Mechanisms underlying the activation of L-type calcium channels by urocortin in rat ventricular myocytes

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Aims
The aim of this study was to elucidate the signalling pathways implicated in the modulation of cardiac L-type Ca²⁺ channels by urocortin (Ucn) in ventricular myocytes.

Methods and results
Adult rat ventricular myocytes were stimulated in vitro with Ucn for 20–40 min. L-type calcium currents (I_{CaL}) were measured with the patch-clamp technique, whereas quantification of activation of extracellular signal-regulated kinases 1/2 (ERK1/2) was assessed by sandwich-ELISA. Ucn induced a significant increase in I_{CaL} density that was not prevented by the protein kinase A (PKA) inhibitor KT-5720 or the non-selective antagonist of guanine nucleotide exchange factor brefeldin A. The Ucn effect was antagonized by astressin, a corticotropin-releasing factor receptor-2 (CRF-R2) antagonist, and significantly reduced by protein kinase C (PKC) and ERK1/2 inhibitors. The cyclic AMP (cAMP) analogue 8-pCPT-2′OMe-cAMP, which selectively activates the exchange protein activated by cAMP (Epac), was ineffective in modifying I_{CaL}. Analysis of phospho-ERK1/2 showed that Ucn induced a significant activation of the ERK1/2 pathway in ventricular myocytes and this effect was prevented by pre-incubation with PKC inhibitors.

Conclusion
The present study provides evidence of new mechanisms involved in the modulation of L-type Ca²⁺ channels by Ucn in adult ventricular myocytes. We propose that the marked increase in I_{CaL} density induced by Ucn is mediated through CRF-R2 and involves PKC-dependent activation of the ERK1/2 pathway, whereas PKA and Epac signalling are not implicated.

Keywords
Urocortin • L-type calcium channels • Patch-clamp • PKC • ERK1/2

1. Introduction
Urocortins (Ucn) (I, II, and III) are endogenous peptides, members of the corticotropin-releasing factor (CRF) family, that bind to G-protein-coupled receptors (CRF-R) 1 and 2.¹-⁴ In the heart, only CRF-R2 is expressed, and several studies have demonstrated that Ucn binding to these receptors exerts beneficial action on the myocardium that might have a therapeutic potential in some pathological conditions, including human heart failure.⁵-⁷ In vivo, Ucn is able to induce coronary vasodilatation and it increases cardiac output and heart rate.⁸⁻⁹ Ucn has been shown to possess potent and long-lasting inotropic properties in healthy and failing animals¹⁰,¹¹ which have been postulated to be dependent on CRF-R2 but independent of β-adrenergic receptors.¹² In vitro, Ucn protects the heart from ischaemia and reperfusion injury.¹³,¹⁴ Many signalling pathways have been shown to be involved in the effects of Ucn on the heart, including phosphoinositide-3-kinase (PI3K), protein kinase A (PKA), protein kinase B/Akt, protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs).¹⁵-¹⁷

Recently, we have reported in ex vivo Langendorff-perfused rat hearts that the positive inotropic effect induced by Ucn was mediated through a PKA-independent mechanism that implicated the activation of PKC and MAPK subfamily extracellular signal-regulated kinases 1/2 (ERK1/2) and the activation of the exchange protein activated by
cyclic AMP (Epac). In the same study, Ucn was reported to enhance L-type calcium current ($I_{\text{CaL}}$), although the precise mechanism involved was not addressed.

Ca$^{2+}$ entry through the L-type Ca$^{2+}$ channels ($I_{\text{CaL}}$) is critical in the control of cardiac function. $I_{\text{CaL}}$ contributes to physiological frequency regulation in the sinus node, it is an important parameter for the duration of the plateau phase of the action potential and refractoriness, and plays a fundamental role in the process of Ca$^{2+}$ release from the sarcoplasmic reticulum, raising free intracellular Ca$^{2+}$ concentration [Ca$^{2+}$], and allowing cell contraction. Moreover, it is also clear that [Ca$^{2+}$], serves as an important second messenger in the regulation of gene expression in life-threatening cardiac diseases.

