parvocellular preautonomic [PA, project to the brain stem and spinal cord (Stern, 2001)], and parvocellular neuroendocrine [NE, project to the median eminence to regulate the release of hormones from the anterior pituitary (Luther *et al.*, 2002)] projection neurons. GABA and glutamate PVN interneurons have also been suggested to play a vital role in regulating the excitability of these neuronal subgroups (Bains & Ferguson, 1997; Daftary *et al.*, 1998; Latchford & Ferguson, 2004).

In this study, we report the specific actions of PK2 on electrophysiologically identified subpopulations of PVN neurons, and describe the cellular and membrane events underlying these actions.

Materials and methods

Slice preparation

Experiments were performed using male Sprague–Dawley rats (150–250 g, Charles River, Quebec, Canada) maintained on a 12-h light : 12-h dark cycle (lights on 07:00 h) from which hypothalamic coronal brain slices were prepared as previously described (Li & Ferguson, 1996). Rats were decapitated, their brains quickly removed from the skull and immersed in cold (1–4 °C) artificial cerebrospinal fluid (ACSF). The hypothalamus was blocked and 300- μ m coronal slices including the PVN were cut using a vibratome (Leica Microsystems, Richmond Hill, Ontario, Canada). All slice preparations were performed during the subjective light period (07:00–19:00-h). In addition, the SCN, and its connections to the PVN, were present in one-third of all slices. These sections were incubated in oxygenated ACSF (95% O₂ and 5% CO₂) for at least 60 min at room temperature. Fifteen minutes prior to recording, the slice was transferred to an interface-type recording chamber and continuously perfused with oxygenated ACSF (see below for solution composition) at a rate of 1 mL/min. All

techniques were carried out in accordance with the guidelines of the Canadian Council for Animal Care and were approved by Queen's University Animal Care Committee.

Electrophysiology

Whole-cell patch recordings were obtained using the 'blind' patch-clamp technique to record from PVN neurons (Li & Ferguson, 1996). The majority of recordings were made during the subjective light period; however, there was no difference in the magnitude or duration of responses compared to recordings performed during the subjective dark period. Patch pipettes were pulled to a resistance of 4–8 M Ω and filled with filtered internal solution (see below). Following establishment of a high resistance seal (> 4G Ω), brief suction was applied to rupture the membrane and achieve whole-cell configuration. Signals were processed with an Axoclamp-2B amplifier. A Ag–AgCl electrode connected to the bath solution via a KCl-agar bridge served as reference. Calculated junction potential (–17.2 mV) was corrected for all data presented in this manuscript. Drugs were applied by switching perfusion from ACSF to a solution containing the desired drug. All signals were digitized (5 kHz) using the CED 1401 plus interface (CED, Cambridge, UK) and stored on computer for off-line analysis. Data were collected using a Spike2 (continuous recording) package (CED, Cambridge, UK).

Solutions

The internal pipette solution contained (in mM) K⁺ gluconate, 140; CaCl₂, 0.1; MgCl₂, 2; EGTA, 1.1; Hepes, 10; Na₂ATP, 2; adjusted to pH 7.25 with KOH. The ACSF composition was (mM) NaCl, 124; KCl, 2; KPO₄, 1.25; CaCl₂, 2.0; MgSO₄, 1.3; NaHCO₃, 20; and glucose, 10. Osmolarity was maintained between 285 and 300 mOsm and pH between 7.3 and 7.4.

Peptides and drugs

PK2 was synthesized using recombinant techniques as previously described (Li *et al.*, 2001). Peptide solutions (1 pM to 100 nM) were prepared fresh daily from a 10 μ M stock by dilution in ACSF. The PK2 antagonist A1MPK1 was synthesized using recombinant techniques as described previously (Bullock *et al.*, 2004), and prepared on the day of experimentation to a concentration of 40 nM from a 10 μ M stock solution by dilution in ACSF. Tetrodotoxin (TTX, Alomone Laboratories, Jerusalem, Israel) was stored in 1 mM stock aliquots and made fresh daily by dilution in ACSF to a final concentration of 1 μ M. The MAPK antagonist, PD 98059 (Sigma–Aldrich, Oakville, Canada), was dissolved in dimethyl sulfoxide (DMSO) and stored as a stock solution of 10 mM and made fresh daily to a final concentration of 50 μ M by dilution with ACSF, which was shown to be effective in a slice preparation (Fuller *et al.*, 2001). The glutamate antagonist, KA, (Sigma–Aldrich) was made fresh daily to a final concentration of 1 mM by dilution with ACSF. All drugs were applied to the slice through a bath perfusion system.

