Cardiovascular Research (2009) **83**, 717–725
doi:10.1093/cvr/cvp161

# Urocortin induces positive inotropic effect in rat heart

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Received 22 October 2008; revised 7 May 2009; accepted 15 May 2009; online publish-ahead-of-print 21 May 2009

**Time for primary review: 20 days**

**KEYWORDS**
Urocortin;
Inotropic;
PKC;
ERK1/2;
Epac;
$Ca^{2+}$

**Aims** The aim of this study is to evaluate the positive inotropic effect of urocortin (Ucn) and to characterize its signalling pathways.

**Methods and results** Contractility was measured in *ex vivo* Langendorff-perfused hearts isolated from Wistar rats. Isolated ventricular cardiomyocytes were used to analyse intracellular calcium ($[Ca^{2+}]_i$) transients evoked by electrical stimulation and L-type $Ca^{2+}$ current by confocal microscopy and whole-cell patch-clamping, respectively. The application of Ucn to perfused hearts induced progressive, sustained, and potent inotropic and lusitropic effects that were dose-dependent with an $EC_{50}$ of approximately 8 nM. Ucn effects were independent of protein kinase A (PKA) activation but were significantly reduced by protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) inhibitors and by brefeldin A, an antagonist of guanine nucleotide exchange factor, suggested to be an inhibitor of exchange protein activated by cAMP (Epac). These whole-organ effects were correlated with the inotropic effects observed in isolated cells: Ucn increased $I_{CaL}$ density, $[Ca^{2+}]_i$ transients, cell shortening and $Ca^{2+}$ content of sarcoplasmic reticulum.

**Conclusion** Our results show that Ucn evokes potent positive inotropic and lusitropic effects mediated, at least in part, by an increase in $I_{CaL}$ and $[Ca^{2+}]_i$ transient amplitude. These effects may involve the activation of Epac, PKC, and MAPK signalling pathways.

## 1. Introduction

Urocortin (Ucn) is a 40-amino acid vasoactive peptide which belongs to the corticotropin-releasing factor (CRF) family.[1] Originally identified in rat midbrain, it has been detected in the heart, brain, vasculature, kidney, and digestive system.[1–3] Ucn plays important roles in the regulation of the hypothalamic–pituitary–adrenal axis and in endocrine, behavioural, and autonomic responses to stress.[4,5] Recent studies indicate that Ucn exerts effects beyond the hypothalamo–pituitary–adrenal axis, directly upon cardiac, vascular, and vaso-humoral function in health and cardiac disease.[6,7] Different reports have demonstrated that Ucn induces an increase in heart rate, cardiac output, and coronary blood flow.[8,9] Along with these effects on cardiac function, Ucn has also been shown to protect both cultured cardiac cells and intact heart against the damaging effects of ischaemia/reperfusion injury.[10,11]

Ucn effects on heart contractility could be mediated through modulation of intracellular $Ca^{2+}$ handling. In cardiac myocytes, cellular contraction is initiated by the transient elevation in $[Ca^{2+}]_i$ during the excitation–contraction coupling: during an action potential, $Ca^{2+}$ influx induced by activation of voltage-dependent L-type $Ca^{2+}$ channels upon membrane depolarization triggers the release of $Ca^{2+}$ via intracellular $Ca^{2+}$ release channels (ryanodine receptors) of sarcoplasmic reticulum (SR) through a $Ca^{2+}$-induced $Ca^{2+}$ release mechanism.[12,13] Relaxation follows a decrease in $[Ca^{2+}]_i$ mainly by the $Ca^{2+}$ uptake into the SR through the SR $Ca^{2+}$ ATPase (SERCA) and $Ca^{2+}$ extrusion through the $Na^+/Ca^{2+}$ exchanger.[12] However, it is not clear whether Ucn cardiac actions involve $I_{Ca}$ modulation since both a decrease[14] and an increase[15] of $I_{Ca}$ have been reported. Moreover, the specific mechanisms underlying cardiac actions have not yet been elucidated.

Three distinct peptides—Ucn-I, II, and III—have been identified. They bind to two G protein-coupled CRF receptors.[16,17] CRF-R1 is localized almost exclusively in the central nervous system;[3] meanwhile CRF-R2 is present in both brain and peripheral tissues.[18] In the cardiovascular

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system, CRF-R2 are found in high concentration in the human left ventricle, intramyocardial blood vessels, and medial layers of internal mammary arteries.[4] Binding of Ucn to CRF-R2 increases its affinity for the Gs protein leading to the stimulation of cAMP production. Recently, we unveiled an unexpected mechanism of Ucn-induced relaxation on rat coronary artery, which involves cAMP/PKA regulation of $Ca^{2+}$-independent phospholipase $A_2$ and store operated $Ca^{2+}$ channels.[19] Classically, cardiac effects of cAMP were attributed to phosphorylation by cAMP-dependent PKA, but recently a guanine nucleotide exchange factor (GEF) also directly activated by cAMP, named exchange protein activated by cyclic AMP (Epac) was identified[20] and introduced as a new player in several cAMP-regulated processes such as cell permeability,[21] cardiomyocytes hypertrophy,[22] and $Ca^{2+}$ handling in cardiomyocytes,[23] in a PKA-independent manner. Recently, Epac activation was proposed to activate MAPK (mitogen-activated protein kinase) subfamily ERK1/2 (extracellular signal-regulated kinases 1/2) in endothelial cells. Moreover, CRF receptors in cardiomyocytes are known to couple with signalling cascades other than the cAMP–PKA pathway. For example, Ucn-dependent stimulation of PKCepsilon[24] or ERK[11] has been reported.

In this study we aimed to get further insights into the signalling pathway that mediates Ucn-evoked inotropic effect on rat heart, with special emphasis on cAMP, PKA, Epac, PKC, and MAPK signalling pathways.

## 2. Methods

All the experiments were performed in accordance to the animal care guidelines of the European Communities Council (86/609/EEC). And this study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures were approved by the Committee of Animal Research at the University of Seville.

