Expression of molecular equivalent of hypothalamic–pituitary–adrenal axis in adult retinal pigment epithelium

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Abstract

We have investigated expression of molecular elements of the hypothalamic–pituitary–adrenal (HPA) axis in the human retinal pigment epithelium (RPE) cells. The presence of corticotropin-releasing factor (CRF); urocortins I, II and III; CRF receptor type 1 (CRFR1); POMC and prohormone convertases 1 and 2 (PC1 and PC2) mRNAs were shown by RT-PCR; the protein products were detected by ELISA, western blot or immunocytochemical methods in an ARPE-19 cell line derived from an adult human donor. CRFR2 was below the level of detectability. The CRFR1 was functional as evidenced by CRF stimulation of cAMP and inositol triphosphate production as well as by ligand induction of transcriptional activity of inducible cis-elements cAMP responsive element (CRE), activator protein 1 responsive element (AP-1) and POMC promoter in ARPE-19 using luciferase reporter assay. Immunoreactivities representative of CRF; pre-urocortin, CRFR1 receptor and ACTH were also detected in mouse retina by in situ immunocytochemistry. Finally, using RT-PCR, we detected expression of genes encoding four key enzymes participating in steroids synthesis (CYP11A1, CYP11B1, CYP17 and CYP21A2) and showed transformation of progesterone into cortisol-immunoreactivity in cultured ARPE-19 cells. Therefore, we suggest that ocular tissue expresses CRF-driven signalling system that follows organisational structure of the HPA axis.


Introduction

Corticotropin-releasing factor (CRF) is the most proximal element of the hypothalamic–pituitary–adrenal (HPA) axis, a system that coordinates the body response to systemic stress (Selye 1936, Vale et al. 1981, Chrousos 1995, Aguilera 1998). Physiological actions of CRF and related peptides such as urocortins I–III (Ucn I–III) are mediated through interactions with membrane-bound receptors, CRFR1 and CRFR2 (Perrin & Vale 1999, Grammatopoulos & Chrousos 2002, Hillhouse & Grammatopoulos 2006, Slominski et al. 2006b). Activation of CRF receptors leads to stimulation of adenylate cyclase, phospholipase C and calcium channels (Perrin & Vale 1999, Hillhouse & Grammatopoulos 2006). Subsequent production of cyclic AMP (cAMP) and inositol triphosphate (IP3) activates signal transduction pathway and enhances expression of the proopiomelanocortin (POMC), followed by secretion of POMC-derived adrenocorticotropic hormone (ACTH; Grammatopoulos & Chrousos 2002, Hillhouse & Grammatopoulos 2006). ACTH stimulates the production and release of cortisol from the adrenal cortex, which by a feedback mechanism attenuates hypothalamic CRF and pituitary POMC production (Chrousos 1995). Another POMC product, α-melanocyte-stimulating hormone (MSH), interacts with its melanocortin receptor type 1 (MC1) and stimulates melanogenesis in the skin (Slominski et al. 2004a). There is growing evidence that CRF and related peptides can act as local modulators of stress in the peripheral organs such as skin, gestational tissues, immune system, pancreas, liver, gastrointestinal tract, skeletal muscle, heart, lung and endocrine organs (ovaries, testes, adrenals and thyroid glands; Slominski et al. 2001, 2004b, 2006b, Linton et al. 2001, Kempuraj et al. 2004, Hillhouse & Grammatopoulos 2006). However, the presence of an analogue of the HPA axis in the ocular tissue has not been documented. Nevertheless, CRF was shown to be involved in the development of the retina (Bagnoli et al. 2003) and pathogenesis of experimental autoimmune uveoretinitis in rodents (Mastorakos et al. 1995). CRF immunoreactivity is also found in amacrine cells (Lindqvist et al. 2003), and the expression of POMC and its receptors (MC3, MC4 and MC5) were detected in adult rat retina (Lindqvist et al. 2003). POMC expression, α-MSH immunoreactivity and receptors for POMC peptides have also been found in the developing chick retina (Teshigawara et al. 2001).

The retinal pigment epithelial (RPE) cells form a monolayer of highly specialised pigmented cells located between the neural retina and the vascular choroid that...
influence their structure and function (Sharma & Ehinger 2003). These cells are exposed to pathological stresses, yet the mechanism of stress response has not been well characterised (Sharma et al. 1995).