Cell classification

Extensive efforts have been made to determine the functional and phenotypic neuron subpopulations located within the PVN (Hatton *et al.*, 1976; Tasker & Dudek, 1991; Luther & Tasker, 2000; Stern, 2001; Luther *et al.*, 2002). *In vitro*, it is well established that electrophysiologically identified cells possessing a transient potassium current (I_A) as shown in the inset of Fig. 1, A₁, represent AVP and oxytocin MNC neurons within the PVN (Hatton *et al.*, 1976; Tasker & Dudek, 1991). Parvocellular neurons within the PVN represent a more diverse combination of neuronal subpopulations that include pre-autonomic [projecting to the spinal cord and medulla (Swanson & Sawchenko, 1983; Stern, 2001)], neuroendocrine [projecting to the median eminence (Liposits, 1993)] output neurons, as well as both glutamate and GABA interneurons (Roland & Sawchenko, 1993; Tasker *et al.*, 1998; Daftary *et al.*, 1998). Recent studies have demonstrated that parvocellular neurons can be electrophysiologically differentiated into preautonomic (Stern, 2001), and neuroendocrine

neurons (Luther *et al.*, 2002) by the presence or absence of a low threshold calcium current, respectively. We have utilized these electrophysiological fingerprints to classify these three subpopulations of PVN neurons in this study.

Analysis

All neurons included in this analysis were able to fire spontaneous or current evoked action potentials of at least 50 mV amplitude throughout the recording period. Neurons were required to maintain a stable membrane potential for at least 100 s prior to application of test substances. Following peptide application, responses were assessed as (i) depolarization – an increase in membrane potential of > 3 mV followed by a return to baseline; (ii) hyperpolarization – a decrease of > 3 mV followed by a return to baseline, or (iii) no response – changes in membrane potential < 3 mV.

Membrane potential change was assessed by averaging all digital points [excluding points above -20 mV (action potentials)] in the 100 s prior to peptide application and comparing this value to sequential 100-s periods after peptide administration, with the maximum change period used in our analysis.

Excitatory postsynaptic potentials (EPSPs) and currents (EPSCs) were analysed using the Mini Analysis Program 6.0 (Synaptosoft Inc., USA). Identification of EPSPs/Cs was based on amplitude (minimum 2 mV/4 pA) and shape (rapid rising phase followed by a slow exponential decay). Each analysed event was examined individually to exclude any false EPSPs/Cs (e.g. action potentials) and the frequency and amplitude of events generated in this way were analysed. During continuous voltage clamp recordings to measure EPSCs, cells were held at -80 mV (close to the reversal potential for Cl^-) to ensure that IPSCs were excluded from the study.

Statistics

Changes in mean membrane potential and EPSP/C frequency were compared using a Student's *t*-test unless otherwise indicated. A one-way ANOVA with a Newman–Keuls multiple comparison post test was used to compare response to PK2 alone and while in the presence of A1MPK1 and PD 98059. Control, subjective light phase, and subjective dark phase A1MPK1 experiments were compared using a χ^2 -test. Rate histograms for amplitude and frequency of EPSCs were analysed using the Kolmogorov–Smirnov test. In all statistical tests $P < 0.05$ was set as the level for significance. All values were expressed as mean $\pm$ SEM.

Results

Whole-cell recordings were obtained from a total of 144 PVN neurons. Thirty of these recordings were obtained during the subjective dark phase while the remainder were obtained during subjective light phase. Using electrophysiological fingerprinting techniques described above, 34 of these cells were classified as magnocellular neurons, 52 as preautonomic neurons, and 45 as neuroendocrine neurons. The remaining 13 neurons could not be precisely characterized into any of these three groups and were therefore excluded from further analysis. After obtaining whole cell access, membrane potential was adjusted to between -45 and -60 mV by current injection, before experimental procedures began.

