CREB critically regulates action potential shape and duration in the adult mouse ventricle

J. S. Schulte, M. D. Seidl, F. Nunes, C. Freese, M. Schneider, W. Schmitz, and F. U. Müller

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Submitted 19 January 2011; accepted in final form 5 March 2012

Schulte JS, Seidl MD, Nunes F, Freese C, Schneider M, Schmitz W, Müller FU. CREB critically regulates action potential shape and duration in the adult mouse ventricle. Am J Physiol Heart Circ Physiol 302: H1998–H2007, 2012. First published March 16, 2012; doi:10.1152/ajpheart.00057.2011.—The cAMP response element binding protein (CREB) belongs to the CREB/cAMP response element binding modulator/activating transcription factor 1 family of cAMP-dependent transcription factors mediating a regulation of gene transcription in response to cAMP. Chronic stimulation of β-adrenergic receptors and the cAMP-dependent signal transduction pathway by elevated plasma catecholamines play a central role in the pathogenesis of heart failure. Ion channel remodeling, particularly a decreased transient outward current (Iₒ), and subsequent action potential (AP) prolongation are hallmarks of the failing heart. Here, we studied the role of CREB for ion channel regulation in mice with a cardiomyocyte-specific knockout of CREB (CREB KO). APs of CREB KO cardiomyocytes were prolonged with increased AP duration at 50 and 70% repolarization and accompanied by a by 51% reduction of Iₒ peak amplitude as detected in voltage-clamp measurements. We observed a 29% reduction of Kcnnd2/Kv4.2 mRNA in CREB KO cardiomyocytes mice while the other Iₒ,slow-related channel subunits Kv4.3 and KChIP2 were not different between groups. Accordingly, Kv4.2 protein was reduced by 37% in CREB KO. However, we were not able to detect a direct regulation of Kv4.2 by CREB. The Iₒ,fast-dependent AP prolongation went along with an increase of Iₒ,s and a decrease of Iₒ,fast, respectively (for review, see Ref. 5). The knockout of either Kcna4/Kv1.4 or Kcnd3 reduces Iₒ by 50% in rat ventricular cardiomyocytes (6) whereas the lack of KChIP2 leads to a complete loss of Iₒ in cardiomyocytes and a marked increase in AP duration (14). In mouse ventricle, the delayed rectifiers are represented by the potassium voltage-gated channel subfamily D member 2 (Kcnnd2/Kv4.2) and member 3 (Kcnnd3/Kv4.3), the Kv channel-interacting protein 2 (Kcnip2/KChIP2), and potassium voltage-gated channel, shaker-related subfamily, member 4 (Kcn4/Kvl.4) subunits, respectively (for review, see Ref. 5). The knockout of either Kcnnd2 or Kcnnd3 reduces Iₒ by 50% in rat ventricular cardiomyocytes (16), and the potassium voltage-gated channel Shab-related subfamily member 1 (Kcnb1/Kvl.1)-based Iₒ,slow (16) and the potassium voltage-gated channel Shab-related subfamily member 1 (Kcnb1/Kvl.1)-based Iₒ,slow (53).

Ion channel remodeling, in particular a decreased Iₒ and subsequent AP prolongation are hallmarks of the failing heart and heart failure (HF)-related arrhythmias and are associated with the occurrence of afterdepolarizations and triggered arrhythmia (1, 27). HF is defined as the heart’s inability to cover the body’s blood demand and is the common end stage of various cardiac diseases including ischemic or dilated cardiomyopathy. HF is among the leading causes of death in Western countries, 50% of HF patients die within 3–4 yr after diagnosis. The arrhythmic “sudden cardiac death” accounts for 30–50% of these fatal casualties (1, 19, 24). Chronic stimulation of β-adrenergic receptors and the cAMP-dependent signal transduction pathway by elevated plasma catecholamines play a central role in the pathogenesis of HF (for review, see Ref. 20). However, mechanisms underlying the altered gene regulation in failing hearts including alterations associated with ion channel remodeling are not known in detail. The transcription factors of the cAMP response element binding protein/modulator and activating transcription factor 1 (CREB/CREM/ATF1) family are expressed in the heart (25, 26) and mediate the regulation of gene transcription in response to cAMP by binding to cAMP responsive elements (CREs, TGACGTC, and variants thereof) in the promoter region of target genes (for review, see Ref. 23). The inactivation of CREM partially protects from β-adrenergic-mediated detrimental effects in

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the mouse heart (15). Mice with a cardiomyocyte-specific inactivation of CREB were investigated before in our group. CREB-deficient mice were viable and displayed neither changes in survival, cardiac morphology, nor alterations of basal or isoproterenol-stimulated left ventricular function in vivo at an age of 16–24 wk (22). However, the consequences of CREB inactivation in the heart for cellular electrophysiology were not investigated. Here, we studied whether CREB is involved in the regulation of cardiac ion channels underlying AP shape and duration.

