T-type Ca\textsuperscript{2+} signalling regulates aldosterone-induced CREB activation and cell death through PP2A activation in neonatal cardiomyocytes

Laurent Ferron\textsuperscript{††}, Yann Ruchon\textsuperscript{1,2}, Jean-François Renaud\textsuperscript{1,2}, and Véronique Capuano\textsuperscript{1,2*}

\textsuperscript{1}Département de Recherche Médicale, Remodelage tissulaire et fonctionnel: signalisation et physiopathologie CNRS-UMR8162, Le Plessis Robinson, France; and \textsuperscript{2}INSERM-U999, Université Paris-Sud XI, Hôpital Marie Lannelongue, 133 ave de la Résistance, 9230 Le Plessis Robinson, France

Received 12 May 2010; revised 8 November 2010; accepted 25 November 2010; online publish-ahead-of-print 30 November 2010

Time for primary review: 31 days

Aims

We have investigated Ca\textsuperscript{2+} signalling generated by aldosterone-induced T-type current (I\textsubscript{CaT}), the effects of I\textsubscript{CaT} in neonatal cardiomyocytes, and a putative role for I\textsubscript{CaT} in cardiomyocytes during cardiac pathology induced by stenosis in an adult rat.

Methods and results

Neonatal rat cardiomyocytes treated with aldosterone showed an increase in I\textsubscript{CaT} density, principally due to the upregulation of the T-type channel Ca\textsubscript{v}3.1 (by 80%). Aldosterone activated cAMP-response element-binding protein (CREB), and this activation was enhanced by blocking I\textsubscript{CaT} or by inhibiting protein phosphatase 2A (PP2A) activity. Aldosterone induced PP2A activity, an induction that was prevented upon I\textsubscript{CaT} blockade. I\textsubscript{CaT} exerted a negative feedback regulation on the transcription of the Ca\textsubscript{v}3.1 gene, and the activation of PP2A by I\textsubscript{CaT} led to increased levels of the pro-apoptotic markers caspase 9 and Bcl-x\textsubscript{S} and decreased levels of the anti-apoptotic marker Bcl-2. These findings were corroborated by flow cytometry analysis for apoptosis and necrosis. Similarly, in a rat model of cardiac disease, I\textsubscript{CaT} re-emergence was associated with a decrease in CREB activation and was correlated with increases in caspase 9 and Bcl-x\textsubscript{S} and a decrease in Bcl-2 levels.

Conclusion

Our findings establish PP2A/CREB as targets of I\textsubscript{CaT}-generated Ca\textsuperscript{2+} signalling and identify an important role for I\textsubscript{CaT} in cardiomyocyte cell death.

Keywords

Apoptosis • Cav3.1 channel • Gene expression • Glucocorticoid receptor • Protein phosphatase

1. Introduction

Ca\textsuperscript{2+} entry through T-type channels was reported to have two main roles, one related to cell excitability and the other to the stimulation of a range of Ca\textsuperscript{2+}-dependent biological events.\textsuperscript{1–3} In ventricular cardiomyocytes, both Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channel subunits are responsible for I\textsubscript{CaT}, and the pattern of expression of these channels varies with the stage of heart development.\textsuperscript{4–6} The reappearance of I\textsubscript{CaT} in adult cardiomyocytes was reported, in association with several aetiologies of ventricular hypertrophy.\textsuperscript{3} However, little is known about the mechanism involved in the regulation of T-type channel expression, and the I\textsubscript{CaT}-dependent Ca\textsuperscript{2+} signalling pathway remains to be explored.