Magnocellular neurons

Using whole-cell current-clamp techniques, 17 MNC neurons (mean resting membrane potential of -47.8 ± 2.0 mV, mean action potential amplitude of 78.1 ± 1.9 mV) were tested with bath administration of PK2 (10 pM to 100 nM applied for up to 240 s). The majority (76%) of these neurons demonstrated long-duration (up to 20 min) reversible depolarizations

(10 nM mean 6.8 ± 0.6 mV) in response to PK2 application as illustrated in Fig. 1, A₁ and A₂. Latency to response was longer in some neurons, which is most likely a function of the recorded neurons depth in the slice (neurons located close to the surface will see changes in peptide concentrations before those deeper in the slice). We also examined if there were differential effects of PK2 on oxytocin vs. vasopressin MNCs neurons (classified by I–V plots; Armstrong & Stern 1998), but found that approximately 70% of each of these cell groups were influenced by PK2.

In order to determine whether the observed effects of PK2 were due to direct actions of this peptide on MNC neurons, additional current-clamp recordings were performed in the presence of TTX (1 μ M). The effectiveness of TTX was confirmed by the absence of action potentials (spontaneous or evoked during depolarizing pulses). TTX abolished PK2-responsiveness (Fig. 1B) in MNC neurons (PK2, 6.8 ± 0.6 mV vs. PK2 + TTX, -1.8 ± 1.8 mV, $n = 6$, unpaired t -test, $P < 0.0001$, Fig. 1C).

The observation that the depolarizing effects of PK2 on MNC neurons were blocked by TTX led us to a retrospective analysis of EPSP activity during PK2-induced depolarizations to determine if an increase in postsynaptic potential frequency was associated with this depolarization. Increases in mean frequency of EPSPs were observed in response to PK2 administration in 6 MNC neurons with mean EPSP frequency increasing from control values of 0.23 ± 0.04 Hz (100-s period prior to PK2) to 0.49 ± 0.12 Hz during treatment ($n = 6$, $P < 0.05$, paired t -test, Fig. 1D).

Continuous whole-cell voltage-clamp recordings were used to determine the effects of PK2 administration on postsynaptic currents (Fig. 2, A₁). In five neurons tested, PK2 increased EPSC frequency from control values of 1.47 ± 0.38 Hz to 2.14 ± 0.03 Hz during PK2 application ($n = 5$, $P < 0.05$, paired t -test, Fig. 2, A₃). Mean EPSC amplitude did not change between control and PK2 conditions (control, 8.62 ± 0.82 pA vs. PK2, 8.18 ± 0.76 pA, $n = 5$, paired t -test, $P > 0.5$, Fig. 2, A₃). The broad-spectrum glutamate antagonist, KA, was administered (1 mM) at the end of voltage clamp recordings, and in all cases EPSCs were abolished.

We further tested the hypothesis that increased synaptic activity from glutamatergic interneurons was responsible for the PK2-induced depolarization in MNC neurons by performing current-clamp experiments in the presence of 1 mM KA, as illustrated in Fig. 2, B₁. Pretreatment with KA until all EPSCs (monitored in voltage clamp configuration) were blocked abolished 10 nM PK2-induced depolarizations (PK2, 6.8 ± 0.6 mV vs. PK2 + KA, -0.3 ± 0.9 mV, $n = 7$, unpaired t -test, $P < 0.0001$, Fig. 2, B₂).

Parvocellular neurons

Both PA and NE parvocellular neurons demonstrated depolarizations in response to bath application of PK2 as illustrated in Fig. 3, A₁ and B₁. A total of 25 PA neurons were tested (mean resting membrane potential -55.6 ± 1.9 mV; mean action potential amplitude 80.8 ± 3.0 mV), of which 68% responded to 10 nM PK2 application with a mean depolarization of 8.6 ± 0.6 mV. The depolarizing effects of PK2 on PA neurons lasted approximately 20 min before a return to baseline membrane potential was observed. Recordings were also obtained from 15 NE neurons (mean resting membrane potential and action potential amplitude -54.7 ± 1.7 mV and 79.9 ± 3.0 mV, respectively), of which a similar proportion (67%) were found to depolarize in response to bath administration of 10 nM PK2 with a mean membrane potential change of 8.7 ± 0.6 mV, an effect which lasted on average 23 min prior to a return to baseline membrane potential.