**MATERIAL AND METHODS**

**Experimental animals.** Mice with a cardiomyocyte-specific ablation of CREB (CREB KO) using the Cre-loxP system were described previously (22). They lack Creb1 exon 10 resulting in a truncated CREB protein without DNA-binding and dimerization domains. Mice not expressing Cre recombine (i.e., with functional CREB) from the same breeding colony were employed as controls (CTR). Experimental animals were 12 to 18 wk old and from generation F8 or more to ensure genetic homogeneity. All animal procedures were conducted in accordance with local animal welfare authorities and approved by the Landesamt Für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen.

**Isolation of adult mouse cardiomyocytes.** Adult cardiomyocytes were isolated from adult (12 to 18 wk) CREB KO and CTR mice by enzymatic digestion as described before (12). Briefly, excised hearts were rinsed with heparin and then retrogradely perfused with collagenase solution (Type II, Worthington Biochemical) in a modified Langendorff apparatus (37°C, 2 ml/min). Cells were then separated by mechanical dispersion and prepared as needed for subsequent experimental procedures.

For patch-clamp experiments, cardiomyocytes were gently centrifuged (42 relative centrifugal force/min) to separate vital myocytes from nonmyocytes. After stepwise elevation of extracellular Ca²⁺ to 1 mM, cells were immediately used for experiments within 6 h after isolation.

**Cellular electrophysiology.** APs and membrane currents were recorded in whole cell configuration using the perforated patch technique with ampholytic B (38). Calcium-tolerant, rod-shaped, and clearly striated ventricular myocytes were selected randomly for electrophysiological studies at room temperature (22–24°C). Patch pipettes were pulled (P97; Sutter, Novato, CA) from borosilicate glass capillaries (Science Products, Hofheim, Germany) with a 1.5-mm diameter. Pipette resistances were 3–5 MΩ when filled with pipette solution. Recordings were performed using a List Medical L/M-PC PC (List Medical, Lambrecht, Germany) interfaced and controlled by an ISO2-recording system (MKF Friedrich, Niedernhausen, Germany) or an EPC 800 Patch Clamp Amplifier and PatchMaster Software (HEKA Elektronik, Lambrecht, Germany). Cell capacitance was determined by ramp pulses before capacitance compensation. Cell capacitance and series resistance were carefully compensated before recording start. Recordings with series resistance >20 MΩ were discarded. The recording of sodium currents was performed using a SEC-05X discontinuous single-electrode voltage-clamp amplifier (NPI, Tamm, Germany) to exclude series resistance errors, which are especially problematic when large currents are recorded.

APs were evoked by injecting suprathreshold current pulses in current-clamp mode at a 1-Hz stimulation frequency. Potassium currents were evoked by a step protocol from −40 to +60 mV (Δ10 mV, 4.5-s duration) from a holding potential (HP) of −80 mV. Na⁺ currents were suppressed by applying a brief prepulse to −40 mV (10 ms) before each test pulse. The transient outward current Ito was separated by applying a 100-ms lasting prepulse to −40 mV (HP: −80 mV) and subtracting respective traces from currents recorded without prepulse as described by Brouillet et al. (3) (see Fig. 2). In both experiments, the following solutions were used (in mM): pipette solution: 5 NaCl, 90 KCl, 2.5 MgATP, 1 EGTA, and 5 HEPES pH 7.4; and extracellular solution 135 NaCl, 4 KCl, 1 MgCl₂, 1.8 CaCl₂, 0.33 Na₂HPO₄, 10 HEPES, and 10 glucose pH 7.4. L-type Ca²⁺ currents were blocked by nifedipine (2 μM) when potassium currents were recorded. Sodium currents were evoked by a step protocol from −80 to +50 mV (Δ5 mV, 200-ms duration) from a HP of −80 mV. The pipette solution consisted of the following (in mM): 120 CsCl, 4 MgCl₂, 4 Na₂ATP, 10 EGTA, and 10 HEPES pH 7.2. The extracellular solution contained the following (in mM): 30 NaCl, 107 CsCl, 0.5 CaCl₂, 2.5 MgCl₂, 10 HEPES, 10 glucose, and 2 CoCl₂ pH 7.4. Ca²⁺ currents were evoked by a step protocol from −40 to +65 mV (Δ5 mV, 400-ms duration) following a 200-ms prepulse to −40 mV to inactivate T-type Ca²⁺ and Na⁺ currents. Here the pipette solution was composed of the following (in mM): 120 CsCl, 4 MgCl₂, 4 Na₂ATP, 10 EGTA, and 10 HEPES pH 7.2. The extracellular solution contained the following (in mM): 135 NaCl, 3 CsCl, 1 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose pH 7.4. Peak amplitudes of membrane currents were normalized to cell capacitance and then plotted against test potentials to obtain respective current-voltage (I−V) relationships.