It has repeatedly been demonstrated that Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 are regulated differently and have different cellular functions. Consistent with this observation, mice lacking Ca\textsubscript{v}3.1 have a cardiac phenotype different from that of mice lacking Ca\textsubscript{v}3.2 and, similarly, mice overexpressing the Ca\textsubscript{v}3.1 gene have a response to hypertrophic stimuli very different from that of mice overexpressing the Ca\textsubscript{v}3.2 gene.\textsuperscript{7,8} However, the role of I\textsubscript{CaT} in the development of cardiac hypertrophy remains a matter of debate, as does the identity of the channel subunit involved. Indeed, Chiang et al.\textsuperscript{7} reported that cardiac hypertrophy was
associated with Ca\textsubscript{3.2} expression (but not with Ca\textsubscript{3.1} expression), whereas Nakayama et al.\textsuperscript{8} described an anti-hypertrophic role for Ca\textsubscript{3.1}. Our previous work with an in vivo model provided evidence that the pathological re-expression of Ca\textsubscript{3.1} and, to a lesser extent, Ca\textsubscript{3.2} channels was independent of cardiac hypertrophy per se, and we suggested that \textit{l}\textsubscript{C\textsubscript{a}T} might be associated with end-stage organ damage during cardiac disease.\textsuperscript{9} Consistent with this hypothesis, the use of mibebradil, an \textit{l}\textsubscript{C\textsubscript{a}T} antagonist, was shown to improve survival in a rat model of chronic heart failure such as sudden death and fibrillation, suggesting that \textit{l}\textsubscript{C\textsubscript{a}T} blockade might have a beneficial impact on cardiac diseases.\textsuperscript{10,11}

Aldosterone is known to be involved in the development of cardiac hypertrophy and failure, and was described as participating in electrophysiological remodelling in ventricular myocytes, increasing both L-type Ca\textsuperscript{2+} current (\textit{l}\textsubscript{Ca}) and \textit{l}\textsubscript{C\textsubscript{a}T}.\textsuperscript{12,13} In this study, we used aldosterone to investigate the mechanisms involved in channel gene expression, to identify the signalling pathways triggered by \textit{l}\textsubscript{C\textsubscript{a}T}-generated Ca\textsuperscript{2+} influx, and to determine the cellular impact of \textit{l}\textsubscript{C\textsubscript{a}T}-generated Ca\textsuperscript{2+} signalling in cardiomyocytes. We found that aldosterone-dependent \textit{l}\textsubscript{C\textsubscript{a}T}-generated Ca\textsuperscript{2+} signalling induced negative feedback on cAMP-response element-binding protein (CREB) via protein phosphatase 2A (PP2A). Through its targeting of PP2A, \textit{l}\textsubscript{C\textsubscript{a}T} increased pro-apoptotic markers and cell death. These findings implicate \textit{l}\textsubscript{C\textsubscript{a}T} in the regulation of CREB-dependent gene expression and \textit{l}\textsubscript{C\textsubscript{a}T}/PP2A/CREB signalling in promoting apoptosis.

2. Methods

2.1 Animals and culture conditions

Animals were cared for and used in accordance with the European convention for the protection of vertebrates used for experimental purposes, and institutional guidelines no. 86/609/CEE 24 November 1986 and 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). Primary cultures of ventricular myocytes were each prepared from hearts of 10 1-day-old male Wistar rats (Charles Rivers, France) as described previously.\textsuperscript{9} Following tissue digestion, cells were plated on new dishes, and maintained in serum-free DMEM supplemented with medium 199 (4:1) and 1 µM cytosine β-d-arabinofuranoside (Sigma), to prevent proliferation of residual non-myocytes. Cells were exposed to aldosterone or pre-treated with antagonists or inhibitors prior to addition of aldosterone. The antagonists or inhibitors used were RU 38486 spironolactone, mibebradil, flunarizine, flutamide, or nifedipine all from Sigma, FKS06, or okadaic acid (OA) from Alexis Biochemicals (San Diego, CA, USA) and dexamethasone from Sigma.

The hypertrophic model was described elsewhere.\textsuperscript{5} Briefly, adult male Wistar rats were subjected to abdominal aortic stenosis for 6 (AS6) or 12 weeks (AS12) and controls were age-matched sham-operated rats (Ct6 and Ct12). The left ventricles were isolated, rapidly frozen, and stored at −80°C.

2.2 Electrophysiological recordings

Ca\textsuperscript{2+} currents were recorded by whole-cell patch-clamp technique, at room temperature (22–24°C). The fire-polished pipettes used had a resistance of 1–3 MΩ when filled with pipette solution: 10 mM CsCl, 120 mM Cs-aspartate, 3 mM MgCl\textsubscript{2}, 10 mM HEPES, 15 mM EGTA, 1.8 mM CaCl\textsubscript{2}, 10 mM glucose, 3.6 mM creatine phosphate, 5 mM MgATP, 0.2 mM Tris-GTP, pH 7.2, adjusted with CsOH. Ca\textsuperscript{2+} currents were recorded in an external solution containing: 135 mM TEA-Cl, 1 mM MgCl\textsubscript{2}, 10 mM CaCl\textsubscript{2}, 10 mM HEPES, 10 mM glucose, 3 mM 4–aminopyridine, 20 mM CsCl, 0.03 mM tetrodotoxin, pH 7.4, adjusted with CsOH. Additional details of data recording were reported elsewhere.\textsuperscript{5}

