Modulation of Calcium Movements by Urocortin II in Endothelial Cells

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Key Words

Endothelial cells • Calcium movements • Potassium channels • Urocortin II

Abstract

Background: In endothelial cells urocortin II has recently been found to activate nitric oxide synthase through cAMP-dependent and Ca^{2+} -related pathway. **Aim:** The present study was therefore planned to determine the mechanisms of urocortin II effect on Ca^{2+} movements. **Methods.** In Fura-2 loaded porcine aortic endothelial cells (PAE), the effects of urocortin II on $[\text{Ca}^{2+}]_i$ were analyzed and compared with those of various K^+ channels agonists/antagonists. **Results:** In Fura-2 loaded PAE, urocortin II promoted a transient increase of $[\text{Ca}^{2+}]_i$ mainly originating from an intracellular pool sensitive to thapsigargin and slightly from the extracellular space. In addition, urocortin II caused the hyperpolarization of plasma membrane through the opening of K^+ channels, which contributed to the increased $[\text{Ca}^{2+}]_i$. These effects were abolished by the corticotropin releasing factor receptors (CRFR2) blocker, the adenylyl cyclase and Ca^{2+} -calmodulin-kinase (CaMKII) inhibitors and by blockers of K^+ channels. In addition, in PAE cultured

in Na^+ -free medium or loaded with the plasma-membrane Ca^{2+} pump inhibitor the urocortin II-evoked Ca^{2+} transient was slower. **Conclusion:** The results obtained show that urocortin II affects intracellular Ca^{2+} homeostasis in PAE by both promoting a discharge of intracellular pool and by interfering with the operation of store-dependent channels through CRFR2-cAMP-CaMKII related signalling and K^+ channels opening.

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Introduction

In addition to exerting beneficial effects on cardiac contractility and relaxation, urocortin II (ucn II) and other corticotropin-releasing factor (CRF) family peptides have been reported to be extremely potent vasodilators [1], acting either through endothelial or non-endothelial factors [2, 3]. In anesthetized pigs, the coronary vasodilatation caused by intracoronary ucn II administration has recently been found to be abolished by the nitric oxide synthase (NOS) inhibitor L-NAME [4]. Moreover, in porcine aortic endothelial cells (PAE) the interaction of ucn II with the subtype 2 of CRF receptors (CRFR2) has

been found to cause eNOS activation and NO production through the involvement of cAMP-PKA signalling leading to the phosphorylation of p38, ERK and Akt [5]. It is also noteworthy that the effects of ucn II on NO release were potentiated by pretreatment of PAE with Ca^{2+} ionophore A23187 and abolished by Ca^{2+} -calmodulin kinase II (CaMKII) inhibitor KN-93 [5], thus indicating the involvement of intracellular Ca^{2+} signalling in the effects of ucn II on NO production.

Hence, in endothelial cells the production of vasodilator factors such as NO through constitutive NO synthase (eNOS) can be regulated not only through genomic and non-genomic targeting at the level of phosphorylation/dephosphorylation [6-8] at specific sites but also through Ca^{2+} -dependent processes [9-11]. The increase in intracellular Ca^{2+} occurs following the activation of membrane receptors which initiate signal transduction pathways that lead to Ca^{2+} release from the endoplasmic reticulum and entry from the extracellular compartment. In many cell types, including endothelial cells, the release of intracellular Ca^{2+} has been shown to be coupled to subsequent Ca^{2+} entry by store depletion or “capacitative Ca^{2+} entry” [12].

Such coupling has been demonstrated in response to receptor-mediated stimuli [12-15] and receptor-independent emptying of intracellular stores by agents such as thapsigargin, ionomycin and cycloviobuxine D [12-16]. Regarding CRF family peptides, ucn II has been shown in cardiomyocytes to increase Ca^{2+} transients through CRFR2-PKA signalling by means of L-type Ca^{2+} channels opening and an increase in fractional sarcoplasmic reticulum Ca^{2+} release [17].

As previously reported eNOS might represent a direct protein substrate for CaMKII, which is involved in various Ca^{2+} -mediated mechanisms and is also expressed in endothelial cells [18]. Moreover, Ca^{2+} transients could be involved in NO production through changes of membrane potential [19] caused by large-conductance Ca^{2+} -activated potassium (BKCa) channels opening which are widely expressed in endothelial cells [20-22] and participate in the regulation of vascular tone [23]. Indeed, members of CRF family peptides have been shown to cause endothelium-dependent and independent relaxation and hyperpolarization of rat mesenteric and coronary arteries, through the activation of BKCa channels [24, 25].

Thus, in an extension of earlier works from our group demonstrating an effect of ucn II on NO production, the aim of the present study was to investigate in PAE the mechanisms involved in the effect of ucn II on Ca^{2+} movements.

Materials and Methods

Culture of PAE

The experiments were performed in PAE, which were isolated from pigs, supplied by an accredited dealer (Azienda Invernizzi, Olengo, Novara, Italy), which were housed, anesthetized and sacrificed in our laboratory in accordance with national guidelines (DLGS 27/01/1992, license no. 116) and with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). PAE were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, Milan, Italy) supplemented with 10% fetal calf serum (FCS; Sigma), 2 mM L-glutamine (Sigma), 1% penicillin-streptomycin (Sigma) at 37°C with 5% CO_2 in incubator. PAE (1.5×10^6 cells/ml) were plated into 0.1% gelatine-coated coverslips with DMEM 10% FCS supplemented with L-glutamine, penicillin-streptomycin for 4 h. After this time, the cells were used for experiments. For the analysis by fluorescence microscopy, the cells were plated on 60 mm dishes and maintained with DMEM 10% FCS (Sigma) supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin at 37°C with 5% CO_2 in incubator.