Little information is available for the mechanism involved in the effect of Ucn on $I_{\text{CaL}}$ in the heart. Therefore, the present study was undertaken to further elucidate the signalling pathways implicated in cardiac L-type Ca$^{2+}$ channel modulation by Ucn in adult rat ventricular myocytes.

2. Methods

An expanded Methods section detailing protocols and solutions used in this study can be found in the Supplementary material online.

2.1 Isolation of ventricular myocytes

All the experiments were performed in accordance with 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 Institute of Health (NIH Publication No. 85-23, revised 1996). All procedures were approved by the Bioethical Committee of the Complutense University of Madrid. Ventricular myocytes were isolated from the hearts of adult male Wistar rats (250–300 g of weight), as described previously.

2.2 Electrophysiological recording technique

Electrophysiological experiments were performed at room temperature (24–26 °C) on Ca$^{2+}$-tolerant rod-shaped ventricular myocytes. Conventional whole-cell and perforated patch-clamp recordings were performed to measure $I_{\text{CaL}}$.

In a previous study, we have demonstrated in ex vivo perfused heart that Ucn induced a positive inotropic effect with a slow time course that began 40 min. in ventricular myocytes incubated with vehicle or with Ucn for 20–40 min.

The decay of $I_{\text{CaL}}$ as shown in Supplementary material online, Figure S1C shows representative examples of $I_{\text{CaL}}$ density traces obtained in three independent myocytes. The upper panel shows $I_{\text{CaL}}$ traces obtained in a control myocyte. The middle panel shows higher values of $I_{\text{CaL}}$ density obtained in one myocyte incubated 30 min with 100 nM Ucn and the bottom panel illustrates $I_{\text{CaL}}$ traces obtained in one ventricular myocyte pre-incubated for 15 min with the CRF-R2 antagonist astressin (0.5 μM) and then treated for 30 min with Ucn. $I_{\text{CaL}}$ traces in this myocyte were similar to those obtained in control myocytes. Figure 1B illustrates the current density–voltage relations from 18 control myocytes (non-treated, open circles), 19 ventricular myocytes incubated with 100 nM Ucn (closed circles) and from nine myocytes pre-incubated for 15 min with 0.5 μM astressin and then treated with Ucn (closed squares). $I_{\text{CaL}}$ density was significantly higher in cells treated with Ucn (closed circles) compared with control cells (open circles). The presence of the CRF-R2 antagonist astressin (closed squares) prevented the effect of Ucn on $I_{\text{CaL}}$ density. The maximum value of $I_{\text{CaL}}$ density in control was obtained at 0 mV (−11.1 ± 0.7 pA/pF), whereas in the presence of Ucn, the maximum value of $I_{\text{CaL}}$ density was observed at −10 mV (−16.8 ± 1.4 pA/pF). The bar graph in Figure 1C shows the concentration dependence of the effect of Ucn on $I_{\text{CaL}}$. Ventricular myocytes were exposed to vehicle (control) or to four different concentrations of Ucn, 10, 50, 70, and 100 nM for 20–40 min, and $I_{\text{CaL}}$ was recorded at −10 mV. The bar graph shows the percentage of $I_{\text{CaL}}$ increase induced by Ucn with respect to the control group in cells exposed to 10 nM (102.2 ± 8.6%, n = 6), 50 nM (114.1 ± 13.7%, n = 6), 70 nM (135.2 ± 25.5%, n = 7), and 100 nM (154.5 ± 13.4%, n = 9) of Ucn.

The voltage dependence of $I_{\text{CaL}}$ activation and inactivation was also measured in control (open circles) and in Ucn-treated myocytes (closed circles). Similar values for the maximal activation and inactivation voltage ($V_{\text{50}}$) and slope factor ($k$) were obtained in both groups (see Supplementary material online, Figure S1A). In addition, the time course for activation of $I_{\text{CaL}}$ was analysed by measuring the time to peak and, the time course for inactivation by analysing the decay of $I_{\text{CaL}}$ as shown in Supplementary material online, Figure S1C. The time to peak obtained at −10 mV was similar in both groups. The decay of $I_{\text{CaL}}$ was best fitted by a bi-exponential model and non-significant differences were observed when both experimental groups were compared (see Supplementary material online, Figure S1B).

