Orexin-stimulated MAP kinase cascades are activated through multiple G-protein signalling pathways in human H295R adrenocortical cells: diverse roles for orexins A and B

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Abstract

Orexins A and B (ORA and ORB) are neuropeptide hormones found throughout the central nervous system and periphery. They are required for a host of physiological processes including mitogen-activated protein kinase (MAPK) regulation, steroidogenesis, appetite control and energy regulation. While some signalling mechanisms have been proposed for individual recombinant orexin receptors in generic mammalian cell types, it is clear that the peripheral effects of orexin are spatially and temporally complex. This study dissects the different G-protein signalling and MAPK pathways activated in a pluripotent human adrenal H295R cell line capable of all the physiological steps involved in steroidogenesis. Both extracellular receptor kinase 1/2 (ERK1/2) and p38 were phosphorylated rapidly with a subsequent decline, in a time- and dose-dependent manner, in response to both ORA and ORB. Conversely, there was little or no direct activation of the ERK5 or JNK pathway. Analysis using signalling and MAPK inhibitors as well as receptor-specific antagonists determined the precise mediators of the orexin response in these cells. Both ERK1/2 and p38 activation were predominantly Gq- and to a lesser extent Gs-mediated; p38 activation even had a small Gi-component. Effects were broadly comparable for both orexin sub-types ORA and ORB and although most of the effects were transmitted through the orexin receptor-1 subtype, we did observe a role for orexin receptor-2-mediated activation of both ERK1/2 and p38. Cortisol secretion also differed in response to ORA and ORB. These data suggest multiple roles for orexin-mediated MAPK activation in an adrenal cell-line, this complexity may help to explain the diverse biological actions of orexins with wide-ranging consequences for our understanding of the mechanisms initiated by these steroidogenic molecules.


Introduction

Orexins A and B (ORA and ORB) are two hypothalamic peptides that originate from the post-translational proteolytic cleavage of a common precursor, prepro-orexin. They articulate their signalling cascades via two G-protein coupled receptors (GPCRs), orexin receptor-1 (OX1R) and orexin receptor-2 (OX2R; Sakurai et al. 1998) and are involved in many physiological processes including feeding, mitogen-activated protein kinase (MAPK) regulation and reproductive behaviour, adrenal function and energy homeostasis (Flier & Maratos-Flier 1998, Lubkin & Stricker-Krongrad 1998, Muschamp et al. 2007).

The intracellular signalling pathways mediating the effects of orexins have been under intense investigation (Malendowicz et al. 1999, Mazzocchi et al. 2001a,b, Randeva et al. 2001). Expression of both orexin receptor subtypes has been shown in both the human and rodent adrenal gland (Sakurai et al. 1998, Johren et al. 2001, Randeva et al. 2001, Spinazzi et al. 2005a). These receptors couple to multiple G-proteins and activate intracellular signalling pathways within the adrenal gland (Karteris et al. 2001, 2005, Malendowicz et al. 2001, Randeva et al. 2001) leading to the production of both cAMP and inositol trisphosphate (IP3) in human adrenal cells (Randeva et al. 2001). The physiological effects of these different pathways are unknown.

Recent studies have shown involvement of individual signalling pathways by OX1R-induced MAPK activation, including extracellular receptor kinase 1/2 (ERK1/2) in CHO cells over-expressing OX1R (Ammoun et al. 2006a). Furthermore, roles for intracellular signalling molecules including cAMP (up- and down-regulation) and IP3/Ca2+ were also observed in CHO cells (Holmqvist et al. 2005) and Odora cells (Gorojankina et al. 2007). It has been suggested...
that ORA-stimulated OX1R signalling through the ERK1/2 and p38 MAPK pathways in CHO cells, which mediates cellular processes such as cell growth and apoptosis (Ammoun et al. 2006b). Orexins also caused caspase-dependent cell death by apoptosis through activation of OX2R in CHO cells (Voisin et al. 2006). The involvement of OX2R-mediated ERK1/2 and p38 MAPK pathways in biological responses has also been reported in rat adrenocortical cells (Spinazzi et al. 2005b). The localisation of the orexin proteins is widespread, but there are distinct differences between the two orexin subtypes and their receptors. While ORA is equipotent for OX1R and OX2R, ORB has an ∼10-fold higher affinity for the OX2 receptor subtype (Ammoun et al. 2003). Additionally, the two receptor types appear to have differentially regulated β-arrestin-binding and internalisation profiles (Pfleger et al. 2006). We have previously described differential effects of ORA and ORB, as well as a clear Gq-independent p38 activation profile in contrast to Gq-mediated ERK1/2 activation on a stable OX2R transfection in HEK293 cells (Tang et al. 2008).