Cytosolic calcium ($[\text{Ca}^{2+}]_c$) measurement

The coverslips were washed twice with sterile PBS 1X and incubated with Fura-2/acetoxymethyl ester (AM; 5 μM final concentration, Sigma) for 30 min in the dark in DMEM 0% FCS and without red phenol supplemented with 1% penicillin-streptomycin and 2 mM L-glutamine. In some experiments PAE were incubated in zero Na^+ PSS [N-methyl-D-glucamine (NMDG) 126 mM, KCl 1.5 mM, MgCl_2 1.2 mM, HEPES 10 mM, D-glucose 10 mM, and CaCl_2 mM; Sigma]. After further washing with DMEM, the coverslips in DMEM without Ca^{2+} were mounted in agitation at 37°C in thermostated quartz cuvette in a Hitachi F-4500 Fluorescence Spectrometer, operating continuously for 300 s at the wavelength pair 340 nm excitation/510 nm emission. Ucn II (0.001-1 μM ; Sigma) was added to the suspension of Fura-2/AM loaded PAE, in the presence or absence of Ca^{2+} in the incubation medium (obtained with 50 mM EGTA). In some experiments the effects of ucn II were compared with those of the Ca^{2+} ATPase inhibitor thapsigargin (1 μM ; Sigma), ATP (10 μM ; Sigma), thrombin (0.5 U/ml; Sigma). Moreover, some experiments were performed by ucn II administration in absence or presence of the CRFR2 inhibitor astressin-2B (1 μM ; Sigma), the PKA inhibitor H89 (1 μM ; Sigma), the CaMKII inhibitor KN93 (1 μM ; Sigma), the plasma-membrane Ca^{2+} -ATPase (PMCA) pump, 5- (and 6) blocker, carboxyeosin diacetate (25 μM ; Invitrogen, San Giuliano Milanese; Italy), the non specific K^+ channels blocker tetraethylammonium chloride (TEA; 10 mM; Sigma), the K_{ATP} channels agonists cromakalim (1 μM ; Sigma) and P1075 (10 μM ; Sigma) and the K_{ATP} channels antagonist, glybenclamide (1 μM ; Sigma), the selective activator and inhibitor of BKCa, NS1619 (30 μM ; Sigma) and iberiotoxin (300 nM; Sigma). The experiments were performed in presence of low K^+ (1.5 mM) or 5 mM K^+ in the medium. In some cells the effects of ucn II were examined in presence of various K^+ concentrations in the incubation medium (10 mM, 20 mM, 30 mM, 40 mM, 60 mM).

Analysis of "capacitative" calcium entry

In cells, the major Ca^{2+} entry pathway is the store-operated one, in which the emptying of intracellular Ca^{2+} stores activates Ca^{2+} influx ("capacitative" calcium entry).

The effects of ucn II on the "capacitative" Ca^{2+} entry through the plasma membrane Ca^{2+} channels were examined through the evaluation of the rate of Ca^{2+} overshoot in PAE. The cells on coverslips were pretreated with EGTA (50 mM) and were subsequently exposed to the Ca^{2+} ATPase inhibitor, thapsigargin (1 μM), and ucn II (1 μM) alone or in co-stimulation for 5 min. Finally, 60 mM CaCl_2 was added to the samples and the effects on Ca^{2+} overshoot were analyzed.

Measurement of plasma membrane potential

The PAE on coverslips were loaded with the potential-sensitive fluorescent dye bis-oxonol (0.3 μM ; Sigma) [12] and suspended in DMEM supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin without red phenol and FCS (incubation medium) at 37°C in a thermostated quartz cuvette in a Hitachi F-4500 Fluorescence Spectrometer, operating at the wavelength pair 530 nm excitation/570 nm emission.

In PAE, changes of membrane potential caused by ucn II (1 μM) were tested in absence or presence of the same agents used with Fura-2/AM; the incubation medium contained either 1.5 mM or 5 mM K^+ or high K^+ (10 mM, 20 mM, 30 mM, 40 mM, 60 mM).

Fluorescence microscopy

The PAE were washed twice with sterile PBS 1X and incubated with DMEM without FCS and red phenol but supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin and were loaded with Fura-2/AM for 30 min at 37°C in the dark. After this time the cells were stimulated with ucn II (1 μM) either in the absence or presence of the K_{ATP} channels agonists, cromakalim (1 μM), P1075 (10 μM), and of the selective activator of BKCa, NS1619 (30 μM). The medium was then removed, PBS 1X was added and then the dishes were analyzed by fluorescence microscopy (Zeiss Axiovert 40 CFL inverted microscope; Carl Zeiss, Oberkochen, Germany) using 40X object with filter for 340 nm excitation/510 nm emission. The images were analyzed through Image Pro-Plus (Media Cybernetics, Inc., Silver Spring, MD, USA).

Statistical analysis

Quantification of $[\text{Ca}^{2+}]_c$ was conventionally obtained by measuring the Fura-2/AM fluorescence in Ca^{2+} -free (0.1 M EGTA) and Ca^{2+} -saturated conditions by the equation $[\text{Ca}^{2+}]_c = K_d [(R - R_{\text{min}})/(R_{\text{max}} - R)]$ [16, 26]. The fluorescence intensities obtained were corrected for cell autofluorescence at the wavelengths employed [26].

