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RESEARCH PAPER

cAMP- and Ca^{2+}/calmodulin-dependent protein kinases mediate inotropic, lusitropic and arrhythmogenic effects of urocortin 2 in mouse ventricular myocytes

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BACKGROUND AND PURPOSE

Urocortin 2 is beneficial in heart failure, but the underlying cellular mechanisms are not completely understood. Here we have characterized the functional effects of urocortin 2 on mouse cardiomyocytes and elucidated the underlying signalling pathways and mechanisms.

EXPERIMENTAL APPROACH

Mouse ventricular myocytes were field-stimulated at 0.5 Hz at room temperature. Fractional shortening and $[Ca^{2+}]_i$ transients were measured by an edge detection and epifluorescence system respectively. Western blots were carried out on myocyte extracts with antibodies against total phospholamban (PLN) and PLN phosphorylated at serine-16.

KEY RESULTS

Urocortin 2 elicited time- and concentration-dependent positive inotropic and lusitropic effects (EC_{50}: 19 nM) that were abolished by antisauvagine-30 (10 nM, $n = 6$), a specific antagonist of corticotrophin releasing factor (CRF) CRF_2 receptors. Urocortin 2 (100 nM) increased the amplitude and decreased the time constant of decay of the underlying $[Ca^{2+}]_i$ transients. Urocortin 2 also increased PLN phosphorylation at serine-16. H89 (2 μM) or KT5720 (1 μM), two inhibitors of protein kinase A (PKA), as well as KN93 (1 μM), an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), suppressed the urocortin 2 effects on shortening and $[Ca^{2+}]_i$ transients. In addition, urocortin 2 also elicited arrhythmogenic events consisting of extra cell shortenings and extra $[Ca^{2+}]_i$ increases in diastole. Urocortin 2-induced arrhythmogenic events were significantly reduced in cells pretreated with KT5720 or KN93.

CONCLUSIONS AND IMPLICATIONS

Urocortin 2 enhanced contractility in mouse ventricular myocytes via activation of CRF_2 receptors in a cAMP/PKA- and Ca^{2+}/CaMKII-dependent manner. This enhancement was accompanied by Ca^{2+}-dependent arrhythmogenic effects mediated by PKA and CaMKII.

Abbreviations

ASV, antisauvagine-30; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CRF, corticotrophin releasing factor; CRF_1 receptor, CRF type 1 receptor; CRF_2 receptor, CRF type 2 receptor; Epac, exchange protein activated by cAMP; FS, fractional shortening; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; PLN, phospholamban; RT50, time required from peak shortening to 50% relengthening; SERCA, SR Ca^{2+}-ATPase; SR, sarcoplasmic reticulum

Introduction

Corticotrophin releasing factor (CRF), a 41 amino-acid peptide hormone (Spiess *et al.*, 1981), is a critical component of stress responses mediated by the endocrine and autonomic nervous system and participates in the regulation of behaviour and mood (Dunn and Berridge, 1990; Chang *et al.*, 1993; Chen *et al.*, 1993; Perrin *et al.*, 1993; Vita *et al.*, 1993; Eckart *et al.*, 2002). Moreover, CRF and the urocortins, endogenous CRF-like peptides, exert powerful cardiovascular actions in animals and men (Parkes *et al.*, 2001; Weisinger *et al.*, 2004; Stiedl *et al.*, 2005; Rademaker *et al.*, 2005b; Nazarloo *et al.*, 2006; Davis *et al.*, 2007b; Grossini *et al.*, 2008; Calderon-Sanchez *et al.*, 2009; Yang *et al.*, 2010). Systemic administration of urocortins can modulate heart rate, cardiac contractility and peripheral resistance via activation of CRF receptors (Mackay *et al.*, 2003; Bale *et al.*, 2004; Weisinger *et al.*, 2004). It is of particular clinical and pharmacological relevance that urocortins exert beneficial effects in heart failure (Rademaker *et al.*, 2005a,b; Davis *et al.*, 2007b; Yang *et al.*, 2010), a major cardiac condition with a prevalence of 1–2% and – despite recent advances in pharmacotherapy – a particularly poor prognosis (McMurray and Pfeffer, 2005). Several studies have shown that urocortins improve cardiovascular and renal function in experimental heart failure (Rademaker *et al.*, 2002; 2005a; 2006; Mackay *et al.*, 2003;). Furthermore, small trials in humans have suggested that urocortins are safe in healthy subjects (Davis *et al.*, 2004; 2007a) and, in addition, that they exert beneficial effects in heart failure patients, in part resembling those observed in experimental heart failure (Davis *et al.*, 2005; 2007b). Thus, urocortins exhibit therapeutic potential for the treatment of heart failure. Despite this therapeutic potential, however, the cellular actions of urocortins on cardiac myocytes remain only poorly understood.

Urocortins act through two G protein-coupled CRF receptor subtypes, CRF type-1 receptor (CRF_1 receptor) and CRF type-2 receptor (CRF_2 receptor) (Eckart *et al.*, 2002; Perrin and Vale, 1999; receptor nomenclature follows Alexander *et al.*, 2009). In rodents, the CRF_1 receptor is mainly expressed in the central nervous system, whereas CRF_2 receptors are found also in peripheral tissue including heart (Spiess *et al.*, 1998; Van Pett *et al.*, 2000; Bale *et al.*, 2004; Yang *et al.*, 2010). Urocortin 2 binds selectively to CRF_2 receptors, with no appreciable activity on CRF_1 receptors (Reyes *et al.*, 2001). In a previous study, we demonstrated in rabbit ventricular myocytes that urocortin 2 elicits positive inotropic and lusitropic effects (Yang *et al.*, 2006). Urocortin 2 acted via CRF_2 receptors to stimulate protein kinase A (PKA) activity. PKA, in turn, elevated L-type Ca^{2+} current and sarcoplasmic reticulum (SR) Ca^{2+} content via increased SR-Ca^{2+}-ATPase (SERCA) activity to augment intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) transients and enhance cardiomyocyte contractility (Yang *et al.*, 2006). Similarly, a recent study in rat ventricular myocytes observed Ca^{2+}-dependent positive inotropic and lusitropic effects induced by urocortin 1 (Calderon-Sanchez *et al.*, 2009). Urocortin 1 also increased PKA activity in rat cardiac myocytes. However, unlike rabbit cardiac myocytes, in rat cardiac myocytes, this increased PKA activity did not appear to underlie the positive inotropic and lusitropic effects. Rather the exchange protein activated by cAMP (Epac), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) signalling pathways contributed to the positive inotropic and lusitropic effects of urocortin 1 (Calderon-Sanchez *et al.*, 2009). These findings suggest that, in cardiac myocytes, urocortins may act via multiple signalling pathways to elicit inotropic and lusitropic effects and, in addition, that the relative contribution of these signalling pathways to the inotropic and lusitropic effects may be species-dependent.

Transgenic mouse models have become a powerful and widely used tool to study the physiological and pathophysiological roles of proteins in cardiac function, including the cardiac actions of urocortins (Bale *et al.*, 2004). Importantly, however, the cellular actions of urocortins on mouse cardiac myocytes have not been studied yet. Therefore, we decided to characterize the functional effects of urocortin 2 on isolated mouse ventricular myocytes and to elucidate the underlying signalling pathways and mechanisms. Our results show that urocortin 2 exerted powerful positive inotropic and lusitropic effects in mouse ventricular myocytes and that both cAMP-PKA and Ca^{2+}/calmodulin-Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) signalling participated in these effects. Furthermore, urocortin 2 also elicited Ca^{2+}-dependent arrhythmogenic effects mediated by the same signalling pathways. Qualitatively similar effects were observed in human atrial myocardium.

