Intracellular calcium measurements of single human skin cells after stimulation with corticotropin-releasing factor and urocortin using confocal laser scanning microscopy

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Summary

Using confocal laser scanning microscopy we investigated the Ca^{2+} distribution in single corticotropin releasing factor- and urocortin-stimulated human skin cells. The models tested included melanoma cells, neonatal melanocytes and keratinocytes, and immortalized HaCaT keratinocytes. The changes in intracellular Ca^{2+} signal intensities observed after stimulation of different cell types with corticotropin releasing factor and urocortin showed that: (1) the increase of intracellular Ca^{2+} concentration was caused by a Ca^{2+} influx (inhibition by EGTA); (2) this Ca^{2+} influx took place through voltage-activated Ca^{2+} ion channels (inhibition by d-cis-diltiazem, verapamil) and (3) cyclic nucleotide-gated ion channels were not involved in this process (no effect of Mg^{2+}). The effects were also observed at very low peptide concentrations (10^{-13} M) with no apparent linear correlation between peptide dosage and increase of fluorescence intensity, which implied co-expression of different corticotropin releasing factor receptor forms in the same cell. Immortalized (HaCaT) keratinocytes exhibited the strongest differential increases of a Ca^{2+} fluorescence after peptide-stimulation. Corticotropin releasing factor induced Ca^{2+} flux into the cytoplasm, while urocortin Ca^{2+} flux into the nucleus with a remarkable oscillatory effect. The latter indicated the presence of an intracellular urocortin-induced signal transduction pathway that is unique to keratinocytes.

Key words: CRF, Urocortin, Keratinocytes, Melanocytes, Calcium signaling, Calcium oscillation

Introduction

Corticotropin releasing factor (CRF), the proximal neuroendocrine element in the activation of the hypothalamic-pituitary-adrenal axis in mammals (HPA), coordinates the complex array of behavioral, autonomic and endocrine responses to stress. CRF was identified in 1981 as a 41 amino acid peptide originating in the hypothalamus (Vale et al., 1981). Two additional CRF-related peptides, urocortin (URC) and urocortin II (URC II), have been characterized (Reyes et al., 2001; Vaughan et al., 1995). Receptors for CRF and CRF-related peptides belong to the family of seven-transmembrane-domain G_s-protein-coupled receptors and are encoded by two genes: CRF receptors type 1 and 2 (CRF-R1 and CRF-R2) (Chalmers et al., 1996; Speiss et al., 1998). In humans several alternatively spliced variants of CRF-R1 and three of CRF-R2 (α, β and γ) have been identified to date (Linton et al., 2001; Perrin and Vale, 1999; Pisarchik and Slominski, 2001). The phenotypic effects of CRF and URC are mediated by CRF-R1 and CRF-R2; in general, both peptides are equipotent towards CRF-R1, while CRF-R2 has a greater affinity for URC than for CRF (Wei et al., 1998). URC II, however, is active only towards CRF-R2 (Reyes et al., 2001).

It is currently accepted that signal transduction through CRF-R is primarily linked to the activation of adenylate cyclase followed by the production of cyclic adenosine monophosphate (cAMP) (Perrin and Vale, 1999; Speiss et al., 1998). However, phospholipase C may also be activated, producing inositol triphosphate (IP_3), which in turn activates PKC-dependent and calcium-activated pathways (Chalmers et al., 1996; Speiss et al., 1998). CRF, URC and CRF-Rs are widely expressed in peripheral tissues, where CRF function may be important in the local regulation of homeostasis (Linton et al., 2001; Slominski et al., 2000d; Slominski et al., 2001). The intracellular signaling pathway activated through peripheral CRF receptors involves the production of cAMP and subsequent activation of protein kinase A (PKA) (Linton et al., 2001; Slominski et al., 2001). The involvement of calcium-activated pathways via phospholipase C or membrane-bound calcium channels has also been demonstrated, and CRF receptor-mediated activation of mitogen activated protein (MAP) kinase signal transduction pathways has been reported in several cell types (Linton et al., 2001; Slominski et al., 2001).

Mammalian skin is both a source of and a target for CRF-

related peptides (Slominski et al., 2000c; Slominski et al., 2001), which led to the proposal that the CRF/URC signaling system may play a central role in the skin's response to stress (Slominski et al., 1999; Slominski et al., 2000d; Slominski et al., 2001); skin produces CRF and URC and expresses CRF-R1 (Pisarchik and Slominski, 2001; Roloff et al., 1998; Slominski et al., 1995; Slominski et al., 1996; Slominski et al., 1998; Slominski et al., 2000a; Slominski et al., 2000c). These CRF-Rs are functional, e.g., they respond to CRF and URC through activation of receptor(s)-mediated pathways to modify skin cell phenotype (Fazal et al., 1998; Quevedo et al., 2001; Slominski et al., 1999; Slominski et al., 2000b; Slominski et al., 2001). Human keratinocytes, both normal immortalized (HaCaT) and neonatal express CRF-R1 and bind CRF (Quevedo et al., 2001; Slominski et al., 1999; Slominski et al., 2000b; Slominski et al., 2001). Furthermore, CRF stimulated cAMP production and inhibited the proliferation of HaCaT keratinocytes with a higher potency than either urocortin or sauvagine (Slominski et al., 2000b). Similarly, CRF modulated the interferon induced expression of hCAM and ICAM-1 adhesion molecules and of the HLA-DR antigen in neonatal keratinocytes (Quevedo et al., 2001). In melanoma cells, CRF binding sites were detected, and CRF and URC, added to cell suspensions, produced dose-dependent increases in intracellular calcium. The effect showed a fairly rapid onset (within a second) (Fazal et al., 1998; Slominski et al., 1999); the Ca^{2+} signal did not, however, return to the basal level, but rather continued to increase gradually with time. To understand this phenomenon better we investigated in detail the mechanism of CRF- and URC-mediated calcium signaling in skin cells in situ, using laser confocal microscopy. This technique has a high space resolution, which we exploited to investigate the Ca^{2+} distribution in single peptide-stimulated cells.