**SDS-PAGE and quantitative immunoblotting.** Mouse ventricles were prepared and immediately frozen in liquid nitrogen. After mechanical homogenization with a pestle, 30–40 mg of the tissue were sonicated three times for 7 s on ice in 400 μl of buffer containing 0.193 M histidine and 0.73 M sucrose at pH 7.5; thereafter, SDS was added to a final concentration of 1%. Homogenates were centrifuged at 14,000 rpm for 20 min and 4°C, and protein content of the supernatant was determined according to Lowry with BSA as standard. Proteins were loaded at 50–100 μg/lane for detection of cardiac calsequestrin (CSP), GAPDH, and Kv4.2, Kv4.3, and KChIP2. After SDS-PAGE on 8–10% polyacrylamide gels, the transfer onto nitrocellulose membranes (BA 85 Protran Nitrocellulose, 0.45-μm pore size; Schleicher and Schüll, Dassel, Germany) was performed by tank blotting in 50 mmol/l sodium phosphate buffer (pH 7.4) for 180 min at 1.5 at 4°C. Immunodetection was performed according to the manufacturer’s instructions for Kv4.2 (1:200, APC-023; Alomone, Jerusalem, Israel), Kv4.3 (1:150, Ab5194; Millipore, Schwalbach/Ts., Germany) was treated the same as Kv4.2. Briefly, membranes were blocked with 5.0% dry fat milk in PBS with 0.025% sodium azide overnight at 4°C. The first antibody was pretreated 1 h in 0.5 ml PBS-1% BSA, centrifugated, and diluted with 4.5 ml blocking solution. The blocked membranes were incubated for 2 h in the antibody-blocking solution and washed four times in PBS-0.1% Tween-20 without sodium-azide. Secondary anti-rabbit antibody (1:5,000, ECL rabbit IgG, HRP-Linked Whole Ab; GE Healthcare, Freiburg, Germany) was applied for 1 h at room temperature in PBS-0.1% Tween-20. For blocking GAPDH (1:10,000, ab9485; Abcam, Cambridge, MA), COX (1:2,500, PA1–913; Thermo Fisher, Bonn, Germany), and KChIP2 (1:200, KChIP2 H-100, sc-25685; Santa Cruz Biotechnology, Heidelberg, Germany) membranes were treated in PBS containing 5.0% dry fat milk for 2 h and washed four times for 10 min in PBS-0.1% Tween-20 (PBS-T), incubated 2 h at room temperature or overnight at 4°C with the primary antibody in PBS, followed by being washed four times with PBS-T. The corresponding secondary antibodies anti-mouse-IgG (1:20,000, ECL Mouse IgG, HRP-Linked Whole Ab; GE Healthcare) for detection of GAPDH and anti-rabbit-IgG (1:10,000 for detection of CSQ and 1:3,000 for detection of KChIP2) were incubated for 2 h at room temperature and washed four times with PBS-T. The amount of protein loaded was within linear range of the determination. The detected bands of Kv4.2 and Kv4.3 were blocked by the specific antibodies provided by the manufacturer’s instructions (not shown). Signals were visualized and quantified by using the ECL plus detection system (Amersham ECL Plus; GE Healthcare) by using the STORM blot imaging system (Molecular Dynamics, Sunnyvale, CA). CREB KO and CTR cardiac left ventricular homogenates were loaded in an alternating manner, and variabil-
ity was controlled by using CSQ and GAPDH as standard proteins and presented relative to the CTR homogenates.