In order to determine whether the observed effects of PK2 were due to direct actions of this peptide on PA and NE neurons, 11 parvocellular neurons were tested (four PA and seven NE)

with PK2 in the presence of TTX (1 μ M), of which eight depolarized (three PA and five NE) as illustrated in Fig. 3, A₂ and B₂. Neither the magnitude of these responses (PA, PK2, 8.6 ± 0.6 mV vs. PK2 + TTX, 13.7 ± 2.5 mV, unpaired *t*-test, $P > 0.1$, Fig. 3, A₄; NE, PK2, 8.7 ± 0.6 mV vs. PK2 + TTX, 8.5 ± 2.0 mV, unpaired *t*-test, $P > 0.5$, Fig. 3, B₄), nor the proportion of neurons influenced (PA, PK2, 17 of 25 vs. PK2 + TTX, three of five, χ^2 , $P > 0.5$; NE, PK2, 10 of 15 vs. PK2 + TTX, four of seven, χ^2 , $P > 0.5$) by PK2 were significantly different to effects observed in ACSF. In contrast to MNC neurons, the observation that responses to PK2 are maintained in the presence of TTX indicates that PK2 exerts direct effects on both PA and NE neurons.

In view of the observations that both PA and NE neurons responded to PK2 in a similar manner (similar mean magnitudes of depolarization, percentages of responders, and effects maintained in TTX), they were grouped together for further analysis. The PK2-induced depolarization of parvocellular neurons was found to be concentration-dependent, as illustrated in Fig. 4, with peak effects observed at 1 nM to 10 nM with an EC₅₀ of 2.3 pM (Fig. 4D).

In order to confirm that the PK2-induced depolarization of parvocellular neurons was receptor-mediated, experiments were performed in the presence of A1MPK1, a PK2 receptor antagonist (Bullock *et al.*, 2004). Neurons pretreated with A1MPK1 (300 s at 40 nM) showed significant differences in response magnitude (PK2, 7.6 ± 3.5 mV vs. PK2 + A1MPK1, 0.4 ± 0.8 mV, $n = 9$, ANOVA, $P < 0.01$, Fig. 5C) and proportion of neurons influenced (PK2, four of six vs. PK2 + A1MPK1, zero of nine, χ^2 , $P < 0.05$) to 1 nM PK2 administration (Fig. 5, A₁ and A₂). In three of nine (33%) neurons tested, a partial agonist effect of A1MPK1 was observed (8.4 ± 2.4 mV) prior to PK2 administration (data not shown). However, the mean change in membrane potential of all cells tested with A1MPK1 was 3.1 ± 1.9 mV. This effect was not statistically different from normal changes in ACSF (A1MPK1, 3.1 ± 1.9 mV vs. ACSF, -0.3 ± 0.6 mV, $n = 12$, unpaired *t*-test, $P > 0.1$).

Previous data showing PK2-activated p44/p42 MAPK led us to investigate the role of MAPK in the observed PK2-mediated effects in this study (Masuda *et al.*, 2002; Lin *et al.*, 2002). In order to determine if p44/p42 MAPK signalling pathways are activated by PK2 in parvocellular neurons, experiments were conducted in the presence of the MAPK inhibitor, PD 98059. PD 98059 was applied for a minimum of 500 s prior to PK2 application at a concentration of 50 μ M (Fuller *et al.*, 2001). In eight cells tested, PD 98059 abolished the 1 nM PK2-evoked depolarization (Fig. 5, B₁ and B₂) in 100% of the neurons tested (PK2, 7.6 ± 3.5 mV vs. PK2 + PD 98059, 0.4 ± 0.5 mV, $n = 8$, ANOVA, $P < 0.001$, Fig. 5C) and also showed a significant difference in the proportion of neurons influenced (PK2, four of six vs. PK2 + PD 98059, zero of eight, χ^2 , $P < 0.05$). In three of eight (38%) neurons tested, a partial agonist effect of PD 98059 was observed (5.4 ± 2.1 mV). DMSO (0.25%), the vehicle for PD 98059 had no effect on four parvocellular neurons tested (ACSF, 0.3 ± 0.5 mV vs. DMSO, -1.3 ± 1.0 mV, $n = 4$, paired *t*-test, $P > 0.05$, data not shown). These results suggest that PK2 acts directly on PVN parvocellular neurons to depolarize them in a concentration-dependent manner via an intracellular MAPK signalling mechanism.