All results were obtained from five different cell cultures for each experimental protocol. The results obtained were examined through one-way ANOVA followed by Newman-Keuls *post hoc* test. The non-parametric Mann Whitney U test for unpaired data was used to compare percentage responses. A simple-regression analysis was performed to examine the correlation between the concentration of ucn II administrated and the observed $[\text{Ca}^{2+}]_c$ effects in the concentration-response study.

Fig. 1. Concentration-dependent effects of ucn II on $[\text{Ca}^{2+}]_c$ in Fura-2/AM loaded PAE in presence of 1.5 mM K^+ (A) or 5 mM K^+ (B) in the medium. (C) Percentage effects of 1 μM ucn II on Ca^{2+} movements in presence of 5–60 mM K^+ in the medium. In (A), (B), an example taken from one of the five different experiments for each experimental protocol. Reported data in (C) are means $\pm$ SD (n=5).

All data are presented as means $\pm$ SD (range). A value of $P < 0.05$ was considered statistically significant.

Results

Concentration-response study

As shown in Fig. 1 A, ucn II (0.001–1 μM), used at similar concentrations to those that were able to gradually increase NO release in PAE [5], caused a transient and concentration-dependent increase of $[\text{Ca}^{2+}]_c$ ($P < 0.05$). The maximum effect was obtained at 1 μM ucn II after 30 s stimulation and amounted to $14.3\% \pm 1.7$ ($P < 0.05$) of control values; this concentration was maintained for all successive experiments. A similar concentration-dependent effect and time-course was obtained in presence of 5 mM K^+ in the medium (Fig. 1 B). As shown in Fig. 1 C, increasing the K^+ in the medium up to 20 mM potentiated the effects of ucn II on Ca^{2+} release

Fig. 2. Analysis of Ca^{2+} stores mobilized by ucn II in Fura-2/AM loaded PAE. (A) Effects of $1\ \mu\text{M}$ ucn II on $[\text{Ca}^{2+}]_c$ in absence of Ca^{2+} in the medium. (B) Effect of ucn II given before/after $10\ \mu\text{M}$ ATP or $0.5\ \text{U/ml}$ thrombin (C) on $[\text{Ca}^{2+}]_c$. (D) Effects of $1\ \mu\text{M}$ thapsigargin given alone (first trace) or with $1\ \mu\text{M}$ ucn II administered either at the peak of effect of thapsigargin (middle trace) or before thapsigargin (last trace). In (A), (B), (C), an example taken from one of the five different experiments for each experimental protocol. Thr, thrombin; Tap, thapsigargin.

($P < 0.05$). A linear relationship was observed between the administrated concentrations of ucn II and the effects on $[\text{Ca}^{2+}]_c$ ($P < 0.05$). All the effects were virtually abolished when K^+ in the medium was higher than $30\ \text{mM}$ (Fig. 1 C).

Analysis of calcium stores mobilized by ucn II

As depicted in Fig. 2 A, the increase of $[\text{Ca}^{2+}]_c$ caused by ucn II in PAE cultured in Ca^{2+} -free medium was lower than that observed in presence of Ca^{2+} ($P < 0.05$). The $[\text{Ca}^{2+}]_c$ increase caused by ucn II in PAE treated with EGTA amounted to $99.3 \pm 6.2\ \text{nM}$ (93-106) from control values of $95.3 \pm 5.4\ \text{nM}$ (90-101). These results demonstrate that ucn II caused an increase of $[\text{Ca}^{2+}]_c$ mainly by promoting Ca^{2+} mobilization from intracellular stores (as suggested by the persistence of Ca^{2+} signal even in Ca^{2+} -free medium) and, marginally, from extracellular milieu.

In order to achieve more details on the nature of the intracellular store affected by ucn II, experiments were performed using extracellular ATP and thrombin, which

mobilizes Ca^{2+} from IP_3 -sensitive pools and thapsigargin, a powerful inhibitor of non-plasma membrane Ca^{2+} -ATPases.

As shown in Figs. 2 B, C, the amount of Ca^{2+} mobilized by either ucn II and ATP or thrombin did not significantly differ irrespective of the sequence of addition ($P > 0.05$). The $[\text{Ca}^{2+}]_c$ increase caused by ATP given before and after ucn II amounted to $121.8 \pm 5.6\ \text{nM}$ (116-130; $P < 0.05$) and 120.5 ± 5 (116-127; $P < 0.05$), respectively from control values of $107.4 \pm 4.8\ \text{nM}$ (102-114) and $106.4 \pm 4.3\ \text{nM}$ (102-112). The $[\text{Ca}^{2+}]_c$ increase caused by ucn II given before and after ATP amounted to $122.6 \pm 5.6\ \text{nM}$ (117.3-132; $P < 0.05$) and 121.4 ± 4.6 (117-129; $P < 0.05$), respectively from similar control values as those reported before. The $[\text{Ca}^{2+}]_c$ increase caused by thrombin given before and after ucn II amounted to $121.7 \pm 5.2\ \text{nM}$ (115-128; $P < 0.05$) and $121.5 \pm 6\ \text{nM}$ (114.5-131; $P < 0.05$), respectively from control values of $106.7 \pm 6\ \text{nM}$ (100-115) and $106.9 \pm 5.4\ \text{nM}$ (101-114). The $[\text{Ca}^{2+}]_c$ increase caused by ucn II given before and after thrombin amounted to 121.4 ± 5.9