Since other pigmented cells such as the skin melanocytes express a functional analogue of HPA axis (Slominski et al. 2000, 2004a, 2005), we investigated whether the human adult ARPE-19 express molecular elements of this axis. In vitro observations from cell line model were further confirmed in vivo by immunohistochemical analysis of mouse eye.

Materials and Methods

Cell culture and tissues

A widely researched human adult RPE cell line, ARPE-19, was obtained from American Type Culture Collection (ATTC, Manassas, VA, USA) and maintained in T-25 culture flasks (Becton Dickinson, Franklin Lake, NJ, USA) at 37 °C in an atmosphere of 95% air and 5% CO2. The culture medium contained Dulbecco’s modified Eagle’s medium (DMEM) with 5% foetal bovine serum (FBS), insulin (50 µg/ml) and an antibiotic–antimycotic mixture (10 000 units/penicillin G Sodium, 10 mg/streptomycin sulphate and 25 µg/amphotericin B; Gibco, Invitrogen Corp.). Cells were passaged weekly and fed every second day. Culture passages 25 were used in the experiments. Cells were plated at 250 000 cells/cm2 on 75 cm3 flasks and allowed to become confluent. The cells were detached from the flask by trypsinisation (0.05% trypsin/EDTA for 5 min) and washed with an ice-cold 20 mM Tris, pH 7.4, 1% Triton X-100 buffer (Sigma). Cellular homogenates were centrifuged at 16 000 g for 10 min at 4 °C and the supernatants used for assays were stored at −80 °C. Forty micrograms of total protein extract were resolved on 12% SDS-PAGE, transferred to Immobilon-P poly(vinylidene difluoride) membrane (Millipore Corp, Bedford, MA, USA) for 1.5 h at 4 °C and blocked overnight at cold room in 5% non-fat powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween 20). For protein isolation, frozen cell pellets were solubilised by pipetting into an ice-cold 20 mM Tris, pH 7.4, 1% Triton X-100 buffer supplemented with Protease inhibitor cocktail (Sigma). Cellular homogenates were centrifuged at 16 000 g for 10 min at 4 °C and the supernatants used for assays were stored at −80 °C. Forty micrograms of total protein extract were resolved on 12% SDS-PAGE, transferred to Immobilon-P poly(vinylidene difluoride) membrane (Millipore Corp, Bedford, MA, USA) for 1.5 h at 4 °C and blocked overnight at cold room in 5% non-fat powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween 20). For immunodetection of proteins, membranes were incubated with goat anti-CRF-R1 (C20) antibodies (dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 3 h, then washed once with 5% non-fat, dry milk in TBST, twice with TBST, and then incubated for 1 h with anti-goat antibodies coupled to horseradish peroxidase (dilution 1:2000; Santa Cruz Biotechnology). Membranes were washed twice in TBST and once in TBS and bands were visualised by Super Signal West Pico (Pierce Biotechnology, Rockford, IL, USA). The protein concentration was determined with a BCA protein assay kit (Pierce Biotechnology) and BSA used as a positive control. Protein ladder was purchased from Fermentas.