### 2.1 *Ex vivo* Langendorff-perfused rat heart

Adult male Wistar rats weighing 250–450 g were heparinized (4 IU/g i.p.) and anaesthetized by intraperitoneal administration of ketamine hydrochloric acid (1–2 mL/250 g). The hearts were quickly removed, mounted on the aortic cannula of the Langendorff perfusion system apparatus, and perfused with an oxygenated Krebs–Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 5 mM glucose) as described previously.[25] The buffer was equilibrated with 95% $O_2$ and 5% $CO_2$. To obtain an isovolumetrically beating preparation, a latex balloon filled with water and connected by a catheter to a transducer (Abbott, IR), was inserted through the left atrium into the left ventricle and inflated to provide an end-diastolic pressure between 8 and 12 mmHg. Before each experimental protocol was initiated, the isolated hearts were set at a mean arterial pressure of 60–80 mmHg and were allowed to stabilize at 37°C for 40–60 min. Chart Powerlab software (ADInstruments) was used for continuous recording throughout the experiments of heart rate, left ventricular pressure (LVP), and maximum positive and negative derivative of left ventricular pressure ($\pm dP/dt$) as shown in *Figure 1*. The heart contractility under different treatments was evaluated by the analysis of $+dP/dt$, which corresponds to % increase of $+dP/dt$ normalized to basal $+dP/dt$ after stabilization period. Ucn was also studied in rate-paced hearts without any significant change in the contractility when compared with no-paced hearts.

### 2.2 Isolation of ventricular myocytes

The hearts were removed and mounted on a Langendorff-perfusion apparatus. The ascending aorta was cannulated, and a retrograde perfusion was set-up. The heart was first perfused for 2 min at 36–37°C with a nominally calcium-free Tyrode solution containing 0.2 mM ethylene glycol tetraacetic acid (EGTA), and then for 10 min approximately with the same Tyrode solution containing collagenase type II (Worthington) plus 1 mg/mL of bovine serum albumin (BSA; Sigma), all in the presence of 50 μM $CaCl_2$. After perfusion, the hearts were removed from the Langendorff apparatus and the left and right ventricles were cut-off, chopped into small pieces, and gently shaken for 3 min in a standard Tyrode solution containing 0.5 mM of $CaCl_2$ to disperse the isolated cells. Subsequently, isolated cells were filtered, centrifuged, and suspended in the Tyrode solution containing 1 mM $CaCl_2$. All the experiments were performed at room temperature (24–26°C) on $Ca^{2+}$-tolerant rod-shaped myocytes. The nominally calcium-free Tyrode solution for isolation of myocytes contained (mM): 130 NaCl, 0.5 $MgCl_2$, 5.4 KCl, 22 glucose, 25 HEPES, 0.4 $NaH_2PO_4$, 5 $NAHCO_3$; pH was adjusted to 7.4 with NaOH.

### 2.3 Intracellular $Ca^{2+}$ measurement

$[Ca^{2+}]_i$ transients were recorded in freshly isolated cardiomyocytes loaded with the fluorescent $Ca^{2+}$ dye fluo-3AM as previously described.[26] Cells were incubated for 20–40 min in the presence or absence of 100 nM Ucn. During experiments, cells were continuously superfused with normal Tyrode (mM): 140 NaCl, 4 KCl, 1.1 $MgCl_2$, 10 HEPES, 10 glucose, 1.8 $CaCl_2$ (pH was adjusted to 7.4 with NaOH), as previously described.[23] To evoke $[Ca^{2+}]_i$ transients, cardiomyocytes were stimulated at 1 Hz by field stimulation using two parallel platinum electrodes. SR $Ca^{2+}$ load was estimated by rapid caffeine application (10 mM) on isolated myocytes. Images were obtained with confocal microscopy (Meta Zeiss LSM 510, objective W.I. 63X and N.A. 1.2) by scanning the cell with an Argon laser every 1.54 ms. Fluorescence was excited at 488 nm, and emission was collected at >505 nm. The line of scan was selected parallel to the longitudinal cell axis to measure cell shortening. Image analysis was performed using IDL software (Research System Inc.). The fluorescence values ($F$) were normalized by the basal fluorescence ($F_0$) to obtain the fluorescence ratio ($F/F_0$), and decay time constant ($\tau$) was calculated by fitting the descending phase of the fluorescence trace to a single exponential in each cell.

### 2.4 Electrophysiological studies

Cell membrane currents were recorded with the whole cell configuration of the patch-clamp technique using an Axopatch-200B amplifier controlled by a computer, which was equipped with pClamp (version 6.0-Axon Instruments) and interfaced to the amplifier with a Digidata 1322A (Axon Instruments). The recording pipettes filled with internal solution had tip resistances ranging from 0.9 to 2 MΩ. Whole-cell recordings were started 5 min after seal disruption, to allow cell dialysis and stabilization of $I_{Ca}$.[27] $I_{CaL}$ was determined from a holding potential of −50 mV, depolarizing pulses from −40 mV to +60 mV with 10 mV increments for 300 ms at a frequency of 0.2 Hz were applied. The $I_{CaL}$ amplitude was measured as the difference between peak current and the current at the end of the pulse. For comparison between different cells, whole cell currents were expressed as current densities that were calculated from the current amplitude normalized by the membrane capacitance ($C_m$), which was determined by applying ±10 mV voltage steps from −60 mV and $C_m$ was calculated according to the equation:

$$\frac{C_m = \tau I_0}{\Delta V_m(1 - I_\infty/I_0)}$$

where $\tau$ is the time constant, $I_0$ the maximum capacitative current value, $V_m$ the amplitude of the voltage step, and $I_\infty$ the

