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CRH Functions as a Growth Factor/Cytokine in the Skin

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Abstract

We tested the effect of CRH and related peptides in a large panel of human skin cells for growth factor/cytokine activities. In skin cells CRH action is mediated by CRH-R1, a subject to posttranslational modification with expression of alternatively spliced isoforms. Activation of CRH-R1 induced generation of both cAMP and IP3 in the majority of epidermal and dermal cells (except for normal keratinocytes and one melanoma line), indicating cell type-dependent coupling to signal transduction pathways. Phenotypic effects on cell proliferation were however dependent on both cell type and nutrition conditions. Specifically, CRH stimulated dermal fibroblasts proliferation, by increasing transition from G₁/0 to the S phase, while in keratinocytes CRH inhibited cell proliferation. In normal and immortalized melanocytes CRH effect showed dichotomy and thus, it inhibited melanocyte proliferation in serum containing medium CRH through G₂ arrest, while serum free media led instead to CRH enhanced DNA synthesis (through increased transition from G₁/G₀ to S phase and decreased subG₁ signal, indicating DNA degradation). CRH also induced inhibition of early and late apoptosis in the same cells, demonstrated by analysis with the annexin V stains. Thus, CRH acts on epidermal melanocytes as a survival factor under the stress of starvation (anti-apoptotic) as well as inhibitor of growth factors induced cell proliferation. In conclusion, CRH and related peptides can couple CRH-R1 to any of diverse signal transduction pathways; they also regulate cell viability and proliferation in cell type and growth condition-dependent manners.

Corticotropin releasing hormone (CRH) is a 41-amino acid peptide that coordinates those central responses to stress mediated by the hypothalamic-pituitary-adrenal (HPA) axis (Chrousos, 1995; Perrin and Vale, 1999; Hillhouse et al., 2002). In addition, CRH together with the related peptides urocortin I–III, has been implicated at the central level, in the functional regulation of behavioral, autonomic, endocrine, reproductive, cardiovascular, gastro-intestinal, metabolic, and immune systemic activities (Linton et al., 2001; Grammatopoulos and Chrousos, 2002; Hillhouse et al., 2002; Theoharides et al., 2004). In peripheral organs including skin similar activities for the CRH peptides family have been reported, occurring mostly through para or autocrine mechanisms supporting a local functional (Slominski et al., 1999b, 2000c, 2001, 2003, 2004a; Linton et al., 2001; Zouboulis et al., 2002; Ito et al., 2004; Kempuraj et al., 2004; Theoharides et al., 2004; Zbytek et al., 2004; Zbytek and Slominski, 2005). CRH may also have a role in the regulation of cell differentiation and proliferation (Slominski et al., 2003; Zbytek and Slominski, 2005). Nevertheless, the skin is the main barrier against environmental stressors that include solar radiation, mechanical and thermal energy, and biological agents (Slominski and Wortsman, 2000).

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The action of CRH and related peptides is mediated by interactions with membrane-bound G protein-coupled receptors (GPCRs) receptors: CRH-receptor type 1 (CRH-R1) and type 2 (CRH-R2) which through alternative splicing of their genes generates diverse receptors isoforms (Perrin and Vale, 1999; Pisarchik and Slominski, 2001; Grammatopoulos and Chrousos, 2002; Hillhouse et al., 2002; Slominski et al., 2004a). In the skin, CRH is a component of a system organized similarly to the HPA (Slominski et al., 1999b, 2000c, 2005b). Thus skin melanocytes treated with CRH responds with ACTH-mediated increases in cortisol production (Slominski et al., 2005b). CRH and related peptides and the corresponding CRH-Rs are expressed in the skin in species and cell type associated manners (Slominski et al., 2001, 2003, 2004a). CRH-R1 is expressed in the epidermis, dermis, and subcutis with CRH-R1 α being the most prevalent isoform. CRH-R2 is expressed in hair follicle keratinocytes and papilla fibroblasts, sebaceous and eccrine glands, muscle and dermal blood vessels. In mouse skin, the *CRH-R2* gene and protein are more widely expressed than in humans, being detected in all cutaneous compartments and in cultured normal and malignant melanocytes. Human, but not mouse skin cells can produce CRH (Slominski et al., 1996, 1999b, 2000b, 2001; Roloff et al., 1998), urocortin I is produced in both human and mouse skin (Slominski et al., 2000a), and urocortin II mRNA is detected in both species, although actual protein production has been only documented in rodent skin (Slominski et al., 2004a). Both CRH and urocortin can modify function of the skin immune system and activate mast cells (Slominski et al., 2003; Zbytek et al., 2003; Kempuraj et al., 2004; Theoharides and Cochrane, 2004; Theoharides et al., 2004). CRH can also modify thymidine incorporation in the dermal and epidermal compartments of histocultured mouse skin depending on the phase of the hair growth cycle (Slominski et al., 1999b). Urocortin in combination with bFGF stimulates hair growth in the mouse (Vale et al., 2004).