ELISA assays

ARPE-19 p24, p30 and human immortalised HaCaT keratinocyte cells were grown in DMEM with 5% charcoal
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer location</th>
<th>Fragment size</th>
<th>Reference</th>
</tr>
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<td>CRF</td>
<td>MZ024 5'-CACCTCTAGCCCTGGATTTC-3'</td>
<td>Exon 2</td>
<td>413 bp</td>
<td>Slominski et al. (1995)</td>
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<td>MZ025 5'-GCCCTGGAATTTGCAAGG-3'</td>
<td>Exon 2</td>
<td>145 bp</td>
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<td>MZ027 5'-CTTGCCACCGAGGTGAAAT-3'</td>
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<td>479 bp</td>
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<td>MZ104 5'-ACTGATAGATCACGATGACAGTCG-3'</td>
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<td>P575 5'-TGAGGACCCACTGTCGACAGGGA-3'</td>
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<td>318 bp</td>
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<td>P576 5'-TCCCTCCAAATTTGCGACAGCT-3'</td>
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<td>CRFR1</td>
<td>CRFR1 fragment spanning exons 2–7</td>
<td>Exon 2</td>
<td>PCR fragments after second round of amplification</td>
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<td>P110 5'-CTCGCTCTGCTAGGCGCCCTCC-3'</td>
<td>Exon 7</td>
<td>479 bp Insertion of cryptic exon between exons 4 and 5 (CRFR1h)</td>
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<td>P111 5'-GGCTGATGATGTGACAGCT-3'</td>
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<td>P112 5'-CTCGCTCTGCTAGGCGCCCTCC-3'</td>
<td>Exon 7</td>
<td>163 bp exons 3, 4 and 6 are absent (frameshift, CRFR1e)</td>
<td>Pisarchik &amp; Slominski (2001)</td>
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<tr>
<td></td>
<td>P113 5'-GGCTGATGATGTGACAGCT-3'</td>
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<td>PCR fragments after second round of amplification</td>
<td>Pisarchik &amp; Slominski (2001)</td>
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<td>P114 5'-CCATGGGAAGCTGTAATCCAC-3'</td>
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<td>200 bp Exon 12 is absent (frameshift, CRFR1f)</td>
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<td>P117 5'-CCATGGGACCCACTGTCGACAG-3'</td>
<td>Exon 7</td>
<td>114 bp Exon 11, 27 bp of exon 10 and 28 bp of exon 12 are absent (CRFR1g)</td>
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<td>MZ128 5'-CTGGGACATTGCGGAGATG-3'</td>
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<td>Slominski et al. (1995)</td>
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<td>P597 5'-CTACGGGGGACTGACAGCT-3'</td>
<td>Exon 7</td>
<td>222 bp</td>
<td>Slominski et al. (1995)</td>
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*refers to current paper.
FBS and antibiotic mixture until 80% confluent. Conditioned media and cells were collected separately. Media from ARPE-19 cells were concentrated in C18 Sep-columns (Peninsula Laboratories, San Carlos, CA, USA). CRF peptide concentration in the supernatants was measured with ELISA kit for CRF (Phoenix Pharmaceuticals, Belmont, CA, USA) and normalised for total protein content in cell lysates (quantified with BCA reagent; Pierce Biotechnology). In order to detect cortisol production in human ARPE-19 (passage 28), cells were seeded at a concentration of 500 000 cells/10 cm Petri dish in DMEM supplemented with 5% FBS and antibiotic mixture. After overnight incubation, cultures were washed with PBS and media were replaced with serum-free DMEM containing progesterone $10^{-6}$ or $10^{-5}$ M. After 24 h of incubation with CRF or ACTH ($10^{-7}$ M), medium was collected and the steroids were extracted with methylene chloride as described previously (Slominski et al. 2004c, 2005). The extracts were dried under nitrogen and then reconstituted in assay buffer and used for cortisol quantification with the BioQuant Cortisol ELISA kit (Bio-Quant, San Diego, CA, USA). The amount of cortisol was calculated from cortisol standard curve and presented as nanograms per millilitre of the medium.

**CRF treatment and cAMP assays**

ARPE-19 cells were grown on 96-well plates until confluent (2–3 days) in DMEM containing 5% FBS and antibiotics. Twelve hours prior to experiments, the medium was replaced with DMEM containing 5% FBS, antibiotics and 0.5 mM 3-isobutyl-1-methylxanthine. The cells were incubated with serial dilutions of the CRF (Sigma) for 1 h at 37°C and 5% CO$_2$ as described previously (Slominski & Slominski 2004, Slominski et al. 2006a). Cyclic AMP concentration in cell lysates was measured by cAMP functional assay kit (Packard BioScience, Meriden, CT, USA). The assay was based on the competition between endogenous and exogenous cAMP (biotinylated) to a specific antibody (acceptor beads) in the presence of streptavidin-coated donor beads. The signal (excitation 680 nm and emission 520–620 nm) was measured by universal microplate reader Fusion x (Packard BioScience) and was indirectly proportional to the concentration of endogenous cAMP. The concentration of endogenous cAMP was corrected to a standard curve prepared by serial dilution of cAMP (Packard BioScience).