Fig. 3. Effects of 1 μ M ucn II and 1 μ M thapsigargin on Ca^{2+} overshoot in Fura-2/AM loaded PAE. Example taken from one of the five different experiments for each experimental protocol. The abbreviations are the same of Fig. 2.

nM (115-131; $P < 0.05$) and 121.8 ± 4.8 (116-127; $P < 0.05$), respectively from similar control values as those reported before. These findings demonstrated that the pool mobilized by ucn II was different from that mobilized by either ATP or thrombin. The fact that the response of PAE to ucn II and to agents acting through IP_3 generation like ATP or thrombin did not significantly differ irrespective of the sequence of addition, suggests that ucn II doesn't act through IP_3 related signaling.

As reported in Fig. 2 D (first trace) the administration of thapsigargin caused a persistent increase of $[\text{Ca}^{2+}]_c$ ($P < 0.05$). The $[\text{Ca}^{2+}]_c$ increase caused by thapsigargin amounted to 396 ± 34.5 nM (345-435; $P < 0.05$) from control values of 99.2 ± 12.6 nM (85-115). The addition of 1 μ M ucn II after thapsigargin did not elicit any further increase in $[\text{Ca}^{2+}]_c$ as reported in Fig. 2 D (middle trace). Moreover, ucn II promoted a decrease of the $[\text{Ca}^{2+}]_c$ to 251 ± 15.9 nM (230-270; $P < 0.05$) which was statistically different from the one caused by thapsigargin alone (Fig. 2 D). In further experiments, pretreatment of PAE with ucn II markedly changed the kinetics of cytosolic Ca^{2+} fluctuations promoted by thapsigargin (Fig. 2 D, last trace). The increase of $[\text{Ca}^{2+}]_c$ elicited by thapsigargin after ucn II was statistically different from the one observed when thapsigargin was given alone. The peak effect of thapsigargin on $[\text{Ca}^{2+}]_c$ amounted to 311.4 ± 11.6 nM (298-329) and in the plateau phase the $[\text{Ca}^{2+}]_c$ amounted to 245.6 ± 11.2 nM (230-260) from control values of 101.8 ± 5.6 nM (95-108). This finding strongly supports the conclusion that ucn II mobilizes Ca^{2+} from a store which is depleted by thapsigargin treatment.

Mechanisms of action involved in calcium movements by ucn II

The depletion of thapsigargin-sensitive intracellular stores activates channels in the plasma membrane which are permeable to Ca^{2+} . This phenomenon can be easily investigated through Ca^{2+} overshoot.

In PAE treated with thapsigargin or ucn II in Ca^{2+} -free medium, the readmission of Ca^{2+} in the incubation medium caused a Ca^{2+} overshoot amounting to 600 ± 10 nM (585.1-609; $P < 0.05$) and 250 ± 6 nM (240-256; $P < 0.05$), respectively, from control values of 97 ± 5 (92-101). The Ca^{2+} overshoot caused by the concomitant

Fig. 4. Effects of ucn II on $[\text{Ca}^{2+}]_c$ after the inhibition of Ca^{2+} extrusion mechanism. (A) Effects of 1 μ M ucn II on Ca^{2+} movements in PAE cultured in Na^+ -free medium (PSS- Na^+). (B) Effects of 1 μ M ucn II on Ca^{2+} movements in PAE cultured in Na^+ -free medium and loaded with 25 μ M carboxyeosin (PSS- Na^+ + CarE). Example taken from one of the five different experiments for each experimental protocol. CarE, carboxyeosin.

Fig. 5. Effects of ucn II on $[Ca^{2+}]_i$ in presence of CRFR2, PKA and CaMKII blockers in cells cultured with 1.5 mM K^+ (A) or 5 mM K^+ (B). C= control; ucn II= urocortin II (1 μ M), As= astressin 2B (1 μ M); H89= H89 (1 μ M); KN-93= KN93 (1 μ M). As + ucn II= co-stimulation; H89 + ucn II= co-stimulation; KN-93 + ucn II= co-stimulation. (A) a $P < 0.05$ versus control; b, c, d $P < 0.05$ versus a. Reported data are means $\pm$ SD (n= 5).

administration of the two substances amounted to 380 ± 8 nM (368-386.7; $P < 0.05$), which was statistically different from the one caused by thapsigargin alone ($P < 0.05$). An example of these findings taken from one of the five different experiments performed in PAE is depicted in Fig. 3.

Since Na^+/Ca^{2+} exchanger (NCX) and PMCA have been shown to contribute to lower $[Ca^{2+}]_i$ in vascular endothelial cells [27, 28], we designed experiments to abolish their contribution to Ca^{2+} extrusion. As reported in Fig. 4 A in PAE cultured in Na^+ free medium, ucn II

Fig. 6. Effects of ucn II on $[Ca^{2+}]_i$ in presence of K^+ channels agonists and antagonists in cells cultured with 1.5 mM K^+ (A) or 5 mM K^+ (B). C= control; ucn II= urocortin II (1 μ M); Cro=cromakalim (1 μ M); Cro + ucn II=co-stimulation; P1075= P1075 (10 μ M); P1075 + ucn II= co-stimulation; NS=NS1619 (30 μ M); NS + ucn II=co-stimulation. (A) a, b, c, d, e, f, g $P < 0.05$ versus control; c $P < 0.05$ versus a, b; e $P < 0.05$ versus d (B) e $P < 0.05$ versus a. The other significances are the same as in (A). (C) Percentage effects of NS1619 alone or in co-stimulation with ucn II in presence of 5-60 mM K^+ in the medium. Reported data are means $\pm$ SD (n= 5).