2.5 Drugs and statistical analysis

Drugs were purchased from Sigma, Promega, and Invitrogen. Ucn (rat) is a synthetic peptide purchased from Sigma. Data are presented as means ± SEM. Statistical significance was evaluated by one-way ANOVA followed by the Newman–Keuls multiple comparison tests. A value of $P < 0.05$ was considered significant.

3. Results

3.1 Ucn increases $I_{\text{CaL}}$ density in rat ventricular myocytes

Figure 1A shows representative examples of $I_{\text{CaL}}$ density traces obtained in three independent myocytes. The upper panel shows $I_{\text{CaL}}$ traces obtained in a control myocyte. The middle panel shows higher values of $I_{\text{CaL}}$ density obtained in one myocyte incubated 30 min with 100 nM Ucn and the bottom panel illustrates $I_{\text{CaL}}$ traces obtained in one ventricular myocyte pre-incubated for 15 min with the CRF-R2 antagonist astressin (0.5 μM) and then treated for 30 min with Ucn. $I_{\text{CaL}}$ traces in this myocyte were similar to those obtained in control myocytes. Figure 1B illustrates the current density–voltage relations from 18 control myocytes (non-treated, open circles), 19 ventricular myocytes incubated with 100 nM Ucn (closed circles) and from nine myocytes pre-incubated for 15 min with 0.5 μM astressin and then treated with Ucn (closed squares). $I_{\text{CaL}}$ density was significantly higher in cells treated with Ucn (closed circles) compared with control cells (open circles). The presence of the CRF-R2 antagonist astressin (closed squares) prevented the effect of Ucn on $I_{\text{CaL}}$ density. The maximum value of $I_{\text{CaL}}$ density in control was obtained at 0 mV (−11.1 ± 0.7 pA/pF), whereas in the presence of Ucn, the maximum value of $I_{\text{CaL}}$ density was observed at −10 mV (−16.8 ± 1.4 pA/pF). The bar graph in Figure 1C shows the concentration dependence of the effect of Ucn on $I_{\text{CaL}}$. Ventricular myocytes were exposed to vehicle (control) or to four different concentrations of Ucn, 10, 50, 70, and 100 nM for 20–40 min, and $I_{\text{CaL}}$ was recorded at −10 mV. The bar graph shows the percentage of $I_{\text{CaL}}$ increase induced by Ucn with respect to the control group in cells exposed to 10 nM (102.2 ± 8.6%, n = 6), 50 nM (114.1 ± 13.7%, n = 6), 70 nM (135.2 ± 25.5%, n = 7), and 100 nM (154.5 ± 13.4%, n = 9) of Ucn.

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3.2 Signalling pathways involved in the Ucn-induced increase in $I_{\text{CaL}}$ density

3.2.1 cAMP/PKA pathway

Ucn binds to CRF-R2, which couples to Gs–cAMP–PKA signalling. Therefore, we examined whether Ucn action is mediated by the activation of PKA. We assayed the activity of PKA in isolated...
ventricular myocytes treated 10 min with Ucn 100 nM. Figure 2A illustrates that Ucn increased PKA activity and this effect was largely inhibited by the PKA inhibitor KT-5720. To explore the implication of PKA in the modulation of Ucn on $I_{\text{CaL}}$, ventricular myocytes were pre-incubated for 15 min with KT-5720 (1 μM) and then exposed to Ucn 100 nM. The upper panel in Figure 2C shows representative $I_{\text{CaL}}$ traces obtained at $-10 \text{ mV}$ in two different myocytes. In the presence of the PKA inhibitor KT-5720, Ucn induced a significant increase in $I_{\text{CaL}}$ density obtained at $-10 \text{ mV}$ in control cells, in cells incubated with KT-5720, in cells pre-treated with KT-5720 and then exposed to Ucn 100 nM, and in cells only treated with Ucn 100 nM. Mean values of $I_{\text{CaL}}$ density in ventricular myocytes pre-treated with KT-5720 ($-10.6 \pm 1.5 \text{ pA/} \mu\text{F}, n = 11$) were similar to those obtained in control myocytes ($-10.9 \pm 0.6 \text{ pA/} \mu\text{F}, n = 18$). In the presence of KT-5720, Ucn induced a significant increase in $I_{\text{CaL}}$ ($-15.5 \pm 0.8 \text{ pA/} \mu\text{F}, n = 12$) which was independent of PKA activation. In order to further demonstrate that KT-5720 effectively blocks PKA activity in our experimental conditions, ventricular myocytes were incubated 20–40 min with the β-adrenergic agonist, ISO 10 nM in the presence and in the absence of KT-5720 1 μM. The bottom panel in Figure 2C shows examples of $I_{\text{CaL}}$ traces at $-10 \text{ mV}$ in two different myocytes. In the presence of KT-5720, the effect of ISO on $I_{\text{CaL}}$ was largely inhibited. Figure 2B shows the mean values of $I_{\text{CaL}}$ obtained at $-10 \text{ mV}$ in ventricular myocytes incubated with ISO ($-18.9 \pm 2.7 \text{ pA/} \mu\text{F}, n = 8$) and in myocytes treated with ISO but pre-incubated with KT-5720 ($-11.7 \pm 1.5 \text{ pA/} \mu\text{F}, n = 12$).