Additional recordings were carried out during the subjective light (07:00–19:00 h) and dark (19:00–07:00 h) period in slices (prepared during subjective light period) that were known to contain the SCN (Fig. 6C), in which we examined the effects of A1MPK1 alone on the membrane potential of parvocellular neurons. During subjective light, four of six cells tested (66%) showed clear hyperpolarizations in response to A1MPK1 (A1MPK1, -4.4 ± 0.4 mV vs. ACSF, -0.3 ± 0.6 mV, unpaired *t*-test, $P < 0.001$) as illustrated in Fig. 6, A₁. In contrast, no such hyperpolarizing responses to A1MPK1 were observed in cells tested during the subjective dark period (1.8 ± 1.4 mV; $n = 10$) as illustrated in Fig. 6, A₂, effects that were not statistically different from normal spontaneous changes in membrane potential observed in ACSF ($-0.3 \pm$

0.6 mV, $n = 10$, unpaired t -test, $P > 0.1$). A partial agonist effect was still observed in three of these neurons (30%, 6.8 ± 1.9 mV) during the subjective dark period. Chi-squared tests revealed that while there was no significant difference in responses between neuronal populations of the control and dark period A1MPK1 groups ($P > 0.5$), the responses of neurons in the light period was significantly different to both the control ($P < 0.05$) and dark period ($P < 0.05$) groups (Fig. 6B). The observed hyperpolarizing effects of A1MPK1 on parvocellular neurons during subjective light suggests a PK2-ergic excitatory tonic input to these neurons may originate from PK2-ergic neurons in SCN during the subjective light period when PK2 expression in SCN is high.

Finally, depolarizing effects of 10 nM PK2 were maintained during the subjective dark period in three of five cells tested, showing a mean membrane potential change of 10.9 ± 4.2 mV (not significantly different from 10 nM PK2 tested during the subjective light period, unpaired t -test, $P > 0.5$). These observations are in accordance with data showing that the receptor mRNA expression in PVN does not change during the circadian cycle (Cheng *et al.*, 2002).

Hippocampal neurons

The depolarizing effects of PK2 on PVN neurons appear to be specific to cells in this location, as zero of eight cells recorded from the CA1 region of hippocampal slices showed no response to bath administration of PK2 (PK2, 1.4 ± 0.5 mV vs. ACSF, 1.8 ± 0.7 mV, unpaired t -test, $P > 0.5$, data not shown).

Discussion

With its expression in SCN directly tied to the normal circadian cycle with daytime levels over 50 times greater than those at night, PK2 represents a SCN messenger with the potential to relay circadian signals to important hypothalamic targets, such as the PVN. Our studies have shown that PK2 depolarizes the majority of magnocellular, neuroendocrine and preautonomic PVN neurons, suggesting a mechanism through which this peptide may exert circadian control over a diverse array of autonomic and neuroendocrine systems. Furthermore, we have utilized the electrophysiological fingerprints of these subpopulations of neurons within PVN (see Materials and methods) to identify separate mechanisms of PK2 action on magnocellular (indirect effects that appear to be the result of activation of glutamate neurons within our slice preparation), and parvocellular (direct, MAPK mediated action) neurons suggesting the possibility of potentially complex and diverse interactions between PK2-ergic neurons originating in the SCN and their efferent targets in the PVN.

Magnocellular neurons

The observed influence of PK2 on both oxytocin and vasopressin secreting MNC neurons may at least partially explain the circadian secretion from the posterior pituitary and the circadian variation of these hormones in the circulation (Windle *et al.*, 1992; Hermes & Renaud, 1993). We demonstrated that PK2 exerts indirect effects on MNCs, which is in accordance with anatomical literature suggesting a lack of direct projections from the SCN to this specific cell population (Watts *et al.*, 1987; Buijs *et al.*, 1994).