Materials and Methods

Reagents

Human CRF and the α-helical CRF(9-41) were obtained from Michael Beyermann (Institute of Molecular Pharmacology, Berlin, Germany). Human URC were obtained from Edward Wei (University of California, Berkeley, CA) and J. K. Chang (Phoenix Pharmaceuticals, Mountain View, CA) and M. Beyermann. The purity of the custom synthesized peptides was 95-99% as determined by high performance liquid chromatography (HPLC) and mass spectrometry. Dulbecco's modified minimal essential medium (DMEM), Ham's F10 medium, fetal bovine serum (FBS), antimycotic/antibiotic mixture, trypsin (0.25%) and calcium/magnesium-free Hank's solution were from Gibco BRL Life Technologies (Gaithesburg, USA). Low-calcium (0.15 mM) serum-free keratinocyte growth medium (KGM) culture medium containing human recombinant epidermal growth factor, insulin, hydrocortisone, bovine pituitary extract (BPE) and antibiotics, MCDB 154 medium and BPE were from Clonetics Corp. (San Diego, CA). The cell-permeant acetomethoxy ester of FLUO-3 (Haugland, 1992; Minta et al., 1989; Thomas and Devaille, 1991) (Fluo-3/AM, MoBiTech GmbH Göttingen, Germany) was used in the presence of Pluronic F-127 (MoBiTech GmbH, Göttingen, Germany) as Ca^{2+} indicator. All other reagents were purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany).

Cells

Semi-confluent HaCaT immortalized human keratinocytes were maintained at 37°C and 5% CO_2 in DMEM medium supplemented with antibiotics in the presence of 10% FBS as described previously (Slominski et al., 2000b). Human melanoma cells (SKMEL188) were grown in Ham's F10 medium following standard methods; the media were supplemented with 10% fetal bovine serum and antibiotics (Slominski et al., 1998). Normal human neonatal keratinocytes were a gift from Mitchell Denning, Loyola Medical Center (Quevedo et al., 2001). Primary cell cultures were established from foreskin as described previously (Quevedo et al., 2001). The cells were propagated in low-calcium (0.15 mM), serum-free KGM containing BPE (Quevedo et al., 2001). Normal human neonatal melanocytes (a gift of Z. Abdel-Malek, University of Cincinnati, OH) were cultured in MCDB 153 supplemented with 4% FBS, 13 μg/ml BPE, 8 nM TPA, 1 μg/ml α-tocopherol, 0.6 ng/ml basic fibroblast growth factor, 1 μg/ml transferrin, 5 μg/ml insulin and 1% antibiotic-antimycotic mixture as described previously (Abdel-Malek et al., 1995).

For optical measurements, the cells were cultivated for 2 days on glass cover slips (30 mm diameter). After washing with phosphate buffered seline (PBS), cells were loaded with the Ca^{2+} indicator Fluo-3/AM (4.4×10^{-6} M in the culture medium (see above) in the presence of 0.01% Pluronic F-127) for 30 minutes at 37°C in darkness, then washed three times and incubated in the medium (see below for the measurements).

Optical Ca^{2+} measurements using confocal laser scanning microscope

The present studies were performed utilizing an LSM 410 and an LSM 510 invert confocal laser scanning microscope (Carl Zeiss Jena, Germany) with a ×100/1.3 oil immersion objective and an argon-krypton laser (488 nm) excitation source. The excitation wavelength was selected by a dichroitic mirror (FT510). The fluorescence intensities were detected at wavelengths greater than 515 nm using an additional cut-off filter (LP515) in front of the detector. Fluorescence images were scanned and stored as a time series (approx. 11 minutes). The peptides were applied to the cells 1 minute after scanning began. Regions of interest (ROI's) were subsequently selected for determination of the fluorescence intensities in the cytosol and the nucleus. These data were stored as ASCII files and computed off-line. Different solutions were used as the extracellular medium:

medium 1 [Ca^{2+}] in mM: 140 NaCl, 4.6 KCl, 2 $CaCl_2$, 10 glucose;

medium 2 [0 Ca^{2+}] in mM: 140 NaCl, 4.6 KCl, 0.55 EGTA, 10 glucose;

medium 3 [Mg^{2+}] in mM: 140 NaCl, 4.6 KCl, 2 $CaCl_2$, 15 $MgCl_2$, 10 glucose.