2.3 Transfection and luciferase assay

The 3 kb region upstream from cacna1g was isolated from Wistar rat genomic library (λ DASH II, Stratagene) and inserted into the luciferase reporter pgL3-basic vector (Promega, Madison, WI, USA) as described previously.\textsuperscript{14} The resulting plasmid was used to generate chimeric pGL promoter/luciferase constructs (pGL4100–pG4L600). The GRE1 mutant (853 bp upstream from the translation start site) resulted from a point mutation generated in pGL4400 by PCR with 5’TGGAGGG CCCGTC(T)A(C)TGGAA3’ used as the primer. Neonatal myocytes were cotransfected using luciferase construct and pSVβgal (Promega) with FuGENE 6 (Roche, Indianapolis, IN, USA). Luciferase and β-galactosidase activities were determined in a related reporter assay system (Promega). All experiments were performed on at least three cultures set up in triplicate.

2.4 RNA isolation and mRNA quantification

Total RNA samples were prepared from ventricular tissues using Trizol (Invitrogen). The Ca\textsubscript{3.1}, Ca\textsubscript{3.2}, cyclophylin primers, and quantitative RT–PCR procedures have been described elsewhere.\textsuperscript{5,15} Briefly, quantitative RT–PCR was performed with a normalized RNA aliquot (1 µg) and a known amount of cRNA internal control (2.5 × 10\textsuperscript{6} molecules). The internal control differed from the target counterpart by only one restriction site. PCR was performed with a trace amount of 32P-labelled S’ primer. The number of copies per micrograms of total RNA was calculated using the following equation: \[\frac{\text{[nucleotides} \times 330]}{6.02 \times 10^{23}} \times 0.62 \times 10^{17}\], and from the relationship [molecules cRNA control/cpm cRNA control] × cpm target = target molecules per normalized RNA aliquot.

2.5 Protein extraction and western blot analysis and phosphatase activity assay

The preparation of cytosolic, nuclear, and total extracts were described elsewhere.\textsuperscript{14} Antibodies were guaumericocitocoid receptor (GR), Bcl-x\textsubscript{L}, Bcl-X\textsubscript{L}, Bcl-2 (Santa Cruz Biotecnology, CA, USA), total CREB, Ser-133-phosphorylated CREB (pCREB), caspase 9 (Cell Signaling Technology, MA, USA), which detects the pro-caspase 9 as well as the cleaved fragment of caspase 9, catalytic subunit of PPI (Santa Cruz Bio-technology), PP2AC, and PP2B (Cell Signaling Technology). (Actin (Santa Cruz) was used for normalization. Protein bands were visualized with the Lumi-light\textsuperscript{24,25} blotting Kit (Roche) and analysed with Gene Tools from Syngen (Cambridge, UK). The activity of PP2A was measured with the Ser/Thr phosphatase assay system (Promega). Phosphatase activity was calculated as the rate of Pi release from pre-phosphorylated peptide and normalized with respect to basal levels (sample without phosphopeptide).

2.6 Flow cytometry

For each treatment, cells were collected after digestion with trypsin. Samples from each cell suspension were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V, propidium iodide (PI) exclusion assay (PI-Pharmingen) was carried out according to the manufacturer’s protocol. For each sample, 30 000 cells were analysed on a FACSCalibur machine, with CELLQuest software (Becton Dickinson, Franklin Lakes, NJ, USA). Counts are expressed as a percentage of the total number of cells counted.
activates PP2A and cardiomyocyte death

I

I

i

cytos, control conditions indicated that I

I

Ni2

Current induced by aldosterone was determined on the basis of the mode, with an IC50 of 300 nM, whereas, in aldosterone-treated myocytes, I