In culture of human skin cells represented by normal and immortalized keratinocytes CRH and urocortin inhibit cell proliferation (Slominski et al., 2000a; Quevedo et al., 2001), while stimulating keratinocyte cell differentiation (Zbytek et al., 2005; Zbytek and Slominski, 2005). They also modulate expression of cell surface adhesion molecules (Quevedo et al., 2001) and production of cytokines (Zbytek et al., 2002; Slominski et al., 2003; Orłowski et al., 2004). These phenotypic effects may be related to effects on the intracellular concentrations of cAMP, IP3, Ca, or on NF-kappaB activity (Fazal et al., 1998; Slominski et al., 1999b; Slominski et al., 2000d; Wiesner et al., 2003; Zbytek et al., 2003; Zbytek et al., 2004). These data define CRH and related peptides as cutaneous growth factor/pleiotropic cytokines that can regulate proliferation, differentiation, and immune interactions (Slominski et al., 2003). To further clarify this role for CRH we have now tested a large panel of human skin cells.

MATERIALS AND METHODS

Peptides

CRH (Sigma, St. Louis, MO) or (American Peptide Company, Sunnyvale, CA), acetyl-CRH {D-Phe12, D-Glu20, L-Nle21, Lys33, D-His32, D-Nle38}(4-41), human [cyclo30-33] (UCW 4938) (California Peptide Research, Inc., Napa, CA) or urocortin I (California Peptide Research, Inc.) was added every 12 h for indicated time periods (see figures). CRH-R1 agonist (UCW 4938) and urocortin I were obtained from Dr. Edward Wei, University of California, Berkeley, CA.

Cell culture

Normal and malignant skin cell lines (Table 1) were cultured as described previously (Slominski et al., 1999a, 2003; Coburn et al., 2003; Zbytek and Slominski, 2005). Normal keratinocytes, melanocytes, and dermal fibroblasts were purchased from Cascade Biologicals (Portland, OR). Keratinocytes were propagated in Epilife medium supplemented with 0.06 mM

calcium chloride and EpiLife® defined growth supplement containing purified bovine serum albumin, purified 5 µg/ml bovine transferrin, 0.18 µg/ml hydrocortisone, recombinant human insulin-like growth factor type-1, prostaglandin E₂, 0.2 ng/ml recombinant human epidermal growth factor, and antibiotics (Cascade Biologicals). Normal epidermal melanocytes and the immortalized human epidermal melanocytes line PIG-1 (gift of Dr. Caroline LePoole, Loyola Medical Center, IL) were cultured in Medium 154 (Cascade Biologicals) supplemented with 0.2% v/v bovine pituitary extract, 0.5% v/v fetal bovine serum, 5 µg/ml bovine insulin, 5 µg/ml bovine transferrin, 3 ng/ml basic fibroblast growth factor, 0.18 µg/ml hydrocortisone, 3 µg/ml heparin, 10 ng/ml phorbol 12-myristate 13-acetate. Dermal adult fibroblasts were cultured in Cascade 106 medium plus Cascade Low Serum Growth Supplement (containing fetal bovine serum, 2% v/v; hydrocortisone, 1 µg/ml; human epidermal growth factor, 10 ng/ml; basic fibroblast growth factor, 3 ng/ml; and heparin, 10 µg/ml), and antibiotics.

Cell proliferation assays

Proliferation analyses were as described (Slominski et al., 2003; Zbytek et al., 2005; Zbytek and Slominski, 2005). Briefly, SKMEL-188 melanoma cells (5,000 cells/well) were seeded in growth medium in a 96-well plate. After 12 h the medium was replaced with growth supplements free medium 154 and graded concentrations of UCW peptide were added every 12 h for 48 h, prior to MTT assay (Slominski et al., 2003). The data was expressed as percent of control.

³H-thymidine incorporation into DNA was measured in cells seeded into 96-well plates (5,000 or 2,000 cells/well) for dermal fibroblasts and immortalized epidermal (PIG-1) melanocytes or normal epidermal (neonatal) melanocytes, respectively (Slominski et al., 2003; Zbytek and Slominski, 2005). After 24 h the media were changed to serum free Ham's F10 for 12 h to synchronize the cell cycle. Thereafter, the medium was changed to Ham's F10 medium with or without 5% FBS (containing growth factors); graded dilutions of CRH were added, and the cultures were incubated for another 24 h. For the last 8-6 h, the medium was supplemented with methyl-³H thymidine (1 µCi/ml) (Amersham, Piscataway, NJ), and the incorporation was measured as described (Zbytek and Slominski, 2005).