**Inositol triphosphate (IP$_3$) assays**

IP$_3$ was measured by an amplified luminescent proximity homogenous assay (AlphaScreen Glutathione–S-Transferase (GST) detection kit and AlphaScreen IP$_3$ Assay Supplement, both from Packard BioScience) as described previously (Zbytek & Slominski 2005, Slominski et al. 2006a). In brief, cells were detached with a trypsin/EDTA solution, washed once with PBS and suspended at a concentration of 2500 cells/10 µl per well in 96-well white opaque plates in PBS buffer containing 15 mM HEPES, pH 7.4. Cells were stimulated with CRF (Sigma) at the indicated concentrations for 0–120 s. The reaction was stopped with 1-05% perchloric acid. Production of IP$_3$ was assayed by incubation of the reaction mixture with IP$_3$-binding protein followed by detection with a solution containing biotinylated IP$_3$ analogue, streptavidin-coated donor beads and anti-GST acceptor beads. The signal (excitation 680 nm and emission 520–620 nm) was measured by universal microplate reader Fusion x (Packard BioScience) and was indirectly proportional to the concentration of endogenous IP$_3$. The raw data (n=3 experiments) were correlated with a standard curve generated with d-myo-inositol 1,4,5-triphosphate (Sigma).

**Reporter gene constructs activity**

ARPE-19 cells were transfected using lipofectamine and PLUS reagents (Invitrogen) with firefly luciferase reporter gene plasmids pCRE-Luc, pAP1-Luc, pPOMC-Luc and with phRL-TK plasmid (coding Renilla luciferase and used as normalisation control; Promega). Plasmids pCRE-Luc (containing four cAMP responsive elements) and pAP1-Luc (five AP1 responsive elements) were described previously (Pisarchik & Slominski 2004). Plasmid pPOMC-Luc contained the sequence −771 to −8 of the human POMC promoter (Slominski et al. 2005, 2006b, Zbytek et al. 2006). After transfection, the cells were treated with serial dilution of CRF in DMEM medium (5% charcoal FBS and antibiotics) for 24 h. The firefly luciferase and Renilla luciferase signals were recorded with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) using Dual-Luciferase Reporter Assay System (Promega). After subtracting background luminescence, the ratio of firefly signal to Renilla signal was calculated. The values obtained were divided by the mean of control (untreated) cells.

**Statistical analyses**

Data were presented as means±S.E.M. (n=3–4) and analysed with Student’s t-test (for two groups) or one-way ANOVA with appropriate post hoc tests (for more than two groups) using Prism 4.00 (GraphPad Software, San Diego, CA, USA). Statistically significant differences were denoted with asterisks or P values were shown. The dose–response curve fitting and EC$_{50}$ calculations were also performed using Prism 4.0 software.

**Immunocytochemistry and histochemistry**

Cells were seeded onto 8-well Lab-Tek II chamber slides (Nalge Nunc, Inc., Naperville, IL, USA). Subconfluent cells were fixed with 4% parformaldehyde in PBS for 10 min. The cells were permeabilised with 0-1% Triton X–100 (in PBS) for 5 min and blocked with 1% BSA (in PBS) for 30 min. Immunostaining was performed as described previously (Slominski et al. 2004b, 2006a). Briefly, immunostaining was carried out with antisera: goat anti-CRF–R1 (1:200) and anti-urocortin I (1:100; both from Santa Cruz Biotechnology).
ACTH immunoreactivity was detected by rabbit anti-ACTR antibody (1:200, AFP173P, NIADDK, Bethesda, MD, USA). The antigens were visualised with an fluorescein isothiocyanate (FITC)-conjugated secondary anti-goat or anti-rabbit antibody (1:200 in 1% BSA in PBS for 1 h). The slides were extensively washed with PBS between staining and then mounted with Vectashield Mounting Medium with propidium iodide or 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Background controls were performed by omitting the primary antibodies.

To determine in situ antigen expression, formalin-fixed cryosections were first incubated in the normal serum of the animal, in which the secondary antibody was raised, followed by incubation with the primary antibody as above (except that goat anti-CRF-R1 antibody was used at 1:100 dilution) in a humidified chamber overnight. Optimum working concentration and incubation time for the antibody were determined earlier in pilot experiments. After incubation, the slides were rinsed with PBS and incubated for 1 h in an appropriate secondary antibody (anti-goat or anti-rabbit) conjugated with Cy3 (Jackson Laboratories, West Grove, PE, USA). The slides were then rinsed again in PBS and mounted with Vectashield (Vector Laboratories). At least three slides from each specimen were stained, and in each experiment, controls were obtained by omitting the primary antibody. Slides were examined under a fluorescent microscope. Digital fluorescence and bright field micrographs were taken using a Nikon camera.