All three mediums (≈290 mOsm/kg) were adjusted to pH 7.4 (with NaOH). Medium 1 represented a normal extracellular solution for intracellular Ca^{2+} measurements (Weisner and Hagen, 1999). EGTA (in medium 2) has a high affinity for Ca^{2+} and complexes the free Ca^{2+} in the medium; under these conditions a Ca^{2+} influx into the cells is impossible (Bers, 1998; McGuian et al., 1991; Miller and Smith, 1984; Williams and Fray, 1990). The function of cyclic nucleotide-gated (CNG) ion channels is blocked by extracellular Mg^{2+} (three to five times more effectively than Ca^{2+}) (Frings et al., 1995; Weyland et al., 1994). With medium 3 we have the possibility of testing whether the increase of intracellular Ca^{2+} concentration is caused by a Ca^{2+} influx through such CNG channels. The extracellular Mg^{2+} concentrations (10-15 mM) were selected so as not to cause blockage, however, of voltage activated Ca^{2+} ion channels (McDonald et al., 1994). The cells were variously preincubated for 30 minutes with:

(1) d-cis-diltiazem [DIL]: 2.5×10^{-5} M in medium 1;

(2) verapamil [VER]: 2.5×10^{-6} M in medium 1;

(3) staurosporine [STA]: 1×10^{-6} M in medium 1.

D-cis-diltiazem (Ferry et al., 1993; Matlib and Schwartz, 1983) and verapamil (Atlas and Adler, 1981; Fleckstein, 1977) cause the blockage of voltage gated ion channels in the plasma membrane. Staurosporine is an effective inhibitor of the cyclic guanosine

monophosphate (cGMP)- and the cAMP-dependent protein kinases, as described previously (Matsumoto and Sasaki, 1989; Tamakori et al., 1996). These media and active compounds afforded various possibilities of investigating changes in the intracellular Ca^{2+} signal.

Results

Cytosolic calcium levels after application of CRF

Changes in intracellular Ca^{2+} signal intensity after CRF stimulation are shown in Fig. 1. An increase in $[Ca^{2+}]_i$ is evident for all cell types at peptide concentrations of 10^{-11}-10^{-7} M; in the case of HaCaT cells this is seen at concentrations as low as 10^{-13} M (P<0.05, Fig. 1A,C,E,G). However, the increase in Ca^{2+} signal appears to be independent of the concentration of ligand used. Fig. 1B,D,F,H illustrate that an increase of intracellular Ca^{2+} signal (shown for a concentration of 10^{-10} M CRF) is found when the medium containing Ca^{2+} and Mg^{2+} is used, and is inhibited in the calcium-free medium (EGTA is present). Pre-exposure of the cells to the voltage-gated Ca^{2+} channel blockers d-cis-diltiazem (DIL) and verapamil (VER) and an effective inhibitor of the cGMP- and the cAMP-dependent protein kinases staurosporine (STA) significantly inhibited the stimulatory effect of CRF. In addition, DIL, VER and STA inhibited basal intracellular calcium in HaCaT cells, which suggested a possible attenuation of autocrine stimulation of intracellular calcium levels in this cell type

Cytosolic calcium levels after application of URC

The data for calcium level changes in HaCaT and melanocytes after URC treatment (Fig. 2) shows lack of dose-dependent responses for URC. Which is similar to that presented for CRF in Fig. 1. Thus, URC significantly stimulated intracellular $[Ca^{2+}]_i$ in HaCaT keratinocytes at concentrations of 10^{-13}-10^{-7} M and in normal melanocytes at 10^{-10} M. The effects were significantly inhibited by EGTA, DIL, VER and STA.

Characterization of the receptor-mediated Ca^{2+} response

The receptor antagonist α-helical CRF(9-41) was used to clarify the nature of the receptor-mediated signal pathway (Chalmers et al., 1999). Human normal melanocytes were pre-incubated with 10^{-6} M antagonist for 20 minutes in cell medium, which was subsequently replaced by medium 1 for the measurements. The pre-treatment with α-helical CRF(9-41) abolished or significantly inhibited Ca^{2+} uptake in melanocytes after CRF or URC stimulation (Fig. 3).

Measurements of intra-nuclear Ca^{2+} indicator fluorescence

Intra-nuclear fluorescence intensity changes, measured after peptide stimulation, are depicted in Fig. 4. The nuclear fluorescence values are expressed as ratios to the corresponding cytosolic values. The data from HaCaT