Flow cytometry analyses

Dermal fibroblasts (2,50,000 cells per 25 cm² flask) and PIG-1 melanocytes (5,00,000 cells per 10 cm² dish) were seeded in growth media. After 12 h the media were changed to serum free Ham's F10 for 12 h to synchronize the cell cycle. Thereafter, media were changed to Ham's F10 with (PIG-1) or without (fibroblasts) 5% FBS and cultures were incubated with 100 nM CRH or vehicle for another 24 h or 36 h (Zbytek and Slominski, 2005). SKMEL-188 cells were seeded in 10 cm² Petri dishes (5,00,000 cells/dish). After 12 h media were removed and medium 154 (without growth supplements), with or without 100 nM CRH was added for 48 h incubation period. Cells were processed for analysis with a FacsCalibur cytometer as described (Slominski et al., 2003; Zbytek et al., 2005; Zbytek and Slominski, 2005), gated to exclude debris and doublets. A total of 10,000 events corresponding to the G₁ region of DNA histogram were collected. Data were analyzed with the ModFit 2.0 software package (Verity Software House, Topsham, ME).

To measure apoptosis, PIG-1 melanocytes were seeded in Petri dishes (5,00,000 cells per 10 cm²); after 12 h media were changed to serum free Ham's F10 medium with or without CRH 100 nM and incubated for an additional 48 h (adding CRH every 12 h). Apoptosis was determined with FITC-conjugated annexin V binding, while sub-G₁ analysis was performed after propidium iodide staining of live cells as described (Slominski et al., 2003; Zbytek and Slominski, 2005). Results were expressed as percent of untreated cells using the FITC+/PI— and FITC+/PI— windows. Graphical representation of FL-1 and FL-2 signals was prepared

with the WinMdi 2.8 (shareware from Joseph Trotter, The Scripps Research Institute, San Diego, CA).

cAMP and inositol triphosphate (IP₃) assays

The assays were performed as described previously (Pisarchik and Slominski, 2004; Zbytek and Slominski, 2005). Briefly, cells cultured in growth factors supplemented media were detached with trypsin/EDTA, washed with PBS, and suspended in Ham's F10 containing 0.5 mM IBMX or in PBS buffer containing 15 mM HEPES, pH 7.4 for cAMP, and IP₃ assays, respectively. cAMP concentration was measured in cell extracts with a cAMP functional assay kit; Packard BioScience, Meriden, CT (Pisarchik and Slominski, 2004). IP₃ was measured by amplified luminescent proximity homogenous assay (AlphaScreen™ Glutathione-S-Transferase (GST) detection kit and AlphaScreen™ IP₃ Assay Supplement) (Zbytek and Slominski, 2005).

Western blot analysis

Cell pellets were solubilized in PBS buffer containing 2% Triton X100 and Proteases cocktail (Sigma), and the homogenates centrifuged at 16,000g for analyses on the supernatants (Pisarchik and Slominski, 2004). Fifty micrograms of protein suspended in Laemmli's buffer were resolved on 12% polyacrylamide/1%SDS gel, transferred to immobilion-p (polivinylidene difluoride) membrane (Millipore Corp., Bedford, MA) and blocked with 5% nonfat milk in 50mM Tris, pH 7.5, 150mM NaCl, 0.01% Tween-20. Detection of the CRH-R1 immunoreactive proteins was performed with goat anti-CRHR1 antibodies (1:200; sc-1757, Santa Cruz Biotechnology, Santa Cruz, CA); the membranes were washed and incubated for 1 h with anti-rabbit antibodies coupled to horseradish peroxidase (1:1,000; Santa Cruz Biotechnology) and the bands visualized by Super Signal West Pico (Pierce).

PNGase F treatment

The lysates (50 µg protein per sample) were denatured with 0.5% SDS and 1% β-mercaptoethanol for 10 min at 100°C, and then treated with 500 U of PNGase F (Biolabs) for 2 h. The proteins were suspended in Laemmli's buffer and processed as described above

Immunohistochemical detection of CRH-R1 in cultured skin cells

Cells were seeded in 16 well Lab-Tek II chamber slides (Nalge Nunc, Inc., Naperville, IL). Subconfluent cultures were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (in PBS), blocked with 1% BS, and immunostained with goat anti-CRHR1 antibody at dilution 1:100 (sc-1757, Santa Cruz Biotechnology). Cells incubated with nonimmune goat serum were nonspecific immunostaining controls. After washing, slides were incubated with FITC-conjugated anti-goat antibody (1:500 in 1% BSA in PBS), washed again, and mounted using VECTASHIELD Mounting Medium with Propidium Iodide (Vector Laboratories, Burlingame, CA). Images collected with NIKON Eclipse TE300 microscope (Melville, NY) were recorded and analyzed with the MetaValue software.