Quantitative real-time RT-PCR. Total RNA was extracted from cardiac ventricles or cardiomyocytes of CREB KO and CTR mice with the use of Trizol (Life Technologies, Darmstadt, Germany). Total RNA (0.5 μg) was randomly reversely transcribed to cDNA using the first-strand cDNA synthesis kit for RT-PCR AMV (Roche, Mannheim, Germany). The real-time RT-PCR was carried out using a LightCycler 1.5 System (Roche), and the detection was performed by measuring the binding of the fluorescence dye SYBR Green I to double-stranded cDNA at 530 nm (QuantiFast SYBR Green PCR kit; Qiagen, Hilden, Germany). Primers (Life Technologies) were designed using Primer3 (http://frodo.wi.mit.edu/; Ref. 40) with standard configuration except: “Mispriming Library” was set to “Rodent end Simple” and “Product Size” was limited to 100–300 bp. The PCR reactions were set up in microcapillary tubes in a volume of 20 μl. The reaction components were 2 μl undiluted cDNA, 10 μl QuantiFast SYBR Green PCR Master Mix, 4 μl H2O, and 2 μl for each primer (10 μM). Reactions were incubated at 95°C for 5 min followed by 50 cycles at 95°C for 5 s, 60°C for 1 s, and 72°C for 10 s. Relative levels of particular cDNAs were determined with the help of LightCycler software (version 3.5) with appropriate calibration curves obtained with different amounts of control cDNAs. Crossing points were determined by using the second derivative method. On completion of the PCR amplification, a melting curve analysis was performed. Relative quantification was performed by calculating relative expression (ChIP) was adapted from published protocols (31, 44). Mouse ChIP was performed as described in the manufacturer’s protocol. Luciferase activities were assayed using the Dual-Luciferase Reporter Assay System (Promega). Additional proteins were determined by using the manufacturer’s protocol. Luciferase activities were determined by using a Mithras LB 990 Microplate Analyser (Berthold

For reporter plasmid construction: a genomic fragment of the Kcn2 gene promoter was amplified via PCR using the following primers: (Kv4.2-XhoI) F-5′-ggggctgagttgcatagaga-3′ and (Kv4.2-HindIII) R-5′-ttttgggaaggtgacaaggag-3′; and for the FBJ osteosarcoma oncogene (Fos) promoter F-5′-tgccaaagcagttgaagaaag-3′, R-5′-cctactaaccgccgagccg-3′. To detect a possible enrichment of the precipitated DNA fragments from the potassium voltage-gated channel, Shal-related family, member 2 (Kcn2Δ) gene promoter, the following three primer pairs were used: −2,131 to −1,940 bp (Kcn2Δ-2,131) F-5′-ttgatatgatggccaggtatg-3′, R-5′-attacccgtccaacttgctg-3′; −1,052 to −874 bp (Kcn2Δ-1,052) F-5′-tttgggaaggtgacaaggag-3′, R-5′-ggggctgagttgcatagaga-3′; and −694 to −495 bp (Kcn2Δ-694) F-5′-aggggtcagcttcgcttg-3′, R-5′-cattaccgccgagggacag-3′. Numbers indicate the distance to the ATG start codon. Since precipitated DNA fragments have an approximate size of 500- to 700-bp detection by real-time RT-PCR with the chosen primer pairs (amplifying −200-bp regions) would detect an enrichment of DNA fragments in the whole promoter region.

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two groups, a Mann-Whitney-rank sum test was conducted. Statistical
revealed significant differences between groups. For comparison of
two-way ANOVA with post hoc testing according to Bonferroni. Post
independent experiments. Statistical evaluation was performed using
experiments are reported as
boxes) and the median (horizontal line in the box). The number of
means