I

CaT was blocked in the monophasic inactivation parameters (B) that was not associated with changes in activation or in steady-state inactivation (circles) curves. Activation: V0.5 = −270 ± 26 mV, k = 5.8 ± 0.5 mV for Ctl (n = 6); V0.5 = −31.4 ± 0.4 mV, k = 4.8 ± 0.1 mV for Aldo (n = 11). Inactivation: V0.5 = −63.7 ± 1.5 mV, k = −4.3 ± 0.2 mV for Ctl (n = 6); V0.5 = −60.4 ± 0.5 mV, k = −4.7 ± 0.1 mV for Aldo (n = 8). (D) Dose–response curves for Ni2+ on Icat recorded from a holding potential of −100 mV to a test pulse of −20 mV, n = 5–10 cells for each Ni2+ concentration. For Ctl, the ICGO is 300 µM. For aldosterone, two ICGO values were determined: 1.5 and 300 µM, *P < 0.05 vs. Ctl.

Figure 1 Aldosterone increases principally Ca3.1-generated T-type calcium current. (A) Typical recordings are shown for control (Ctl) and aldosterone treatment (Aldo, 1 µM). (B) Icat density—voltage relationships in Ctl (n = 8) and Aldo (n = 15). (C) Voltage-dependent activation (squares) and steady-state inactivation (circles) curves. Activation: V0.5 = −270 ± 26 mV, k = 5.8 ± 0.5 mV for Ctl (n = 6); V0.5 = −31.4 ± 0.4 mV, k = 4.8 ± 0.1 mV for Aldo (n = 11). Inactivation: V0.5 = −63.7 ± 1.5 mV, k = −4.3 ± 0.2 mV for Ctl (n = 6); V0.5 = −60.4 ± 0.5 mV, k = −4.7 ± 0.1 mV for Aldo (n = 8). (D) Dose–response curves for Ni2+ on Icat recorded from a holding potential of −100 mV to a test pulse of −20 mV, n = 5–10 cells for each Ni2+ concentration. For Ctl, the ICGO is 300 µM. For aldosterone, two ICGO values were determined: 1.5 and 300 µM, *P < 0.05 vs. Ctl.

2.7 Statistical analysis

Data are expressed as means ± SEM (n ≥ 3). The statistical significance of differences between groups was estimated by one-way ANOVA, followed by Dunnett’s test or two-way ANOVA, followed by Bonferroni’s test. Differences were considered significant if P < 0.05.

3. Results

3.1 Aldosterone increases Ca3.1-related current

Cultures of ventricular myocytes treated with aldosterone for 48 h displayed an increase in Icat current density (5.7 ± 0.5 pA/pF n = 15 vs. 3.6 ± 0.3 pA/pF n = 8 in the control, P < 0.01; Figure 1A and B) that was not associated with changes in activation or in steady-state inactivation parameters (Figure 1C). The nature of the Ca3.X-related current induced by aldosterone was determined on the basis of the Ni2+ sensitivity of Icat (Figure 1D). The Ni2+ dose–response in control conditions indicated that Icat was blocked in the monophasic mode, with an ICGO of 300 µM, whereas, in aldosterone-treated myocytes, Icat was blocked in the biphasic mode, with ICGO values of 1.5 and 300 µM (Figure 1D), corresponding to the sensitivities described for Ca3.2- and Ca3.1-related currents, respectively.16,17 The relative contributions of these currents were assessed from the Ni2+ dose–response relationship, and we found that Ca3.2 and Ca3.1 contributed 20 and 80% of the total current, respectively. These relative contributions of functional Ca3.X channels correspond to observations reported in pathological conditions.9

3.2 Aldosterone increases transcription of the Ca3.1 gene by activating the GR

Quantitative RT–PCR showed that Ca3.1 and Ca3.2 mRNA levels were higher (by factors of 2.4 and 1.4, respectively) in aldosterone-treated cells than in control cells (Figure 2A). These results contrast with those of Lalèvée et al.13 who found that aldosterone-induced Icat was associated with an increase in the amount of Ca3.2 mRNA (up to 410%) and a non-significant increase in the Ca3.1 mRNA level. This discrepancy is probably due to the use of an inappropriate normalization marker, cyclophilin, which is repressed in aldosterone-treated cells, and the use of primers that do not recognize the relevant isoform (data shown and discussed in Supplementary material online, Figure S7). Thereafter, we have focused our study on the regulation of Ca3.1 gene expression.