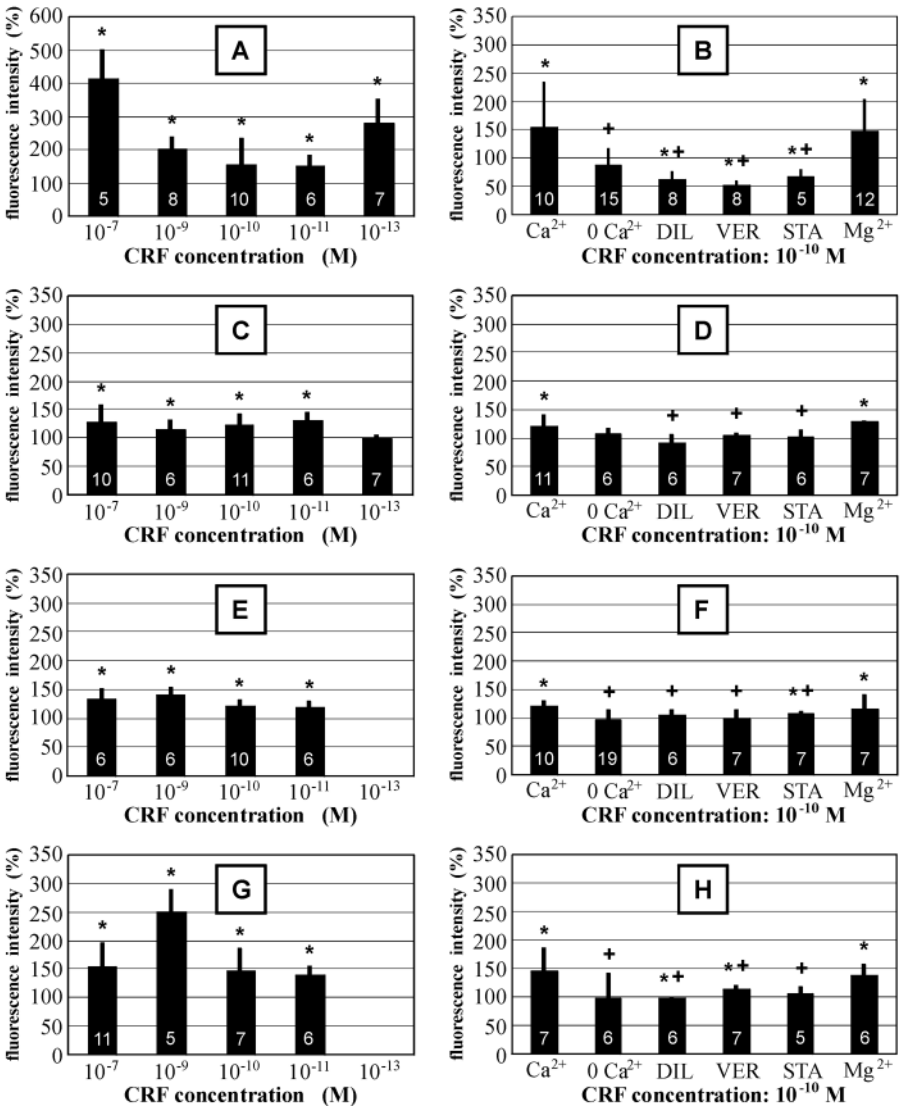

Fig. 1. Relative cytosolic fluorescence intensities of the Ca^{2+} indicators (mean and s.d.; control=fluorescence intensity before the application of the peptide=100%) at the final values of the time-dependent response (see Fig. 6) in HaCaT keratinocytes (A,B), neonatal normal keratinocytes (C,D), human melanoma cells (E,F) and neonatal normal melanocytes (G,H) after stimulation with different concentrations of CRF (A,C,E,G) and with different extracellular mediums and preincubation regimes (B,D,F,H). *Statistically significant differences (P<0.05, unpaired t-test) from the control; +, statistically significant differences (P<0.05, unpaired t-test) from the effect after stimulation with 10^{-10} M CRF. Number of cells recorded (n) is shown in the respective columns.

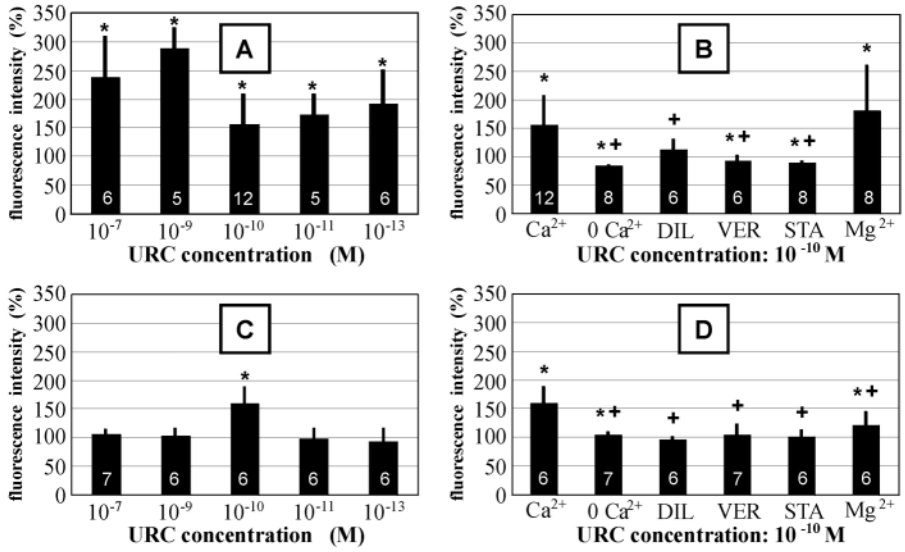

Fig. 2. Relative cytosolic fluorescence intensities of the Ca^{2+} indicators (mean and s.d.; control=fluorescence intensity before the application of the peptide=100%) at the maximal values of the time-dependent response (Fig. 6) in HaCaT keratinocytes (A,B) and human melanocytes (C,D) after stimulation with different concentrations of URC (A,C) and using different extracellular medium and preincubation conditions (B,D). *Statistically significant differences (P<0.05, unpaired t-test) from the control; +, statistically significant differences (P<0.05, unpaired t-test) from the effect after stimulation with 10^{-10} M URC. Number of cells recorded (n) is shown in the respective columns.