Table 1. Quantification of mRNA levels in cardiomyocytes of CREB KO and CTR mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reaction Efficiency</th>
<th>Relative Expression</th>
<th>SE</th>
<th>95% Confidence Interval</th>
<th>Primer Pairs</th>
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</thead>
<tbody>
<tr>
<td>Hprt</td>
<td>0.75</td>
<td>1.00</td>
<td></td>
<td></td>
<td>F-5'ggagtcctggttaggtttgccagta-3'</td>
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<tr>
<td>Kcn2 (Kv4.2)</td>
<td>0.75</td>
<td>0.71*</td>
<td>0.48–1.11</td>
<td>0.37–1.61</td>
<td>R-5'gggacccaagacctgtcacttta-3'</td>
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<tr>
<td>Kcn3 (Kv4.3)</td>
<td>0.85</td>
<td>0.87</td>
<td>0.54–1.41</td>
<td>0.36–2.12</td>
<td>R-5'ttgtacagcccttcacccatca-3'</td>
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<tr>
<td>Kcn2p (KChip2)</td>
<td>0.83</td>
<td>0.93</td>
<td>0.62–1.39</td>
<td>0.44–2.00</td>
<td>R-5'ctgtcaagctcctccttgc-3'</td>
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<tr>
<td>Cacna1c (Cav1.2)</td>
<td>0.89</td>
<td>0.79*</td>
<td>0.57–1.07</td>
<td>0.43–1.45</td>
<td>R-5'gctccccacccctcctc-3'</td>
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<tr>
<td>Cacnb1</td>
<td>0.83</td>
<td>1.02</td>
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<td>0.56–2.36</td>
<td>R-5'gcacccgatgtccttgaag-3'</td>
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<tr>
<td>Cacnb2</td>
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<td>0.97</td>
<td>0.67–1.39</td>
<td>0.53–2.04</td>
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<td>Scn5a (Nav1.5)</td>
<td>0.86</td>
<td>0.91</td>
<td>0.71–1.42</td>
<td>0.33–2.49</td>
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<td>Scn8a (Nav1.6)</td>
<td>1.00</td>
<td>2.52*</td>
<td>1.26–4.99</td>
<td>0.66–7.50</td>
<td>R-5'tgacgctgctgtgctgtc-3'</td>
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<tr>
<td>Cre3'-UTR</td>
<td>1.00</td>
<td>2.14*</td>
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<td>Crem ICER</td>
<td>0.82</td>
<td>0.71*</td>
<td>0.50–1.10</td>
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<td>Atf1</td>
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<td>1.06</td>
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<td>0.63–1.97</td>
<td>R-5'gatcctgatctctgtggagtttg-3'</td>
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</table>

Calculation of relative expression ratios using hypoxanthine guanine phosphoribosyl transferase (Hprt) mRNA as reference gene and statistical randomization test analysis (10,000 iterations) were performed by the relative expression software tool (REST 2009 Version 2.0.13). Note the significant downregulation of Kcn2, Cacna1c, and Crem ICER and the upregulation of Scn8a and Cav 3'-untranslated region (UTR) in the cAMP response element binding protein knockout (CREB KO) mice compared with the control (CTR) mice. F, forward; R, reverse. *P < 0.05 vs. CTR.

RESULTS

APs are prolonged in CREB KO cardiomyocytes. Figure 1A shows superimposed representative AP recordings from CREB KO and CTR cardiomyocytes and displays AP prolongation in CREB KO myocytes. Analysis of AP duration parameters (Fig. 1D) revealed an increased AP duration at 50 and 70% repolarization (APD50 and APD70) in CREB KO while APD90 was not significantly different from control. A significant upregulation of ICER and downregulation of CREM and the upregulation of ICER and the upregulation of ICER was observed in CREB KO cardiomyocytes compared with CTR.

Table 2. Quantification of mRNA levels in the cadiac ventricle of CREB KO and CTR mice