Methods

Myocyte isolation

All animal care and experimental procedures were in accordance with local guidelines for animal care. Ventricular myocytes were isolated from adult mouse hearts using enzymatic dissociation. Briefly, mice (46 in total; NMRI and C57BL/6; age: 8–20 weeks; weight: 24–35 g; either sex) were

injected with heparin (1000 U·kg^{-1}, i.p.) 15 min prior to the isolation procedure and anaesthetized with ketamine/xylazine (0.1 mL·100 g^{-1}, i.p.). Afterwards, hearts were rapidly removed and mounted on a temperature-controlled (37°C) Langendorff perfusion system. After 3 min perfusion with Ca^{2+}-free Tyrode's solution equilibrated with carbogen, hearts were perfused for 20 min with collagenase II-containing Tyrode's solution. Tyrode's solution contained (in mM): 126 NaCl, 4.4 KCl, 1.2 $MgCl_2$, 0.12 NaH_2PO_4, 4.0 $NaHCO_3$, 10 HEPES, 5.5 glucose, 1.8 pyruvate, adjusted with NaOH to pH 7.4. After this perfusion, left ventricles were minced into small pieces and incubated with the collagenase-containing Tyrode's solution for an additional 15 min. Following a centrifugation step, myocytes were filtered through 200 µm nylon mesh, re-suspended in Tyrode's solution containing 0.0125 mM $CaCl_2$ besides the components listed above and allowed to settle for another 15 min at room temperature. During this time, extracellular [Ca^{2+}] was slowly raised to 1.2 mM. Only viable, regularly rod-shaped cells with clear cross-striations were used for experiments.

Contractility and Ca^{2+} measurements

Measurements of unloaded cell shortening were performed as described previously (Yang *et al.*, 2006). Briefly, myocytes were transferred into a glass bottomed chamber and examined with an inverted microscope. Cells were bathed in Tyrode's solution containing 2 mM $CaCl_2$ and field-stimulated at 0.5 Hz at room temperature. Cell length was measured by video edge detection (Crescent Electronics, Sandy, UT, USA). Contractile parameters analyzed included: fractional shortening (FS), maximal rates of cell shortening (+dL·dt^{-1}) and relengthening (–dL·dt^{-1}), time-to-peak shortening and the time required from peak shortening to 50% relengthening (RT50).

Transient changes in intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) ([Ca^{2+}]$_i$ transients) were measured using indo-1, as described previously (Suarez *et al.*, 2004). Briefly, cells affixed to laminin-coated glass cover slips were loaded with indo-1/AM (3 µM, 30 min). Following dye loading, the glass cover slips were rinsed to remove excess indo-1/AM and mounted on the stage of a Nikon Diaphot epifluorescence microscope equipped with a x40 oil immersion objective lens (N.A. = 1.3), which was interfaced with a dual-emission photometry system (Photon Technology International). Myocytes were field-stimulated at 0.5 Hz. Indo-1 was excited at 365 nm. Fluorescence emission was directed to two photomultiplier tubes and collected at 405 nm (F405) and 485 nm (F405) respectively. The ratio F405/F485 represents a measure for [Ca^{2+}]$_i$. During these measurements, cells were bathed in Tyrode's solution containing 2 mM $CaCl_2$.

Finally, combined shortening and [Ca^{2+}]$_i$ measurements were conducted in cells loaded with Fluo-4/AM (2 µM, 20 min) using the same video edge detection system as described above. In addition, cells were illuminated with light from an attached mercury arc lamp. Fluo-4 was excited at 485 ± 10 nm and fluorescence emission collected at >515 nm. These measurements were conducted in Tyrode's solution containing 1 mM $CaCl_2$.

Western blot analysis

Myocyte extracts were prepared in cell lysis buffer (20 mM Tris, 20 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, pH 7.4). After determination of protein content, adjusted equal loads of protein were separated by 4–20% Bis-Tris polyacrylamide gel electrophoresis (Invitrogen, San Diego, CA, USA), and transferred to a nitrocellulose membrane. The membrane was incubated with 5% milk powder in Tris-buffered saline containing 0.05% Tween, overnight at 4°C. Then, the following primary antibodies were added: antibody against phospholamban (PLN) phosphorylated at serine 16 (rabbit polyclonal IgG; 07-052, Millipore, Billerica, MA, USA) or antibody against PLN (mouse monoclonal IgG2a; MA3-922, Affinity Bioreagents, Golden, CO, USA). Afterwards, the blots were washed, exposed to secondary antibody conjugated to horseradish peroxidase to drive a chemiluminescence reaction (Perkin Elmer Inc., Waltham, MA, USA), and exposed to film. Densitometric analysis of bands was performed using the Image Software for PC.

Human atrial trabeculae

Samples of human atria were obtained from patients (21 in total; 44–82 years; 14 males) with informed written consent and approval by the local ethics committee. This investigation conformed to the principles outlined in the Declaration of Helsinki. Right atrial appendages were obtained from hearts of patients undergoing cardiac bypass or valve replacement surgery.

Atrial trabeculae were prepared as described previously (Kockskamper *et al.*, 2008). Trabeculae (n = 34 from 21 patients) were mounted on hooks in a temperature-controlled (37°C) recording chamber and electrically stimulated at 1 Hz in Tyrode's solution containing (mM): Na^+ 152, K^+ 3.6, Cl^- 135, HCO_3^- 25, HEPES 5, Ca^{2+} 2.5, Mg^{2+} 0.6, $H_2PO_4^-$ 1.3, SO_4^{2-} 0.6, pH 7.4. Isometric contractions were recorded using a force transducer and displayed on a chart recorder and a computer for further analysis. Trabeculae were gradually stretched to the length at which maximal force development was observed (L_{max}). Following an equilibration period of >15 min at L_{max}, the experimental protocol was started.

Data analysis

Data are expressed as the mean ± SEM of n measurements. Differences between groups were evaluated by Student's t-tests or ANOVA. Details are provided in the figure legends. $P < 0.05$ was considered to indicate significant differences.

Materials

Antisauvagine-30 (ASV), a selective CRF_2 receptor antagonist (Ruhmann *et al.*, 1998), was synthesized at the Department of Molecular Neuroendocrinology (Max Planck Institute for Experimental Medicine, Göttingen) (Eckart *et al.*, 2001). Urocortin 2 was either synthesized at the Department of Molecular Neuroendocrinology, Göttingen, or purchased from Sigma (St Louis, MO, USA). Urocortin 1, urocortin 3 and H89 were from Sigma and KT5720 and KN93 were from Calbiochem (San Diego, CA, USA).

Results

Urocortin 2 elicits positive inotropic and positive lusitropic effects in mouse ventricular myocytes via activation of CRF_2 receptors

Figure 1A shows an original recording of unloaded cell shortening of a mouse ventricular myocyte electrically stimulated at 0.5 Hz and challenged with urocortin 2 (100 nM), which caused a gradual increase in FS. Following urocortin 2 exposure, FS increased from 9.2% to 20.5% over a time course of 20 min. On average, the maximal increase in FS was reached 20 min after urocortin 2 application and it amounted to approximately 240% of the initial control (Figure 1C). By contrast, untreated control myocytes showed a small rundown in FS over the same time course (Figure 1C). Comparison of individual cell shortenings obtained at 0 and 20 min after urocortin 2 exposure revealed that, in addition to the increase in FS, urocortin 2 also accelerated the kinetics of shortening and relengthening (Figure 1B) and mean values from these experiments are shown in Figure 1D. The urocortin 2-induced increase in FS was steeply concentration-dependent with an EC_{50} of 19 nM urocortin 2 and a Hill coefficient of 3.2 (Figure 1E). It was abolished by pretreatment of the cells with 10 nM ASV, a selective antagonist of CRF_2 receptor (Figure 1E). Moreover, ASV also suppressed the urocortin 2 effects on shortening and relengthening velocities (Figure 1D). Taken together, these results show that, in mouse ventricular myocytes, urocortin 2 elicited time- and concentration-dependent positive inotropic and lusitropic effects that were mediated by activation of CRF_2 receptors.

As observed with urocortin 2, urocortin 1 and urocortin 3 also elicited positive inotropic and lusitropic effects (not shown). At 100 nM, urocortin 1 and urocortin 3 increased FS to 152 ± 15% and 157 ± 22% of the initial control respectively (both $P < 0.05$ versus initial control, tested by paired Student's *t*-test; data from $n = 6$ myocytes from 3 mouse hearts for each series).