Statistical analyses

Data were tested for distribution and homogeneity of variances, and statistical significance was calculated independently for parametric and non-parametric data, with the Statistica-5.0 software package (Statsoft, Tulsa, OK) (Slominski et al., 2003; Zbytek and Slominski, 2005). Dose-response plots and EC₅₀ were fitted with the Prism 4.0 software package (GraphPad Software, Inc., San Diego, CA). Data is the mean ± SEM; for n = 12-16 (MTT and [³H] thymidine assays) or n = 3-4 (IP₃ and cAMP assays). Flow cytometry data (n = 4) was analyzed with Student's *t*-test.

RESULTS AND DISCUSSION

Characterization of CRH-R1 protein(s) in skin cells

Using cell culture models, immunofluorescence and Western blot analyses we found the CRH-R1 protein present in all skin cells including normal and malignant melanocytes, and keratinocytes as well as dermal fibroblasts (Figs. 1 and 2, Table 1). However, a protein of specifically 45-47 kD (corresponding to theoretical mass of unprocessed CRH-R1) was detected only in dermal fibroblasts, squamous cell carcinoma cells, and in two melanoma lines (Fig. 2A, arrow). Instead, the most abundant and prevalent are CRH-R1 forms (glycosylated, see below) had higher molecular weight (mw) (60-70 kD) (Fig. 2A, asterisk and arrow-head). These were uniformly detected in all skin cells, while a form of 55 kD that is characteristic of brain tissue (positive control) was found only in normal melanocytes, squamous cell carcinoma, and two melanoma lines (Fig. 2A, arrow-head). To test whether immunoreactive proteins of higher mw represent glycosylated receptor, we treated cell lysates with PN Gase F, *N*-glycosidase to remove *N*-glycan chains from glycosylated form of the receptor (Ruhmann et al., 1996; Sydow et al., 1997; Hauger et al., 2000). This procedure resulted in reduction of mw of the CRH-R1 immunoreactive proteins from melanocytes and keratinocytes, from 65 kD to 45-47 kD (Fig. 2C); the latter matches the predicted molecular weight of unprocessed CRHR1 α (45.3 kD). An additional CRH-R1 immunoreactive protein of 37 kD (detected in keratinocytes, squamous cell carcinoma, and melanoma cells) was resistant to PN Gase F treatment (Fig. 2), indicating that this form is either directly processed CRHR1 α or, that it represents the described alternatively spliced form CRHR1 g of 36.8 kD (Pisarchik and Slominski, 2004).

In summary, the pattern described above is in agreement with data in literature from other tissues that express the CRHR1 receptor showing forms with varying mobility on SDS—PAGE (Grigoriadis and De Souza, 1988, 1989; Ruhmann et al., 1996; Hauger et al., 2000). For example, glycosylated forms of CRH-R1 of 70 kD or of 53 kD have been detected in the in pituitary or brain tissues, respectively (Grigoriadis and De Souza, 1988, 1989). Furthermore, detection of multiple bands immunoreactive with antibodies against CRH-R1 have also been reported in AtT20, Y-79 cell lines, and in rat pituitary (Sydow et al., 1997). Thus, products found in skin cells, of higher mw (55 kD and 60-70 kD) would arise by cell type-dependent posttranslational modification (glycosylation) of the translated CRH-R1 α form (47 kD). Furthermore, the increased heterogeneity of CRH-R1 protein products found in squamous cell carcinoma and melanoma cells may have resulted from a combination of posttranslational modifications of CRH-R1 α and of alternatively spliced isoforms (Pisarchik and Slominski, 2004).

Signal transduction

We found that cutaneous CRH-R1 are coupled to production of the second messengers, for example, cAMP (Fig. 3) and IP3 (Fig. 4) (Table 1). CAMP production was stimulated by CRH and urocortin in almost all tested skin cells, except for normal adult epidermal keratinocytes (Fig. 3) and a single melanoma SKMEL-188 line (not shown) that expressed CRH-R1d instead of the CRH-R1 α isoform (Pisarchik and Slominski, 2001). Thus, in almost all skin cells expressing CRH-R1 α (normal adult epidermal keratinocytes are the sole exception) binding of the ligand to the receptor activates adenylate cyclase (Fig. 3, Table 1). Furthermore and similar to the experimental model of COS cells overexpressing CRH-R1 (Wei et al., 1998) CRH also has higher potency than urocortin in stimulation of cAMP production in skin cells (see Fig. 3A). This indicates that the CRH-R1 α coupling to adenylate cyclase characteristic of the central level structures (brain and pituitary) (Perrin and Vale, 1999; Slominski et al., 2000c; Grammatopoulos and Chrousos, 2002; Hillhouse et al., 2002) is conserved in the peripheral tissues of which skin is an example. The phenotypic significance of this coupling

is most evident in pigmentary system where cAMP dependent-pathways play a dominant role in positive regulation of melanocyte differentiation (Slominski et al., 2004b). In addition, most recently we have reported that CRH can stimulate POMC expression, but solely in melanocytes and fibroblasts where CRH-R1 α is coupled to cAMP production, but not in normal epidermal keratinocytes, where such coupling is absent (Slominski et al., 2005a,2005b). Thus CRH signaling in melanocytes and fibroblasts initiates the same signaling pathway as in central axis structures where CRH-R1-mediated stimulation of cAMP production leads to activation of kinase A (PKA) with subsequent enhancement of POMC expression and ACTH production (Perrin and Vale, 1999;Slominski et al., 2000c;Grammatopoulos and Chrousos, 2002;Hillhouse et al., 2002).