The additional observation that PK2 induced increases in EPSP and EPSC frequency in MNCs and exhibited kynurenic acid-sensitive effects, argues for the involvement of glutamate interneurons within our slice preparation in mediating these effects. Such integrative roles for glutamate interneurons in the PVN have previously been demonstrated to mediate effects of noradrenaline (Daftary *et al.*, 1998), orexin (Follwell & Ferguson 2002), and angiotensin II on MNCs. The fact that EPSC amplitude was not changed by PK2, while depolarizing effects were blocked by TTX, suggests that the peptide acts at glutamate neuron cell bodies. It could

also be argued that PK2 would thus increase numbers of action potential induced EPSCs, which would thus result in a greater proportion of larger events; effects which were not observed in our recordings. This suggests that the majority of events counted in our experiments were in fact action potential-induced PSCs, although effects of PK2 on presynaptic terminals cannot be totally ruled out in the absence of a complete analysis of PK2 effects on mini EPSCs recorded in TTX. Such analysis will be the subject of future focused voltage-clamp studies.

Parvocellular neurons

The parvocellular regions of PVN are usually functionally subdivided into NE cells projecting to the median eminence and PA neurons, which send efferent projections to medullary and spinal autonomic centres. Anatomical studies have shown direct SCN projections to PA PVN neurons (Larsen *et al.*, 1998; Teclemariam-Mesbah *et al.*, 1999; Buijs *et al.*, 2003). These PA neurons project to the intermediolateral column of the spinal cord (IML), and autonomic centres in the brain stem such as the nucleus tractus solitarius (NTS) and rostral ventrolateral medulla (RVLM), where preganglionic sympathetic neurons influence the cardiovascular, respiratory, and gastrointestinal systems (Schlenker *et al.*, 2001; Stern, 2004), suggesting that projections from SCN to PVN are fundamental in the modulation of daily autonomic biological patterns. In contrast to PA neurons some controversy still exists with regard to the demonstration of direct anatomical connections between SCN and NE neurons of PVN, with separate studies suggesting the presence (Vrang *et al.*, 1995) or absence (Buijs, 1996) of such connections. However, it is attractive to speculate that such projections, if they do exist, represent a mechanism through which SCN neurons control the excitability of corticotrophin releasing hormone (CRH) and other NE PVN neurons and consequently, circadian regulation of the pituitary hormones which they control.

Although we initially examined effects of PK2 on these two populations of neurons separately, the fact that similar excitatory effects were observed in both groups of cells, led us to group these cell groups together for subsequent analysis. In contrast to effects on MNCs, excitatory effects on parvocellular neurons were maintained in TTX, supporting the conclusion of direct postsynaptic effects of the peptide on these cells. Such observations also raise the interesting possibility that a proportion of these parvocellular neurons may in fact be the glutamate interneurons driving the excitatory effects on MNCs, although to date there is no direct immunocytochemical evidence to support such a possibility. We were also able to show that these excitatory effects were concentration-dependent and receptor mediated as they were abolished by pretreatment of slices with the PK2 receptor antagonist A1MPK1. As a point of interest, in some cases this antagonist showed partial agonist effects prior to PK2 administration; effects that have been reported previously (Bullock *et al.*, 2004).

Intriguingly, we have also demonstrated that in slices that specifically included SCN, and thus presumably functional SCN connections with PVN, the PK2 antagonist A1MPK1 hyperpolarized 66% of parvocellular neurons tested during the subjective light period. Combined with the finding that these effects were not maintained during the subjective dark phase, these observations suggest that during the period of high PK2 mRNA expression (Cheng *et al.*, 2002) PK2 tonically excites NE and PA PVN neurons.