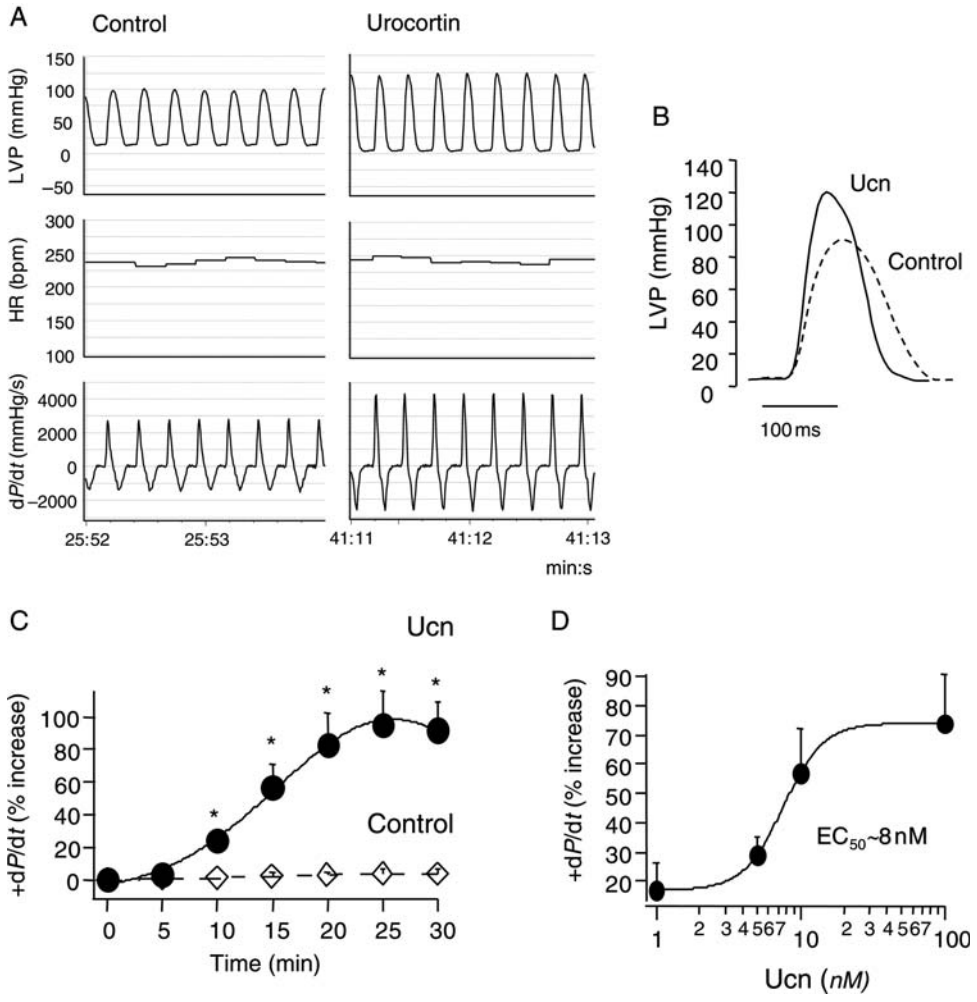


**Figure 1** Effect of urocortin on contractility in *ex vivo*-isolated perfused rat hearts. (*A*) Representative original recording of the left ventricular pressure (LVP, upper tracing), the heart rate (HR, middle tracing), and the derivative of left ventricular pressure (d*P*/d*t*, lower tracing) in Langendorff perfused heart before (*control*) and after urocortin (*Ucn*, 10 nM) addition. (*B*) Superimposed traces of LVP from untreated (*control*) and urocortin-treated heart (*Ucn*, 10 nM). (*C*) Time-course of the inotropic effect of urocortin (*Ucn*, 10 nM) on contractility. The summary data are mean ± SEM ($n = 8$–9) from the urocortin (*Ucn*, 10 nM)-treated and untreated hearts (*control*), and are expressed as a percent change vs. baseline values. (*D*) Dose-dependent effect of urocortin (*Ucn*, 1–100 nM) on +d*P*/d*t* in perfused rat hearts. The fit was generated with Hill equation and the $EC_{50}$ was approximately 8 nM ($n = 4$). Data are significantly different at $P < 0.05$ when indicated by asterisks.

amplitude of the steady-state current. The *I*–*V* relationships obtained in the isolated cardiomyocytes under different conditions were fitted with a function combining the Goldman–Hodgkin–Katz equation.

$Ca^{2+}$ recordings solution contained (mM): 140 NaCl, 1.1 $MgCl_2$, 5.4 CsCl, 10 glucose, 5 HEPES, 1.8 $CaCl_2$; pH was adjusted to 7.4 with NaOH. The intracellular recording pipette solution contained (mM): 100 CsCl, 20 TEA, 5 EGTA, 10 HEPES, 5 $Na_2ATP$, 0.4 $Na_2GTP$, 5 $Na_2$-creatine phosphate, 0.06 $CaCl_2$; pH was adjusted to 7.2 with CsOH.

## 2.5 PKA activity assay

The Kinase-Glo® Luminescent Kinase Assay (Promega) was used to measure the PKA activity in homogenates from perfused hearts and isolated cardiomyocytes as described previously.[19] The PKA activity was measured in each sample according to the manufacturer's instruction, and luminescence was recorded on a GloMax™ 96 Microplate Luminometer (Promega). The activity is expressed as $RLU^{-1}$ (relative light units)/amount of protein.

## 2.6 Drugs and statistical analysis

Drugs were purchased from Sigma, Promega, and Invitrogen. Group data are presented as mean ± SEM. Single or paired Student's *t*-test was used to determine the statistical significance of the obtained data. The significance between multiple groups was evaluated using analysis of variance followed by Tukey test. Data marked with asterisks (*) were considered significantly different.

# 3. Results

## 3.1 Urocortin evokes positive inotropic and lusitropic effects

Acute effects of Ucn on LVP and contractility were analysed in *ex vivo*-perfused heart. *Figure 1A* shows that Ucn (10 nM) induced a strong increase in heart contractility indicated by the enhancement of the positive and negative maximum derivative of left ventricular pressure (±d*P*/d*t*). The comparison of superimposed LVP obtained right before Ucn application and after 25 min in the presence of Ucn, revealed that the peptide decreased the time-to-peak of LVP, increased its amplitude, and accelerated its relaxation (*Figure 1B*). In the presence of Ucn, +d*P*/d*t* increased 95.6 ± 20.1% ($n = 9$); meanwhile, it barely changed in untreated hearts (+d*P*/d*t* = 2.9 ± 1.4%, $n = 8$). The time

course of Ucn increased heart contractility was rather slow and gradual; the augmentation of $+dP/dt$ began within 5 min, peaked between 20 and 30 min, and was still significantly high 40 min after Ucn application (*Figure 1C*). Moreover, the cumulative administration of Ucn (1–100 nM) to isolated hearts showed a dose-dependent increase of $+dP/dt$ (*Figure 1D*). Maximal response to Ucn was observed at 100 nM, and the half-maximal effective concentration ($EC_{50}$) of Ucn was about 8 nM.