The amount of Ca3.1 mRNA increased in aldosterone-treated cells, reaching a plateau 9 h after the beginning of treatment (Figure 2B). Cycloheximide treatment did not prevent aldosterone-induced increases in mRNA levels during the first hour (Supplementary material online, Figure S2), suggesting that aldosterone activated pre-existing regulatory factors. However, protein synthesis was required for a sustained effect of aldosterone, since the Ca3.1 mRNA amount gradually declined after the first hour of aldosterone plus cycloheximide treatment. The effect of the pre-existing transcriptional factor was no longer visible after 9 h of treatment.

We further assessed cacna1g regulation by investigating promoter activity through unidirectional deletions and point mutations. Aldosterone treatment induced an increase in promoter activity, which was maximal for sequences up to 1.5 kb long (pGL4400) (Figure 2C). Aldosterone had a dose-dependent effect on Cacna1a upregulation, and we found that when compared with control, 1 µM induced an 87% increase of pGL4400 activity and 0.1 µM only 20% increase of pGL4400 activity (Supplementary material online, Figure S3).

Treatment with the GR antagonist RU 38486 almost completely turned off the transcription of pGL4400 and pGL4600, data not shown) in both aldosterone-treated and untreated cells (Figure 2D). In contrast, the mineralocorticoid receptor (MR) antagonist spironolactone simply decreased the level of transcription. Several putative glucocorticoid response elements (GREs) are present in pGL4400.14 Mutations in GRE1 abolished aldosterone-induced transcription of the Ca3.1 gene, cacna1g (Figure 2E), but did not alter basal levels of transcription or the levels of transcription induced by a synthetic glucocorticoid analogue, dexamethasone. Consistent with these findings, the rapid (10 min) translocation of GR from the cytosol to the nuclear compartment (Figure 2F) was observed in aldosterone-treated cells, supporting the role of GR in the genomic response of aldosterone.

After 2 h of incubation with aldosterone, the amount in cytosolic GR returned to control levels, probably due to de novo GR synthesis. These findings are consistent with the involvement of pre-existing regulatory factors and the need for de novo synthesis for sustained increases in mRNA levels.

3.3 Icat modulates cacna1g expression and CREB activation

To test the hypothesis that Icat is likely to play a role in the control of gene expression, we investigated the effect of the Icat blockers
mibefradil, fluoxetine, and flunarizine on caca1tg expression (Figure 3A). During the exponential phase of mRNA accumulation (until 6 h—Figure 2B), Ca2+ current blockers had no effect on the amount of Caca3.1 mRNA (Figure 3A). However, during the plateau phase (9 h), Caca3.1 mRNA levels were higher with aldosterone plus mibefradil (AM) or flunarizine (AFluna) [or fluoxetine (AFluo), data not shown] than with aldosterone alone. Blocking ICaT with nifedipine had no additional effect. The aldosterone-induced increase in ICaT density triggered negative feedback control over caca1tg expression that was confirmed by the stronger promoter activity observed in pGL4400-transfected cells exposed to AM than in transfected cells exposed to aldosterone alone (Figure 3B). We then focused on the transcription factors regulated by ICaT expression by investigating the involvement of the serine threonine phosphatases modulating CREB activation during aldosterone treatment. Aldosterone-induced increases in CREB phosphorylation were prevented in conditions in which ICaT re-expression was diminished (Supplementary material online, Figure S5).

### 3.4 ICaT modulates CREB activation via PP2A

Many signalling pathways are thought to control CREB phosphorylation status, depending on cell and physiopathological status. We investigated the involvement of the serine threonine phosphatases 1, 2A, and 2B (PP1, PP2A, and PP2B), to identify the protein phosphatases modulating CREB activation during aldosterone treatment (Figure 4). Cells were treated with the PP2B (calcineurin) inhibitor FK506 (1 µM) or with the PP2A inhibitor OA (5 nM) (Figure 4A). OA treatment increased phosphorylated CREB levels in control and aldosterone-treated cells. In contrast, FK506 treatment prevented the aldosterone-induced increase in phosphorylated CREB levels but had no effect on these levels in control cells. Neither OA nor FK506 affected total CREB levels (data not shown).

