Effect of CRF and related peptides on calcium signaling in human and rodent melanoma cells

Nadeem Fazal[a], Andrzej Slominski[a,*], Mashkoor A. Choudhry[b], Edward T. Wei[c], Mohammed M. Sayeed[b]

[a] *Department of Pathology, Medical Center, Loyola University, 2160 First South Avenue, Maywood, IL 60153, USA*
[b] *Department of Physiology, Medical Center, Loyola University, 2160 First South Avenue, Maywood, IL 60153, USA*
[c] *School of Public Health, University of California, Berkeley, CA, USA*

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Abstract Corticotropin releasing factor (CRF) induces a rapid, within seconds, and dose-dependent increase in the intracellular Ca^{2+} in both human and hamster melanoma cells. This effect is inhibited by depletion of extracellular calcium using 3 mM EGTA and is attenuated by the CRF receptor antagonist, α-helical-CRF(9-41). Other peptides of the CRF superfamily, sauvagine and urocortin, also induce increases in cytoplasmic calcium concentration but at higher concentrations than CRF. We conclude that malignant melanocytes express CRF receptors, which are coupled to activation of plasma membrane calcium channels.

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Key words: Corticotropin releasing factor; Urocortin; Sauvagine; Receptor; Calcium; Malignant melanocyte

1. Introduction

It has been proposed that mammalian skin contains an equivalent of 'hypothalamic-pituitary axis' composed of locally produced corticotropin-releasing factor (CRF) and pro-opiomelanocortin (POMC) derived peptides as an important mechanism of skin response to stress [1,2]. It is already well documented that skin acts not only as a target for POMC derived peptides including ACTH, MSH, and β-endorphin but also as sites of their production [2–5]. Skin cells also express the CRF gene and produce CRF peptide, which is stimulated by ultraviolet radiation (UVR) [6–9]. Most recently, expression of CRF-receptor (CRF-R) subtype-1 mRNA and protein was detected in the mammalian skin [6,8–10].

The CRF signal is translated through an interaction with G-protein coupled CFR-R [11]. At the central level this mechanism involves stimulation of adenylate cyclase, phospholipase C or activation of calcium channels [11,12]. The CRF receptors include those which show high specificity towards CRF and receptors for which the specific ligands are urocortin or sauvagine peptides [13,14]. A potential functional role, if any, of CRF receptors in the skin remain enigmatic. Therefore, we have undertaken an effort to define mechanisms of signal transduction through the CRF receptors in malignant human and hamster melanocytes.

*Corresponding author. Fax: (1) (708) 3272620.
E-mail: aslomin@wpo.it.luc.edu

2. Materials and methods

2.1. Materials

Human CRF synthesized by standard solid phase method was purchased from Molecular Research Lab (North Carolina). Urocortin and sauvagine were purchased from Peninsula Labs (Belmont, CA, USA), α-helical-CRF(9-41) was a gift from Dr. Nicholas Ling (Neurocrine Biosciences, San Diego, CA, USA). Ham F-10, Dulbecco's Modified Essential Medium (DMEM), fetal bovine serum, Hanks' balanced salt solution (HBSS), antibiotic antimycotic mixture and 0.25% trypsin+1 mM EDTA were purchased from Gibco (Grand Island, NY, USA). Fura-2/AM was from Molecular Probes (Oregon).

2.2. Cell culture

HaCaT immortalized keratinocytes (obtained from Dr. Higgins, Albany Medical College, Albany, NY, USA), human melanoma SK-MEL188 line (obtained from Dr. Chakraborty, Yale University, New Haven, CT, USA) and hamster melanoma AbC1 were used for the experiments. Semi-confluent cultures of cells were maintained in Ham F-10 or DMEM media with antibiotics in the presence of 10% fetal bovine serum in 75-cm^2 flasks at 37°C in the presence of 5% CO_2, as described previously [7,15]. Media were changed every second day. Melanoma cells were detached from the substratum by HBSS+1 mM EDTA and HaCaT keratinocytes were detached with 0.25% trypsin+1 mM EDTA, then after centrifugation cells were washed in HBSS and processed for calcium analysis.