It is becoming apparent that the complexity of orexin physiology and pharmacology is reflected in the complex downstream MAPK cascades being activated and that the MAPK profile is crucial for many cellular processes including cell growth, differentiation, gene regulation and steroidogenesis (Werry et al. 2005, Otis & Gallo-Payet 2007). We have recently demonstrated the pathways required for orexin-mediated up-regulation of the steroidogenically acute regulatory (StAR) protein, a key requirement mediating the metabolism of cholesterol (Ramanjaneya et al. 2008).

Despite the important steroidogenic action of orexins, the intracellular signalling mechanisms by which OXRs activate MAPK cascades in human adrenal cells are not well understood. Therefore, the aim of this study was to elucidate the mechanisms of OXRx-activation by ORA and ORB using the immortalised adrenal cell line H295R. H295R cells act as pluripotent adrenocortical cells capable of being directed to produce each of the zone-specific steroids derived from cholesterol metabolism (Rainey et al. 1994). We assessed the orexin-mediated activation of the terminal serine/threonine (MAP) kinases of the four major MAPK/MAPKK pathways, namely ERK1/2, p38, ERK5 and JNK (Roberts & Der 2007). We also compared the relative mRNA transcription with protein expression of the two orexin receptors OX1R and OX2R. Our studies demonstrate that multiple G-protein signalling pathways are involved in the activation of ERK1/2 and p38 MAPK by ORA and ORB in H295R cells and we have examined further the precise pathway components using a combination of selective inhibitors of adenylyl cyclase (AC), protein kinase A (PKA), protein kinase C (PKC), MAPK kinase (MEK) and OX1R-specific antagonist SB334867. In this study, we demonstrate a general Gq-mediated requirement, a Gi-element for p38 activation and an interesting Gq-mediated p38 activation, which appears to be orexin-specific. These data also show clear receptor-specificity, particularly involved in the OX1R-activation of p38 through ORB. These findings provide a novel analysis of the signalling cascades and subsequent cortisol secretion mediated through ORA and ORB and their receptors, with important implications towards our understanding of orexin signalling in steroidogenically active human cells.

Materials and Methods

Biochemical reagents

Human ORA and ORB were obtained from Phoenix Pharmaceuticals (Belmont, CA, USA). Insulin-transferring-selenium (ITS) and Ultraserum-G from PALL Life Sciences (Cergy, France), and growth supplement for H295R cells was obtained from Sigma–Aldrich Company Ltd. Accutase required for cell passage was obtained from TCS cell works (Bucks, UK), ECL plus western blotting detection reagents from Amersham Biosciences. Agarose was obtained from MBI Fermentas (York, UK), Helena Biosciences (Gateshead, UK).

Inhibitors

The following inhibitors were obtained from Calbiochem (Darmstadt, Germany). 2',5'-Dideoxyadenosine (DDA) is a potent and specific inhibitor of adenylyl cyclase, the enzyme catalysing cAMP production; Myristoylated PKA inhibitor amide 14–22 (MIP), a selective inhibitor of PKA; 2-[1-[2-(1-methylpyrrolidino)ethyl]-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide (Bis), selective inhibitor of PKC (PKCi); 1,4-diamino-2,3-dicyano-1,4-Bis (2-aminophenylthio)butadiene (U0126), a potent and specific inhibitor of MEK1 and MEK2; pertussis toxin (PTX), an enzyme derived from the bacterium Bordetella pertussis, blocks the action of Gi family G-proteins. Concentrations used in this study are described in the figure legends.

Antibodies

Goat polyclonal anti-OX1R antibody (Santa Cruz, UK), Mouse monoclonal anti-OX2R antibody, anti-β-actin antibody were purchased from Abcam (Cambridge, UK). Polyclonal HRP-conjugated goat anti-rabbit and anti-mouse IgG/HRP were from Dakocytomation (Glostrup, Denmark).