Table 1. Changes in cytosolic Ca^{2+} (nM) induced by various agents in Fura-2/AM loaded PAE. Values are means $\pm$ SD of five different observations for each experimental protocol. Ucn II= urocortin II (1 μM); As= astressin 2B (1 μM); H89= H89 (1 μM); KN-93= KN93 (1 μM); Cro= cromakalim (1 μM); P1075= P1075 (10 μM); Glib= glybenclamide (1 μM); Ib= iberiotoxin (300 nM); TEA= tetraethylammonium chloride (10 mM); NS=NS1619 (30 μM). * $P < 0.05$ versus control; ** $P < 0.05$ versus ucn II; + $P < 0.05$ versus P1075.

Samples	1.5 mM K^{+}	5 mM K^{+}
control	107 $\pm$ 3	114.9 $\pm$ 1
ucn II	122.3 $\pm$ 1.6*	134.7 $\pm$ 0.9*
As	108 $\pm$ 0.3	109.2 $\pm$ 0.5*
H89	102.4 $\pm$ 0.9	108.3 $\pm$ 1*
KN-93	104.4 $\pm$ 1.9	107.5 $\pm$ 1.2*
Cro	125 $\pm$ 1.8*	149.4 $\pm$ 1.9*
Cro+ucnII	130.8 $\pm$ 2.2* **	166.6 $\pm$ 1.3* **
P1075	115.5 $\pm$ 0.5*	132.1 $\pm$ 3.4*
P1075+ucnII	122.6 $\pm$ 0.4* +	149.4 $\pm$ 4.1* ** +
Glib	106.2 $\pm$ 1.5	111 $\pm$ 3.9
Glib+ucnII	105.2 $\pm$ 0.7	107.4 $\pm$ 1.6
Glib+Cro+ucnII	104.6 $\pm$ 1.3	107 $\pm$ 1.5
Ib	97.4 $\pm$ 1.6*	111.6 $\pm$ 0.4
Ib+ucnII	98.9 $\pm$ 1.1	111.8 $\pm$ 0.9
TEA	103.1 $\pm$ 1.9	112.5 $\pm$ 2.5
TEA+ucnII	104.9 $\pm$ 0.1	109.5 $\pm$ 3.3
NS	112.4 $\pm$ 3.7*	126 $\pm$ 4*
NS + ucnII	115.6 $\pm$ 2.9*	129.1 $\pm$ 1.8*
As+Cro+ucnII	103.8 $\pm$ 1	109.1 $\pm$ 0.2
As+P1075+ucnII	103.9 $\pm$ 2	108 $\pm$ 4

Table 2. Changes in membrane potential (AUF min^{-1}) induced by various agents in bis-oxonol loaded PAE. Values are means $\pm$ SD of five different observations for each experimental protocol. Ucn II= urocortin II (1 μM); As= astressin 2B (1 μM); Cro= cromakalim (1 μM); P1075= P1075 (10 μM); Glib= glybenclamide (1 μM); Ib= iberiotoxin (300 nM); TEA= tetraethylammonium chloride (10 mM); NS=NS1619 (30 μM). * $P < 0.05$ versus control; ** $P < 0.05$ versus ucn II; + $P < 0.05$ versus K^{+} channels agonists given alone.

Samples	1.5 mM K^{+}	5 mM K^{+}	20 mM K^{+}	30 mM K^{+}
control	60.8 $\pm$ 0.2	86 $\pm$ 2	89.04 $\pm$ 2	61.3 $\pm$ 1.3
ucn II	57.8 $\pm$ 0.4*	75.6 $\pm$ 0.6*	76.6 $\pm$ 0.4*	59 $\pm$ 1.7
As	61.9 $\pm$ 1	90.2 $\pm$ 0.8*		
As+ucnII	62.3 $\pm$ 2.7	89.6 $\pm$ 0.5		
Cro	55.7 $\pm$ 2.2*	73.9 $\pm$ 0.9*	72.8 $\pm$ 7.2*	61 $\pm$ 4
Cro+ucnII	53 $\pm$ 2.4* **	69.3 $\pm$ 1.1* ** +	68.1 $\pm$ 0.9* ** +	59.2 $\pm$ 2.6
P1075	56.5 $\pm$ 2.5*	75 $\pm$ 1.2*	73.7 $\pm$ 1.2*	60 $\pm$ 2.3
P1075+ucnII	53.1 $\pm$ 1.5* **	71 $\pm$ 3* ** +	69.4 $\pm$ 2.7* ** +	59.5 $\pm$ 3
Glib	65.2 $\pm$ 1.1*	90.9 $\pm$ 2.1*		
Glib+ucnII	65.9 $\pm$ 2.9	91.2 $\pm$ 1.2		
Glib+Cro+ucnII	66.3 $\pm$ 1.9	92 $\pm$ 1.2		
Ib	61.4 $\pm$ 1.6	84.9 $\pm$ 1.2		
Ib+ucnII	61 $\pm$ 0.5	84.5 $\pm$ 1		
TEA	61 $\pm$ 1.3	84.8 $\pm$ 1.2		
TEA+ucnII	61.2 $\pm$ 0.3	84.9 $\pm$ 0.1		
NS	52.3 $\pm$ 0.7*	72.1 $\pm$ 0.4*	71.1 $\pm$ 16*	60.8 $\pm$ 3
NS+ucnII	49.8 $\pm$ 1.1* **	67.1 $\pm$ 1.1* ** +	66.4 $\pm$ 0.5* ** +	59 $\pm$ 1.8
As+NS	61.6 $\pm$ 0.3	91.4 $\pm$ 0.8		
As+NS+ucnII	61.9 $\pm$ 1.1	90.1 $\pm$ 1.4		