### Results

**CRF and urocortins are expressed in ARPE-19 cells**

By RT-PCR, we detected the mRNAs for CRF, Ucn I, Ucn II and Ucn III in adult ARPE-19 cells (Fig. 1A). The RT-PCR product of 413 bp corresponding to the predicted fragment of exon 2 of the CRF gene (Slominski et al. 1995) was detected in p24, but not in p30 of ARPE-19 cells. Brain and HaCaT keratinocyte cDNAs were used as positive controls. The observation was confirmed in three independent experiments, indicating that the expression of CRF gene is influenced by a number of passages in culture. As predicted for Ucn I, Ucn II and Ucn III genes, the corresponding fragments of 145 bp (Bamberger et al. 1998), 195 bp (Imperatore et al. 2006) and 318 bp (Table 1) were detected in p24 and p30 of ARPE-19 cells (Fig. 1B–D). Sequences of the amplified fragments had 100% homology with corresponding genes as documented previously (Pisarchik et al. 2004, Slominski et al. 2004).

CRF and Ucn I mRNAs were translated into corresponding protein products as demonstrated in Fig. 1A, E and F. Specifically, the presence of CRF peptide in the ARPE-19 cells was detected by ELISA assay in both early and late passages with concentrations only slightly lower than those in control HaCaT keratinocytes (Fig. 1E). Immunocytochemical analysis has also demonstrated the presence of Ucn I immunoreactivity (Fig. 1A and F).

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**Figure 1** Expression of CRF, urocortins I, II and III (stresscopin) in human ARPE-19 cells. RT-PCR detection of mRNA of CRF (A), Ucn I (B) Ucn II (C) and Ucn III (D). For panels A–D: 100 kb DNA ladder, M; brain, B; immortalised HaCaT keratinocytes, H; ARPE-19 passages 24, p24; and 30, p30; negative control without cDNA, C. (E) Detection of CRF in whole cell lysates of ARPE-19 cells in comparison with HaCaT keratinocytes by ELISA. *P<0.05, †P<0.01 (n=3). (E) Immunocytochemical detection of urocortin I in human ARPE-19 cells, passages 24 (p24) and 30 (p30). Colour panels and control experiments are shown in supplementary Fig. 1 (see supplementary data in the online version of Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol193/issue1/). Bar, 25 μm.
POMC, PC1 and PC2 genes and ACTH antigens are expressed in ARPE-19 cells

Similar levels of POMC gene expression were detected independently, with two sets of primers in early and late passages of ARPE-19 line, being slightly lower than those in the brain (Fig. 2A and B). RT-PCR products of 261 bp (Slominski et al. 1995) and 100 bp (Slominski et al. 2005) were specific for exon 3 of the POMC gene, e.g. 100% of their sequence homology with POMC was shown in previous experiments (Pisarchik & Slominski 2004, Slominski et al. 2005). Since processing of POMC precursor requires the presence of both prohormone convertases 1 and 2 (PC1 and PC2), we have performed RT-PCR analysis of RNA isolated from p24 and p30 of ARPE-19 cells. PC2 mRNA was detected in both passages, while PC1 was detected only in p24, indicating a potential for age-dependent differences in POMC processing (Fig. 2D and E). Immunocytochemical analyses detected ACTH immunoreactivity in both passages of ARPE-19 cells (supplementary figure 1C and Fig. 2C) indicating that POMC message is translatable in RPE cells.

Expression of CRFR1 in ARPE-19 cells

The unique set of nested PCR primers spanning exons 2–7 and 9–14 of CRFR1 were used to detect expression of splicing variants of CRFR1 as described previously (Pisarchik & Slominski 2001). p24 of ARPE-19 cells expressed isoforms CRFR1α, e, f and g, while p30 expressed only CRFR1α (Fig. 3A and B). The expression of CRFR2 was below the level of detection by RT-PCR using primers amplifying common fragment of CRFR2 (Slominski et al. 2004b); however, the CRFR2 transcript was detected in the brain mRNA used as a positive control (data not shown).