Other materials

Precision Plus Protein Standard was from Bio-Rad Laboratories Ltd. The mammalian expression vector pcDNA3.1(+) was from Invitrogen. Polyvinylidene difluoride (PVDF) membrane was purchased from Amersham Biosciences. All the primers were obtained from TAGN (Gateshead, UK).

Cell culture

H295R human adrenocortical cells (NCI-H295 cells, originally cultured from a human adrenocortical tumour in

1980, continued to express the three major pathways of adrenal steroidogenesis 10 years later (Gazdar et al. 1990). NCI-H295 cells, obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in complete media containing DMEM/F12 (1:1) supplemented with 2% Ultroser G (Biosepra, Villeneuve-la-Garenne, France) and ITS (Discovery Labware, Bedford, MA, USA) in 6-well plates for 24 h after reaching confluence. Following 24 h serum-starvation, media was replaced with 3 ml fresh media containing different agents and cultured for 4 h. At the end of the incubation period, cells were washed with ice-cold PBS and subjected to protein extraction and analysis as described below.

**Western blotting**

Protein lysates were prepared by adding equal amounts of Laemmli buffer to each well, and samples were denatured by sonication and boiling. Samples were separated by SDS-PAGE (10% resolving gel) and transferred to PVDF membranes at 100 V for 1 h in a transfer buffer containing 20 mM Tris, 150 mM glycine and 20% methanol. The PVDF membranes were incubated with primary antibody for phospho-ERK1/2 or phospho-p38 or phospho-ERK5 or phospho-JNK (Cell Signalling Technology) raised in rabbit at a 1:1000 dilution in tris-buffered saline (TBS)-0.1% Tween (TBST) and 5% BSA overnight at 4°C. The membranes were washed and incubated with a secondary anti-rabbit HRP-conjugated antibody (1:2000) for 1 h at room temperature, and washed for 1 h with TBST. Antibody complexes were visualised using ECL Plus, chemiluminescence detection kit. The densities were measured using a scanning densitometer coupled to Scion Image scanning software (Scion Corporation, Frederick, MD, USA). Membranes were stripped and reprobed for total ERK1/2 or p38 or ERK5 or JNK with antibody raised in rabbit at 1:1000 dilution in TBS TBST 5% BSA overnight at 4°C. Antibody complexes were visualised and measured as above.

**Using pathway inhibitors/receptor antagonists to compare ERK1/2 and p38 activation**

AC inhibitor (DDA), PKA-inhibitor (MIP), PKC-inhibitor (Bis), Gs-inhibitor (Ptx), MEK/ERK1/2 inhibitor (U0126) and p38 inhibitor (SB203580), as well as the OX1R antagonist SB334867, were added to 3 ml fresh media and added to confluent, cultured cells for 4 h. Inhibitors were added at least 30 min before the other reagents and remained during the incubation period. Cells were then processed as above.

**Cortisol secretion assay**

Cells were cultured in 6-well plates. Forty-eight hours before the experiment, cells were starved overnight in serum-free media. Cells were then washed and incubated in fresh serum-free media with different concentrations of ORA or ORB for both 4 and 24 h. After each time point, the supernatant was removed and frozen immediately in liquid nitrogen. Cortisol content of the medium was determined from three different experiments using a commercial RIA kit (Immunotech, Marseille, France) according to the manufacturer’s instructions and normalised to the basal content (considered as 100%). Each column is calculated and presented as percentage change over basal.

**Calcium measurements**

NCI H295R cells were grown to confluence and plated in 96-well black-walled tissue culture plates 24 h prior to the experiment. Relative levels of intracellular Ca²⁺ were measured