The positive inotropic and positive lusitropic effects of urocortin 2 are Ca^{2+}-dependent

To determine whether the inotropic and lusitropic effects of urocortin 2 were Ca^{2+}-dependent, the transient changes in $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$ transients) and FS were measured

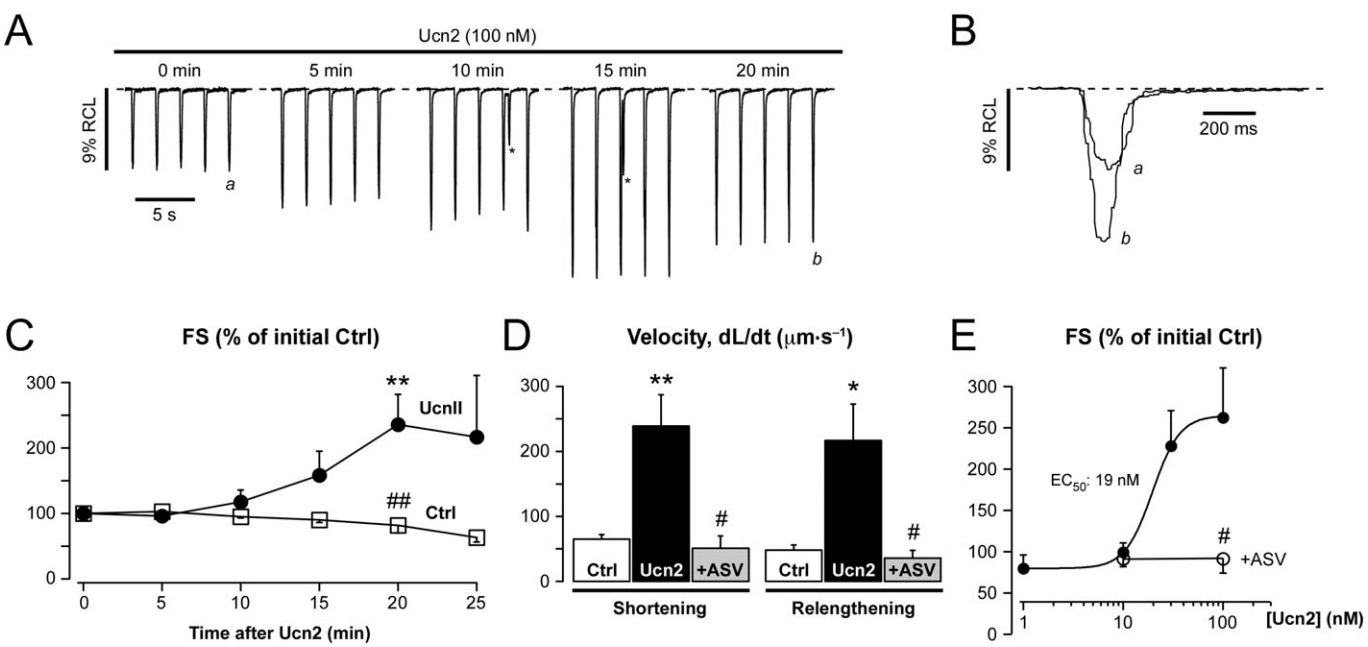

Figure 1

Urocortin 2 (Ucn2) elicits positive inotropic and lusitropic effects in mouse ventricular myocytes. (A) Fractional shortening (FS, % of resting cell length, RCL) 0, 5, 10, 15 and 20 min after application of 100 nM Ucn2. Asterisks indicate two extra shortenings not elicited by the electrical stimulation. (B) Direct comparison of individual shortenings from (A) after 0 (a) and 20 (b) min of Ucn2 exposure. (C) Time course of FS (normalized to the initial control) in untreated control myocytes (Ctrl, $n = 5$ myocytes from three mouse hearts) and in myocytes challenged with 100 nM Ucn2 ($n = 10$ myocytes from five mouse hearts). $^{**}P < 0.01$ versus 0 min Ucn2; $^{\#\#}P < 0.01$ versus Ucn2; tested by two-way ANOVA for repeated measures, followed by Tukey's range test. (D) Maximal velocities of shortening and relengthening in untreated control cells (Ctrl, $n = 5$ myocytes from three mouse hearts) and in myocytes treated with 100 nM Ucn2 in the absence (Ucn2, $n = 10$ myocytes from five mouse hearts) and presence of 10 nM antisauvagine-30 (+ASV, $n = 6$ myocytes from five mouse hearts). $^{**}P < 0.01$ and $^{*}P < 0.05$ versus Ctrl; $^{\#}P < 0.05$ versus Ucn2; tested by one-way ANOVA followed by Tukey's range test. (E) Concentration-response curve of the Ucn2-induced increase in FS. Values were obtained from 3–9 ventricular myocytes from 3–5 mouse hearts. Line is a fit of the Hill equation to the Ucn2 data yielding an EC_{50} of 19 nM Ucn2 and a Hill coefficient of 3.2. In the presence of 10 nM antisauvagine-30 (+ASV, $n = 6$ myocytes from five mouse hearts), the Ucn2 effect was blocked. $^{\#}P < 0.05$ versus Ucn2 alone, tested by unpaired Student's *t*-test.