Coupling to a second signaling system, production of IP₃ has also been demonstrated in all the tested cells where CRH and urocortin stimulated its accumulation in time and dose dependent-manners (Fig. 4). The EC₅₀ was similar between cell lines (in the range of 10-9 M) indicating comparable intercellular potencies for the CRH activation of phospholipase C. This stimulation is also in agreement with previous findings in normal epidermal keratinocytes (Zbytek and Slominski, 2005) and squamous cell carcinoma (Kiang, 1995,1997). Since IP₃ mobilizes Ca²⁺ from intracellular stores, the above coupling would explain observations of CRH activated cytosolic calcium flux in normal and immortalized (HaCaT) keratinocytes, and in normal and malignant melanocytes (Wiesner et al., 2003). In turn, these findings uncover a phenotypic significance for the CRH-R1 coupling to IP₃ production, since the Ca²⁺ signal is fundamental for keratinocytes differentiation (Eckert et al., 1997;Bikle et al., 2004). In fact, we recently documented that activation of CRH-R1 α stimulates the differentiation program in both normal epidermal and immortalized keratinocytes (Zbytek et al., 2005;Zbytek and Slominski, 2005).

It is therefore significant that activation of CRH-R1 α in normal epidermal keratinocytes is coupled to IP₃ but not cAMP (Figs. 3A and 4F), indicating specificity for CRH signaling through channeling it to Ca²⁺ mobilization to induce the cell differentiation program (cf (Zbytek et al., 2005;Zbytek and Slominski, 2005). It is possible that the same pathway may be probably involved in the regulation of keratinocyte immune activity (Slominski et al., 2003; Zbytek et al., 2003,2004,2005;Zbytek and Slominski, 2005). Similar restrictive transductions of the CRH-R1 signal have been noted in the feto-placental unit (Karteris et al., 2000). Bypassing of such a restriction may be possible, for example by immortalization (HaCaT keratinocytes) or by malignant transformation (C1-4 cells); these cells have restored coupling to the cAMP signaling system (Table 1, Fig. 3B). It must be noted that CRHR1 α can couple to different G proteins, apparently in cell type dependent-manner, by using alternative signal transduction systems even within the same cell type (for example protein kinase A and protein kinase C) (Karteris et al., 2000;Grammatopoulos et al., 2001;Blank et al., 2003;Papadopoulou et al., 2004;Pisarchik and Slominski, 2004;Wietfeld et al., 2004). As regards the isoform CRH-R1d, solely expressed by SKMEL-188 (Pisarchik and Slominski, 2001), our detailed studies document that it must be coupled exclusively to the IP₃—Ca signaling system (Fig. 4; Table 1). Thus, depending on cell type and functional status the same receptor isoform can be connected preferentially to the IP₃ and Ca²⁺ second messengers or to both cAMP and IP₃/Ca²⁺ signal transduction pathways.

Proliferation and apoptosis in non-malignant skin cells

Activation of CRH receptors led to regulation of cell proliferation in skin cells in cell type and growth condition dependent-manners (Table 2). Dermal fibroblasts cultured in growth factor deficient medium showed stimulation of cell proliferation by CRH with EC₅₀ = 0.5 × 10⁻⁹ M (Fig. 5), which is similar to EC₅₀ (1.2 × 10⁻⁹ M) for IP₃ production but much lower than that for cAMP (1.9 × 10⁻⁸ M) (Figs. 3A and 4E). This suggests that the signal cascade initiated by