cells (Fig. 4A) indicate a nuclear Ca^{2+} fluorescence intensity 1.25-1.45-fold higher than that of the cytosol after URC stimulation at 10^{-10}-10^{-7} M, and a ratio close to 1 at 10^{-13}-10^{-11} M. In contrast, after CRF stimulation, the ratios were consistently below 0.5 over the complete concentration range employed, i.e. they were only approximately one-third as high as those calculated for URC stimulation. This striking difference between nuclear and cytosolic signals was not observed in the case of human malignant melanocytes (Fig. 4B), in which the median relative intensity increases were lower, e.g., at 1.3 after URC (comparable to HaCaT cells), and 1.0 after CRF stimulation. Although at peptide concentrations of 10^{-11}-10^{-9} M the ratios were significantly higher for URC than for CRF, these differences did not reach the striking values observed for HaCaT keratinocytes. In addition, the ratios were observed on neonatal epidermal keratinocytes and neonatal normal melanocytes. These yielded median relative ratios (Fig. 4C) of 0.81 and 0.87, respectively, after CRF stimulation, emphasizing again the differences between these cell lines and HaCaT keratinocytes. Furthermore, at concentrations of 10^{-11} and 10^{-10} M of CRF the ratios were significantly lower in neonatal keratinocytes than in neonatal melanocytes, indicating a difference between epithelial and pigment cells. The time-gallery of confocal images (Fig. 5) of HaCaT cells confirms the effcets shown in Fig. 6A and B.

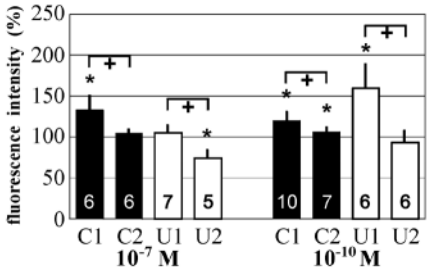

Fig. 3. Relative cytosolic fluorescence intensities of the Ca^{2+} indicators (mean and s.d.; control=fluorescence intensity before the application of the peptide=100%) at maximal (URC) or final (CRF) values of the time-dependent response (see Fig. 6) in human melanocytes after preincubation with the specific antagonist α-helical-CRF(9-41) (2) and stimulation with CRF (shaded, C) and URC (unshaded, U). *Statistically significant differences (P<0.05, unpaired t-test) from the control (control=100%); +, statistically significant differences (P<0.05, unpaired t-test) from the effects after stimulation with either peptide in the absence of antagonist (1). Number of cells recorded (n) is shown in the respective columns.

Time course of the changes of intracellular Ca^{2+}

Fig. 5 illustrates the variable intranuclear and cytosolic fluorescence changes following CRF and URC stimulation. Thus, URC predominantly stimulates $[Ca^{2+}]_i$ flux into the nucleus (right panel), while CRF into the cytoplasm (left panel). Note that there is an oscillatory effect of URC on $[Ca^{2+}]_i$ in HaCaT cells, while CRF induces a linear accumulation of Ca^{2+}. The relative fluorescence intensities (cytosolic and intra-nuclear) from the images in Fig. 5 over an 11 minute period of observation are summarized in Fig. 6A and B. Cytosolic fluorescence (Fig. 6A) is seen to rise continuously over the time period after CRF application, while that within the nucleus follows only after an apparent lag of around 200 seconds. After 11 minutes, a significant increase in cytosolic fluorescence is established. The signal pattern is, however, different after URC stimulation (Fig. 6B). In this case, the increase of intra-nuclear fluorescence intensity appears stronger from the beginning than was indicated by the difference in relative intensities (Fig. 4A). We have assumed that calcium increases in the cytosol before reaching the nucleus; however, the sensitivity of the time of measurements does not allow accurate accurate assessment of intracellular localization.