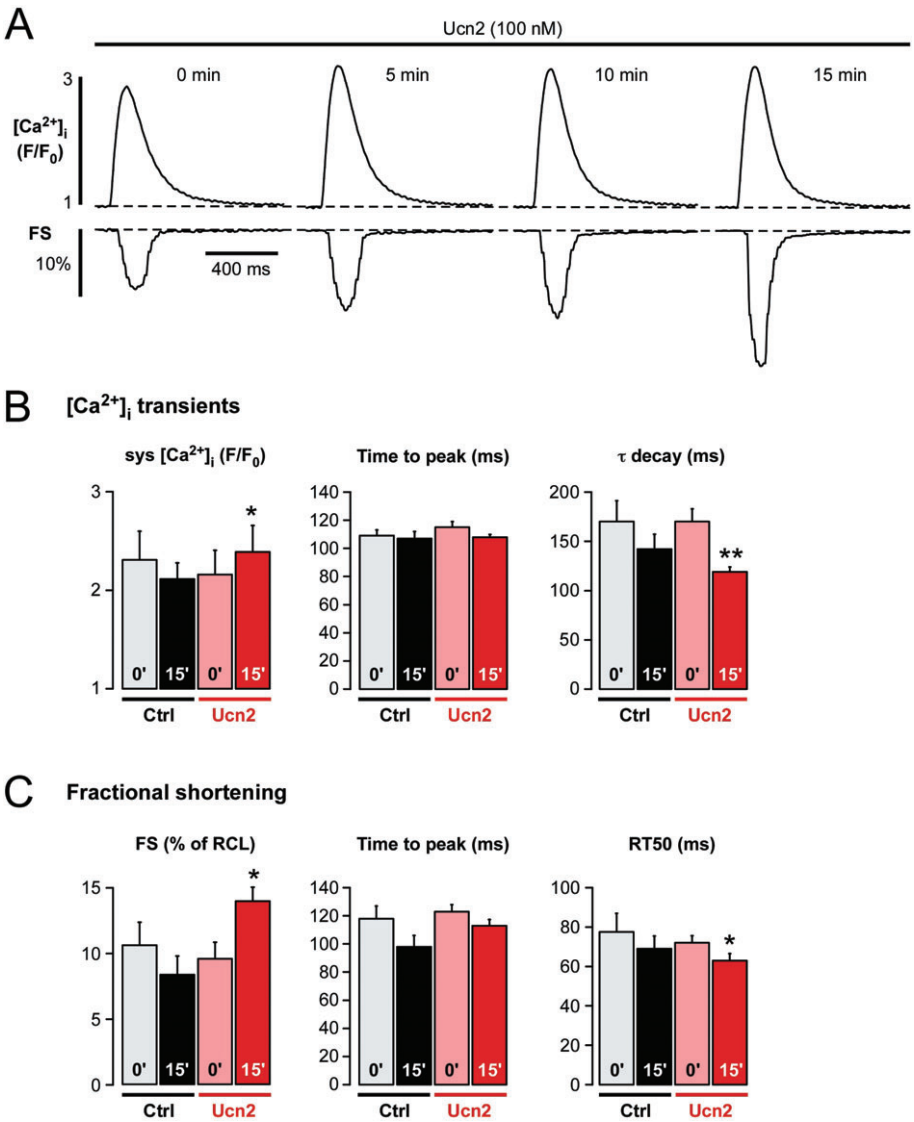

Figure 2

The positive inotropic and lusitropic effects of urocortin 2 (Ucn2) are Ca^{2+}-dependent. (A) Original recordings of $[Ca^{2+}]_i$ transients, measured as normalized Fluo-4 fluorescence, F/F_0 (top) and fractional shortening (FS), measured as % of resting cell length (bottom) 0, 5, 10 and 15 min after application of 100 nM Ucn2. (B) Systolic $[Ca^{2+}]_i$ and kinetics of $[Ca^{2+}]_i$ transients and (C) FS and shortening kinetics in untreated control myocytes (Ctrl, n = 10 myocytes from eight mouse hearts) and in myocytes exposed to 100 nM Ucn2 (n = 19 myocytes from 10 mouse hearts). $[Ca^{2+}]_i$ transient decay and relaxation were quantified by the time constant τ of fluorescence decrease and the time required from peak shortening to 50% of relengthening (RT50), respectively, *$P < 0.05$ versus 0 min Ucn2; **$P < 0.01$ versus 0 min Ucn2, tested by paired Student's t-test.

simultaneously in Fluo-4-loaded ventricular myocytes. Figure 2A shows individual $[Ca^{2+}]_i$ transients (top) and FS (bottom) of a ventricular myocyte 0, 5, 10, and 15 min after the addition of 100 nM urocortin 2. The $[Ca^{2+}]_i$ transients were augmented and accelerated by urocortin 2. These effects were accompanied by increased cell shortening and accelerated relengthening. Average values for $[Ca^{2+}]_i$ transients and shortening kinetics are presented in Figures 2B and C. By contrast, untreated control myocytes (Ctrl) showed a small rundown of both systolic $[Ca^{2+}]$ and FS. The time-to-peak $[Ca^{2+}]_i$ transient and FS remained unchanged, whereas the time constant τ for $[Ca^{2+}]_i$ transient decay (Figure 2B, $P < 0.01$) and the RT50 (Figure 2C, $P < 0.05$) were significantly reduced by 15 min exposure to urocortin 2. These effects were not seen in untreated control myocytes.

We also measured the effects of urocortin 2 on $[Ca^{2+}]_i$ transients using the ratiometric dye Indo-1 (Figure 4). In Indo-1-loaded myocytes, urocortin 2 significantly increased systolic $[Ca^{2+}]_i$ and the amplitude of the $[Ca^{2+}]_i$ transients. Diastolic $[Ca^{2+}]_i$ remained unchanged. Taken together, these data indicate that the positive inotropic and positive lusitropic effects of urocortin 2 were Ca^{2+}-dependent

and mediated by alterations in the underlying $[Ca^{2+}]_i$ transients.

PKA and CaMKII mediate the positive inotropic and lusitropic effects of urocortin 2

The CRF_2 receptor couples to the G_s-cAMP-PKA pathway. Furthermore, in rabbit, but not in rat (Calderon-Sanchez *et al.*, 2009) ventricular myocytes, the positive inotropic and lusitropic effects of urocortins are mediated by PKA (Yang *et al.*, 2006). Therefore, we investigated the involvement of PKA in the positive inotropic effect of urocortin 2 in mouse ventricular myocytes. Experiments were conducted on either Fluo-4- or Indo-1-loaded myocytes, and two different PKA inhibitors were used, KT5720 (1 μM) and H89 (2 μM). Figure 3A shows original recordings of $[Ca^{2+}]_i$ transients and cell shortenings obtained at 0 min and 15 min after exposure to urocortin 2 (100 nM). Urocortin 2 alone (left) increased the $[Ca^{2+}]_i$ transients and cell shortening and accelerated $[Ca^{2+}]_i$ transient decay and relengthening (see also Figure 2). In the presence of KT5720 (1 μM), however, these effects of urocortin 2 were blocked and the $[Ca^{2+}]_i$ transients as well as cell shortening was reduced, similar to the rundown observed in untreated control myocytes (compare Figures 1 and 2). Average values are shown in Figure 3B,C confirming the lack of effect of urocortin 2 in myocytes pretreated with KT5720. Similarly, in Indo-1-loaded myocytes, the increase in systolic $[Ca^{2+}]_i$ (Figure 4A) and amplitude of $[Ca^{2+}]_i$ transients (Figure 4B), elicited by urocortin 2 was abolished by pretreatment with H89 (2 μM). The results show that the urocortin 2-induced positive inotropic effect was mediated by stimulation of PKA.

Protein kinase A acts via phosphorylation of several key proteins involved in intracellular Ca^{2+} homeostasis. One target is PLN, a regulator of the SERCA. PLN is phosphorylated by PKA at serine-16 and this causes de-repression of SERCA activity by PLN. Therefore, we tested whether urocortin 2 phosphorylates PLN at serine-16 in mouse ventricular myocytes. Figure 5 shows original immunoblots of phosphorylated and total PLN (A, B) as well as average results (C, D) of ventricular myocytes treated with urocortin 2. In myocytes exposed to 100 nM urocortin 2, a time-dependent increase in PLN phosphorylation that was maximal after 20 min was observed (Figure 5A,C). This increase in PLN phosphorylation was concentration-dependent with a maximum occurring at 100 nM urocortin 2 (Figure 5B,D). Furthermore, it was suppressed by pre-incubation of the myocytes with 2 μM H89 (Figure 5D). Thus, urocortin 2 increased PLN phosphorylation