PP2A and PP1 activities were determined in vitro for cardiomyocyte lysates and could be separated on the basis of sensitivity to OA concentration (Figure 4B). In aldosterone-treated cells, total PP2A—PP1 activity was twice that in control cells, mostly due to an increase in PP2A activity. Indeed, 80 ± 5% of phosphatase activity was abolished by PP2A blockade (OA—12 nM). The aldosterone-induced increase in PP2A—PP1 activity was prevented by the ICaT blockers. The small contribution of PP1 in aldosterone-treated cells was confirmed using an inhibitor of PP1, and its activation shown to depend on ICaT-generated Ca2+ influx (Figure 4B, bottom panel). Finally, we were unable to detect a significant difference in the amounts of the catalytic subunits of PP1, PP2A, and PP2B, under the different culture conditions.
conditions tested (Supplementary material online, Figure S6). Therefore, aldosterone-induced \( I_{CaT} \) upregulates the level of phosphatase activity through post-transcriptional regulation rather than changes in phosphatase gene expression.

### 3.5 \( I_{CaT} \) modulates cell-death markers via PP2A

An increase in PP2A activity was associated with cell death.\(^{23}\) We investigated whether \( I_{CaT} \)-induced PP2A activity might be involved in cardiomyocyte death (Figure 5). An increase in activated caspase 9 levels occurred only 9 h after the start of the treatment (Figure 5A) in parallel with a time-dependent effect of \( I_{CaT} \) blockade (Figure 3A). At this time point, treatment with OA prevented the aldosterone-induced caspase 9 activation. Aldosterone also induced a switch between the Bcl-xL and Bcl-xS variants, which was prevented by the use of OA or OA plus mibefradil (Figure 5B). These results indicated that the timing of PP2A activation was related to an increase in \( I_{CaT} \)-generated \( Ca^{2+} \) influx. The relationship between \( I_{CaT} \) and the activation of pro-apoptotic markers was confirmed in cells treated for 24 h with aldosterone, in which the increase in caspase 9 and...
Bcl-x₅ levels and the decrease in Bcl-2 levels were reversed by treatment with mibefradil or fluoxetine (Figure 5C).

Finally, using a rat model of cardiac hypertrophy, we have shown in long-term stenosis (12 weeks treatment—AS12) that the levels of caspase 9 and Bcl-x₅ markedly increased, whereas the reciprocal effect was seen on Bcl-2, when compared with the sham group (Ctl12) (Figure 5D). Only the levels of Bcl-x₅ increased during hypertrophy (6 weeks treatment—AS6 vs. Ctl6) or ageing (Ctl12 vs. Ctl6).

### 3.6 Aldosterone-induced I_{CaT} contributes to myocyte apoptosis and necrosis

Apoptosis was quantified by FACs analysis after staining with annexin V-FITC and PI. As previously reported, aldosterone induced cardiomyocyte apoptosis in cells treated for 24 h, as shown by flow cytometry (annexin V-stained cells, Q2+Q4) (Figure 6A). However, longer periods of exposure to aldosterone (48 h) increased the number of necrotic PI-stained cells (Q1) (P<0.05 vs. Q1 aldosterone 24 h), whereas no significant difference was found in the number of annexin V—or annexin V+PI-stained cells (Q2+Q4) between 24 and 48 h of aldosterone exposure. Treatment with AM decreased the effect of aldosterone on cell death, as shown by smaller numbers of necrotic PI-stained cells (Q1) (P<0.05 vs. Q1 aldosterone 48 h), whereas no significant difference was found in the number of annexin V—or annexin V+PI-stained cells (Q2+Q4) between 24 and 48 h of aldosterone exposure. Treatment with AMN enhanced the effect of AM reducing further the number of PI-stained cells (P<0.05, Q1 AMN vs. Q1 AM and Q1 AMN vs. Q1 aldosterone 48 h). In conclusion, the use of mibefradil with or without nifedipine...
reversed the effect of aldosterone on cell viability (Figure 6B). Thus, aldosterone-dependent apoptosis and necrosis are induced by both \( I_{\text{CaT}} \) and \( I_{\text{CaL}} \).

4. Discussion

We have investigated the signalling pathway(s) of \( I_{\text{CaT}} \) and its implication in cardiomyocyte death. In this context, we have focused on the pathway(s) by which aldosterone upregulates \( Ca_{3.1} \) expression to generate \( I_{\text{CaT}} \) and identified mechanisms involved in \( caca1g \) expression.