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<th>Table 1 List of primers used in this study</th>
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Figure 1  Time-dependent effects of orexins on MAPK signalling modulators ERK1/2, p38, ERK5 and JNK in H295R cells. Western blot analysis of protein extracts from H295R cells for MAPK protein expression at different time points. Cells were incubated with serum-free medium containing 100 nM orexin A or orexin B (as specified), a positive control (100 nM angiotensin) or control without stimulus for specified time points, washed with ice cold PBS, and cells lysed using sample buffer. The western blots shown in (a) and (b) used an antibody against phosphorylated and total ERK1/2 which recognised bands with apparent molecular weights of 42 kDa for ERK1 and 44 kDa for ERK2. The corresponding antibodies for p38, ERK5 and JNK detected bands at ~38 kDa (graphs c and d), 115 kDa (graphs e and f) and 50 kDa (graphs g and h) respectively. Densitometric analysis of the ratio of phosphorylated to total protein was calculated using Scion Image software. Data points are means ± S.E.M. from three independent experiment, and significance is calculated as fold increase over basal (*P<0.05, **P<0.01, ***P<0.001).
using the Fluo-4 NW calcium assay kit (Molecular Probes, Invitrogen) according to the manufacturer’s protocol. Cells were loaded with fluo-4 AM in the presence of 2.5 mM probenicid in a commercial phenol-free, magnesium-free Hank’s balanced salt solution (Invitrogen). ORA and ORB were automatically injected at various concentrations and fluorescence was measured using a Fluostar Optima fluorescence plate reader (BMG Labtech, Offenburg, Germany, excitation at 485 nm and emission at 520 nm) at room temperature.

**RT-PCR measurements of gene expression**

Total RNA was extracted using the Qiagen RNeasy Mini Kit and reverse-transcribed into cDNA as previously described (Randeva et al. 2001). Steroidogenic gene expression was measured by RT-PCR, using 3 μg RNA and random primers as reverse transcription primers. A control reaction that omitted reverse transcriptase was included to check for the presence of genomic DNA. Steroidogenic gene expressions were amplified using a Hybaid Thermal Cycler in 50 μl reaction medium containing 1 unit Taq polymerase (Fermentes, York, UK), 20 pmol of each sense and anti-sense primer and dNTP (10 mmol/l each), using the following cycling conditions: 94 °C for 1 min; then 38 cycles of 94 °C for 60 s, 60 °C for 45 s and 72 °C for 30 s, followed by a 10 min extension at 72 °C. The sequences for the sense and anti-sense primers are summarised in Table 1. PCR products were stained with ethidium bromide and visualised by electrophoresis through 2% agarose gels. Sequencing of the PCR products confirmed the sequence identities. OX1R and OX2R gene expressions were also measured in a similar way.

**Statistical analysis**

Non-parametric tests were used for statistical analysis. All the data in the present study are expressed as mean ± S.E.M. unless otherwise indicated. Data involving more than two groups were assessed by Friedman’s ANOVA followed by post hoc Tukey’s test. For western immunoblotting experiments, the densities were measured using a scanning densitometer coupled to scanning software Scion Image (Scion). Cortisol significance was determined by ANOVA and Student’s t-test against control cells (*P < 0.05). All statistical analyses were performed using GraphPad (San Diego, CA, USA) software (version 4.0) and P value < 0.05 was considered to be significant in each case.

**Results**

**ORA and ORB stimulated activation profiles of the four major classes of signalling MAP kinase, ERK1/2, P38, ERK5 and JNK in an adrenal cell-line (H295R)**

The downstream effects of ORA and ORB are known to be mediated by two GPCRs (OX1R and OX2R), which can
Figure 3  Effect of G-protein coupled pathway and MAPK inhibitors on orexin-regulated ERK protein activation in H295R cells. Cells were cultured as described in the methods. Cell lysates were analysed by immunoblotting for phospho-ERK1/2 protein with total ERK1/2 and used as a loading internal control. Graph (a) represents 100 nM ORA-stimulated cells comparing the inhibition of adenylate cyclase (using 50 μM DDA), MEK1,2/ERK1/2 (using 10 μM U0126) and PKA (using 1 μM MIP); (b) 100 nM ORA-stimulated cells comparing the inhibition of PKC (1 μM Bis) and Gi-mediated signalling (200 ng/ml PTX). Graphs (c) and (d) are the equivalent experiments for 100 nM ORB-stimulated cells. A positive control (100 nM angiotensin) was also included where indicated on the graphs. Quantitative analysis of phospho-ERK1/2 in reference to total-ERK1/2 was performed using Scion Image scanning densitometry. Relative density ratios were calculated using the inhibitor alone treated group value or control group value as one. The data represent the mean ± S.E.M. of three measurements from three independent experiments. **P<0.01 and ***P<0.001, orexin treatment compared with basal and #P<0.05 and ###P<0.001, orexin compared with inhibitor plus orexin.
activate several members of the G-protein machinery (including Gq, Gi and Gs) and subsequently regulate MAPK cascades (Sakurai et al. 1998, Karteris et al. 2001, Karteris & Randeva 2003).