Western blotting analysis (Fig. 3C) revealed that lysates of ARPE-19 cells (p24 and p30) express a different pattern of CRFR1 isoforms in accordance with RT-PCR experiments. The bands of 37, 45 and 64 kDa corresponded to processed, full-length and glycosylated CRFR1α isoform respectively, as documented previously (Slominski et al. 2006a). In passage p24, we also detected bands of approximately 24 and 52 kDa, which could represent other isoforms of CRFR1 detected by RT-PCR (Fig. 3A and B).
The 24 kDa band could represent glycosylated form of CRFR1e, which had a calculated molecular mass of 14 kDa before glycosylation. The 52 kDa band might correspond to the glycosylated CRFR1f and/or CRFR1g, which were detected by RT-PCR (Fig. 3A and B). The molecular masses of CRFR1f and CRFR1g before glycosylation are 39.1 and 43.13 kDa respectively. It could be suggested that glycosylation would lead to approximately 10–15 kDa shift in the molecular mass, similar to what was previously shown for CRFR1a (Slominski et al. 2006a). Further confirmation of CRFR1 receptor protein expression in both passages of ARPE-19 cells was provided by immunocytochemistry (supplementary figure 1B and Fig. 3D).

**CRFR1 expressed in ARPE-19 cells is functional**

Stimulation of ARPE cells with CRF elevated the production of cAMP and IP3 in dose-specific and cell passage-specific manner (Fig. 4), indicating that CRFR1 receptor was functionally active. CRF triggered cAMP and IP3 production in ARPE-19 cells with the highest efficiency in late passages.
Figure 4 CRF stimulates production of cAMP (A) and IP₃ production (B–E) in ARPE-19 cells. (A) Left panels present dose–response curves. Right panels, stimulation of CRF 100 nM (10 nM for HaCaT) is compared with stimulation with forskolin (0.1 mM). Dose–response curves are obtained after 30 s (B) or 60 s (C) after stimulation with CRF. Time-dependent stimulation of IP₃ production in ARPE cells is shown for CRF doses of 10⁻⁷ M (D) or 10⁻⁸ M (E). *$P<0.05$. 

Specifically, the EC_{50} (effective concentration) for p25 was $1.3 \times 10^{-11}$, while in p30 EC_{50} was $2.25 \times 10^{-11}$, when compared with $8 \times 10^{-11}$ for control HaCaT keratinocytes (Fig. 4A). CRF also stimulated IP$_3$ production in dose- and time-dependent manner (Fig. 4B-E), with the highest activity 30 s after addition of the ligand (EC$_{50} = 7 \times 10^{-9}$), but only in late passages of ARPE-19 cells (p30; Fig. 4B-E). The stimulation of IP$_3$ production for p24 of ARPE-19 cells was below detectable levels (data not shown). The EC$_{50}$ for IP$_3$ production in ARPE-19 was comparable to that observed in normal epidermal keratinocytes (Zbytek & Slominski 2005, Slominski et al. 2006a) and squamous cell carcinoma (Kiang 1995).

We also studied transcriptional activity of CRE and AP1 responsive elements and of human POMC promoter (~771 to ~ 8) in human ARPE-19 cells, p25–28, using dual luciferase assay system and plasmids pCRE-Luc, pAP1-Luc and POMC-Luc as described (Pisarchik & Slominski 2004, Slominski et al. 2005, Zbytek et al. 2006). CRF-stimulated transcriptional activity of CRE and AP1 reached maximal responses at different dose range, e.g. at $10^{-11}$ and $10^{-8}$ M for CRE (Fig. 5A) and $10^{-10}$ and $10^{-7}$ M for AP1 (Fig. 5B). These assays indicate that activation of second messengers’ production by CRF leads to transcriptional activity of the corresponding regulatory elements (CRE and AP1). Dual effect at different dose range is unusual and may reflect co-expression of different isoforms of CRFR1 in ARPE-19.