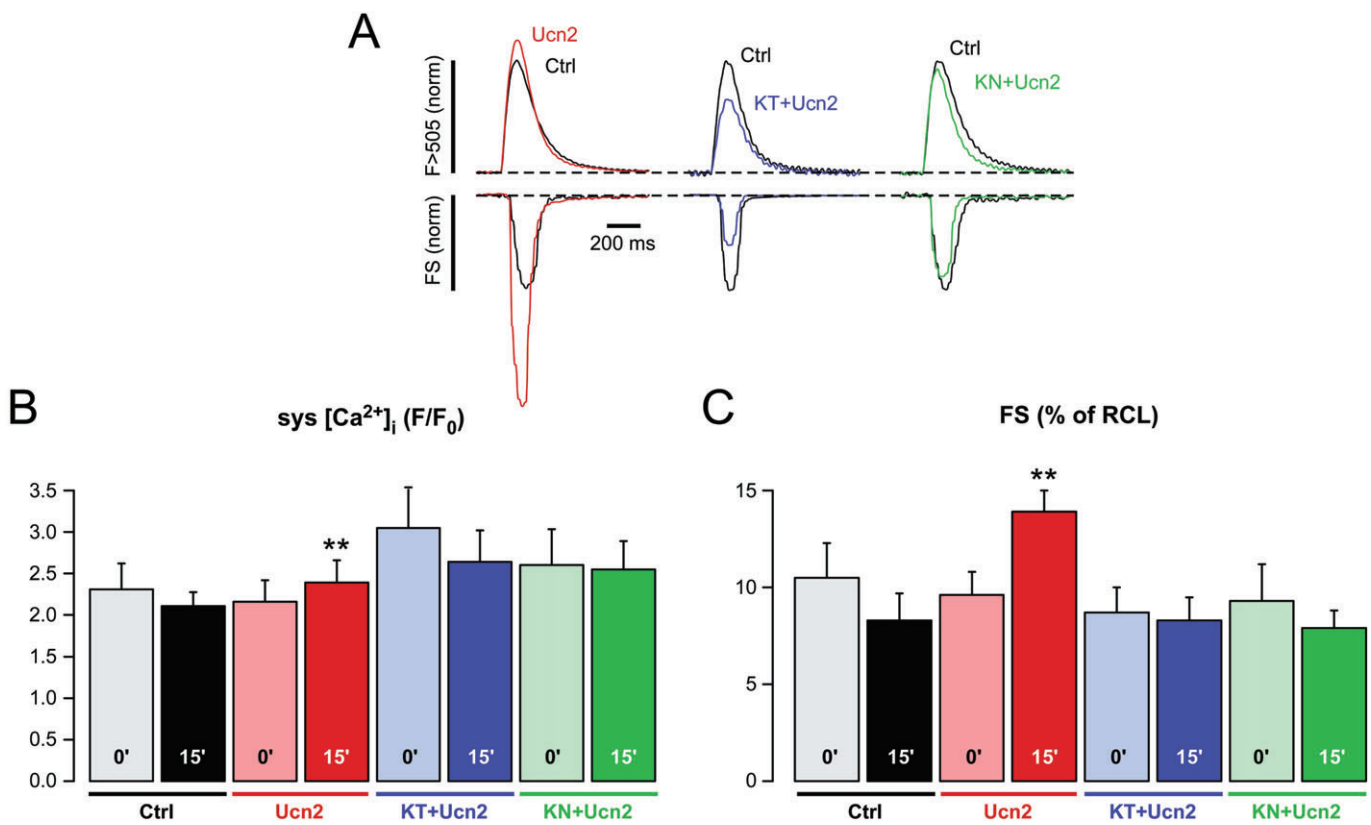

Figure 3

KT5720 and KN93 abolish the positive inotropic effect of urocortin 2 (Ucn2). (A) Original recordings of Fluo-4 fluorescence (i.e. $[Ca^{2+}]_i$ transients; top) and cell shortenings (bottom) of myocytes treated with 100 nM Ucn2 in the absence (Ucn2) and presence of 1 μM KT5720 (KT + Ucn2) or 1 μM KN93 (KN + Ucn2). Recordings were obtained after 0 and 15 min of Ucn2 exposure and have been normalized to the amplitude at 0 min. Average values of systolic $[Ca^{2+}]_i$ (B) and fractional shortening (FS) (C) obtained after 0 and 15 min of Ucn2 exposure in the absence (Ucn2, red, $n = 19$) or presence of 1 μM KT5720 (KT + Ucn2, $n = 14$) or 1 μM KN93 (KN + Ucn2, $n = 6$) or in time-matched controls (Ctrl, $n = 10$). Results from a total of 15 mouse hearts. $^{**}P < 0.01$ versus 0 min Ucn2, tested by paired Student's *t*-test.

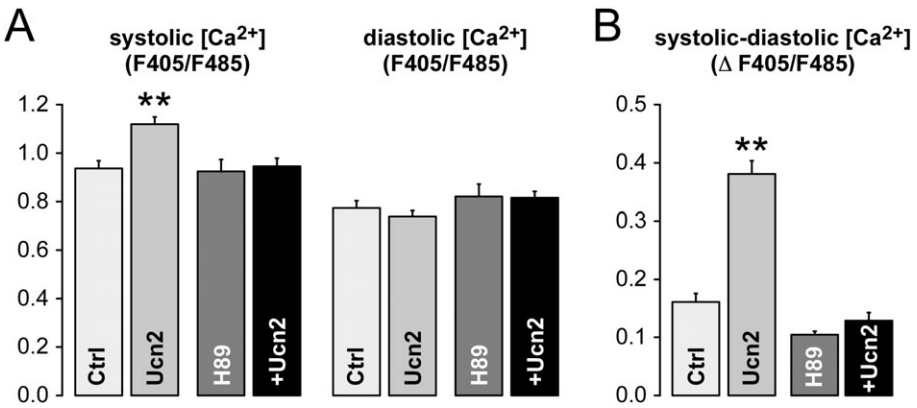

Figure 4

Urocortin 2 (Ucn2) increases $[Ca^{2+}]_i$ transients in Indo-1-loaded myocytes in an H89-sensitive manner. (A) Systolic (left) and diastolic (right) $[Ca^{2+}]_i$, measured as the ratio F405/F485 of the Indo-1 fluorescence signal in myocytes left untreated (Ctrl, $n = 13$ myocytes from three mouse hearts), treated with 100 nM Ucn2 ($n = 15$ myocytes from four mouse hearts), treated with 2 μM H89 (H89, $n = 7$ myocytes from three mouse hearts), or treated with 100 nM Ucn2 in the presence of 2 μM H89 (+Ucn2, $n = 10$ myocytes from three mouse hearts). (B) Amplitude, that is systolic minus diastolic Indo-1 fluorescence ratio, of the $[Ca^{2+}]_i$ transients in the same myocytes. $^{**}P < 0.01$ versus Ctrl, H89, and +Ucn2, tested by one-way ANOVA followed by Tukey's range test.

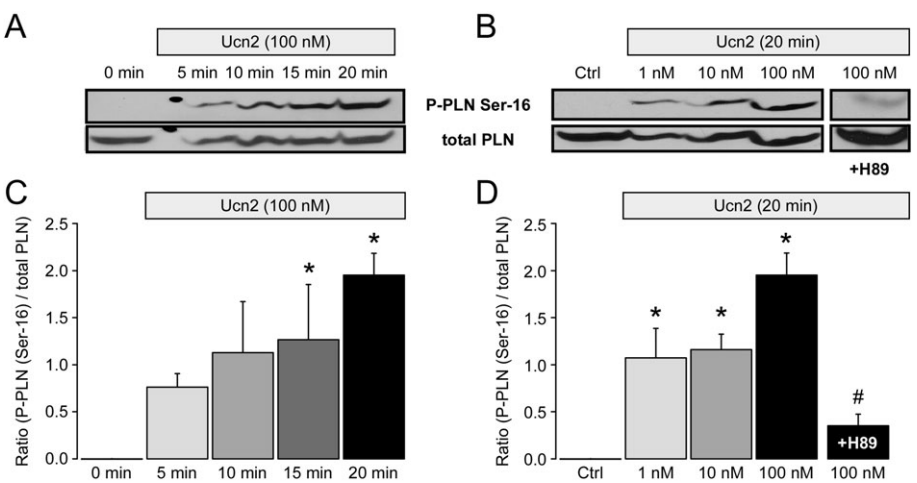

Figure 5

Urocortin 2 (Ucn2) increases phospholamban (PLN) phosphorylation at serine-16 in a time- and concentration-dependent manner. Original immunoblots of phosphorylated (P-PLN Ser-16) and total PLN following exposure to Ucn2 for various times (A) or at various concentrations (B). Average values of the ratio of P-PLN to total PLN from $n = 3$–8 independent experiments (i.e. mouse hearts) are shown in (C) and (D) respectively. Results with H89 (2 μM) were obtained from an additional series of experiments ($n = 3$ mouse hearts). $^{*}P < 0.05$ versus 0 min Ucn2 (C) or Ctrl (D) respectively. $\#P < 0.05$ versus 100 nM Ucn2 alone, tested by one-way ANOVA followed by Tukey's range test.

at serine-16 in a PKA-dependent manner and with a time and concentration dependence resembling the positive inotropic and lusitropic effects (compare Figures 1 and 2).

A previous study has shown that β-adrenoceptors, which also couple to cAMP-PKA signalling, may stimulate CaMKII via a yet to be defined (PKA-independent) mechanism (Curran *et al.*, 2007). Furthermore, CaMKII may also be stimulated via increased $[Ca^{2+}]_i$ transients secondary to stimulation of the cAMP-PKA pathway. Therefore, we also tested the possible involvement of CaMKII in the positive inotropic effect of urocortin 2 in mouse ventricular myocytes. CaMKII was inhibited using 1 μM KN93. As with KT5720, KN93 also abolished all urocortin 2 effects on cell shortening and $[Ca^{2+}]_i$ transients (Figure 3A–C). In the presence of KN93, amplitude and kinetics of $[Ca^{2+}]_i$ transients and cell shortenings remained unaltered after urocortin 2 exposure. The results suggest that, in addition to cAMP-PKA signalling, urocortin 2 also stimulates CaMKII, which contributes to the positive inotropic and lusitropic effect in mouse ventricular myocytes.