2.3. Measurement of intracellular calcium flux

The measurements of $[Ca^{2+}]_i$ were performed using methods described previously [16]. Briefly, cells were loaded with 2 M Fura-2/AM (Molecular Probes, OR) for 1 h at room temperature. The unbound Fura-2 was washed using HBSS. Fura-2 loaded cells were transferred to a fluorometer cuvette. Fluorescence signals were recorded using Hitachi Spectrofluorometer (Model F-2000) at excitation wavelengths of 340 and 380 nm, and the emission measured at 540 nm. The cells were stimulated with different peptides after 90–100 s of the initial response (basal). A peak elevation in $[Ca^{2+}]_i$ occurred immediately after the addition of peptide. This peak elevation in $[Ca^{2+}]_i$ was taken as the peptide-induced elevation and was used for calcium analysis. The results were digitized and imported into a statistical analysis program (SigmaPlot) for quantitative analysis. Integrated $[Ca^{2+}]_i$ was estimated by calculating the area under the $[Ca^{2+}]_i$ response curve over a period of 300–600 s after stimulation of the cells.

Statistical analyses were performed using the Student's *t*-test. The differences were considered significant when $P < 0.05$.

3. Results and discussion

The basal $[Ca^{2+}]_i$ in control (unstimulated) human melanoma cells and HaCaT keratinocytes is 120 ± 20 nM (mean ± S.E.) and in hamster melanoma cells is 200 ± 20 nM (mean ± S.E.) per 2.5×10^6 cells. The addition of the peptides to the cells results in a significant elevation in the $[Ca^{2+}]_i$ (400–650 nM). As shown in Fig. 1A, an increase in $[Ca^{2+}]_i$ is observed within 1–3 s following different doses of CRF (10^{-7} to 10^{-12} M) in melanoma cells, and in HaCaT kerati-

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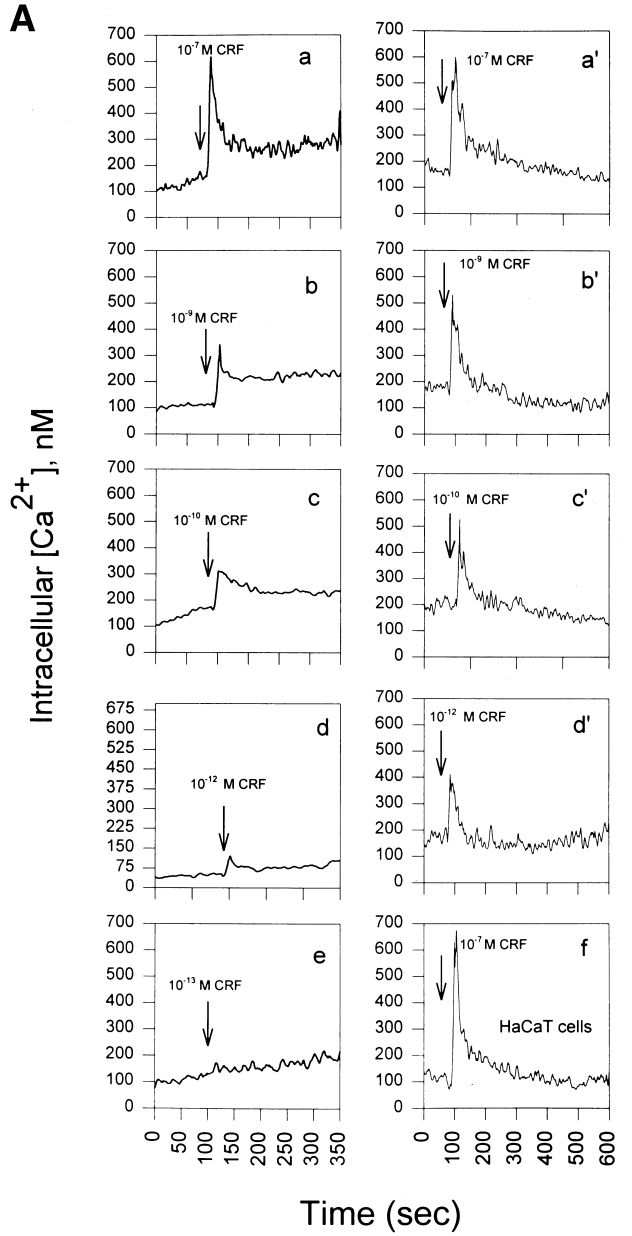