IP3 is involved in that proliferative response. Flow cytometric DNA content analysis showed that proliferation resulted from increased transition from the G₁/G₀ to the S phase of the cell cycle (Fig. 5B). This proliferative effect was abolished by the addition of either serum or growth factors (not shown). Therefore, it appears that CRH can act as a growth factor for cells of mesenchymal origin, an effect that is opposite to that noted in cells of ectodermal origin (epidermal keratinocytes). In the latter, CRH and related peptides at low ligand concentrations inhibited cell proliferation (whether in the presence or absence of growth supplements in the medium) (Slominski et al., 2000d; Quevedo et al., 2001; Zbytek et al., 2005; Zbytek and Slominski, 2005). Since both cell types express predominantly the same receptor isoform, CRH-R1 α , those opposite effects must result from differential coupling to the downstream signal transduction cascade. Indeed, CRH signal transduction in keratinocytes is coupled to IP3 and Ca²⁺ as second messengers (Wiesner et al., 2003; Zbytek and Slominski, 2005), which regulates their differentiation (Eckert et al., 1997; Bikle et al., 2004). Insofar, the available evidence strongly suggests that activation of CRH-R1 α in keratinocytes releases a sequential program of signal transduction, resulting in a biochemical cascade that simultaneously inhibits cell proliferation while stimulating differentiation, perhaps through overlapping mechanisms (Zbytek and Slominski, 2005). The biochemical cascade includes Ca²⁺, phospholipase C, protein kinase C, and AP1 (Zbytek et al., 2005; Zbytek and Slominski, 2005). Similar analysis for fibroblasts is still incomplete; nevertheless, data obtained in other systems such as serotonin receptors demonstrate that coupling to IP3 in fibroblasts results in growth stimulation (Slominski et al., 2005). This evidence further supports cell type restriction (downstream of IP3 signaling) for CRH-R1 phenotypic effect.

Interestingly, cells of neural crest origin (normal and immortalized melanocytes) show dichotomy in the effect of CRH on cell proliferation, depending on the presence or absence of growth factors. Thus, when normal melanocytes are cultured in serum-containing medium CRH inhibits cell proliferation in a dose dependent manner through G₂ arrest (Fig. 6). This is demonstrated by a significant increase ($P < 0.005$) in the proportion of cells in G₂/M phase without change in the S phase (Fig. 6B). Conversely, in cells starved by incubation in serum free media, CRH instead prevents attenuation of DNA synthesis (Fig. 7), and flow cytometry analysis shows not only increased transition from G₁/0 to S phase but also decreased subG₁ signal (not shown). SubG₁ signal strength is proportional to DNA degradation and characteristic of cells undergoing necrosis/apoptosis (Gong et al., 1994; Ormerod, 2000). Indeed, flow cytometry analysis of cells stained with annexin V confirmed a protective role of CRH against serum deprivation induced cell death ($P < 0.05$) and also documented inhibition of early and late apoptosis (Fig. 7C,D). Thus, in epidermal melanocytes CRH acts as both survival factor (anti-apoptotic agent) under stressful conditions (starvation) or as inhibitor of cell proliferation, when provided with growth factors (counteracting their activity). Although the specific roles of CRH activated IP3- and cAMP-dependent pathways remain to be tested in this cell group, others have already documented important roles for these intracellular pathways in melanocyte proliferation and differentiation (Pawelek et al., 1992; Busca and Ballotti, 2000; Park and Gilchrist, 2002), it is also possible that additional indirect mechanisms may be involved acting via stimulation of production of POMC derived ACTH or MSH (Slominski et al., 2005b).

Melanoma cell proliferation

The effect of CRH on melanoma cells was variable, for example, some cell lines showed growth inhibition, while others showed lack of effect or even lack of reproducibility of the effects (not shown). This instability could be related to variable co-expression of CRH-R1 isoforms in these neoplastic cells (Pisarchik and Slominski, 2001), or to concomitant variability in induced POMC expression (Sato et al., 2002; Slominski et al., 2004b). Therefore, we restricted our analysis to a cell line expressing under non-induced conditions only one receptor isoform, SKMEL-188 (Pisarchik and Slominski, 2001), and used modified CRH peptide (UCW), which

show high selectivity for CRH-R1 (Wei et al., 1998; Carlson et al., 2001). In SKMEL-188 cells, when maintained in low calcium 154 medium we found inhibition of cell proliferation by CRH-R1 peptide agonist (UCW) (Fig. 8A). The inhibitory effect was connected to inhibition of transfer from G₁/0 to S phase of the cell cycle (Fig. 8B). Since in these cells the activation of the CRH-R1 is coupled solely to Ca and IP₃ signaling, those pathways could be predominantly involved in negative regulation of melanoma cell proliferation.

General implications

The present studies document that CRH-R1 expressed in all major skin cellular populations (physiological or pathological) are functional. Dual coupling of these receptors involves coupling to both the adenylate cyclase and phospholipase C pathways in the majority of the tested populations is in agreement with the recently demonstrated activities CRH-R1, which even when derived from the same membrane preparation, can activate Gs, Gi, or Gq (Karteris et al., 2000; Grammatopoulos et al., 2001; Blank et al., 2003; Papadopoulou et al., 2004; Pisarchik and Slominski, 2004; Wietfeld et al., 2004). Functional assays with transfected COS cells have shown the CRH-R1 α dependent-activation of varied *cis*-elements (CRE, CaRE, SRE, AP1, or NF κ B) (Pisarchik and Slominski, 2004). Thus, the same CRH-R1 isoform (α) can regulate diverse transcriptional activity via activation of different signal transduction pathways within a single cell type (Pisarchik and Slominski, 2004). Phenotypic expressions of the activation of the CRH-R1 are changes in cell cycling and cell viability that are cell type specific and growth condition dependent. These phenotypic manifestations in addition to the previously reported immunomodulatory actions of CRH (cf (Slominski et al., 2003; Theoharides et al., 2004; Zbytek et al., 2004)) indicate novel roles for CRH with cytokine/growth factor pleiotropic activities.