Studies showing that PK2 stimulates the phosphorylation of p44/p42 MAPK in both types of G-protein-coupled receptors activated by prokineticins (Masuda *et al.*, 2002; Lin *et al.*, 2002), suggested that MAPK may play a similar signalling role in PVN neurons. Our demonstration that PK2-evoked depolarization of both PA and NE PVN neurons was abolished in the presence of the MAPK pathway inhibitor, PD 98059, supports a role for this signalling pathway in mediating PK2 effects on these neurons. The downstream events following MAPK activation have not been examined in the present study although previous studies showing MAPK modulation of potassium and L-type calcium channel currents (Fitzgerald, 2000; Yang

et al., 2001; Dolmetsch *et al.*, 2001; Yuan *et al.*, 2002), suggest these to be important areas for future study.

Physiological relevance – the SCN–PVN connection

The SCN is responsible for driving a circadian-mediated rhythm of neuroendocrine output from the PVN, which maintains normal daily metabolic function (Moore & Eichler, 1972). In the rat, CRH levels are high during the subjective light period, and decline towards the onset of the dark period in response to rises in both adrenocorticotropin (ACTH) and corticosterone (Watts *et al.*, 1987; Kwak *et al.*, 1992, 1993). This pattern appears to be approximately coupled with that of PK2, which exhibits increased expression during daytime hours (Cheng *et al.*, 2002). Our present study, demonstrating excitatory effects of PK2 on putative CRH neurons in the PVN during the subjective light period, supports a physiological role for PK2 in regulating CRH output. The excitatory cellular effects of PK2 observed during daytime hours correlate with expected hormonal release observed in the intact rat during this period (Kwak *et al.*, 1993). However, it is interesting to note that peak levels of CRH expression occur at night, along with observed increases in blood pressure and heart rate (Watts *et al.*, 1987; Zhang & Sannajust, 2000). It is possible that PK2 synthesis, followed by axonal transport from the SCN to the PVN during the day, delays peak CRH expression, such that a lag is created between the timing of secretion of the two peptides. Hence, although PK2 mRNA expression within the SCN is high during daylight hours (Cheng *et al.*, 2002), its synthesis and presence within the PVN would logically be phase locked to this mRNA expression but time delayed to later in the light cycle or even early in the dark phase. Clearly future studies will need to address this issue of the temporal relationship of PK2 mRNA expression in SCN and physiological effects in PVN. As it is peptide, rather than receptor expression that varies diurnally, the cellular effects of PK2 demonstrated in the present study likely reflect *in vivo* effects of PK2 when its expression within the PVN reaches a peak. As such, the clear effects of PK2 demonstrated in the present study argue strongly in favour of the potential physiological relevance for PK2 as a regulator of the excitability of magnocellular, neuroendocrine and preautonomic PVN neurons.

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Abbreviations

ACSF	artificial cerebrospinal fluid
AVP	vasopressin
CRH	corticotrophin releasing hormone
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
KA	kynurenic acid
MAPK	

	mitogen activated protein kinase
MNC	magnocellular
NE	neuroendocrine
PA	preautonomic
PK2	prokineticin 2
PVN	paraventricular nucleus
SCN	suprachiasmatic nucleus
TTX	tetrodotoxin

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Fig. 1. PK2 depolarizes magnocellular neurons. (A₁ and A₂) Current-clamp recordings showing depolarization of MNC neurons in response to 10 nM PK2 (bath administration indicated by the black bar), showing full reversibility to the peptide. (A₁ inset) Double-pulse protocol used to classify MNC neurons based on electrophysiological characteristics (arrow indicates delay to first spike indicating the presence of I_A). (B) MNC current-clamp recording showing the lack of PK2 evoked depolarization in the presence of TTX. (C) Mean depolarization (± SEM) of MNC neurons in the presence of ACSF + PK2 (black), and TTX + PK2 (grey), showing lack of PK2 effects in the presence of TTX (***P < 0.0001, unpaired Student's *t*-test). (D) Current clamp recording showing EPSPs (top-before peptide application, bottom-after peptide application) taken from a single MNC neuron that responded to 10 nM PK2. Note the increase in EPSP frequency after PK2 administration (*asterisk denotes events identified by Mini Analysis Program 6.0).

Fig. 2.

PK2 depolarizes magnocellular neurons as a result of activation of glutamatergic neurons.