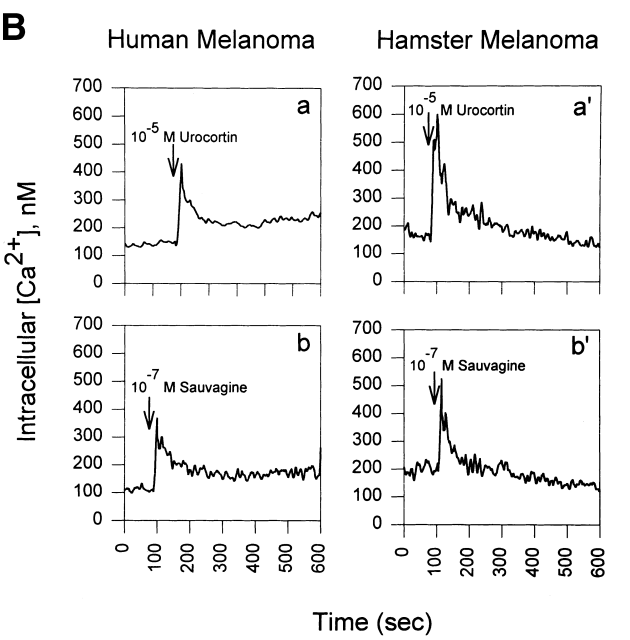

Fig. 1. The effect of CRF and related peptides on $[Ca^{2+}]_i$. A: Elevation of $[Ca^{2+}]_i$ stimulated by different doses of CRF in human (a–e) and hamster (a′–d′) melanoma cells, and HaCaT keratinocytes (f), measured by Fura-labeling at the time indicated by the arrow. B: Elevation of $[Ca^{2+}]_i$ stimulated by urocortin and sauvagine in human (left panel) and hamster (right panel) melanoma cells.
←

nocytes at concentration 10^{-7} M. There is no stimulation after addition of the solvent alone. Thus, this is a first demonstration that CRF has any effect on malignant melanocytes including an increase in $[Ca^{2+}]_i$. The stimulatory effect of CRF on HaCaT keratinocytes is in agreement with data reported by Kiang [17,18] who showed that CRF and CRF related peptides can stimulate intracellular calcium raises in squamous cell carcinoma line A431. However, we have discontinued further experiments with HaCaT cells because of a difficulty in maintaining them in single cell suspension.