Furthermore, URC-induced oscillations in Ca^{2+} levels are observed in both the cytosol and nucleus, as indicated in Fig. 5B. Additional data pertaining to these oscillations are presented in Fig. 6. It can be seen that the frequency of the oscillations is independent from the peptide concentration (Fig. 6C). It begins to change only at the concentration of 10^{-15} M. In addition to this concentration-independent variation in

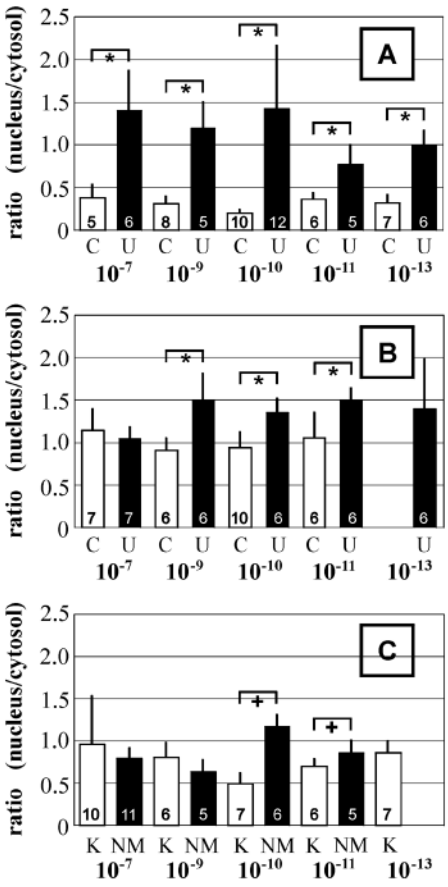

Fig. 4. Ratio of the relative fluorescence intensities (nucleus/cytosol; mean and s.d.) of the Ca^{2+} indicators at maximal (URC) or final (CRF values of the time-dependent response (see Fig. 6) in HaCaT keratinocytes (4A) and human malignant melanocytes (4B) after stimulation with URC (U, filled bars) and CRF (C, unfilled bars) and in neonatal keratinocytes (K, unfilled) and normal melanocytes (NM, filled) after stimulation with CRF (4C). *Statistically significant differences (P<0.05, unpaired t-test) between the ratios after stimulation with URC and with CRF; +, statistically significant differences (P<0.05, unpaired t-test) between the ratios in keratinocytes and normal melanocytes. Number of cells recorded (n) is shown in the respective columns.

oscillation frequency, the numbers of cells displaying such oscillations remain rather constant with a decrease only at concentration of 10^{-15} M (Fig. 6D). The observed oscillatory behavior appears to be a specific characteristic of the response of HaCaT keratinocytes to URC stimulation, since it has not been seen in any other cell type.

Discussion

Receptor-mediated changes in cytosolic Ca^{2+} levels after application of CRF and URC

The changes in intracellular Ca^{2+} signal intensities observed after stimulation of different cell types with CRF and URC imply that: 1) the increase of intracellular Ca^{2+} concentration is caused by a Ca^{2+} influx (results of EGTA experiments), 2) this Ca^{2+} influx takes place through voltage-activated Ca^{2+} ion channels (results with DIL, VER, STA) and 3) cyclic nucleotide-gated ion channels do not have an important role in this process (results with Mg^{2+}). These conclusions are in agreement with studies with non-skin models, which showed a CRF-stimulated Ca^{2+} influx through voltage-activated ion channels of the L-, P- or T-type in other cell lines (Guerineau et al., 1991; Kiang, 1994; Kuryshev et al., 1996). Of the cell types used in this study, the HaCaT keratinocytes consistently exhibited the strongest increase of Ca^{2+} fluorescence intensity after peptide-stimulation, which is in agreement with a central role of calcium in the regulation of differentiation and proliferation of keratinocytes.

The results with both peptides showed no correlation between peptide dosage and increase of the fluorescence intensities (dose-effect-correlation). We also observed the increase in intracellular Ca^{2+} levels at very low peptide concentrations, e.g., 10^{-13} M (see Figs 1, 2), which contrasted

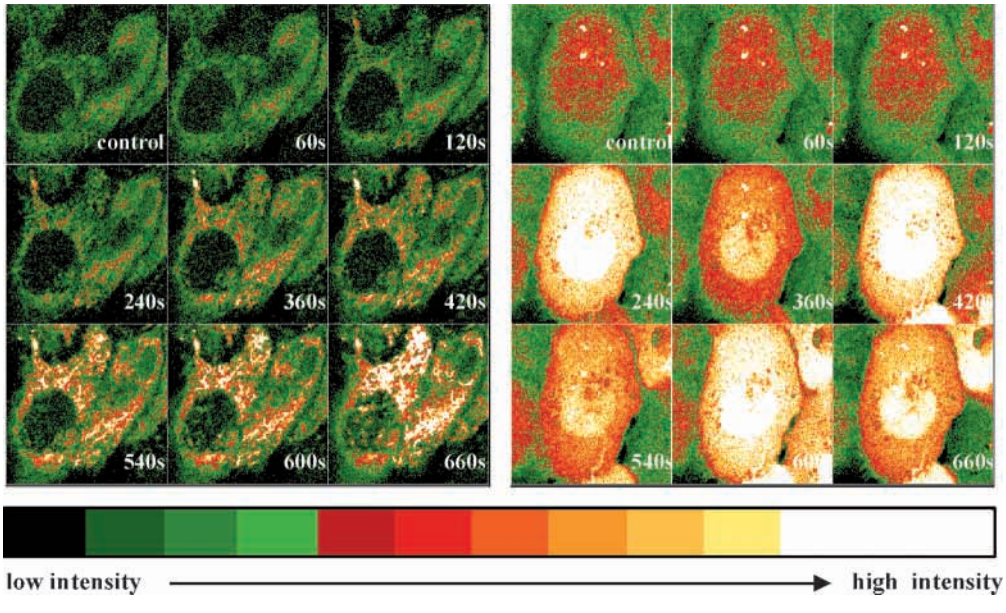

Fig. 5. Time galleries of fluorescence images of the Ca^{2+} indicators in HaCaT after stimulation with CRF (10^{-7} M, left panel) and URC (10^{-7} M, right panel). The intensity range (8 bit) is indicated below the images galleries.