Urocortin 2 elicits arrhythmogenic events

In addition to the inotropic and lusitropic effects, urocortin 2 also induced arrhythmogenic events (Figure 6). Figure 6A

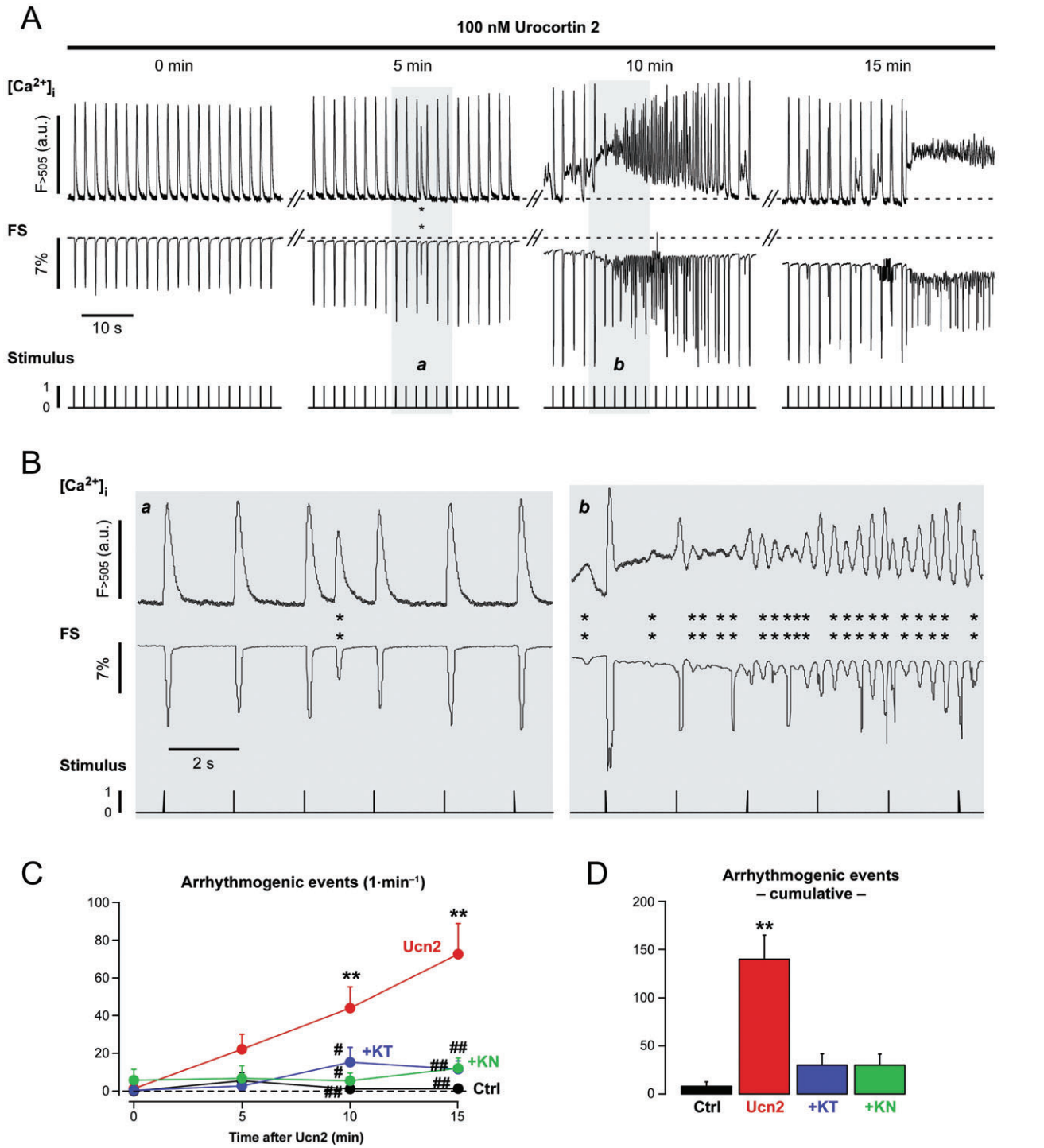

Figure 6

Urocortin 2 (Ucn2) elicits arrhythmogenic events. (A) $[Ca^{2+}]_i$ signals (top), that is raw Fluo-4 fluorescence in arbitrary units (a.u.), fractional shortening (FS) (middle), and the electrical stimulation (bottom) following application of 100 nM Ucn2. $[Ca^{2+}]_i$ signals and shortening are rhythmic in the beginning and become progressively more irregular, that is arrhythmogenic, after 5–15 min Ucn2 exposure. The first extra $[Ca^{2+}]_i$ transient and shortening not caused by the electrical stimulation is marked by the asterisks. (B) Enlarged traces corresponding to the shaded areas *a* and *b* in (A). Extra $[Ca^{2+}]_i$ transients and shortenings are marked by the asterisks. (C) Arrhythmogenic events in untreated control myocytes (Ctrl, $n = 18$) and in myocytes treated with 100 nM Ucn2 in the absence (Ucn2, $n = 20$) and presence, respectively, of 1 μM KT5720 (+KT, $n = 14$) or 1 μM KN93 (+KN, $n = 6$). Results from a total of 17 mouse hearts. $^{**}P < 0.01$ versus 0 min Ucn2; $\#\#P < 0.01$ and $\#P < 0.05$ versus Ucn2; tested by two-way ANOVA of repeated measures followed by Tukey's range test. (D) Cumulative number of arrhythmogenic events within the 15 min observation period in the same myocytes shown in (C). $^{**}P < 0.01$ versus Ctrl, +KT and +KN, tested by one-way ANOVA followed by Tukey's range test.

shows $[Ca^{2+}]_i$ signals (top), FS (middle) and the electrical stimulation (bottom) of a ventricular myocyte 0, 5, 10 and 15 min after exposure to 100 nM urocortin 2. Immediately after urocortin 2 exposure (0 min), $[Ca^{2+}]_i$ transients and shortenings were rhythmic; each stimulus elicited a $[Ca^{2+}]_i$ transient and shortening of the myocyte. Urocortin 2 elicited increases in $[Ca^{2+}]_i$ transients and FS. Furthermore, after 5 min, a spontaneous extra $[Ca^{2+}]_i$ transient and shortening was observed (marked by the asterisks). This is illustrated in more detail in the enlarged tracings in Figure 6Ba. Ten and 15 min after the application of urocortin 2, more spontaneous $[Ca^{2+}]_i$ increases and shortenings developed and progressively increased in frequency. The enlarged tracings in Figure 6Bb indicate that each spontaneous $[Ca^{2+}]_i$ increase was associated with a shortening (marked by the asterisks). Detailed analysis of the time course of the arrhythmogenic events revealed that the spontaneous $[Ca^{2+}]_i$ increases preceded the shortenings, suggesting spontaneous SR Ca^{2+} release as the underlying mechanism.

The incidence of the urocortin 2-induced arrhythmogenic events in mouse ventricular myocytes was concentration-dependent. Arrhythmogenic events were observed in 20% (one out of five) of the myocytes studied at 1 nM urocortin 2, in 40% (two out of five) of the myocytes studied at 10 nM urocortin 2, and in 75% (15 out of 20) of the myocytes studied at 100 nM urocortin 2.

The time course of the average rate of the spontaneous arrhythmogenic events elicited by 100 nM urocortin 2, that is extra $[Ca^{2+}]_i$ increases and shortenings not triggered by the electrical stimulation, is shown in Figure 6C. Untreated control myocytes exhibited hardly any arrhythmogenic events. By contrast, urocortin 2 (100 nM) induced arrhythmogenic events, which progressively increased with time. In addition (Figure 6D), the cumulative arrhythmogenic events, that is the cumulative average of extra $[Ca^{2+}]_i$ increases and shortenings during the 15 min observation period, were much more frequent in cells challenged with urocortin 2 than in untreated controls. Thus, urocortin 2 exerted an arrhythmogenic effect in mouse ventricular myocytes. Furthermore, urocortin 1 and urocortin 3 also elicited arrhythmogenic effects (not shown). At 100 nM, urocortin 1 caused arrhythmogenic events in 67% and urocortin 3 in 50% of the cells studied ($n = 6$ myocytes from three mouse hearts for each series). Cumulative arrhythmogenic events after 15 min amounted to 96 ± 39 for urocortin 1 and to 51 ± 27 for urocortin 3 ($n = 6$ each).