To further characterize the specificity of the effect of Ucn, we tested whether β-adrenergic pathway was linked to the positive inotropic effect. We applied isoproterenol (ISO) to activate β-adrenergic receptors in hearts at the time of maximum response to 100 nM Ucn. We observed that 1 μM of ISO produced an additional rapid increase in heart contractility (*Figure 2A*). Furthermore, in the presence of propranolol, the widely used inhibitor of β-adrenergic receptors,[28] Ucn increased $+dP/dt$ to practically the same level as in untreated heart (*Figure 2B*). The further addition of ISO (1 μM) failed to produce any increase of heart contractility as shown in *Figure 2B*. All together suggests that Ucn exerts strong positive inotropic effect independent of the β-adrenergic pathway.

### 3.2 Ucn increases heart contractility independently of PKA and seems to involve Epac pathway

Ucn binds with high affinity to the CRF-R2, which couples to Gs/cAMP/PKA signalling.[2,18] Therefore, we examined whether Ucn action is mediated by the activation of PKA. We assayed the activity of PKA in perfused hearts and in isolated cardiomyocytes treated 10 min with Ucn (10 nM). *Figure 3A* shows that indeed Ucn increased PKA activity

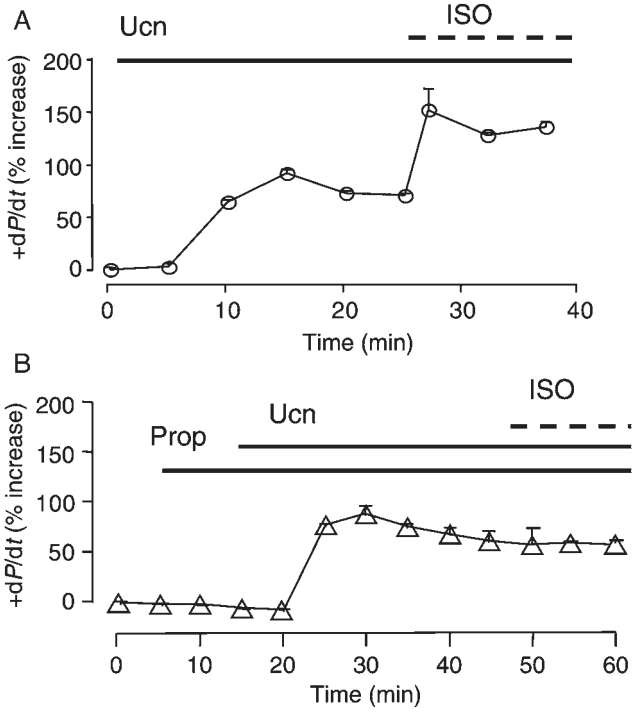


**Figure 2** β-Adrenergic receptor activation in hearts treated with urocortin. (*A*) Summary data of $+dP/dt$ time-course from perfused hearts treated with urocortin (*Ucn*, 100 nM). Isoproterenol (*ISO*, 1 μM) was added at the end as indicated. (*B*) Same as in (*A*) but in heart pretreated with propranolol (*Prop*, 1 μM). Data are from four different hearts.

significantly in isolated perfused hearts. Heart pretreatment with H89 (1 μM), a widely used inhibitor of PKA,[15] significantly reduced Ucn activation of PKA. In addition, *Figure 3B* shows that PKA activation was also observed in isolated cardiomyocytes treated with Ucn (10 nM), which was largely inhibited by H89 (1 μM) and by astressin (0.5 μM), specific antagonist of CRF-R2.[29] However, in *ex vivo*-perfused hearts, the inhibition of PKA had little effect on Ucn-increased contractility. We observed that the infusion of H89 alone decreased heart contractility, and maximum $+dP/dt$ enhanced by Ucn in the presence of H89 reached $82.9 \pm 10.9\%$ ($n = 4$) that is not statistically different from the maximum $+dP/dt$ in Ucn-treated hearts ($95.6 \pm 20.1\%$, $n = 9$) as shown in *Figure 3C*. Furthermore, the analysis of superimposed LVP obtained from heart treated first with H89 and then with Ucn, revealed that the peptide increased LVP amplitude and accelerated its relaxation (*Figure 3D*) independent of PKA inhibition. Taken together, these data indicate that while Ucn is able to stimulate PKA, Ucn-induced positive inotropism and lusitropism appear to be independent of cAMP/PKA pathway, suggesting that other cAMP-binding protein and subsequent signalling pathway may mediate the effects of Ucn.

Recently, a GEF called Epac has been shown to be involved in several cAMP-regulated processes in the heart.[21–23] Although there is no specific Epac antagonist yet, the small GEF antagonist brefeldin A (BFA) has been used to effectively antagonize Epac action on synaptic transmission at the crayfish neuromuscular junctions.[30,31] So, we used BFA to gain insights into the possible role of Epac in Ucn-induced heart contractility. First, we observed that BFA alone (20 μM) had no effect on hearts basal contractility. Meanwhile, 5 min pretreatments with BFA (20 μM) before Ucn application largely inhibited the positive inotropic effect. $+dP/dt$ increase reached only $20.4 \pm 7.6\%$ ($n = 4$) as shown in *Figure 3E*. These data indicate that a PKA-independent and BFA-sensitive pathway is involved in Ucn inotropic effect, which could be due, at least in part, to Epac-dependent pathway activation.

### 3.3 Role of PKC and ERK1/2 in the positive inotropic effect induced by Ucn

To assess the possible contribution of PKC to the positive inotropic action of Ucn, we evaluated the effects of the broad spectrum PKC inhibitor chelerythrine, and the specific PKC inhibitor GF109203X[28] in *ex vivo*-perfused hearts. *Figure 4* shows that both chelerythrine (10 μM) and GF109203X (8 nM) applied 5 min before Ucn significantly prevented the increased contractility. Increase in $+dP/dt$ were $33.7 \pm 2.7\%$ ($n = 5$) and $21.0 \pm 7.9\%$ ($n = 5$), respectively (*Figure 4A*). The effect of GF109203X was more potent than chelerythrine inhibition, and their infusion alone had no influence on hearts contractility.