The finding that CRH can act as a pleiotropic cytokine in the skin provide additional framework for our hypothesis that a cutaneous CRH-based homeostatic response system represents an evolutionary preservation of an ancestral stress response mechanism. By necessity, such a system probably developed initially in the periphery, to then evolve after adoption by the neuroendocrine elements to reach its present level of specialization at the central level (Slominski and Wortsman, 2000; Slominski et al., 2000c; Slominski et al., 2003). Thus, the skin is continuously facing a hostile environment assembled locally neuroendocrine elements to maintain local, and hence global homeostasis, through stress buffering activities or restoration of the skin's structural and functional integrity. Within this context, regulation of viability and proliferative/differentiation activity of the epidermis, functional activity of adnexal structures, and activity of the pigmentary and immune systems, and dermal mesenchymal elements are all vital components. Precise coordination of many of those responses would be served by a multipurpose CRH signaling system, such as that being defined in the skin. Thus, the skin, because of its strategic location and intrinsic properties represents an excellent model for testing an evolutionary conserved important role for a CRH based signaling system for the regulation of homeostasis through mechanisms of almost infinite regulation in the level of complexity.

In summary, we provide evidence that CRH and related peptides can couple CRH-R1 to diverse signal transduction pathways, and show that CRH-R1 can regulate cell viability and proliferation in a cell type and nutrient availability restricted fashion.

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Fig. 1.

Immunocytochemical detection of CRH-R1 protein in cultured skin cells: **A, B**—normal (neonatal) melanocytes; **C, D**—immortalized (PIG-1) melanocytes; **E, F**—melanoma SKMEL-188; **G, H**—melanoma WM164; **I, J**—immortalized epidermal keratinocytes (HaCaT); **K, L**—dermal fibroblasts. **A, C, E, G, I, K**— cells were incubated with goat anti-CRHR1 antibody (*sc-1757*, Santa Cruz Biotechnology) with following detection by FITC-conjugated anti-goat antibody. Background controls (primary antibody was omitted) are in **B, D, F, H, and L**. Red fluorescence show nuclei stained with propidium iodide. Bar —100 μ m.

Fig. 2.

Western blot detection of CRH-R1 proteins in skin cells. **A:** Membranes were incubated with goat anti-CRH-R1 antibodies (*sc-1757*). MW, molecular weight marker; 1—human brain (cortex); 2—dermal fibroblasts; 3—immortalized epidermal keratinocytes (HaCaT); 4—normal (neonatal) melanocytes; 5—immortalized (PIG-1) melanocytes; 6—squamous cell carcinoma (C₄₋₁); 7—melanoma WM164; 8—melanoma WMSCBE2; 9—melanoma SKMEL-188. **B:** Membranes incubated with secondary antibodies (control). *glycosylated CRH-R1 of 60-70 kD, arrowhead—glycosylated CRH-R1 of 55 kD, arrow—unprocessed CRH-R1 of 45-47 kD, double-arrowhead—processed CRH-R1 of 37 kD. **C:** Evidence that immunoreactive CRH-R1 proteins of higher mw represent glycosylated CRH-R1. Cell lysates of normal (neonatal) melanocytes and immortalized epidermal keratinocytes (HaCaT) were treated with PNGase F and then subjected to Western blot analysis (See material and methods for details). Arrows—proteins detected with goat anti-CRH-R1 antibodies (*sc-1757*).

Fig. 3. Activation of CRH-R1 stimulates cAMP in skin cells. Panels present dose-response curves for CRH and urocortin (**A**) and for CRH only (**B**). HACAT, HaCaT keratinocytes; SCC, squamous cell carcinoma; * $P < 0.05$; ** $P < 0.001$.

Fig. 4.

CRH stimulates IP₃ production in skin cells. Cells were stimulated with 1-100 nM CRH for 0-150 sec. Panels present dose-response curves: **A:** Innortalized keratinocytes (HaCaT) at 30 sec, **(B)** SK-MEL188 melanoma at 60 sec, **(C)** PIG-1 immortalized melanocytes at 30 sec, **(D)** epidermal (neonatal) melanocytes (passage #4) at 90 sec, **(E)** dermal fibroblasts (passage #4) at 120 sec. **F:** Epidermal keratinocytes (passage#4) at 60 sec. Inserts show time dependent-curves obtained with CRH at 100 nM.* $P < 0.05$.

Fig. 5.