We initially used a western blot analysis to test whether the two orexin peptides signalled through different downstream MAPK elements in the immortalised adrenal cell line, H295R. The time-dependent effects of 100 nM ORA and

![Graphs showing western blot analysis results](attachment:image.png)

**Figure 4** Effect of G-protein coupled pathway and MAPK inhibitors on orexin-regulated p38 protein activation in H295R cells. Cells were cultured as described in the methods. Cell lysates were analyzed by immunoblotting for phospho-p38 protein with total p38 used as the loading internal control. Graph (a) represents 100 nM ORA-stimulated cells comparing the inhibition of adenylate cyclase (using 50 μM DDA), PKA (using 1 μM MIP), PKC (1 μM Bis) and MEK1,2/ERK1/2 (using 10 μM U0126); (b) Inhibition of Gi-mediated signalling (using 200 ng/ml PTX) for both 100 nM ORA and ORB stimulation; and (c) ORB-stimulated cells comparing the inhibition of adenylate cyclase (using 50 μM DDA), PKA (using 1 μM MIP), PKC (1 μM Bis) and MEK1,2/ERK1/2 (using 10 μM U0126). A positive control (100 nM angiotensin) was also included where indicated on the graphs. Quantitative analysis of phospho-p38 in reference to total-p38 was performed using Scion Image scanning densitometry. Relative density ratios were calculated using the inhibitor alone treated group value or control group value. The data represent the mean ± S.E.M. of three measurements from three independent experiments. **P<0.01 and ***P<0.001 represents the fold increase over controls compared to orexin and ###P<0.001 represent orexin compared with inhibitor plus orexin.
ORB treatment on the activation of ERK1/2, p38, ERK5 and JNK are shown in Fig. 1. Both ORA and ORB had a significant ($P<0.001$), rapid elevation of ERK1/2 and p38 in the range of approximately from 3.5 to 7-fold increase over basal levels (Fig. 1a–d). Over an hour, the initial 5 min spike then receded. This decline reached the basal levels for ERK1/2, but remained significantly above basal for p38 activation (1.68-fold ±0.19; $P<0.05$ and 2.02-fold ±0.29; $P<0.01$ for ORA and ORB respectively). ERK5 activation also significantly increased to a maximum of 3.34-fold ±0.38 ($P<0.001$) for ORA and 2.05-fold ±0.10 ($P<0.05$) for ORB, however, this was a more gradual incline and the fold-increase was not as pronounced as for ERK1/2 or p38 (Fig. 1e and f). We observed no increase in JNK activation in response to either orexin peptide (Fig. 1g and h). The immediate, significant effects of orexin-stimulated activation of ERK1/2 and p38 suggest a likely direct physiological role for these MAPK elements.

The dose-dependent effects of ORA and ORB stimulation on ERK1/2 and p38 activation are shown in Fig. 2. Significant stimulation was seen at 10 and 100 nM ($P<0.001$), concomitant with the reported affinity for these peptides at their receptors. Interestingly, 1 nM activation of both ERK1/2 and p38 was only significant ($P<0.05$) for ORA (Fig. 2a and c). ORA and ORB have different binding profiles to the two orexin receptors and ERK1/2 has previously been shown to be activated by several different secondary messenger systems employed by the orexin receptors (Werry et al. 2005). This prompted us to consider that there may be multiple activators of MAPK, possibly drawing on different subtypes of the heterotrimeric G-protein complex released following orexin interaction with their GPCRs.

Effect of pathway inhibitors of AC (DDA), PKA (MIP), PKC (Bis), $G_i$ (Ptx), MEK/ERK1/2 (U0126) and p38 (SB203580) on ERK1/2 and p38 activation in adrenal H295R cells stimulated with ORA and ORB

As all three G-protein families have been implicated in MAPK activation in this study. We assessed the levels of ERK1/2 and p38 activation in the presence of a comprehensive series of pathway inhibitors.