Activation of PKC leads to phosphorylation of various cellular proteins including ERK1/2. To assess the contribution of ERK1/2 into the positive inotropism induced by Ucn, we tested PD98059, the specific inhibitor of ERK1/2,[11] in perfused hearts. *Figure 4B* shows that Ucn failed to increase the contractility in hearts pretreated 5 min with PD98059 (5 μM). The inhibitory effect was very strong, and $+dP/dt$ increased by Ucn in PD98059-treated hearts reached only $13.9 \pm 9.6\%$ ($n = 5$) indicating the important role of

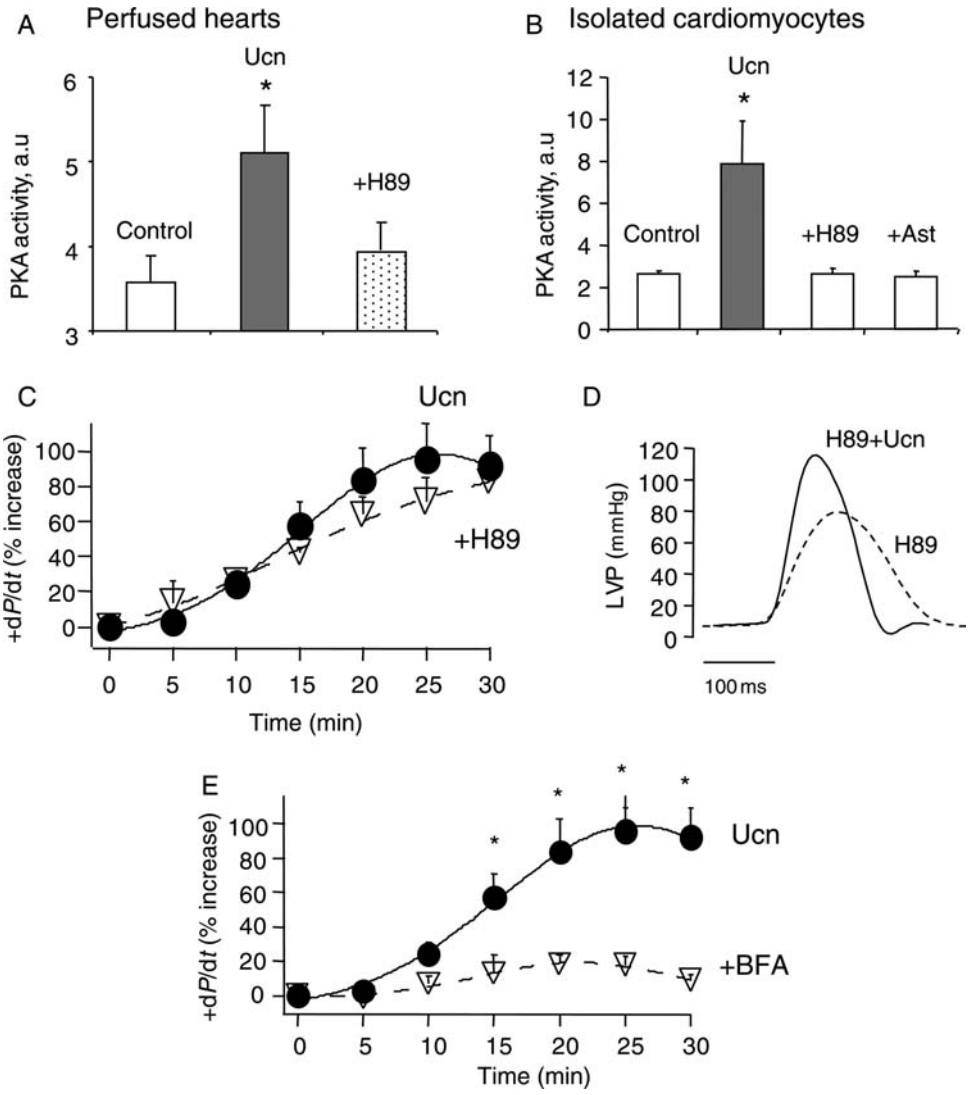


**Figure 3** Role of protein kinase A (PKA) and Epac in urocortin effect. (*A*) Bar graph showing the activity of PKA in untreated hearts (*control*); in hearts perfused 10 min with urocortin (10 nM, *Ucn*); and in hearts pre-incubated 5 min with H89 (1 μM) and then 10 min with urocortin (10 nM, *+H89*; $n = 9$ samples from three different hearts). (*B*) PKA activity in isolated cardiomyocytes in different conditions as in (*A*). *+Ast* is for cells treated 5 min with astressin (500 nM) and then 10 min with urocortin (10 nM; $n = 8$ samples from three separate cultures). (*C*) Shows the effect of PKA inhibition with H89 on urocortin-evoked contractility (*+H89*). H89 (1 μM) was applied 5 min before the addition of urocortin (10 nM; $n = 4$). (*D*) Superimposed traces of LVP from H89-treated heart before (*H89*, 1 μM) and after urocortin addition (*H89+Ucn*, 10 nM). (*E*) Time-course of urocortin (10 nM) effect on $+dP/dt$ in untreated hearts (*Ucn*) and in hearts pretreated 5 min with brefeldin A (*+BFA*, 20 μM) to inhibit Epac. The summary data are from four experiments. *$P < 0.05$.

ERK1/2 in the positive inotropic effect induced by Ucn. PD98059 alone had no effect on basal contractility of the heart.

### 3.4 Urocortin evokes positive inotropic and lusitropic effects at the cellular level

Since intracellular $Ca^{2+}$ increase plays a critical role in heart contraction, we examined whether Ucn inotropic actions were because of an enhancement in intracellular $Ca^{2+}$ mobilization. We investigated the effect of Ucn on $[Ca^{2+}]_i$ transients and cell contraction in isolated cardiomyocytes. *Figure 5A* shows representative examples of line scan images taken from a control cardiomyocyte (top) and a Ucn-incubated cardiomyocyte (bottom) under field stimulation. Ucn (100 nM) incubation for 20–40 min increased the amplitude of the $[Ca^{2+}]_i$ transient (*Figure 5B*) and accelerated its decay (*Figure 5C*). Furthermore, cellular contraction measured as % of cell shortening was also increased by Ucn incubation (*Figure 5D*). This increase in $[Ca^{2+}]_i$ transient amplitude was not prevented by PKA inhibition but was largely blocked by PKC inhibition (data not shown). $[Ca^{2+}]_i$ transient amplitude depends on SR $Ca^{2+}$ load, hence we next analysed whether Ucn could modify SR $Ca^{2+}$ content. After field-stimulating the cell to reach the steady state, caffeine was applied to empty the SR and estimate the SR $Ca^{2+}$ content in a control cardiomyocyte (top) and in the incubated one during 20–40 min in the presence of 100 nM Ucn (bottom) as shown in *Figure 5E*. The caffeine-evoked $[Ca^{2+}]_i$ transients were significantly higher in cardiomyocytes incubated with Ucn (*Figure 5F*). A rise in $[Ca^{2+}]_i$ transients and the subsequent higher contraction may be explained by an increase in SR $Ca^{2+}$ load observed in cardiomyocytes incubated with Ucn. Taken together, these results suggest that the Ucn inotropic effects in the whole heart could be because of an increase in $[Ca^{2+}]_i$ and cellular contraction.