It is known that ISO, through PKA activation, induces a rapid increase in $I_{\text{CaL}}$ in ventricular myocytes. We carried out a group of experiments using the perforated patch configuration of the patch-clamp technique to compare the time course of Ucn and ISO on $I_{\text{CaL}}$ in ventricular myocytes. Figure 3A and B illustrates representative experiments in which peak $I_{\text{CaL}}$ at $-10 \text{ mV}$ was continuously monitored every 6 s. Figure 3A shows the effect of Ucn perfusion on $I_{\text{CaL}}$. Ucn was able to increase $I_{\text{CaL}}$ and the effect started 5 min after Ucn application reaching a maximal level at 14–15 min. Figure 3B shows the effect of ISO on $I_{\text{CaL}}$. Application of ISO induced a very rapid effect (<1 min) on $I_{\text{CaL}}$ that reached a maximal level at 3–4 min. Similar results were obtained in other four ventricular myocytes.

3.2.2 Epac pathway

Epac belongs to a family of cAMP-regulated guanine nucleotide exchange factors (GEFs) that mediate PKA-independent signal transduction properties of the second messenger cAMP.

In order to analyse the possible participation of the Epac pathway in the effect of Ucn on $I_{\text{CaL}}$, we carried out two types of experiments. In the first one, ventricular myocytes were pre-treated for 15 min with brefeldin A (BFA), a non selective inhibitor of Epac, and then treated with 100 nM Ucn. Figures 4A illustrates superimposed $I_{\text{CaL}}$...
traces obtained at $-10$ mV in one myocyte treated with BFA and in another myocyte pre-treated with BFA and then with Ucn. Under these conditions, Ucn was able to induce a significant increase in $I_{\text{CaL}}$.

Figure 4B summarizes the results obtained in 10 myocytes pre-treated with BFA and in 14 myocytes pre-treated with BFA and then treated with Ucn 100 nM. Mean values of $I_{\text{CaL}}$ density at $-10$ mV obtained in ventricular myocytes pre-treated with BFA 20 $\mu$M were not different ($-10.9 \pm 0.6$ pA/pF, $n = 10$) from those obtained in control myocytes ($-10.9 \pm 0.6$ pA/pF, $n = 18$). In the presence of BFA, exposure of ventricular myocytes to Ucn induced a significant increase in $I_{\text{CaL}}$ density ($-15.6 \pm 0.7$ pA/pF, $n = 14$) which suggests that BFA-sensitive pathway was not involved in the effect of Ucn on $I_{\text{CaL}}$.

In a second group of experiments, ventricular myocytes were incubated for 20–40 min with the cAMP analogue 8-pCPT-2′OMe-cAMP (8-CPT) 20 $\mu$M in the presence of KT-5720 in order to prevent any effect of 8-CPT on PKA. Figure 4C shows superimposed $I_{\text{CaL}}$ traces obtained at $-10$ mV in one control myocyte and in another myocyte incubated with 20 $\mu$M 8-CPT + KT. Figure 4D summarizes the results obtained in 18 control and in 13 8-CPT-treated myocytes. Mean values of $I_{\text{CaL}}$ density ($-10.9 \pm 0.6$ pA/pF, $n = 18$) were not modified by 8-CPT + KT ($-11.4 \pm 1.1$ pA/pF, $n = 13$), which suggests that the Epac pathway is not involved in the effect of Ucn on $I_{\text{CaL}}$.