PKC-inhibition reduced ERK1/2 activation to basal levels with either orexin peptide ORA or ORB (Fig. 3b and d). p38 activation was also reduced by PKC-inhibition to basal levels with both orexin peptides (Fig. 4a and c) suggesting a fundamental importance for the $G_i$-mediated signalling pathway in both MAPK cascades. By contrast, the Ptx-treatment for inhibition of $G_i$-signalling did not have any significant effect on ORA- or ORB-activation of ERK1/2 (Fig. 3b and d), whereas there was a complete reduction in activated p38 from a maximum activation of 3.1-fold ±0.25 over basal down to 1.1-fold ±0.08 for ORA and a significant reduction ($P<0.01$) from 2.9-fold ±0.17 over basal down to 2.3-fold ±0.11 for ORB (Fig. 4b). The inhibition of $G_i$-signalling on ERK1/2 and p38 activation was surprising.

PKA-inhibition had a significant ($P<0.05$), but not full, deleterious effect on ERK1/2 activation from 6.5-fold ±0.61 over basal down to 4.5-fold ±0.22 for ORA and from 5.4-fold ±0.51 over basal down to 4.9-fold ±0.41 for ORB (Fig. 3a and c). However, p38 activation displayed orexin-specific effects of PKA-inhibition, with no effect using ORA and a complete inhibition with ORB (Fig. 4a and c).

These controversial observations were supported using the AC-specific inhibitor (DDA). Again, ERK1/2 activation was significantly reduced ($P<0.01$) from 5.7-fold ±0.94 over basal down to 4.1-fold ±0.39 for ORA but the reduction did not reach significance for ORB (from 7.1-fold ±0.67 over basal down to 4.7-fold ±0.56; Fig. 3a and c). AC inhibition on p38 activation mirrored the orexin-specific contrast seen for PKA-inhibition, with no effect using ORA and a complete inhibition with ORB (Fig. 4a and c). Predictably, the ERK1/2-specific inhibitor (U0126) showed consistent discrimination between ERK1/2 activation (which was completely blocked; Fig. 3a and c) and p38 activation (which was unaffected; Fig. 4a and c).

It is possible that the effects of MAPK activation seen here are discriminating between the ORA and ORB peptides, or theOX1R and OX2R receptors. Evidence for dominant effects of OX1R in the adrenal cells has been observed (Ziolkowska et al. 2005), but mRNA has been shown for both OX1R and OX2R in these cells (Ramanjaneya et al. 2008).

Figure 5 Orexin-mediated intracellular calcium levels. NCI H295R cells were cultured as described in the methods. (a) Stimulation with 100 nM orexins A and B (as indicated) increased intracellular Ca$^{2+}$ levels as measured by Fluo-4 fluorescence. (b) Calcium signalling data compared using the area under the curve for dose comparison and subsequent estimation of EC50 values.
Calcium signalling data

The particular relevance of the Gq pathway observed above was assessed further using intracellular calcium fluorescence. Both ORA and ORB showed a clear, relatively low potency calcium response (Fig. 5), with approximate pEC50 values of \(-6.10\) and \(-6.65\) respectively. ORB was approximately threefold more potent than ORA.

Western blot analysis of orexin receptor expression in H295R cells

Western analysis showed clear expression of both OX1R and OX2R in the adrenal cell-line H295R (Fig. 6), which confirms that seen in the primary human and rat adrenal cortex (Randeva et al. 2001, Johren et al. 2006) as well as the mRNA levels observed previously by our group using RT-PCR and illustrated in Fig. 6.

Dose-dependent effects of the OX1R-specific antagonist SB334867 on ORA and ORB-mediated activation of ERK1/2 and p38

SB334867 is a high-affinity OX1R-specific non-peptide antagonist, effectively blocking the interaction between OX1R and either orexin peptide (ORA and ORB; Smart et al. 2001). Figure 7 demonstrates that in the presence of SB334867, there was a dose-dependent reduction in both ORA- and ORB-stimulated activation of ERK1/2 and p38. Inhibition of OX1R on ERK1/2 activation was consistent for both ORA and ORB but p38 activation through ORB was almost entirely channelled through the OX1R.

Orexin-mediated cortisol secretion of H295R cells

A RIA was used to measure secreted cortisol from the adrenal H295R cells following dose-dependent stimulation with orexins (according to the manufacturer’s instructions; Immunotech). The intra-assay variability was \(\sim 5.6\%\).