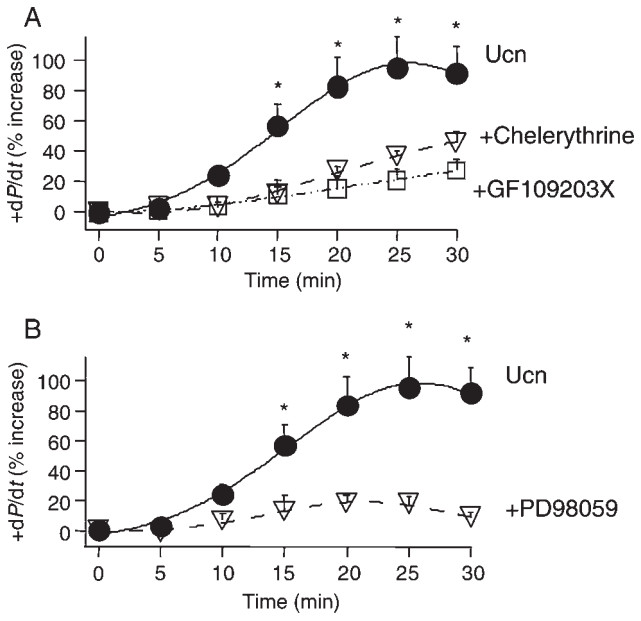


**Figure 4** Urocortin-evoked positive inotropic effect involves Protein kinase C and ERK1/2 activation. (*A*) Time-course of urocortin (10 nM) effect on $+dP/dt$ in hearts treated with urocortin (*Ucn*, 10 nM); in hearts pretreated 5 min either with chelerythrine (+*Chelerythrine*, 10 μM); or GF109203X (+*GF109203X*, 8 nM) to inhibit PKC, before the addition of urocortin (10 nM; $n = 5$ hearts). (*B*) Summary data of $+dP/dt$ increase in hearts treated with urocortin (*Ucn*, 10 nM); and in hearts pretreated 5 min with PD98059 (+*PD98059*, 5 μM), to inhibit ERK1/2, before urocortin infusion ($n$=5). *$P < 0.05$.

### 3.5 Urocortin activates L-type $Ca^{2+}$ channels

L-type $Ca^{2+}$ current ($I_{CaL}$) is the main trigger of $[Ca^{2+}]_i$ transient.[12] Hence, we investigated if Ucn effects in $[Ca^{2+}]_i$ could be owing to modulation of the L-type $Ca^{2+}$ current ($I_{CaL}$) by Ucn. *Figure 6A* shows representative recordings of $Ca^{2+}$ currents from two isolated voltage-clamped cardiomyocytes. Depolarizing steps evoked an inward current with maximal values near −10 mV, which was enhanced in cardiomyocytes incubated with Ucn (100 nM) compared with the magnitude of $I_{CaL}$ in untreated cardiomyocytes. *Figure 6B* illustrates the mean current density voltage relations of $I_{CaL}$ ($I/V$) recorded in non-treated (open circles; $n = 15$) and Ucn-treated isolated cardiomyocytes (closed circles; $n = 10$). The maximal $I_{CaL}$ density at −10 mV was $-11.1 \pm 0.7$ pA/pF in control and $-18.1 \pm 2.4$ pA/pF ($P < 0.01$) in Ucn-treated cells. Moreover, $I_{CaL}$ increase was prevented in cardiomyocytes pretreated with the specific CRF-R2 receptor antagonist (astressin, 0.5 μM) as represented in *Figure 6C*. Thus, Ucn significantly increased $I_{Ca}$ through a mechanism involving CRF-R2 in cardiomyocytes.

## 4. Discussion

The present study provides new details unveiling the mechanism of the inotropic action induced by Ucn in the isolated *ex vivo* Langendorff-perfused rat hearts. We showed that Ucn exerts potent and gradual positive inotropic and lusitropic effects, which reach maximum level at 20–30 min after Ucn addition. Ucn-evoked inotropic response clearly differed from the classical β-adrenergic effect known to develop usually rapidly. Indeed, we have demonstrated that Ucn modulates the inotropic responsiveness of the heart through different signalling pathway than the β-adrenergic pathway. Ucn was active at the nanomolar range and its effect was dose-dependent with an $EC_{50}$ value of about 8 nM; thus Ucn appears to be among the most potent endogenous positive inotropic substances.

Ucn binds with high affinity to the CRF-R2,[2,18] whose activation leads to an increase in cAMP and consequent PKA activation. In fact, we showed that Ucn increases PKA activity both in perfused hearts and in isolated cardiomyocytes. And we confirmed that Ucn activation of PKA is mediated by CRF-R2 in isolated cardiomyocytes. But PKA inhibition had little effect on Ucn-induced positive inotropic effect, which is not in accordance with the data reported in rabbit cardiomyocytes. Ucn inotropic and lusitropic actions involved PKA pathway, even though the inhibition of basal activity of PKA in those cardiomyocytes was not shown.[15] It is important to stand out that PKA inhibition by itself decreased heart contractility confirming the critical role played by PKA on heart basal activity as described previously.[32] Our data clearly show that Ucn-exhibited positive inotropism is mediated by a PKA-independent mechanism suggesting that another cAMP-binding protein and subsequent signalling pathway may be involved.