Urocortin 2-induced arrhythmogenic events are mediated by PKA and CaMKII

Because the arrhythmogenic events were Ca^{2+}-dependent and both PKA and CaMKII act via phosphorylation of several Ca^{2+}-regulating proteins (including PLN, Figure 5), we hypothesized that the urocortin 2-induced arrhythmogenic effects were mediated by PKA and CaMKII. To test this hypothesis, we investigated the ability of urocortin 2 to induce arrhythmogenic events in the presence of either PKA inhibition by KT5720 (1 μM) or CaMKII inhibition by KN93 (1 μM). As shown in Figure 6C and D, both KT5720 and KN93 largely reduced the urocortin 2-induced arrhythmogenic events (both $P < 0.05$ vs. urocortin 2 alone). These results indicate that urocortin 2 elicits arrhythmogenic events in mouse ventricular myocytes via stimulation of PKA and CaMKII.

Urocortin 2 elicits positive inotropic and arrhythmogenic effects in human myocardium

To test whether urocortin 2 elicits qualitatively similar effects in human myocardium, we assessed its effects on isometric force development in electrically stimulated trabeculae isolated from right atrium of patients undergoing cardiac surgery. To this end, human atrial trabeculae were challenged with either 100 nM urocortin 2 (n = 15 trabeculae from 10 atria) or 300 nM urocortin 2 (n = 7 trabeculae from two atria). In 45% (10 out of 22) of the trabeculae studied, urocortin 2 elicited a positive inotropic effect. Figure 7A shows an individual twitch of an atrial trabecula before and during urocortin 2 exposure. In this sample, urocortin 2 increased twitch force by 15% and mean values are shown in Figure 7B. By contrast, untreated control trabeculae exhibited a small rundown in developed force, over the same time.

In two of the 10 trabeculae (20%) that responded to urocortin 2 exposure with a positive inotropic effect, spontaneous contractions were observed that were not elicited by the electrical stimulation. Figure 7C shows an original chart recording of isometric contractions of an atrial trabecula before (left) and during (right) exposure to 100 nM urocortin 2. Before 100 nM urocortin 2 was applied, each electrical stimulation elicited an isometric contraction. In the presence of urocortin 2, however, spontaneous contractions developed which were not triggered by the electrical stimulation (marked by the asterisks). These spontaneous contractions resembled the extra shortenings observed in the isolated mouse ventricular myocytes (e.g. Figure 6). By contrast, untreated control trabeculae did not exhibited any spontaneous contractions (not shown). Taken together, these data demonstrate that in human atrial myocardium, urocortin 2 was able to elicit a positive inotropic as well as an arrhythmogenic effect.

Discussion and conclusions

Heart failure is one of the leading causes of morbidity and mortality worldwide. Despite great advances in the pharmacotherapy of heart failure, prognosis remains poor pointing to the need for new therapeutic concepts and interventions. In recent years, urocortin 2 has gained attention as a potential new treatment option. It combines beneficial cardiovascular, renal and neurohormonal effects that may make it superior to some of the currently used heart failure medications (Burnett, 2005; Yang *et al.*, 2010). In experimental heart failure as well as in heart failure patients, urocortin 2 has been shown to increase heart rate, left ventricular ejection fraction, and cardiac output and to decrease systemic vascular resistance and mean arterial pressure (Mackay *et al.*, 2003; Rademaker *et al.*, 2005a; Davis *et al.*, 2007b). However, the cellular effects of urocortin 2 on cardiomyocytes are not completely understood. Here we show that urocortin 2 activates CRF_2 receptors in cardiac myocytes to stimulate both cAMP-PKA and Ca^{2+}/calmodulin-CaMKII signalling, and that the two

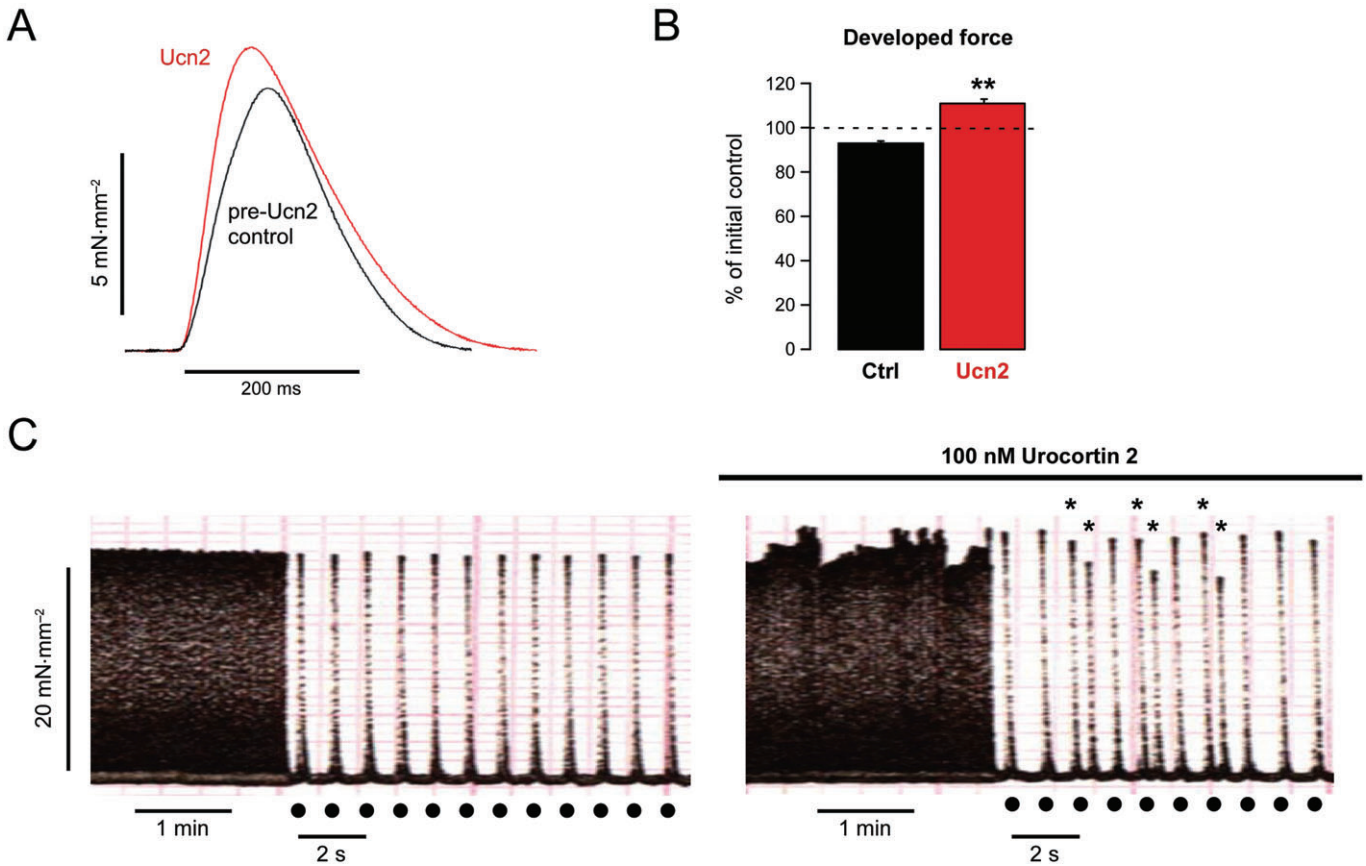

Figure 7

Urocortin 2 (Ucn2) elicits a positive inotropic effect and spontaneous contractions in human atrial trabeculae. (A) Tracings of twitch force of a human atrial trabecula before and during exposure to 300 nM Ucn2. Ucn2 increased twitch force by 15%. (B) Average values of developed force, normalized to the pre-Ucn2 control (=100%), in Ucn2-treated trabeculae (n = 10 trabeculae from seven atria) and in untreated, time-matched control trabeculae (Ctrl; n = 12 trabeculae from nine atria). **, $P < 0.01$ versus Ctrl, tested by unpaired Student's t-test. (C) Original chart recording of isometric contractions of an atrial trabecula before (left) and during (right) exposure to 100 nM Ucn2. Force recording is shown at two time scales. At the faster time scale, individual twitches are visible. Black circles indicate electrical stimulations. Asterisks mark spontaneous contractions not elicited by the electrical stimulation.

kinases combine to elicit a positive inotropic and lusitropic effect as well as an arrhythmogenic effect.