(A₁) Continuous whole-cell voltage-clamp recording of EPSCs from a MNC neuron illustrates that bath application of 10 nM PK2 results in an increase in EPSC frequency (asterisk denotes events identified by Mini Analysis Program 6.0). (A₂) Frequency and amplitude rate histograms from the same neuron, show an increase (frequency; K-S-test, $P < 0.0001$) and lack of change (amplitude; K-S-test, $P > 0.1$) between pre- (black trace) and post- (red trace) 10 nM PK2 application. (A₃) Graphs summarize the mean ($\pm$ SEM) change in EPSC amplitude (left) and frequency (right) in ACSF alone (white) and following 10 nM PK2 administration (black, $n = 5$, $*P < 0.05$, paired t -test). (B₁) Current-clamp recording of a MNC neuron showing a lack of effect to 10 nM PK2 (black bar) in the presence of 1 mM KA (hatched bar). (B₂) Mean depolarization ($\pm$ SEM) of MNC neurons in the presence of ACSF + PK2 (black), and KA + PK2 (hatched, $***P < 0.0001$, unpaired Student's t -test).

Fig. 3.

PK2 depolarizes parvocellular neurons. (A₁) Current-clamp recording showing a depolarization of a PA PK2-responsive (10 nM) neuron with no return to baseline 20 min after peptide administration (black bar). (A₂) PA current-clamp recording showing a PK2 (10 nM) evoked depolarization in the presence of TTX. (A₃) Double-pulse protocol used to identify PA neurons based on electrophysiological characteristics (arrow indicates the presence of LTS). (A₄) Mean depolarization ($\pm$ SEM) of PA cells to PK2 in the presence of ACSF (ACSF + PK2, black) and TTX (TTX + PK2, grey). (B₁) Current-clamp recording showing a depolarization of a NE neuron to 10 nM PK2 (black bar) with partial recovery. (B₂) NE current-clamp recording showing a PK2 (10 nM) evoked depolarization in the presence of TTX. (B₃) Double-pulse protocol used to identify NE neurons based on electrophysiological characteristics (arrow indicates lack of I_A and LTS). (B₄) Mean PK2-mediated depolarization ($\pm$ SEM) of NE neurons in the presence of ACSF (ACSF + PK2, black) and TTX (TTX + PK2, grey).

Fig. 4.

PK2-induced depolarization of parvocellular neurons is concentration-related. (A) Current-clamp recording showing a long-duration depolarization in response to bath application of 100 nM PK2 (black bar) followed by a return to baseline membrane potential. (B) Current-clamp recording showing a depolarization (similar in magnitude to A, but with a shorter duration of response time) in response to bath application of 1 nM PK2 (black bar). (C) Current-clamp recording showing a depolarization [decreased in magnitude to A and B in response to bath application of 10 pM PK2 (black bar)]. (D) Mean depolarization ($\pm$ SEM) in response to increasing concentrations of PK2. Data were fit to a sigmoid concentration–response function.

Fig. 5.

PK2 depolarizes parvocellular neurons through PK2-receptor mediated activation of MAPK. (A₁ and A₂) Current-clamp recordings from two parvocellular neurons showing the abolishment of PK2 effects (black bar) in the presence of 40 nM A1MPK1 (grey bar). (B₁ and B₂) Current-clamp recordings from two parvocellular neurons showing the abolishment of PK2 effects (black bar) in the presence of 50 μM PD 98059 (hatched bar). (C) Mean depolarization (± SEM) of PA and NE cells in the presence of ACSF + PK2 (black), A1MPK1 + PK2 (grey) and PD 98059 + PK2 (hatched, *P < 0.01, **P < 0.001, ANOVA followed by Neuman–Keuls multiple comparison test).

Fig. 6. Endogenous SCN-derived PK2 hyperpolarizes parvocellular neurons. (A₁) Current-clamp recording showing the hyperpolarization of a PVN parvocellular neuron tested during the subjective light phase in response to A1MPK1 (grey bar) application. (A₂) Current-clamp recording of a PVN parvocellular neuron tested with A1MPK1 (grey bar) during the subjective dark phase. (B) Summary data of response types (non-responding-white; depolarizing-grey; hyperpolarizing-black) in parvocellular neurons tested with A1MPK1 under different circadian conditions. (C) Schematic shows location of the SCN relative to the PVN within the intact slice.