Agonist stimulation resulted in a significant increase over basal for ORA at 1 and 100 nM after 24 h (126.7 ± 4.5% and 128.6 ± 7.3% respectively). Only 100 nM ORA stimulation resulted in significant cortisol secretion after 4 h (136.9 ± 8.4%). The ORB-stimulated cortisol increase did not reach significance (Fig. 8).

RT-PCR to assess the effect of orexin-treatment on the gene expression of key steroidogenic molecules

ORA and ORB-mediated up-regulation of key steroidogenic gene-expression is shown in Fig. 9. While four genes showed a significant up-regulation, only \(\beta\)HSD was up-regulated specifically by ORA (and only at 4 h). Additionally, CYP11A was specifically and highly up-regulated by ORB (at both 4 and 24 h).

Discussion

In the present study, we show that key mediators from two of the four major MAPK/MAPKK/MAPKKK signalling cascades are activated by both ORA and ORB in the pluripotent, steroidogenic adrenal H295R cells. Both ERK1/2 and p38 activation was rapid (reaching significance after 5 min), with a subsequent decline from 10 min reaching basal levels for ERK1/2 after an hour. This is broadly consistent with the profiles observed for CHO cells over-expressing recombinant OX1R and OX2R; however, the precise time-lag for MAPK responses are not identical (Sakurai et al. 1998, Ammoun et al. 2006a,b).

It may be relevant that p38 activation did not reduce to basal levels following the 5 min spike, although it is not easy to confirm any physiological consequences. It was proposed that this may be reflective of a long-term activation/p38-link to apoptosis, whereas ERK1/2 is thought to be cell-death protective (Ammoun et al. 2006b). Alternatively, every ERK-signal may well be unique, depending on the receptors involved, the ligands used and the cellular background (Werry et al. 2005).
In contrast to the ERK1/2 and p38 activation profiles, orexin-mediated ERK5 activation showed a small, gradual increase, reflecting a less direct activation, possibly as a result of de novo gene transcription. The western blot suggests that this is not out of the question and orexins are known to initiate transcription of some genes including StAR (Sasson et al. 2006), but the ERK5 effect did begin relatively quickly. This is the first description of ERK5 activation by orexins. As ERK5 is known to regulate cell proliferation in part through the direct activation of serum and glucocorticoid-inducible kinase (SKG; Hayashi et al. 2001), it would be interesting to analyse the possible expression or activation effects of orexin on ERK5 activation as part of a further study.

The activation of both ERK1/2 and p38 was significant at a concentration of 1 nM ORA, but not at this relatively low concentration for ORB. If this observation is indicative of a genuine ORA-bias, it is possibly due to the involvement of OX1R over OX2R in MAPK activation. Clearly, even 1 nM is much higher than physiological concentration, but it is highly likely that the dose-dependent effects observed here are representative of lower concentrations.

The multiple signalling mechanisms previously observed following orexin-treatment in the adrenal gland and cell lines (Karteris & Randeva 2003, Holmqvist et al. 2005) extended to the direct activation of the ERK1/2 signalling cascade. The use of direct protein inhibitors of the G-protein signalling machinery was mostly consistent with these data and also presented a multiple pathway activation of the p38 cascade. These data show that the predominant activation mechanism for both ERK1/2 and p38 involves PKC (likely Gq-mediated) signalling. Gi-mediated signalling was only involved in p38 activation (and was clearly more relevant for ORA than ORB). The Gs/PKA involvement was both orexin- and MAPK-dependent. ERK1/2 was partially activated through adenylate cyclase (cAMP) following stimulation by both ORA and ORB but this was only PKA-dependent for ORA. More surprisingly, p38 activation was strongly activated by both ORA and ORB.
through adenylate cyclase (cAMP) and PKA-dependent for ORB but not at all for ORA.

This Gs-effect was interesting as AC was previously linked with OX1R activation (Holmqvist et al. 2005), whereas in the recombinant expression system from (Ammoun et al. 2006a). ERK-activation of OX1R was cAMP-independent. This seems to reflect, either a predominantly OX2R-effect or a cell-specific role for AC signalling in adrenal cells. Although our experiments demonstrated both receptor sub-types are clearly present in the H295R cell-line, the SB334867 antagonist studies showed a predominant, but not complete requirement for OX1R in the activation of ERK1/2 and p38. It is notable that p38 activation through ORB was almost entirely channelled through the OX1R (which was also responsible for all Gs-mediated p38 activation). This is interesting as the OX2R is known to have a clear PKA- and PKC/Gq-independent p38 activation through these G-proteins when overexpressed in HEK293 cells (Tang et al. 2008). It would be interesting to assess whether there are direct physiological reasons for these differences.