3.2.3 PKC signalling

Several studies have proposed the implication of PKC activation in the cardiac effects of Ucn. In order to analyse whether PKC contributes to the increased density of $I_{\text{CaL}}$ induced by Ucn in our
experiments, ventricular myocytes were pre-treated with the PKC inhibitor chelerythrine (Chel). Figure 5A shows two superimposed traces obtained at $-10$ mV in one myocyte pre-treated with Chel and in another myocyte pre-treated with Chel and then 30 min with Ucn. Under these conditions, Ucn did not affect $I_{\text{Cal}}$ in myocyte pre-treated with Chel. The bar graph in Figure 5B shows the mean values of $I_{\text{Cal}}$ at $-10$ mV obtained in control cells, in cells incubated with Chel, in cells pre-treated with Chel and then exposed to Ucn 100 nM, and in cells only treated with Ucn 100 nM. Mean values of $I_{\text{Cal}}$ density obtained at $-10$ mV in ventricular myocytes pre-treated with Chel 3 μM were not significantly different ($-11.2 \pm 1.3$ pA/pF, n = 13) from those obtained in control myocytes ($-10.9 \pm 0.6$ pA/pF, n = 18). In the presence of Chel, exposure of ventricular myocytes to Ucn did not induce any effect on $I_{\text{Cal}}$ density ($-11.7 \pm 0.9$ pA/pF, n = 10). These results strongly suggest that PKC signalling contributes to the Ucn-induced increase on $I_{\text{Cal}}$.

3.2.4 MAPK/ERK1/2 pathway

It has been postulated that in addition to PKC signalling, some of the physiological effects of Ucn on ventricular myocytes were mediated by MAPK-dependent pathways, including subfamily ERK1/2. To determine the potential involvement of ERK1/2 in the Ucn-induced increase in $I_{\text{Cal}}$, myocytes were pre-treated with a specific inhibitor of ERK1/2, PD98059. Figure 6A shows two superimposed traces obtained at $-10$ mV in one myocyte pre-treated with 20 μM PD98059 and in another myocyte pre-treated 15 min with PD98059 and then 35 min with Ucn. In the presence of PD98059, the Ucn-induced increase in $I_{\text{Cal}}$ was abolished. The bar graph in Figure 6B shows the mean values of $I_{\text{Cal}}$ density at $-10$ mV obtained in control cells, in cells incubated with PD98059, in cells pre-treated with PD98059 and then exposed to Ucn 100 nM, and in cells only treated with Ucn 100 nM. Mean values of $I_{\text{Cal}}$ density obtained in ventricular myocytes pre-treated with 20 μM PD98059 ($-13.7 \pm 0.92$ pA/pF, n = 16) were not significantly different from those obtained in control myocytes ($-10.9 \pm 0.6$ pA/pF, n = 18). In the presence of PD98059, ventricular myocyte exposure to Ucn did not induce a significant change in $I_{\text{Cal}}$ density ($-11.9 \pm 1.1$ pA/pF, n = 17), indicating the important role of ERK1/2 in the Ucn-induced increase in $I_{\text{Cal}}$.

The next step in proving the contribution of ERK1/2 signalling in the Ucn-induced increase in $I_{\text{Cal}}$ density was to investigate the activation of ERK1/2 by Ucn. Figure 7A shows relative values of P-ERK in ventricular myocytes. Ucn promoted an increase in phosphorylation of ERK1/2 that was maximal at 5 min and remained elevated for 20 min of Ucn exposition. This increase in ERK1/2 activation was prevented in myocytes pre-treated with the inhibitor of ERK1/2, PD98059 but also by Chel the inhibitor of PKC as illustrated in Figure 7B. These results point out that activation of ERK1/2 by Ucn requires PKC activity in ventricular myocytes.

4. Discussion

The present study provides new insights into potential mechanisms linking modulation of L-type Ca$^{2+}$ channels by Ucn in adult ventricular myocytes.