Recently, Epac has been involved in several cAMP-dependent but PKA-independent cellular processes. So it is likely that Epac may mediate Ucn effects. However, there is not yet a specific Epac inhibitor. Nevertheless, the small GEF-antagonist, BFA has been shown to be able to inhibit Epac.[30,31] BFA was originally used to interfere with vesicular traffic from the Golgi apparatus by slowing GDP–GTP exchange on ADP ribosylation factors (ARF); the latter are targets of GEF for ARF that are structurally related to Epac, although they are not activated by cAMP (for review see Chardin and McCormick[33]). We showed that BFA did not affect basal contractility of the heart but it did prevented Ucn actions even with smaller concentration when compared with that used in other studies.[30,31] Thus, while there is no specific inhibitor available of Epac, BFA seems to be a useful tool to determine a possible implication of Epac in the signalling pathway. Our results suggest a critical role of a BFA-sensitive pathway, which seems to involve Epac in Ucn-increased heart contractility. This finding is consistent with the increasing numbers of reports that propose an important role of Epac in heart, for example in $Ca^{2+}$ handling in cardiomyocytes[23] or cardiac excitation–contraction coupling.[34]

Activation of PKC has been considered to be an important pathway in cellular responses in cardiac myocytes,[35] and its implication in Ucn effects has been related especially to heart protection against ischaemia.[24,36] The effect of chelerythrine and GF109203X, non-specific and specific PKC inhibitors, in Ucn-positive inotropic effects suggested a role for the conventional isoforms of PKC. Activated PKC can phosphorylate a wide spectrum of cellular proteins such as MAPK. Indeed, classical and novel PKC have been shown to stimulate MAPK in rat ventricular myocytes.[37] Several reports have implicated the sub-family of MAPK, ERK1/2 activation by Ucn as a survival pathway in cardiac cells.[11] Our data show for the first time that the inhibition of ERK1/2 dramatically prevented Ucn effects suggesting a critical role of ERK1/2 in the induced positive inotropism. The contribution of MAPK to the positive inotropism is still

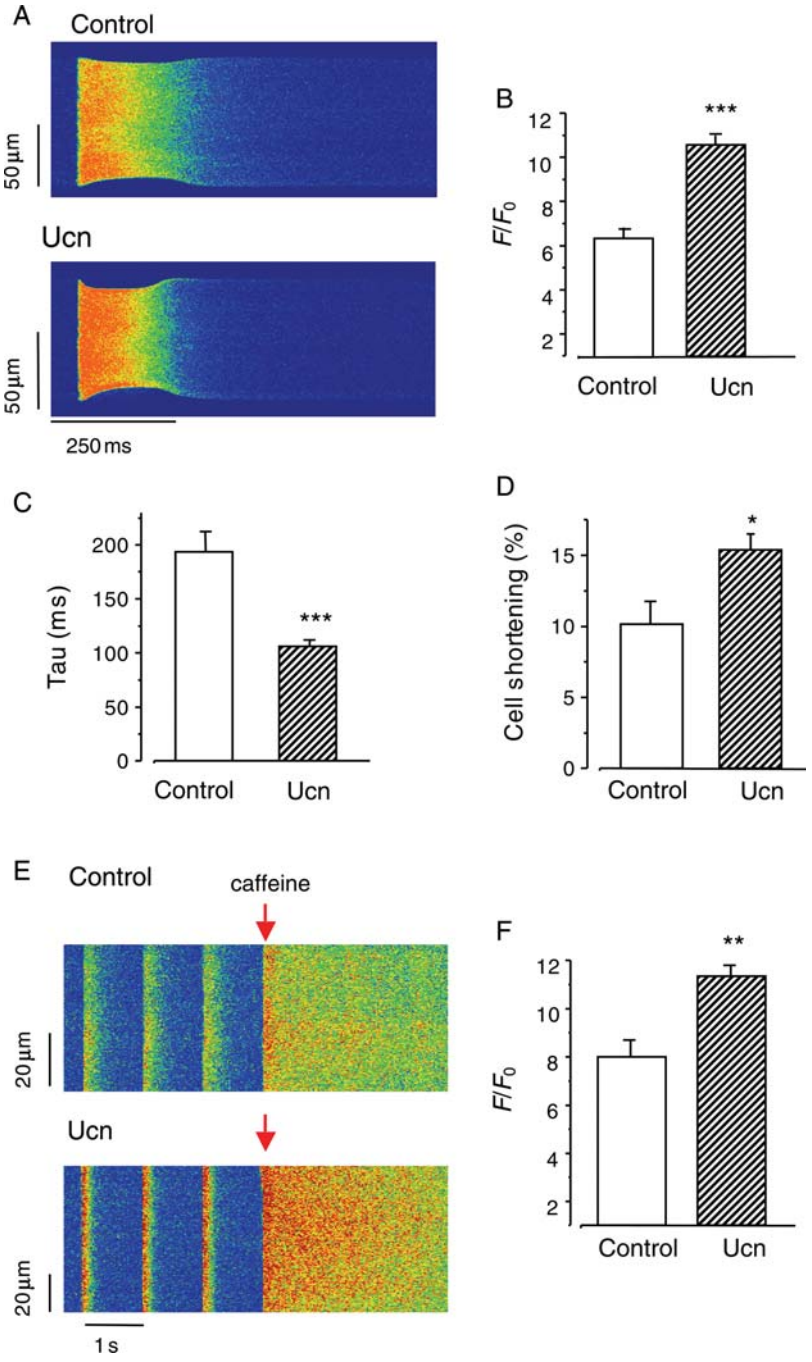


**Figure 5** $[Ca^{2+}]_i$ transients, cell shortening, and sarcoplasmic reticulum $Ca^{2+}$ load were increased by urocortin in isolated cardiomyocytes. (*A*) Representative examples of line-scan confocal images obtained in isolated myocytes field stimulated at 1 Hz in a control (top) and in a cardiomyocyte incubated during 20–40 min with urocortin (100 nM, bottom). (*B*) Amplitude of the fluorescence $[Ca^{2+}]_i$ transients (expressed as $F/F_0$, where $F$ is the peak fluorescence signal and $F_0$ the diastolic fluorescence), their decay time constants were obtained by fitting the decay trace to a single exponential ($\tau$, *C*), and the percentage of cell shortening (*D*) in control cells (white bars) and in cells incubated with urocortin (orange bars); $n = 5$–11; $^*P < 0.05$; $^{***}P < 0.001$ with respect to control cells. (*E*) Line-scan images obtained in a control (top) and in a urocortin incubated (bottom) cell showing intracellular $[Ca^{2+}]_i$ transients during field stimulation at 1 Hz and SR $Ca^{2+}$ release at caffeine (10 mM) application. Caffeine was applied at the moment indicated by the red arrow. (*F*) Bar graphs comparing the amplitude of caffeine-evoked $[Ca^{2+}]_i$ transients in control cells (white bar) and cells incubated during 20–40 min with 100 nM urocortin (orange bar) expressed as $F/F_0$. $n = 7$; $^{**}P < 0.01$ with respect to control cells.