Effect of CRH on adult dermal fibroblasts. **A:** In serum free conditions CRH significantly increased DNA synthesis ($EC_{50} = 0.5 \times 10^{-9}$ M) compared to untreated cells. **B:** Subsequent DNA content analysis yielded increase in transition of cells from $G_1/0$ to S phase cell cycle compartment (see table below). Control: continuous line, CRH (100 nM): dashed line. * $P < 0.05$, ** $P < 0.005$.

Treatment	$G_1/0$ [%]	S [%]	G_2/M [%]
Control	96 ± 0.3	2 ± 0.5	2 ± 0.3
CRH (100 nM)	90 ± 1 **	8 ± 0.5 **	2 ± 1

Fig. 6.

Effect of CRH on melanocytes in medium containing growth factors. In medium containing growth factors CRH significantly decreased DNA synthesis in human epidermal melanocytes ($EC_{50} = 3 \times 10^{-8}$ M) (**B**). **C**: Subsequent DNA content analysis yielded increase in the proportion of PIG-1 cells in G_2/M phase cell cycle compartment (see table below). Control: continuous line, CRH (100 nM): dashed line. * $P < 0.05$, ** $P < 0.005$.

Treatment	$G_1/0$ [%]	S [%]	G_2/M [%]
Control	68 ± 1	13 ± 0.5	19 ± 0.5
CRH [100 nM]	$64 \pm 0.5^*$	14 ± 0.5	$24 \pm 0.5^{**}$

Fig. 7.

Effect of CRH on melanocytes in medium deprived of growth factors. In medium deprived of growth factors CRH significantly increased DNA synthesis in human immortalized PIG-1 melanocytes ($EC_{50} = 3 \times 10^{-8}$ M) (**A**) and epidermal melanocytes ($EC_{50} = 1.3 \times 10^{-9}$ M) (**B**). Subsequent annexin V and propidium iodide staining of PIG-1 cells showed decrease of number of cells in early (FITC+/IP—) and late (FITC+/IP+) apoptosis. Control (**C**) versus CRH at 10 nM concentration (**D**). $*P < 0.05$.

Fig. 8.

CRH inhibits proliferation of melanoma cells. **A:** In medium 154 without growth factors, with 0.2 mM calcium, CRH-R1 agonist (UCW) significantly decreased cell viability (measured with MTT assay) of SKMEL-188 melanoma cells ($EC_{50} = 2.6 \times 10^{-12}$ M). **B:** This was caused by decrease in transition of cells from G₁/0 to S phase cell cycle compartment (see table below). Control, continuous line, CRH (100 nM), dashed line. * $P < 0.05$, ** $P < 0.005$.

Treatment	G ₁ /0 [%]	S [%]	G ₂ /M [%]
Control	61±1	17±1	16±0.2
CRH [100 nM]	67±1*	17±0.4	12±1**

TABLE 1.

Characteristics of cells tested

Cell type	CRH-R1 ^a protein	Second messenger coupling		
		cAMP	IP3	Ca ²⁺
Primary cultures				
Adult epidermal keratinocytes	+	-	+	^b
Neonatal epidermal melanocytes	+	+	+	^b
Adult dermal fibroblasts	+	+	+	ND
Cell lines				
Immortalized (PIG-1) melanocytes	+	+	+	ND
Immortalized (HaCaT) keratinocytes	+	+	+	^b
Squamous cell carcinoma (C ₄ .1)	+	+	ND	ND
WM 35 melanoma (RGP)	+	+	ND	ND
WM 98 melanoma (VGP)	+	+	ND	ND
WM 164 melanoma (metastatic)	+	+	ND	ND
WM 1241D melanoma (metastatic)	+	+	ND	ND
WMSBC2 melanoma	+	+	ND	ND
SKMEL-188 melanoma (hypomelanotic)	+	-	+	^b

ND, not done; RGP, radial growth phase; VGP, vertical growth phase.

^a CRH-R1 immunoreactivity was detected by immunocytochemistry and western blotting.^b Fazal et al. (1998); Slominski et al. (1999b); Wiesner et al. (2003).

TABLE 2.

Effect of ligand activation of CRH-R1 on cell proliferation

Cell type	Effect on cell proliferation in culture media	
	Containing growth factors	Free of growth factors
Primary cultures		
Epidermal keratinocytes	Inhibition. ^{a b}	Inhibition ^a
Epidermal melanocytes	Inhibition	stimulation
Adult dermal fibroblasts	Lack of effect	Stimulation
Cell Lines		
HaCaT keratinocytes	Inhibition ^c	Inhibition ^c
PIG-1 melanocytes	Inhibition	Stimulation
SKMEL-188 melanoma	Variable	Inhibition

ND, not done.

^aZbytek and Slominski (2005).^bQuevedo et al. (2001).^cSlominski et al. (2000d), (2003); Zbytek and Slominski (2005).