It was previously suggested that specific glucocorticoid secretion in response to orexin in adrenal cells was restricted to PKA-dependent pathways acting exclusively through OX1R (Ziolkowska et al. 2005). In our human adrenal cell line, the effects on MAPK activation of ORA and ORB were roughly comparable. This conflicts slightly with what is seen in rat adrenal cells whereby ORA increased the proliferation rate (PR) of cultured cells, while ORB lowered it and the OX1R (proliferative) and OX2-R (anti-proliferative) growth effects involved in the activation of the MAPK p42/p44 and p38 signalling cascades respectively (Spinazzi et al. 2005b). Clearly, the varied biological effects of orexin may be controlled in an orexin-specific, an orexin-receptor specific or indeed a signalling-specific manner. While orexins have been shown to induce apoptosis in a transfected CHO cell line transfected with OX1R (Ammoun et al. 2006b). We have recently published data confirming no significant effect on cell proliferation of H295R cells following orexin treatment (Ramanjane et al. 2008).

Our previous paper also confirmed a significant up-regulation of the StAR protein in response to both ORA and ORB in the same H295R cell line used in this study.
(Ramanjaneya et al. 2008). Both ERK1/2 and p38 inhibition blocked StAR expression. In this study, we have measured a direct functional outcome of steroidogenesis and cortisol secretion. Cortisol is known to increase in primary adrenal cells and \textit{ex vivo} tissue in response to ORA (Spinazzi et al. 2005a). In our H295R cells, this occurred significantly for ORA but not ORB, consistent with the primary cell and \textit{ex vivo} data. It is known that pre-synaptic receptors for orexins in the rat and mouse brain facilitate the release of glutamate and gamma-aminobutyric acid; probably directly activating the vesicle release machinery or acting via a transduction mechanism upstream of the release process (Schlicker & Kathmann 2008). Furthermore, ORA and ORB have recently been shown to have different secretion patterns for glutamate release (Borgland et al. 2008). While this is not directly linked to our experiments, it may reflect different secretion mechanisms, explaining our observed differences in ORA and ORB mediated cortisol release in H295R cells. Furthermore, in this study, we describe different effects of ORA versus ORB treatment on the expression levels of CYP11A and 3\betaHSD, key molecules directly required for steroidogenesis. The calcium signalling data showing an increased potency for ORB over ORA may reflect a non-steroidogenic role for the orexin system in these cells. It is notable that 100 nM orexin treatments did not provide a maximum calcium response. The effects of PKC inhibition at 100 nM orexin treatments on MAPK activation may be due to the comparative experiments used to measure calcium signalling compared with MAPK activation. Alternatively, this could reflect calcium-independent MAPK activation through PKC (either directly or via trans-activation). This has been observed in several previous studies including AngII-stimulation of PKC-mediated ERK1/2 in hepatic C9 cells (Shah & Catt 2002) and the PKC-mediated oxidative stress response of ERK in several cell lines (Hu et al. 2007).

It is important to acknowledge that these data were obtained through analysis of an immortalised adrenocortical carcinoma cell line as a model in our study and not as a direct assessment of the adrenal gland itself. While this is considered as an excellent pluripotent cell line, it is not truly ACTH-sensitive as would be expected for an adrenal gland and we have tried to be cautious in our subsequent physiological interpretation. It would be very useful to compare the signalling responses of orexins in \textit{ex vivo} adrenal tissue samples.

It is clear that the orexin-mediated activation of p38 and ERK1/2 is regulated by at least two MAPK cascades and these appear to be activated by multiple G-protein signalling pathways in H295R adrenal cells. This is a fundamentally important addition to our understanding of the very complex tissue-specific effects of orexins and their receptors. Natural evolution of pathways almost never tolerate inefficiency and redundancy, therefore it will be the next step to determine the precise reasons for such heterogeneity of signal leading to MAPK activation following orexin stimulation.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

The authors are grateful to the Coventry General Charities, who supported this work.

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Received in final form 7 April 2009
Accepted 21 May 2009
Made available online as an Accepted Preprint 21 May 2009