Urocortin 2 enhances contractility of mouse ventricular myocytes via CRF_2 receptors

In the present study, urocortin 2 exerted a powerful, time- and concentration-dependent, positive inotropic and lusitropic effect in mouse ventricular myocytes. Pretreatment of myocytes with ASV, a selective CRF_2 receptor antagonist, largely reduced these effects indicating that they were mediated by the CRF_2 receptor. Similar observations have been made previously in rat and rabbit ventricular myocytes (Yang *et al.*, 2006; Calderon-Sanchez *et al.*, 2009). The EC_{50} value for the positive inotropic effect of urocortin 2 in mouse ventricular myocytes was 19 nM, comparable to values reported from rabbit ventricular myocytes (11 nM urocortin 2) and rat ventricle (8 nM urocortin 1) (Yang *et al.*, 2006; Calderon-Sanchez *et al.*, 2009). The positive inotropic effect elicited by urocortin 2 in mouse ventricular myocytes was large (~140% increase above baseline). It was substantially larger than in either rat (~50% increase) or rabbit (~35% increase) ventricular myocytes (Yang *et al.*, 2006; Calderon-Sanchez *et al.*, 2009) or in human atrial myocardium (~10–15% increase; this study). This may be explained by the high density of CRF_2 receptors in mouse myocardium, which is approximately 10-fold higher than in rat myocardium and approximately 30-fold higher than in human myocardium (Waser *et al.*, 2006). It suggests that the functional effects of urocortin 2 on cardiac contractility may be regulated via alterations in CRF_2 receptor density. This may have direct clinical implications as CRF_2 receptor density is altered in cardiac disease such as left ventricular hypertrophy (Nishikimi *et al.*, 2000). Furthermore, urocortins themselves as well as other hormones (e.g. glucocorticoids) may regulate CRF_2 receptor density (Asaba *et al.*, 2000; Kageyama *et al.*, 2000).

Signalling pathways underlying the inotropic and lusitropic effects of urocortin 2

The CRF_2 receptor is a member of the G protein-coupled receptor subfamily B1 (Harmar, 2001). All members of this

subfamily couple to stimulatory G_s proteins (Harmar, 2001). Previous studies in cardiac myocytes have confirmed that cardiac CRF_2 receptors stimulate cAMP signalling (Nishikimi *et al.*, 2000; Ikeda *et al.*, 2005; Calderon-Sanchez *et al.*, 2009). The current study extends these findings by providing evidence that, in mouse ventricular myocytes, urocortin 2 acts via CRF_2 receptors to activate at least two signalling pathways, that is cAMP-PKA and Ca^{2+}/calmodulin-CaMKII signalling. Both PKA and CaMKII contributed to the positive inotropic and lusitropic effects of urocortin 2, as inhibitors of either kinase abolished the effects of urocortin 2. Stimulation of PKA and CaMKII activity is expected to exert profound effects on intracellular Ca^{2+} homeostasis via phosphorylation of key Ca^{2+}-regulating proteins including the L-type Ca^{2+} channel, the ryanodine receptor SR Ca^{2+} release channel and PLN. The overall effect should be increased Ca^{2+} influx, elevated SR Ca^{2+} content and increased and accelerated $[Ca^{2+}]_i$ transients. In line with these observations, we found that urocortin 2 increased and accelerated $[Ca^{2+}]_i$ transients, as also observed in a previous study in rabbit (Yang *et al.*, 2006). Furthermore, urocortin 2 elevated PLN phosphorylation at serine-16, the site phosphorylated by PKA (Figure 5), augmented SR Ca^{2+} load (Yang *et al.*, 2006) and increased L-type Ca^{2+} current (Yang *et al.*, 2006).

Interestingly, a recent study in rat heart suggested that – unlike in mouse and rabbit ventricular myocytes – the positive inotropic and lusitropic effects of urocortin 1 were mediated by Epac, PKC and MAPK signalling (Calderon-Sanchez *et al.*, 2009). This suggests that some of the cardiac actions of urocortins may be species-dependent and that multiple signalling pathways may be activated in parallel by urocortins to elicit positive inotropic and lusitropic effects in the heart. Moreover, urocortins exhibit additional cardiac effects, most notably cardioprotection during ischemia and reperfusion (Brar *et al.*, 2000; 2002). Therefore, this study and a number of previous studies suggest that urocortins activate various signalling pathways in cardiac myocytes including cAMP-PKA, Epac, Akt/PKB, PKC, MAPK and Ca^{2+}/calmodulin-CaMKII signalling (Brar *et al.*, 2000; 2002; Lawrence *et al.*, 2005; Yang *et al.*, 2006; Calderon-Sanchez *et al.*, 2009). It is likely that there is considerable crosstalk between these various signalling pathways and that they contribute in different degrees to the various effects of urocortins on cardiomyocytes.

Urocortin 2 elicits arrhythmogenic events

This study is the first to demonstrate a direct arrhythmogenic effect of urocortin 2 in adult ventricular myocytes. Arrhythmogenic events elicited by urocortin 2 were Ca^{2+}-dependent, that is triggered by spontaneous diastolic SR Ca^{2+} release. The latter was most likely caused by SR Ca^{2+} overload induced by PKA- and CaMKII-mediated alterations in Ca^{2+} homeostasis (see above). This notion is supported by the observations that urocortin 2 increased phosphorylation of PLN (Figure 5) and that it elevated SR Ca^{2+} load (Yang *et al.*, 2006). Furthermore, inhibition of PKA or CaMKII largely attenuated the urocortin 2-induced arrhythmogenic events indicating that both kinases were involved in this effect and suggesting that the same signalling pathways and mechanisms that underlie the positive inotropic and lusitropic effect of urocortin 2 may also underlie its arrhythmogenic effect. Because any intervention that increases SR Ca^{2+} load may also cause Ca^{2+}-triggered arrhythmias as a potentially adverse effect, the finding of urocortin 2-induced arrhythmogenic events was not unexpected. A similar mechanism has long been recognized to underlie the toxic effects of cardiac glycosides, the first inotropes used in modern medical practice. Similarly, β-adrenoceptor agonists, which are also used as positive inotropes, activate cAMP-PKA and CaMKII signalling to increase SR Ca^{2+} load – with the possible side effect of Ca^{2+}-triggered arrhythmias.

Urocortin 2 effects in human atrial myocardium

As in isolated mouse ventricular myocytes, urocortin 2 elicited positive inotropic and arrhythmogenic effects in human atrial trabeculae under experimental conditions resembling the physiological situation. The fraction of trabeculae responding to urocortin 2 exposure as well as the magnitude of the urocortin 2 effects were smaller than for isolated mouse ventricular myocytes. This may be explained by the lower density of CRF_2 receptors in human myocardium (see above). In addition, higher concentrations of urocortin 2 might be required to elicit a maximal effect in this multicellular preparation as previously reported for endothelin-1 (Talukder *et al.*, 2001). Whatever the cause for the smaller effects in human atrial myocardium, however, the finding that urocortin 2 did exert a positive inotropic and arrhythmogenic effect clearly warrants further studies in animal as well as human myocardium. These studies should be aimed at evaluating in detail the underlying cellular mechanisms not only in normal myocardium but – of particular clinical relevance – also in the diseased heart.

Implications for the use of urocortins in heart failure

Urocortins exert beneficial effects in experimental heart failure as well as in patients. The results obtained so far with heart failure patients are encouraging and clearly warrant further studies (Davis *et al.*, 2007b). The current study sheds new light on the cellular mechanisms underlying some of the observed cardiac effects *in vivo*, for example, increases in ejection fraction and cardiac output. However, they also add a note of caution as they uncover a potential adverse effect of urocortins, that is, Ca^{2+}-triggered arrhythmias. Studies so far have not reported on arrhythmias in humans and it remains to be determined whether or not urocortins also display arrhythmogenic potential *in vivo* in humans.

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Conflicts of interest

None.

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