evoked- Ca^{2+} increase was lower and returned to control values by about 5 min from the start of ucn II administration. The peak effect of ucn II on $[\text{Ca}^{2+}]_c$ amounted to 114.3 ± 9.5 nM (98-122; $P < 0.05$) from control values of 103.6 ± 8 nM (90-110). In PAE cultured in Na^{+} free medium and in presence of the PMCA pump blocker, carboxyeosin, the $[\text{Ca}^{2+}]_c$ increase induced by ucn II, after a peak effect of 115.4 ± 4 nM ($P < 0.05$) from control values of 105.8 ± 6.9 nM (95-114), reached a plateau amounting to 111.4 ± 6.6 nM (100-117; Fig. 4 B; $P < 0.05$). Similar results were found in presence of EGTA in the medium (not shown).

As shown in Figs. 5 A, B, the CRFR2 inhibitor,

astressin-2B, prevented any effect of 1 μM ucn II on Ca^{2+} movements ($P > 0.05$) either in presence of 1.5 mM K^{+} or 5 mM K^{+} in the medium. Similarly, the treatment of PAE with the PKA inhibitor, H89, and the CaMKII inhibitor, KN-93, abolished the response of PAE to 1 μM ucn II ($P > 0.05$). These results confirm the role of CRFR2-PKA and CaMKII in the mechanism of action involved in Ca^{2+} movements activated by ucn II.

To verify the role of K^{+} channels, PAE were treated with various agents alone or in co-stimulation with 1 μM ucn II. The K_{ATP} channels agonist, cromakalim (1 μM), amplified the effects of ucn II ($P < 0.05$), as depicted in Fig. 6 A and reported in Table 1. Moreover, in presence

Fig. 7. Effect of ucn II on membrane potential in bis-oxonol-loaded PAE. (A) The traces represent the effects of 1 μ M ucn II, 10 μ M ATP and 1 μ M thapsigargin on membrane potential. Example taken from one of the five different experiments for each experimental protocol. (B) and (C) Effects of various agents on membrane potential in presence of 1.5 mM K⁺ (B) or 5 mM K⁺ (C) in the medium, respectively. The abbreviations are the same of Figs. 2, 3, 5, 6. (B) a, b, c, d, e, f, g P<0.05 versus control; c, e, g P<0.05 versus a. (C) c P<0.05 versus a, b; e P<0.05 versus a, d; g, P<0.05 versus f. The other significances are the same as in (B). Reported data are means $\pm$ SD (n= 5).

of ucn II the effects of the K_{ATP} channels agonist, P1075, were increased. It is noteworthy that the response of PAE to ucn II in presence of the above K⁺ channels agonists were amplified when 5 mM K⁺ was added in the medium (Fig. 6 B; Table 1; P<0.05). In PAE cultured with 5 mM K⁺ the K_{ATP} channels agonist, P1075, significantly potentiated the effect of ucn II (Table 1, Fig. 6B). As shown in Fig. 6 and Table 1 the effects of 30 μ M NS1619, the selective activator of BKCa, on [Ca²⁺]_c were related to K⁺ in the medium. The maximum response of PAE to NS1619 was found at 20 mM K⁺ (Fig. 6 C; P<0.05). In presence of ucn II the effects of NS1619 were increased in comparison with NS1619 given alone (Fig. 6).

In PAE pre-treated with the non specific K⁺ channels blocker, TEA (10 mM), the K_{ATP} channels antagonist, glybenclamide (1 μ M), and with the selective BKCa inhibitor, iberiotoxin (300 nM), the response of cells to

ucn II was almost abolished (Table 1; P>0.05). Furthermore, the K_{ATP} channels antagonist, glybenclamide and the CRFR2 inhibitor astressin 2B prevented the potentiation of the effects of ucn II in co-stimulation with the K_{ATP} channels agonists cromakalim and P1075 (Table 1; P>0.05).

Fluorescence microscopy analysis confirmed the quantitative data obtained in PAE treated with ucn II alone or in co-stimulation with the K_{ATP} channels agonists, cromakalim and P1075, and the selective activator of BKCa, NS1619, in absence or presence of either 5 mM or 30 mM K⁺ (not shown).

Changes in membrane potential

As shown in Fig. 7 and Table 2, ucn II, the K_{ATP} channels agonists, cromakalim (1 μ M) and P1075 (1 μ M), and the selective activator of BKCa, NS1619 (30 μ M) caused the hyperpolarization of PAE. These effects were

linearly related to K^+ in the medium up to 20 mM K^+ (Table 2; $P < 0.05$). At 30 mM K^+ the changes of membrane potential in response to ucn II were not significant (Table 2; $P > 0.05$).