<table>
<thead>
<tr>
<th>Gene</th>
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<td>0.68*</td>
<td>0.49–0.95</td>
<td>0.34–1.13</td>
<td>R-5'gggacccaagacctgtcacttta-3'</td>
</tr>
<tr>
<td>Kcn3 (Kv4.3)</td>
<td>0.82</td>
<td>0.84</td>
<td>0.63–1.14</td>
<td>0.48–1.65</td>
<td>R-5'gcacagctgagtttggagat-3'</td>
</tr>
<tr>
<td>Kcn2p (KChip2)</td>
<td>0.98</td>
<td>0.82</td>
<td>0.59–1.13</td>
<td>0.44–1.41</td>
<td>R-5'gtgacagctgagtttggagat-3'</td>
</tr>
<tr>
<td>Kcn4 (Kv1.4)</td>
<td>0.88</td>
<td>0.82</td>
<td>0.44–1.58</td>
<td>0.22–2.10</td>
<td>R-5'gacagctgagtttggagat-3'</td>
</tr>
<tr>
<td>Kcn5 (Kv1.5)</td>
<td>0.98</td>
<td>0.95</td>
<td>0.59–1.47</td>
<td>0.41–2.32</td>
<td>R-5'gacagctgagtttggagat-3'</td>
</tr>
<tr>
<td>Kcnb1 (Kv2.1)</td>
<td>0.88</td>
<td>0.82</td>
<td>0.59–1.15</td>
<td>0.47–1.83</td>
<td>R-5'gacagctgagtttggagat-3'</td>
</tr>
<tr>
<td>Cacna1c (Cav1.2)</td>
<td>0.96</td>
<td>0.89</td>
<td>0.68–1.29</td>
<td>0.40–1.72</td>
<td>R-5'gacagctgagtttggagat-3'</td>
</tr>
<tr>
<td>Scn5a (Nav1.5)</td>
<td>1.00</td>
<td>1.01</td>
<td>0.73–1.35</td>
<td>0.59–1.81</td>
<td>R-5'gacagctgagtttggagat-3'</td>
</tr>
</tbody>
</table>

Calculation of relative expression ratios using Hprt mRNA as a reference gene and statistical randomization test analysis (10,000 iterations) were performed by the relative expression software tool (REST 2009 Version 2.0.13). Note the significant downregulation of Kcn2 in the CREB KO mice compared with the CTR. *P < 0.05 vs. CTR.
un altered between groups (CREB KO vs. CTR, in ms: APD_{50} 11.4 ± 1.5*, vs. 7.3 ± 0.9, APD_{70} 24.4 ± 3.6* vs. 13.2 ± 1.6, and APD_{90} 107.0 ± 14.1 vs. 79.2 ± 8.5; *P < 0.05 vs. CTR).

At the same time, the AP amplitude (Fig. 1B) was enhanced in CREB KO while resting membrane potential (RMP; in mV: amplitude 127 ± 3* vs. 117 ± 3 and RMP −72 ± 1 vs. −71 ± 1; *P < 0.05 vs. CTR).

I_{Na} peak amplitude is increased in CREB KO cardiomyocytes. Sodium currents (I_{Na}) were recorded by use of a discontinuous single-electrode voltage-clamp amplifier and a low-sodium extracellular solution to exclude voltage clamp errors caused by series resistance. I_{Na} peak amplitudes recorded under these conditions were increased in CREB KO cardiomyocytes as demonstrated by respective I-V curves (Fig. 2A). The current increase was significant for voltages between −50 and −10 mV (two-way ANOVA with repeated-measures, Bonferroni post hoc test) on average 30% in this range and might explain the enhanced AP amplitude observed in CREB KO cardiomyocytes aside from a contribution to AP prolongation. The increased I_{Na} amplitude was associated with an upregulation of sodium channel, voltage-gated, type VIII, α (Scn8a/Nav1.6) mRNA but an unaltered sodium channel, voltage-gated, type V, α (Scn5a/Nav1.5) mRNA level (Table 1) in CREB KO cardiomyocytes.

I_{Ca,L} peak amplitude is decreased in CREB KO cardiomyocytes. Unexpected with regard to the AP prolongation in CREB KO, we found the peak amplitude of I_{Ca,L} reduced in CREB KO cardiomyocytes. The reduction was significant for voltage steps from −10 mV to +40 mV (two-way ANOVA with repeated-measures, Bonferroni post hoc test) and on average 45% (Fig. 2B). The level of calcium channel, voltage-dependent, L-type, α1C subunit (Cacna1c/Cav1.2) mRNA was at the same time reduced in CREB KO cardiomyocytes, whereas mRNA levels of Cacnb1 and Cacnb2 (L-type calcium channel subunit β-1 and -2) were not altered.

I_{K} peak amplitude is attenuated in CREB KO cardiomyocytes. Recording of total outward potassium currents from CREB KO and CTR cardiomyocytes revealed reduced peak amplitude of total outward potassium currents in CREB KO cardiomyocytes (Fig. 2E). Because the total outward K^+ current amplitude consists of up to four different components, I_{K} was separated from overlaying currents using a prepulse protocol (Fig. 2C and D). The separated I_{K} peak amplitude was decreased in CREB KO cardiomyocytes to average by 51% (Fig. 2F), which is in line with the prolonged early phase of the AP in CREB KO cardiomyocytes.