We show here that, in neonatal cardiomyocytes, aldosterone-induced increase in \( I_{\text{CaT}} \) density results mostly from an increase in \( Ca_{3.1} \) expression. The transactivation of \( caca1g \) by GR was observed with either aldosterone or dexamethasone (1 \( \mu \)M), but aldosterone-activated GRs targeted a GRE (GRE1) that is different from those targeted by dexamethasone-activated GRs. The aldosterone-induced increase in \( I_{\text{CaT}} \)-generated \( Ca^{2+} \) signalling led to molecular remodelling. Indeed, we found that aldosterone-induced CREB phosphorylation was altered by \( I_{\text{CaT}} \)-generated \( Ca^{2+} \) signalling, likely resulting in a reduced transcription of CREB’s target genes. \( I_{\text{CaT}} \)-signalling also exerts a negative feedback regulation on \( caca1g \) expression, which occurs only after exposure to aldosterone for 9 h, suggesting that functional channels are not present at the membrane before that time. While there is still insufficient evidence to infer the existence of a direct interaction between CREB and the GR-binding \( caca1g \) promoter, it seems likely that the \( I_{\text{CaT}} \)-induced decrease in \( caca1g \) expression is due to a defect in CREB interference with the GR-binding activity of GRE1. Further investigations of this issue are required.

We also found that the decrease in phosphorylated CREB levels was mediated by an \( I_{\text{CaT}} \)-induced increase in PP2A and, to a lesser extent, to PP1 activities. However, PP2B exerts opposite effects on CREB phosphorylation because PP2B blockade prevented aldosterone-induced CREB phosphorylation without affecting the total amount of CREB, therefore excluding the possibilities of an increase in ubiquitination and the degradation of phosphorylated CREB.22 In the heart, several isoforms of phosphodiesterase (PDE) hydrolyze cAMP and cGMP,25 and the activities of these enzymes are modulated by phosphorylation.26 Therefore, PP2B blockade may decrease CREB activation by inhibiting PDE dephosphorylation, thereby increasing PDE activity. Indeed, the inhibition of PDE4 was shown to elicit cAMP/protein kinase A-dependent CREB phosphorylation in cardiac cells.27 A priori, our results differ from those describing an inhibitory effect of aldosterone-activated MRs on CREB phosphorylation through PP2B in vascular smooth muscle cells.28 However, in cardiomyocytes, the response to aldosterone is mediated mostly by GRs and our results are thus consistent with the reported positive effect of dexamethasone-activated GRs on CREB signalling.28

A cross-talk between \( I_{\text{CaT}} \) and CREB signalling is consistent with their opposite expression profiles, together with their antagonistic physiological functions. \( I_{\text{CaT}} \) and CREB signalling do not seem to be critical for normal cardiac function6,29 but, in pathological conditions, CREB signalling was implicated in angiotensin II-induced hypertrophy.30 Stenosis-induced hypertrophy showed an opposite pattern in terms of \( I_{\text{CaT}} \) re-emergence and CREB activation. Indeed, hypertrophied cardiomyocytes in the rat 6-week-stenosis model displayed an increase in phosphorylated CREB levels. The phosphorylation of CREB was almost abolished in the 12-week stenosis group and coincided with \( I_{\text{CaT}} \) re-emergence. In addition, when the re-emergence of \( I_{\text{CaT}} \) is reversed, the decrease in \( I_{\text{CaT}} \) density is correlated with a re-increase in phosphorylated CREB protein levels. Therefore, the negative feedback of \( I_{\text{CaT}} \) on CREB signalling observed in neonatal cardiomyocytes also seems to occur in pathological conditions in the adult. This result also suggests that \( I_{\text{CaT}} \) signalling renders cardiomyocytes resistant to hypertrophic factor-induced CREB signalling in the later stages of cardiac hypertrophy. Thus, loss of the negative feedback regulation of CREB signalling by \( I_{\text{CaT}} \) may partly account for the hypersensitivity to hypertrophy described for \( Ca_{3.1}^{-/-} \) mice.3