Moreover, the K^+ channels agonists were able to potentiate the effects of ucn II, as shown in Figs. 7 B, C and Table 2 ($P < 0.05$). Finally, the pre-treatment of PAE with the CRFR2 inhibitor astressin-2B, the K_{ATP} channels antagonist, glybenclamide (1 μ M), the non specific K^+ channels blocker, TEA (10 mM) and the selective BKCa inhibitor, iberiotoxin (300 nM), abolished the response to ucn II (Table 2; $P > 0.05$). Similar effects were observed in presence of H89 (data not shown). Finally, in presence of K^+ in the medium higher than 1.5 mM, the selective activator of BKCa, NS1619 was able to potentiate the effects of ucn II on membrane potential (Fig. 7 C; Table 2; $P < 0.05$).

Discussion

The results of this study show for the first time that ucn II can cause transient increases of intracellular Ca^{2+} in endothelial cells through the activation of a CRFR2-cAMP-PKA signalling and changes of membrane potential caused by the opening of K^+ channels. Both changes in $[Ca^{2+}]_c$ and in membrane potential could be involved in previously described eNOS activation and increased NO production in PAE.

Cytosolic Ca^{2+} homeostasis and signalling is achieved by the complex interplay of Ca^{2+} fluxes between the cytosol, intracellular stores and the extracellular environment [29]. Calcium enters the cytosolic compartment by direct influx from the extracellular milieu through a variety of plasma membrane channels or from intracellular stores, namely mitochondrial and extra mitochondrial pool. Calcium transport may occur via either Ca^{2+} -activated ATPases located in the plasma membrane and in the membrane of non-mitochondrial stores or potential-driven uniporters such as that located in the inner mitochondrial membrane. Transient fluctuations in cytosolic free Ca^{2+} may thus occur following inhibition of Ca^{2+} -ATPases, direct release from intracellular stores and potentiation of Ca^{2+} entry into the cell. Intracellular Ca^{2+} concentration in endothelial cells plays an important role in regulating a variety of cellular functions some of which are related to the production of NO. NO is synthesized in the endothelium by the constitutive (eNOS), Ca^{2+} -dependent, and the inducible, Ca^{2+} -independent, isoforms of the NOS [30, 31].

The eNOS is inactive until intracellular Ca^{2+} levels increase, the Ca^{2+} -binding protein binds to Ca^{2+} and the Ca^{2+} -calmodulin complex activates the eNOS [32]. Many agents known to induce NO release by endothelial cells, stimulate the eNOS by increasing the intracellular concentration of cytosolic free Ca^{2+} either by promoting Ca^{2+} release from intracellular stores and/or by enhancing Ca^{2+} influx from the extracellular compartment [32-34].

In the present report ucn II concentrations similar to those that increased NO production in the same cells [5] were shown to cause a concentration-dependent increase of $[Ca^{2+}]_c$ partly of extracellular origin, as shown by experiments performed in presence of EGTA, and partly mobilized from an intracellular pool depleted by thapsigargin, the Ca^{2+} -ATPase inhibitor which is able to deplete the endoplasmic reticulum Ca^{2+} pool [35-37]. The depletion of this pool is generally associated with the activation of the "capacitative" or store-dependent Ca^{2+} entry, in which the emptying of intracellular Ca^{2+} stores causes the opening of plasma membrane thus promoting Ca^{2+} influx from the extracellular sources [38, 39]. Moreover the results obtained in this paper showed that the intracellular pool mobilized by thapsigargin of reticular origins should contemporarily be functionally distinct from the one mobilized by agents acting through IP₃ generation, like ATP [40] or thrombin [41], which did not change the Ca^{2+} response to ucn II. In endothelial cells, IP₃ is the most common pathway leading to an elevation of $[Ca^{2+}]_c$. At concentrations up to 10 μ M, ATP acting on P2Y purinergic receptors raised cytosolic Ca^{2+} and activated $G_{q/11}$ phospholipase C pathways. Thrombin is another potent agonist that elevates intracellular Ca^{2+} in endothelial cells by a family of G-protein coupled receptors known as protease-activated receptors (PARs). In aortic endothelial cells, activation of PAR-2 or P2Y receptors elevates Ca^{2+} through phospholipase C/IP₃ pathways subsequent to activation of $G_{q/11}$ [41]. In PAE, the effects of ucn II, ATP or thrombin on $[Ca^{2+}]_c$ did not significantly differ irrespective of the sequence of addition, indicating that the peptide does not interfere with the above intracellular signalling.

The increase of Ca^{2+} in response to ucn II was found to be transient and related to CRFR2 and cAMP-PKA signalling. All effects of ucn II were abolished by pre-treatment of PAE with the CRFR2 inhibitor, astressin-2B, and with the PKA blocker, H89, in concentrations previously shown to inhibit the release of NO caused by ucn II in the same cells [5]. Similar effects on Ca^{2+} movements in response to CRF related family peptides have recently been obtained in rat islet beta cells and in rat

lung mast cells [42, 43].