under debate although there are increasing reports that demonstrated ERK1/2 implication in the heart contractility.[38–40]

In addition, we confirmed that the inotropic effect of Ucn could be mediated by an increase in $Ca^{2+}$ handling, possible consequence of the increased $I_{Ca}$.[41] We showed that Ucn increased electrically evoked $[Ca^{2+}]_i$ transients in parallel with the enhancement of SR $Ca^{2+}$ contents, which provides more $Ca^{2+}$ to induce larger $Ca^{2+}$ release from the SR. Ucn acceleration of $[Ca^{2+}]_i$ transient decline may be involved in the Ucn lusitropic effects. On the other hand, Ucn significantly increased $I_{CaL}$ amplitude, which is the main trigger of $[Ca^{2+}]_i$ transient and its amplitude marks the $[Ca^{2+}]_i$ transients amplitude.[12] $I_{CaL}$ potentiation required the activation

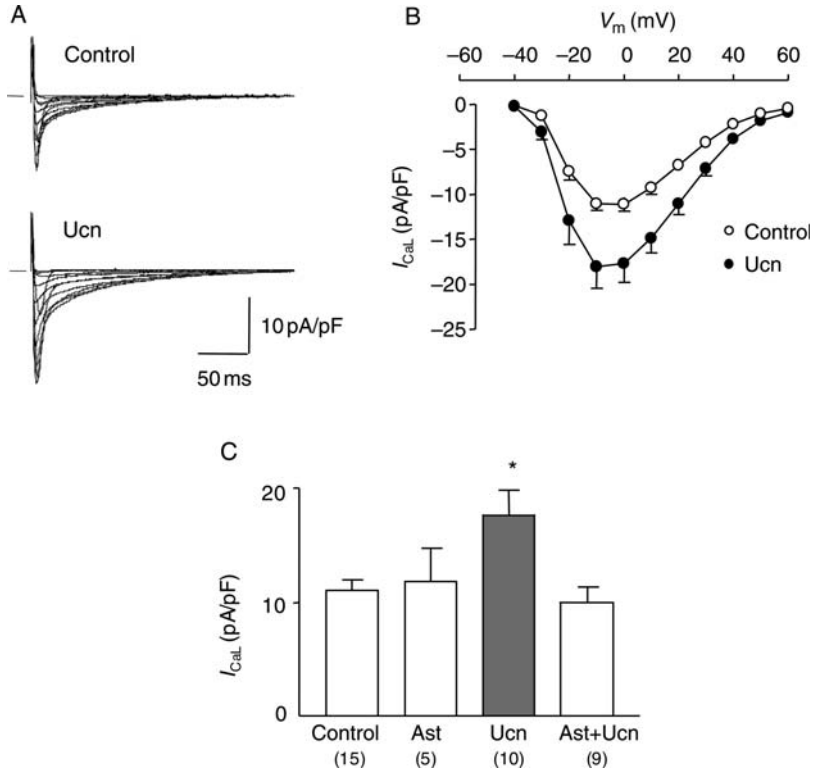


**Figure 6** Upregulation of L-type $Ca^{2+}$ currents ($I_{CaL}$) induced by urocortin in isolated cardiomyocytes. (*A*) Representative traces of $I_{CaL}$ in control cardiomyocytes (*control*) and in cardiomyocytes pre-incubated 20–40 min with urocortin (*Ucn*, 100 nM). (*B*) Summary data of the current/voltage (*I*/*V*) curve in the absence (*control*), and presence of urocortin (*Ucn*, 100 nM). (*C*) Bar graph shows the average of $I_{CaL}$ density recorded at −10 mV in the same condition as in (*B*) and in cardiomyocytes treated with astressin (*Ast*, 500 nM), and in cells incubated 5 min with astressin, then 20–30 min with urocortin (*Ast*+*Ucn*). Data are expressed as mean ± SEM and the number of cells analysed is shown in the bottom of each bar graphs. $^*P < 0.05$ with respect to control cells.

of CRF-R2 as it was inhibited by astressin, which is not consistent with a study in rat cardiomyocytes where Ucn was proposed to decrease $I_{CaL}$ independently of CRF-R2 activation.[14] There are very few studies of Ucn effects on $I_{Ca}$ in cardiomyocytes; thus, the mechanism of Ucn modulation of L-type $Ca^{2+}$ channel deserves further investigations.

In summary, we have demonstrated that the administration of Ucn produced a progressive and long-lasting potent positive inotropic effect in isolated rat heart. These effects were mediated by cAMP through a PKA-independent mechanism. We have shown for the first time a BFA-sensitive pathway, suggesting a possible involvement of Epac in Ucn actions. Moreover, we have demonstrated the important role of PKC and ERK1/2 played in Ucn-evoked positive inotropic effect. In addition, we have shown a significant increase of $I_{CaL}$, $[Ca^{2+}]_i$ transients amplitude, and SR $Ca^{2+}$ content by Ucn, crucial events for the evoked inotropic response. The beneficial action of the positive inotropic effects of Ucn together with the coronary vasodilation in rat heart,[19] may be useful in the treatment of cardiovascular disorders, particularly heart failure, nevertheless extensive clinical studies are needed to warrant Ucn clinical significance.[42]

## Funding

This study was supported by 'Red Cardiovascular RECAVA' of Instituto Carlos III (grant number: RD06-0014-0020, RD06-0014-0007, PI06-0133), Consejerías de Salud, de Innovación Ciencia y Empresa de la Junta de Andalucía (grant numbers: 174/2006, P06-CTS-01711), Inserm, and by Agence Nationale pour la Recherche (grant: Physio2006E-pac). T.S is a 'Ramon y Cajal' Researcher and E.C is a fellow student from RECAVA.

**Conflict of interest.** None declared.

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