Intracellular calcium release following stimulation of different concentrations of CRF in both human and hamster melanoma cells is dose dependent and saturable reaching a plateau at concentrations 10^{-9} to 10^{-7} M (Fig. 1 and Table 1), which suggests that the increase in $[Ca^{2+}]_i$ is due to specific interaction of CRF with its receptor on the cell. This has been confirmed by the use of CRF antagonist α-helical CRF(9-41) [19] at concentrations 10^{-9} and 10^{-8} M. Higher concentrations of α-helical CRF(9-41) (e.g. 10^{-7} M) cause an increase in $[Ca^{2+}]_i$ in a similar manner to CRF (results not shown). The representative experiment showing an inhibitory effect of α-helical CRF(9-41) is presented in Fig. 2. In this assay the average integrated $[Ca^{2+}]_i$ in cells after stimulation with 10^{-9} M of CRF (area under curve 8.9×10^4 nM/s) on human melanoma cells has been inhibited by one minute preincubation with α-helical CRF(9-41) (10^{-8} M) (area under curve 4.87×10^4 nM/s) (Fig. 2a,b). Similarly the effect of 10^{-9} M of CRF (area under curve 5×10^4 nM/s of calcium) on hamster melanoma cells is inhibited by preincubation with α-helical CRF(9-41) (10^{-8} M) (area under curve 3.62×10^4 nM/s of calcium) (Fig. 2a′,b′). Thus, preincubation with α-helical CRF(9-41) reduces the calculated area under the curve by 4×10^4 nM/s of calcium in human melanoma cells and by 2.6×10^4 nM/s of calcium in hamster melanoma cells. This inhibitory effect has been reproduced in 6 independent experiments (not shown). Thus, preincubation with α-helical CRF(9-41) (10^{-8} M) significantly decreases ($P<0.001$) and ($P<0.01$) the CRF induced $[Ca^{2+}]_i$ by $70\pm20\%$ ($n=6$) and $45\pm10\%$ ($n=6$) in human and hamster melanoma cells, respectively. Other peptides of the CRF superfamily, including sauvagine and urocortin, also stimulate intracellular calcium releases, however, at high concentrations, 10^{-5}–10^{-7} M, in human and hamster melanoma cells (Fig. 1B and Table 1). Both peptides are without any effect at concentrations 10^{-9} M. These results in conjunction with previous documentation of the expression of CRF-R_1 mRNA in mammalian skin [6–10] show that the increases in $[Ca^{2+}]_i$ in human and hamster malignant melanocytes are mediated through CRF receptors, most likely CRF-R_1.

The rapid increase of cytosolic Ca^{2+}, which peaks within 1–

Table 1
Stimulation of cytosolic free calcium concentration in melanoma cells by CRF related peptides

Addition (M)	Stimulation above the basal level (%)	
	Hamster melanoma	Human melanoma
CRF		
10^{-13}	No effect	No effect
10^{-12}	302 ± 20	234 ± 40
10^{-10}	303 ± 10	277 ± 10
10^{-9}	368 ± 20	346 ± 20
10^{-7}	490 ± 70	440 ± 30
10^{-5}	ND	ND
Urocortin		
10^{-13}	No effect	No effect
10^{-12}	No effect	No effect
10^{-10}	No effect	No effect
10^{-9}	No effect	No effect
10^{-7}	463 ± 70	310 ± 20
10^{-5}	426 ± 32	326 ± 15
Sauvagine		
10^{-13}	No effect	No effect
10^{-12}	No effect	No effect
10^{-10}	No effect	No effect
10^{-9}	No effect	No effect
10^{-7}	326 ± 10	320 ± 30
10^{-5}	513 ± 30	275 ± 15

ND: not done. The stimulatory effect is shown in relative units as % of basal level (non-induced cells) and represents a mean ± S.D. from four experiments.

3 s of CRF stimulation (Fig. 1), reduces the likelihood that the effect is mediated through cAMP or inositol triphosphate (IP3) mediated mechanisms. These pathways activate calcium influx from the extracellular environment or activate release of Ca^{2+} from internal stores, respectively [12,20]. Stimulation of either pathways, which are coupled to the CRF-R_1 signal