The Ca^{2+} increase caused by ucn II peaked at 30 s and then returned to base level in about 90 s. In the present study the return of $[\text{Ca}^{2+}]_i$ to control values has been shown to be related to the activation of the plasma membrane Ca^{2+} -ATPase (PMCA) pump and NCX activation, which are widely reported to restore the basal intracellular Ca^{2+} levels in endothelial cells [27, 28, 44, 45]. In PAE cultured in Na^+ free medium the decay phase of the ucnII-evoked Ca^{2+} transient was significantly longer; the $[\text{Ca}^{2+}]_i$ virtually returned to control values only at 300 s. Moreover, in PAE cultured in Na^+ free medium and loaded with carboxyeosin the specific PMCA pump inhibitor, the effects of ucn II on $[\text{Ca}^{2+}]_i$ transient reached a plateau.

Hence, our results showed that, during the decay phase of the Ca^{2+} transient, Ca^{2+} was extruded by both the PMCA pump and NCX. Blocking the Na^+ -dependent and Na^+ -independent Ca^{2+} extrusion mechanisms has made the cells unable to extrude the Ca^{2+} released by the thapsigargin-sensitive pool following ucn II stimulation. Moreover, as has been previously described [16, 46-49], ucn II was shown to partly reverse the increase in $[\text{Ca}^{2+}]_i$ promoted by thapsigargin; this finding being related to the inhibition of the "capacitative" opening of plasma membrane Ca^{2+} channels. Indeed, ucn II induced a decrease in the extent of Ca^{2+} influx following treatment with thapsigargin. This effect of ucn II is interestingly similar to the ones previously observed in endothelial cells [16, 48, 49].

Although endothelial cells are non-excitable, membrane potential is a critical determinant of the magnitude of store-mediated Ca^{2+} entry responses [50]. Whereas hyperpolarization promotes Ca^{2+} entry, depolarization reduces Ca^{2+} entry. Activation of store-mediated Ca^{2+} entry by Gq-coupled agonists including bradykinin or Ca^{2+} -ATPase inhibitors like thapsigargin has been reported to cause an initial hyperpolarization attributed to activation of large-or intermediate KCa channels [51]. This hyperpolarization is transient but further promotes Ca^{2+} entry by increasing the electrochemical driving force.

In the present study the effects of ucnII on Ca^{2+} movements were found to be related to K^+ in the incubation medium, which could have changed the rate of membrane potential and, consequently, the response of PAE to ucn II. Indeed, the results of experiments performed with the potential-sensitive fluorescent dye bis-oxonol [12], showed that ucn II caused the hyperpolarization of PAE, which could have been related to the opening of K_{ATP} and BKCa channels. This hypothesis is supported by findings obtained by the experiments with ucn II and K^+ chan-

nels agonists and antagonists, which were able to, respectively, increase and abolish the effects of ucn II. This K^+ channel modulation was found to require CRFR2 activation, since the effects of ucn II in co-stimulation with K^+ channels agonists were inhibited by astressin-2B. In addition, the involvement of BKCa channels is in agreement with previous findings showing that the hyperpolarization and relaxation caused by urocortin have been found to be inhibited by BKCa channels inhibitors [24, 25]. The results obtained in the present study about the role of K_{ATP} channels in the effects of ucn II on Ca^{2+} movements are totally new and are not in agreement with previous reports showing that the K_{ATP} channels blocker, glybenclamide did not affect hyperpolarization and relaxation of rat mesenteric arteries induced by urocortin [24]. The fact that our findings have been obtained in endothelial cells and not in isolated vessels could explain this discrepancy.

Opening K^+ channels by ucn II would have drawn the membrane potential towards the equilibrium potential for K^+ and resulted in hyperpolarization. Indeed in endothelial cells, hyperpolarization is thought to increase the driving force for influx of Ca^{2+} via voltage-independent Ca^{2+} channels and thereby prolong and strengthen the activating Ca^{2+} signal [52].

The raising of extracellular K^+ concentration has been reported to depolarize endothelial cells, limit Ca^{2+} entry [53] and reduce the effects on NO release in cultured endothelial cells [53, 54], which is not different from what was observed in the present study where the effects of ucn II and of K^+ channels agonists on Ca^{2+} movements and membrane potential were abolished at K^+ higher than 30 mM.

Moreover, the experiments performed in presence of the CaMKII blocker, KN93, which abolished the effects of ucn II alone or in co-stimulation the selective BKCa activator, NS1619, provided evidence for involvement of CaMKII in the opening of BKCa channels and subsequent hyperpolarization.

Indeed, among K^+ channels, BKCa channels, in particular, have been reported to be activated by elevated cytosolic Ca^{2+} concentration [55] and to cause strong plasma membrane hyperpolarization. It is noteworthy that also cAMP has been found to activate large conductance K^+ channels in endothelial cells and cause hyperpolarization [56]. The results obtained in the present study are in support of this finding; in PAE the effects of ucn II given in co-stimulation with the selective BKCa activator, NS1619 on Ca^{2+} movements and changes of membrane potential were abolished by the

PKA inhibitor H89.

In conclusion, in the present study concentrations of ucn II recently reported to cause eNOS activation through CRFR2-cAMP and CaMKII-dependent signalling, transiently increased $[Ca^{2+}]_c$. The mechanism of such an effect could be the CRFR2-cAMP dependent depletion of thapsigargin sensitive Ca^{2+} pool and $[Ca^{2+}]_c$ increase. The subsequent opening of K^+ channels and consequent hyperpolarization would increase the driving force for Ca^{2+} entry and contribute to the effects of ucn II on Ca^{2+} movements. Finally, the activation of both NCX

and PMCA, together with the inhibition of the “capacitative” Ca^{2+} entry by ucn II, would restore the basal $[Ca^{2+}]_c$.

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