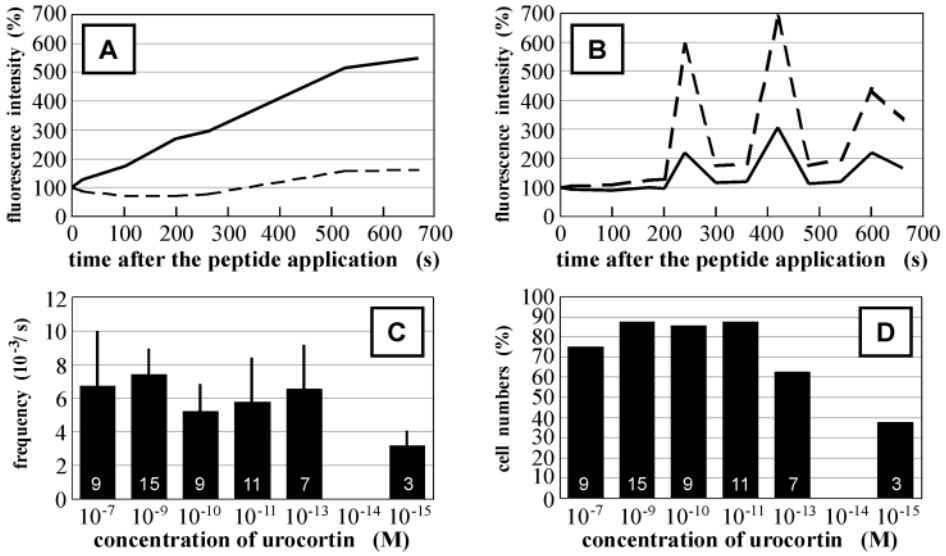

Fig. 6. (A,B) Time course of the relative fluorescence intensities (unbroken line, cytosol; broken line, nucleus) of the Ca^{2+} indicators in two single HaCaT cells after stimulation with CRF (A, 10^{-7} M) and URC (B, 10^{-7} M). (C) Dose-dependent frequency of the oscillations in HaCaT-cells after stimulation with URC (mean and s.d.). Number of cells recorded (*n*) is shown in the respective columns. (D) Dose-dependent numbers of HaCaT cells with oscillations after stimulation with URC (percentage of total number of cells recorded). Number of cells recorded with oscillations (*n*) is shown in the respective columns.

with reported K_D's of 10^{-10}-10^{-9} M and 10^{-8} M for CRF and URC in CHO cells transfected with CRF-R1 and CRF-R2 cDNAs, respectively (Tojo and Abou-Samra, 1993; Vaughan et al., 1995). This raised an important question on the nature of the signal pathways activated by CRF and URC. The preincubation of the cells with the specific CRF-R antagonist α-helical CRF(9-41) reduced CRF or URC induced increases in the Ca^{2+} levels (see Fig. 3). Moreover, previous studies showed the expression of CRF-R1 in melanocytes and keratinocytes and the presence of specific cell surface CRF binding sites (Fazal et al., 1998; Slominski et al., 1995; Slominski et al., 1999; Slominski et al., 2000b). These receptors were functional, i.e. CRF and URC inhibited growth, stimulated cAMP production and modulated the expression of adhesion molecules in cultured human keratinocytes in a dose-dependent fashion (Quevedo et al., 2001; Slominski et al., 2000b; Slominski et al., 2001). Therefore, we conclude that the reported stimulation of Ca^{2+} fluorescence intensity by CRF and URC is mediated through interaction with CRF receptors. In other systems similar effects of CRF appear to be mediated by a pathway involving the adenylate cyclase/cAMP-dependent protein kinase system (Guild and Reisine, 1987; Reisine, 1989; Tojo and Abou-Samra, 1993).