Expression of CYP11A1, CYP11B1, CYP17 and CYP21A2 genes and detection of cortisol in ARPE-19

Using RT-PCR and a set of unique primers, we detected cDNA of four key enzymes, which take part in synthesis of glucocorticoids, namely cytochromes P450 side-chain cleavage (P450scc), 11-β-hydroxylase (P450c11), 17-α-hydroxylase (P450c17) and 21-hydroxylase (P450c21) coded by CYP11A1, CYP11B1, CYP17 and CYP21A2 genes respectively (Payne & Hales 2004). The nested RT-PCR product of 390 bp (Fig. 6A) covering the region of exons 4–7 of CYP11A1 mRNA was as predicted (Slominski et al. 2004c). RT-PCR primers covering splicing sides between exons 8/9 and 9/10 were used to detect 215 bp fragment of CYP11B1 (GenBank Accession number NM_000497, Fig. 6B). Nested primers spanning exons 2–3 for CYP17 (GenBank Accession number NM_000102) and exons 7–8 for CYP21A2 (GenBank Accession number NM_0005002) were used to amplify fragments of 222 and 199 bp respectively. The presence of CYP17 transcript was detected only in early passage of ARPE-19 (p24, Fig. 6C), while the expression of other studied genes was detected in all tested passages (Fig. 6A, B and D).

In order to validate the physiological significance of the expression of the genes studied above, we tested for an accumulation of cortisol in conditioned media of ARPE passage 28. The cortisol-immunoreactivity was detected in ARPE medium and its content was increased by the addition of progesterone. Furthermore, CRH or ACTH ($10^{-7}$ M) also stimulated the accumulation of cortisol-immunoreactivity in culture medium (Fig. 6E).
In situ detection of CRF, Ucn-I, CRFR1 and ACTH antigens in mouse eye

CRF, Ucn-I, CRFR1 and ACTH antigens were detected in formalin-fixed cryosections of mouse eye (Fig. 7). The specific anti-mouse antibodies for corticoliberin (precursor of CRF; sc-1761; Santa Cruz Biotechnology) and urocortin I (Ucn; sc-1825; Santa Cruz Biotechnology) were used to detect unprocessed peptides to assure that they are synthesised locally and were not derived from the central nervous system. Both precursor forms of peptides were detected predominantly in RPE cells and additionally in some cone photoreceptors, especially in the outer segments (Fig. 7A and B). CRFR1 immunoreactivity was predominantly detected in the basal parts of the RPE layer (Fig. 7C). ACTH was localised in the RPE mostly in the apical parts of the cells.

Discussion

Our results show for the first time that ARPE-19 cells, derived from adult human RPE, express the main genes involved in the HPA axis, e.g. CRF, Ucn I-III, CRFR1, POMC, PG1, PC2, CYP11A1, CYP11B1, CYP17 and CYP21A2, and produce CRF, Ucn I, CRFR1 and POMC/ACTH protein products. The CRFR1 was functional as documented by the CRF stimulation of cAMP and IP3 production as well as by the stimulation of transcriptional activity of CRE, AP1 and human POMC promoter. Selected elements of the HPA axis (CRF, Ucn I, CRFR1 and ACTH) were also co-localised in situ in RPE layer of the mouse eye cross-section by immunocytochemistry. Thus, the molecular structure of the HPA axis is duplicated in RPE, which implies a role in the regulation of eye physiology and pathology. This is consistent with a proposed role of peripheral HPA homologue in the regulation of stress responses in another organ exposed to environmental insults – skin (Slominski & Wortsman 2000, Slominski et al. 2000, 2006b, Slominski 2005).

Previous reports in an animal model of autoimmune uveitis have shown the presence of CRF immunoreactivity in the infiltrating immune cells at the site of inflammation including iris, ciliary body, vitreous, retina and choroid, suggesting a pathogenic autocrine or paracrine

Figure 6 Expression of genes for CYP11A1 (A), CYP11B1 (B), CYP17 (C) and CYP21A2 (D) in human ARPE cells. Nested RT-PCRs were performed as described in Materials and Methods section and amplification on predicted fragments of CYP11A1, 390 bp; CYP11B1, 215 bp; CYP17, 222 bp and CYP21A2, 199 bp was detected. 100 kb DNA ladder, M; ARPE-19 passages 24, p24 and 30, p30 (A, C and D) or 26, p26; 31, p31; 37, p37 (B), negative control without cDNA, C. (E) Production of cortisol from progesterone was assayed by ELISA as described in the Materials and Methods section.
proinflammatory role of CRF (Mastorakos et al. 1995). Our results indicate that the CRF detected in the eye, at least partially, originates from the RPE cells. Furthermore, since CRF stimulates cAMP production in the retina of different mammals (Olianas & Onali 1990, Olianas et al. 1993), we propose that retinal cells can also be directly activated by CRF or urocortin synthesised by RPE in addition to the CNS-derived peptides. RPE cells could be a target of CRF and related peptides, since we have shown production of cAMP and IP₃ followed by transcriptional activation of CRE and AP1 responsive elements and, finally, stimulation of POMC promoter.