The CREB and \( I_{\text{CaT}} \)-signalling pathways have opposite effects on cell proliferation. For example, \( I_{\text{CaT}} \) signalling and CREB signalling have antagonistic effects on the pathway mediated by cell-cycle inhibitor p21(CIP1).31 This feature is particularly important in heart, as recent study has reported the persistence in adolescent heart of immature cardiomyocytes with \( I_{\text{CaT}} \)-generated \( Ca^{2+} \) influx.32 The CREB and \( I_{\text{CaT}} \)-signalling pathways also have opposite effects on cell-death processes. CREB signalling was presumed to have anti-apoptotic effects through the upregulation of Bcl-233 and the prevention of NO-induced apoptosis.32 Conversely, the activities of PP2A and PP1 have been associated with cell apoptosis.33 Our study shows that, in aldosterone-treated cardiomyocytes, \( I_{\text{CaT}} \)-induced PP2A activation increases the expression of pro-apoptotic markers, such as caspase 9 and the Bcl-xS variant. Consistent with these findings, cardiomyocyte apoptosis was first detected 24 h after aldosterone exposure and the longer term (48 h) effects of aldosterone involve an increase in necrosis rather than apoptosis. The blockade of \( I_{\text{CaT}} \)-generated \( Ca^{2+} \) signalling decreases cell death by reducing both apoptosis and necrosis, whereas, as previously reported,34 the blockade of \( I_{\text{CaL}} \) decreases necrosis rather than apoptosis. Aldosterone therefore induces different signalling pathways through \( Ca^{2+} \) microdomains, related to either \( I_{\text{CaT}} \) or \( I_{\text{CaL}} \)-generated \( Ca^{2+} \) influx, such as the activation of PP2A–PP1 or PP2B,24 respectively, resulting in temporally regulated cell processes. Consistent with this idea, an increase in the levels of apoptotic markers in the pathological model was associated with the re-emergence of \( I_{\text{CaT}} \) in long-term stenosis (Supplementary material online, Figure S5). Nevertheless, further investigation will be needed to confirm the relationship between PP2A and CREB dephosphorylation in adult rat during heart pathology.

In conclusion, the main finding of our study is that the increase in \( I_{\text{CaT}} \) density induces \( Ca^{2+} \) signalling, which targets PP2A–PP1 and results in a downregulation of CREB target genes such as those associated with cardiac hypertrophy. Through its targeting of PP2A–PP1, \( I_{\text{CaT}} \) increases pro-apoptotic markers and cell death. Although neonatal cardiomyocytes in culture do not completely reflect what takes place in the adult, we confirm that the re-emergence of \( I_{\text{CaT}} \) in a long-term cardiac hypertrophy model is associated with the reduction of CREB-mediated gene expression and the stimulation of expression of apoptotic markers. Thus, our results suggest that, during cardiac pathology, \( I_{\text{CaT}} \) may limit the progression of hypertrophy, thereby corroborating the conclusion of Nakayama et al.35 Whether or not cell death induced by \( I_{\text{CaT}} \) has a ‘protective’ effect against heart disease, such as described by Nakayama et al, is difficult to reconcile and needs to be further investigated. However, it is noteworthy that the protective effect of \( I_{\text{CaT}} \) described by Nakayama et al occurs via an NOS3-dependent signalling, the product of which was shown to result in cardiomyocyte apoptosis.35 The \( I_{\text{CaT}} \)/PP2A/CREB signalling pathway, described in this study, may represent an
alternative to \( I_{CaT}/NOS3/PKG1 \) signalling pathway involved in the anti-hypertrophic effect of \( I_{CaT} \) during heart pathology.

5. Limitations

Most papers describing an effect of aldosterone on ionic currents in cardiomyocyte use a concentration of 1 \( \mu \)M aldosterone, and we found that 1 \( \mu \)M Aldo was indeed the most potent concentration for upregulating the promoter of the \( \text{caca}1g \) gene. We should point out that 1 \( \mu \)M aldosterone is approximately two orders of magnitude higher than physiological concentration. However, in vivo, the impact of paracrine effects, such as those of the renin–angiotensin II–aldosterone system, is thought to occur in the heart, cannot be ignored. Such effects may locally increase aldosterone concentration and increase the impact of plasma aldosterone concentration.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgement

We thank A. Coulombe for his support and Prof. A. C. Dolphin for comments on the manuscript. We also wish to thank A. Capderou for its scientific support and N. Kerlero-de-Rosbo for helpful discussions and critical reading of the manuscript.

Conflict of interest: none declared.

Funding

This work was supported by the Centre National de la Recherche Scientifique, the Association Marie Lannelongue and the Association Française contre les Myopathies (D10581/F11898). Funding to pay the Open Access publication charge was provided by Marie Lannelongue hospital.

References