CRF-R1α is the most efficient receptor isoform for transducing the CRF signal into cAMP mediated pathways, while other isoforms (β, c and d) are inefficiently coupled to cAMP production (Chalmers et al., 1996; Linton et al., 2001; Perrin and Vale, 1999; Speiss et al., 1998). Since the most prevalent form of the CRF receptor expressed in human skin is the R1α isoform (Pisarchik and Slominski, 2001; Slominski et al., 2001), and STA significantly inhibits the stimulatory effect of CRF on Ca^{2+}, we suggest that some of these effects are mediated by CRF-R1α through cAMP-dependent protein kinases. However, the voltage-gated ion channel blockers DIL and VER also block Ca^{2+} influx. The stimulatory effect is observed at ligand concentration 10^{-13} M, i.e. far below the K_D's described for CRF-R1 (see above). The last finding is consistent with previously reported data showing that CRF at 10^{-12} M can induce entry of Ca^{2+} in melanoma cells (Fazal et al., 1998). However, the reported effect was very rapid (within one second), while the current changes were observed only after several minutes of incubation. Referring to the time response similar Ca^{2+} signals were observed after ACh-induced Ca^{2+} release in oocytes expressing two hybrid mAChRs (Lechleiter, 1991). In this investigation a continuous increase in the Ca^{2+} signal around 500 seconds after stimulation was found. As regards to the response to low peptide concentrations another study showed that CRF-like peptides 10^{-15} M stimulated a Ca^{2+} response in human epidermoid A-431 cells (Kiang, 1997). Moreover, stimulus-response curves for muscarinic and α-adrenoreceptors indicate that maximal response can be produced by submaximal stimuli (submaximal receptor occupancy) (Kenakin, 1993). This phenomenon, described as "receptor reserve" (spare receptors, spare capacity), leads to an ultrasensitivity of the system. In this case, the dose-response curve is not a typical Michaelis-Menten curve but rather a nearly digital response (all or nothing) (Germain, 2001).

Therefore, we suggest that skin cells co-express different CRF-R forms, which are coupled to different signal transduction pathways (Pisarchik and Slominski, 2001; Slominski et al., 2001). Although the individual functions of the various receptor subtypes remains to be determined, the differential regulation of Ca^{2+} signal by CRF and URC in HaCaT keratinocytes (Figs 5, 6) show similarities to those described by others (Germain, 2001; Kenakin, 1993; Kiang, 1997; Lechleiter et al., 1991; Reetz and Reiser, 1996).

Unusual ligand induced Ca^{2+} signaling in HaCaT keratinocytes

The strong signal enhancement observed in the nucleus of HaCaT cells after stimulation with URC is in striking contrast with the absence of such an effect after exposure to CRF. It has previously been accepted that the intracellular signal pathway is the same for both peptides. The existence of Ca^{2+}-permeable pores in the nuclear membrane is indisputable (Bhattacharya et al., 1999; Kenakin, 1993), but we observed Ca^{2+} flux into the nucleus only in the case of URC. IP_3

receptors have also been reported on the nuclear membrane. When IP_3 binds to these receptors, a Ca^{2+} influx from the cytosol to the nucleus, or conversely, Ca^{2+} release from the nucleus in the opposite direction are possible (Challis and Wilcox, 1996; Wilcox et al., 1994). In this case, an increase of intracellular IP_3 would be expected after stimulation with URC. Although IP_3 signaling has not been analyzed in HaCaT cells, CRF-related peptides induce IP_3 production and subsequent Ca^{2+} mobilization in A-431 epidermal carcinoma cells (Kiang et al., 1995). Therefore, it is likely that in HaCaT keratinocytes the URC signal would be similarly coupled to production of IP_3. In addition, these data show that CRF and URC signals are transduced through different receptor subtypes in keratinocytes. Furthermore, the nuclear targeting of the URC signal (e.g., intracellular calcium oscillations) suggests a role for this pathway in transcriptional regulation. Others also reported nuclear calcium transient rise in response to integrin-ligand interaction (Shankar et al., 1993) or after stimulation of rat liver nuclear prostaglandin receptors (Bhattacharya et al., 1999).

The differences in time courses of the fluorescence signals after peptide stimulation are intriguing. Stimulation of HaCaT cells with URC produced a remarkable oscillatory effect. Oscillation of intracellular Ca^{2+} levels was previously demonstrated in bovine pulmonary artery endothelial cells over approximately 15 minutes after stimulation with the peptide bradykinin (Sage et al., 1989). A similar oscillatory increase in cytosolic Ca^{2+} concentration was observed after stimulation with CRF (Kuryshev et al., 1996). Furthermore, a long-term perfusion with bradykinin was described as causing oscillation of cytosolic Ca^{2+} activity in rat glioma cells (Reetz and Reiser, 1996). This Ca^{2+} rise was associated with synchronous plasma membrane oscillating hyperpolarization. The authors proposed that the initial transient Ca^{2+} rise induced immediately with bradykinin administration resulted from IP_3-mediated Ca^{2+} release, whereas subsequent oscillations depended mainly on Ca^{2+} influx. Such Ca^{2+} spikes were observed in time intervals from seconds to minutes. This phenomenon was generally discussed as a mechanism of regeneration, e.g. Ca^{2+}influx stimulates an increase in phospholipase C activity whereby an increase of IP_3 occurs and this results in a Ca^{2+} release (Woods et al., 1986). Oscillating Ca^{2+} concentrations might thus have been anticipated after stimulation of CRF receptors. Our data indicate, however, that this was the case only after stimulation with URC i.e. not after CRF stimulation (see Fig. 6). Thus, these results show that HaCaT keratinocytes behave distinctly differently from other cell types and imply the presence of an intracellular signal transduction pathway for URC. In this context, HaCaT keratinocytes serve as an excellent cell model for further characterization of this pathway.

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