The pattern of expression of CRFR1 isoforms changed with the age of ARPE-19 primary culture, which might

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**Figure 7** Localisation of immunoreactivities characteristic for the hypothalamic–pituitary–adrenal axis in mouse eye. Immunohistochemical detection of (A) CRF precursor, (C) pre-urocortin, (E) CRFR1 and (G) ACTH. Panels (A), (C), (E), (G) represent fluorescent and panels (B), (D), (F), (H) bright field images of the same areas. OS, outer segments; ONL, outer nuclear layer; RPE, retinal pigment epithelium.
contribute to the observed changes in CRFR1 signalling. We observed efficient stimulation of cAMP and IP3 production by exogenous ligand only in late passages of APRE-19 cells (p30) after stimulation with CRF. This change in the responsiveness of the receptor may be secondary to either decreased production of CRF by APRE-19 cells, which desensitises receptor to exogenous ligand (paracrine action), or modifying action of isoforms CRFR1f and/or CRFR1g that are expressed only during early passages. The presence of several isoforms of CRFR1 might also explain the dual effect at different dose range observed for CRE and AP1 responsive elements. This is in agreement with our suggestions that the expression of CRFR1f and/or CRFR1g can interfere with CRF signalling through the main isoform – CRFR1ζ, thus modifying its signal transduction pathway (Pisarchik & Slominski 2001, 2004, Slominski et al. 2006b).

Expression of prohormone convertases PC1 and PC2, POMC and detection of ACTH immunoreactivity in human ARPE cells is in agreement with the detection of PC1, PC2 and α-MSH (a direct derivative of ACTH) in avian RPE cells, the cone photoreceptors/nerial retina (Teshigawara et al. 2001) and in the eye sections (Takeuchi et al. 2001, Teshigawara et al. 2001). Additional demonstration of CRF-induced production of second messengers and stimulation of transcriptional activity of CRE, AP1 and POMC promoter in ARPE cells suggest that the production of the neuropeptides can be regulated locally in the eye in response to environmental stimuli similar to that observed in the skin (Slominski et al. 2000, 2001, 2005, 2006b). Interestingly, receptor for melanocortins and ACTH (MC3) was detected in outer segments of photoreceptors (Lindqvist et al. 2003) and MC3 and MC4 receptors were found in other parts of the retina, suggesting that ACTH and/or α-MSH production by RPE cells can affect photoreceptor physiology.

Detection of CYP11A1, CYP11B1, CYP17 and CYP21A2 (crucial genes of the steroidogenic pathway (Payne & Hales 2004)) suggests that human ARPE cells may have corticosteroidogenic capability similar to skin pigment cells (Slominski & Mihm 1996, Slominski et al. 1999, 2004c, 2005). This is further supported by data indicating production of cortisol-immunoreactivity from progesterone in RPE. However, the hypothesis on steroidogenic activity of ARPE requires further validation by testing for regulated enzymatic activity of the protein products of the above genes detected in RPE cells together with assessment of the expression of the 3βHSD in RPE. Expression of the 3βHSD activity has already been documented in skin cells (reviewed in (Slominski & Wortsman 2000, Slominski 2005)).

Lastly, dysfunction of RPE cells plays a pivotal role in the pathogenesis of age-related macular degeneration (AMD), a major cause of visual impairment in ageing population (Sharma et al. 1995), in which inflammatory pathways might be important aetiological factors (Anderson et al. 2002). In this context, regulated production of intermediates of local HPA axis may play a role in eye function including prevention or attenuation of AMD, a subject that deserves further basic and translational studies.

In summary, we show that molecular elements of the HPA axis are expressed in adult human RPE cells indicating a novel mechanism for the local regulation of stress response in the eye and suggesting wider conservation of an HPA-like algorithm in peripheral tissues.

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