Phosphorylation of channel-forming subunits by different kinases is one of the most important ways to change the activity of L-type Ca$^{2+}$ channels. The stimulation of cardiac β-adrenergic receptors and
activation of PKA are the main and most explored ways for Ca\(^{2+}\) channel activation.\(^{36}\) In our study, pharmacological inhibition of the cAMP/PKA pathway failed to prevent the effect of Ucn on \(\mathcal{I}_{\text{CaL}}\). In addition, the time course of the effect of Ucn on \(\mathcal{I}_{\text{CaL}}\) was showed to be slower than the time course of ISO on \(\mathcal{I}_{\text{CaL}}\), suggesting that Ucn via CRF-R2 can enhance \(\mathcal{I}_{\text{CaL}}\) through alternative pathways. Our results differ from those reported in rabbit ventricular myocytes.\(^{23}\) In that study, the increase in \(\mathcal{I}_{\text{CaL}}\) induced by Ucn was hypothesized to involve PKA signalling, although the slow time course of Ucn effect (the maximum effect was reached after 15 min of Ucn perfusion) prompted the authors to hypothesize that other pathways with slower time courses for activation could participate in the effect of Ucn on \(\mathcal{I}_{\text{CaL}}\). Murata et al.\(^{29}\) have demonstrated that cardiomyocytes incubated for 10–30 min with leukaemia inhibitory factor (LIF), a member of the interleukin-6 family, showed a significant increase in \(\mathcal{I}_{\text{CaL}}\) that was independent of the cAMP/PKA pathway, and the time course of which was similar to Ucn effect on \(\mathcal{I}_{\text{CaL}}\). Pre-treatment with PD98059 completely blocked the effect of LIF on \(\mathcal{I}_{\text{CaL}}\), which confirms the role of MAPK/ERK pathway in the modulation of \(\mathcal{I}_{\text{CaL}}\) by LIF. More recently, Takahashi et al.\(^{37}\) have demonstrated that LIF, via ERK activation, phosphorylates the \(\alpha_{1c}\) subunit (Cav1.2) of the L-type Ca\(^{2+}\) channel, in a different serine than the one targeted by PKA, causing \(\mathcal{I}_{\text{CaL}}\) increase. In the present study, we have shown that Ucn is able to activate ERK1/2 in ventricular myocytes. Furthermore, specific inhibition of ERK1/2 completely prevented Ucn-induced increase in \(\mathcal{I}_{\text{CaL}}\) in cardiac myocytes, which strongly suggests that phosphorylation of L-type Ca\(^{2+}\) channels by ERK might be the mechanism involved in the potentiation of \(\mathcal{I}_{\text{CaL}}\) by Ucn. Moreover, the present study revealed that PKC activation is a previous step in the mechanism of Ucn induced ERK1/2 activation in cardiac myocytes. The regulation of L-type Ca\(^{2+}\) channels by PKC is well documented\(^{36}\) and there is evidence for crosstalk with other signalling pathways. In human atrial myocytes, tyrosine kinase stimulates \(\mathcal{I}_{\text{CaL}}\) only after PKC is activated.\(^{38}\) In the human pregnant myometrium, Ucn, via CRF-R1 and -R2, stimulates G\(_3\) with subsequent production of inositol trisphosphates (IP\(_3\)) and PKC activation, which correlated with MAPK/ERK phosphorylation.\(^{39}\) In cardiomyocytes, activation of ERK1/2 by platelet-derived growth factor required PKC activity.\(^{40}\) The mechanism by which PKC might directly activate the MAPK/ERK pathway and activate L-type Ca\(^{2+}\) channels cascade in cardiac myocytes treated with Ucn remains unclear. There is evidence that MAPK/ERK regulates the Kv4.2 channel.
Effects of Ucn on L-type Ca\textsuperscript{2+} channels

Figure 7 Quantification of phosphorylated ERK1/2 in Ucn-treated rat ventricular myocytes. Activation of ERK1/2 by Ucn requires PKC activity. (A) Phosphorylation of ERK1/2 were measured in myocytes unstimulated (control) or exposed to Ucn for 5, 10, or 20 min. (B) Phospho-ERK1/2 measured in myocytes unstimulated (control) or exposed to Ucn for 10 min alone or pre-incubated with PD98059 or Chel (10 min pre-treatment). Data are given as means ± SE from three cultures. *p < 0.01 vs. control.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