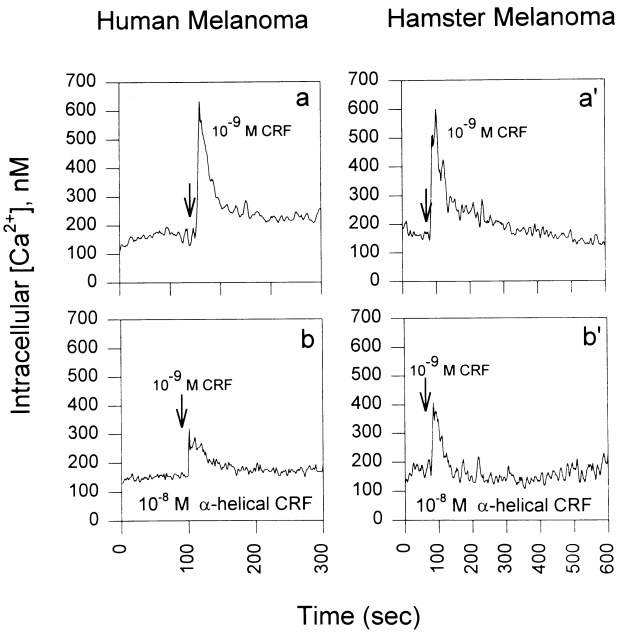

Fig. 2. Inhibitory effect of α-helical CRF(9-41) preincubation on $[Ca^{2+}]_i$ response to CRF stimulation. Changes in $[Ca^{2+}]_i$ were monitored using fluorescene $[Ca^{2+}]_i$ indicator dye, Fura-2, as outlined in Section 2. Upper panel: CRF (10^{-9} M) effect without any pretreatment; lower panel: CRF (10^{-9} M) effect after 1 min preincubation with α-helical CRF(9-41) (10^{-8} M).

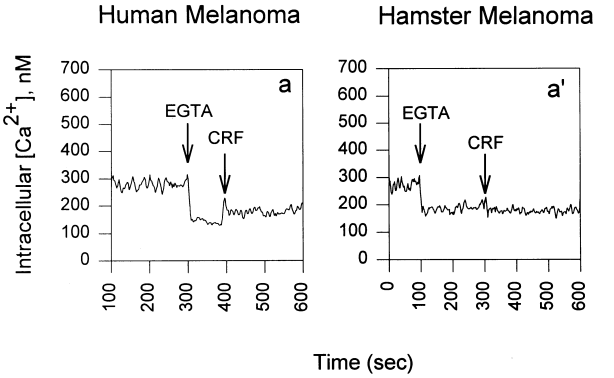

Fig. 3. Elevation of $[Ca^{2+}]_i$ stimulated by CRF depends on external Ca^{2+}. The effect of chelation of external $[Ca^{2+}]_i$ by EGTA (3 mM) (first arrow) inhibited dramatically CRF induced (second arrow) changes in $[Ca^{2+}]_i$ in human and hamster melanoma cells.

transduction system at the central level [11,12], would require a longer delay time of $[Ca^{2+}]_i$ than those observed in our experiments [12,19,20]. The experiments showing that the calcium chelator EGTA (3 mM) significantly inhibits the CRF-induced $[Ca^{2+}]_i$, which is most apparent in hamster cells (Fig. 3), suggests that an influx of calcium through the plasma membrane channel is the major contributor to a prolonged elevation in $[Ca^{2+}]_i$ in malignant melanocytes. Thus, part of the CRF signaling in malignant melanocytes is coupled to direct activation of plasma membrane Ca^{2+} channels, which is similar to effects described in the pituitary cells [12,19,20].

Previously we documented production and release of CRF in normal and malignant melanocytes [5,7], which was accompanied by an expression of CRF-R_1 gene [5–7]. Moreover, mammalian pigment cells express POMC gene and produce POMC-derived ACTH, MSH and β-endorphin peptides [1–4]. In the present study we show that malignant melanocytes contain functional CRF receptors. These observations raise the possibility that the melanocyte/melanoma phenotype may be regulated by autocrine mechanisms directly via an activation of CRF receptors or indirectly by a CRF-stimulated production of MSH or ACTH.

In summary, we have demonstrated that CRF and related urocortin and sauvagine induce changes in the intracellular calcium signaling in skin cells and that this effect in malignant melanocytes is mediated through a CRF-R which is predominantly coupled to the plasma membrane Ca^{2+} channels. These results support our hypothesis that regulated local CRH signals can play a role in skin physiology and pathology and also set the background for further studies on molecular mechanism of CRF signal transduction in this largest organ of the body.

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