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potassium channels by direct phosphorylation of the pore-forming subunit\textsuperscript{41} and phosphorylation of Kv4.2 by PKC-enhanced ERK phosphorylation of the channel in vitro.\textsuperscript{42} A similar mechanism could be proposed for the activation of L-type Ca\textsuperscript{2+} channels by Ucn in cardiomyocytes. On the other hand, it is known that Ucn binding to CRF-R2 activates the Gs-cAMP-PKA cascade in adult cardiomyocytes;\textsuperscript{18} although the results shown in the present study support the idea that PKA is not involved in the enhancement of $\text{I}_{\text{CaL}}$ induced by Ucn, we checked whether the other cAMP-binding protein and subsequent signalling pathway might mediate the effects of Ucn on $\text{I}_{\text{CaL}}$. In this regard, a GEF directly activated by cAMP and named Epac has been identified.\textsuperscript{30,43} There is recent information showing that Epac pathway might participate in the regulation of cardiac myofilament function\textsuperscript{44} and in the Ca\textsuperscript{2+} handling in cardiomyocytes.\textsuperscript{45,46} We have proven previously that pre-treatment with the non-selective Epac antagonist BFA largely inhibited the positive inotropic effect of Ucn in the perfused heart.\textsuperscript{18} Moreover, it is now recognized that Epac can mediate the cAMP-dependent regulation of ionic channel function. For example, Epac mediated inhibition of ATP-sensitive K\textsuperscript{+} channels (K\textsubscript{ATP} channels) in pancreatic β cells,\textsuperscript{47} and in chromaffin cells, Epac induced increase in the low-voltage-activated T-type Ca\textsuperscript{2+} current.\textsuperscript{48} Therefore, we have analysed in the present study, the implication of BFA-sensitive pathway in the effect of Ucn on $\text{I}_{\text{CaL}}$ in ventricular myocytes. Our results demonstrated that Ucn was able to induce a significant increase in $\text{I}_{\text{CaL}}$ in those myocytes pre-treated with BFA, which suggests that the BFA-sensitive pathway is not involved in the effect of Ucn on $\text{I}_{\text{CaL}}$. Moreover, to further discard the implication of Epac in the effect of Ucn on $\text{I}_{\text{CaL}}$, myocytes were pre-treated with the cAMP analogue 8-CPT which selectively activates Epac but not PKA when it was used at low concentrations. Our results showed that 8-CPT has no effect on $\text{I}_{\text{CaL}}$ in adult ventricular myocytes and therefore confirms that cAMP-dependent Epac activation does not modulate L-type Ca\textsuperscript{2+} channels in adult ventricular myocytes. Hence, it appears that the positive inotropic effect induced by Ucn\textsuperscript{18} involves the activation of two independent pathways: Epac signalling and L-type Ca\textsuperscript{2+} channels, and in both mechanisms, PKC plays an important role (see Supplementary material online, Figure S2). We can speculate that the activation of different PKC isozymes could explain distinct effect mediated by Ucn in cardiac myocytes. For example, PKCs is important for Epac regulation of Ca\textsuperscript{2+} signalling in the sarcoplasmic reticulum.\textsuperscript{45} In our studies, we focused on the acute effect of Ucn on heart contractility\textsuperscript{18} and L-type Ca\textsuperscript{2+} channels. However, it is important to keep in mind that chronic activation of some PKC isozymes participate in specific aspects of cardiac remodelling and dysfunction in heart failure, including cardiac hypertrophy.\textsuperscript{49}

In conclusion, the present study provides evidence of new mechanisms involved in the modulation of L-type Ca\textsuperscript{2+} channels by Ucn in adult rat ventricular myocytes. We propose that the marked increase in $\text{I}_{\text{CaL}}$ density induced by Ucn is mediated through CRF-R2 and would involve PKC-dependent activation of MAPK–ERK1/2 pathway, whereas PKA and Epac signalling are not implicated.