Kcnd2 gene expression and Kv4.2 protein content are downregulated in CREB KO mice. Relative quantitative real-time RT-PCR analysis of mRNA levels from mouse ventricles and cardiomyocytes revealed a significant downregulation of Kcnd2/Kv4.2 gene by 27% in homogenates and 29% in cardiomyocytes of CREB KO relative to CTR (Fig. 3, A and B, and Table 1). Genes of other regulatory subunits of the K_{Ca,L} promoter (Kcnd2/Kv4.2, Kcna4/Kv1.4, Kcne2/Kv1.5 and Kcne1/Kv1.4) were not changed in expression levels either in ventricular homogenates or in cardiomyocytes (Fig. 3, A, B, and Table 1). Moreover, no changes in the expression of Kcnq4/Kv7.1, Kcnq1/Kv7.2, and Kcnh2/Kv4.3, the underlying subunits of I_{Ks,slow} and I_{Ks,slow2}, respectively, were observed (Table 2). Consistent, with the mRNA expression analysis, Western blot analysis of Kv4.2 protein levels were significantly downregulated by 37% in the myocardium of CREB KO vs. CTR mice, whereas Kv4.3 and KChIP2 protein content was not different between the genotypes (Fig. 3, C and D).

Direct regulation of the Kcnd2 promoter by CREB was not detected. Downregulation of Kcnd2 mRNA and protein levels raised the question if CREB directly interacts with the Kcnd2 promoter and regulates its activity. The Kcnd2 promoter exhibits no conserved full or half site CRE elements. ChiP with a CREB antibody revealed no enrichment of DNA Kcnd2-promoter fragments in the mouse ventricle, using primer pairs covering the Kcnd2 promoter (Kcnd2−694 1.2 ± 0.6, Kcnd2−1053 1.4 ± 0.7, and Kcnd2−2131 1.3 ± 0.7; n = 4; Fig. 4A; for details, see MATERIALS AND METHODS), whereas a promoter region carrying a CRE-binding CRE site from the Fos gene as a positive control showed a strong enrichment 183 ± 92 fold.

**AJP-Heart Circ Physiol** • doi:10.1152/ajpheart.00057.2011 • www.ajpheart.org
CREB REGULATES $I_{Na}$ CURRENT

Fig. 2. A: representative traces and current-voltage ($I$-$V$) curves of inward Na$^+$ current ($I_{Na}$). $I_{Na}$ peak amplitude was increased in CREB KO cardiomyocytes by up to 31%. (cells/animals: black squares, CTR, n = 27/8; gray squares, CREB KO, n = 20/6). B: representative traces and I-V curves of Ca$^{2+}$ current ($I_{Ca,L}$). $I_{Ca,L}$ peak amplitude was reduced in CREB KO cardiomyocytes by $\sim$45% (cells/animals: black squares, CTR, n = 12/4; gray squares, CREB KO, n = 17/3). C and D: pulse protocol and separation procedure to separate the transient outward current ($I_{to}$). A classic step protocol ($-40$ to $+60$ mV, $\Delta$10 mV, HP: $-80$ mV) was used to evoke total outward potassium currents and then the same protocol was applied a second time with a preceding prepulse to $-40$ mV for 100 ms (C). Resulting currents of both protocols were subtracted from each other (D). Resulting array represents the separated $I_{to}$. E: $I$-$V$ curves of total peak potassium outward current ($I_{K}$) illustrate a reduction of total peak potassium outward current in CREB KO cardiomyocytes. F: $I$-$V$ curves of the separated $I_{to}$. $I_{to}$ peak amplitude was reduced in CREB KO cardiomyocytes. Average reduction in CREB KO amounted to 51% of CTR cardiomyocytes (cells/animals: black squares, CTR, n = 10/6; gray squares, CREB KO n = 8/3). *P < 0.05, two-way ANOVA with repeated-measures, Bonferroni post hoc test.
CREB REGULATES $I_{\text{Na}}$ CURRENT

Fig. 3. Quantification of mRNA and protein levels of potassium channel subunits Kv4.2 (top), Kv4.3 (middle), and KChIP2 (bottom) in CREB KO and CTR mice. Quantitative real-time RT-PCR analysis of mRNA encoding Kcnd2/Kv4.2, Kcnd3/Kv4.3, and Kcnip2/KChIP2 was performed in the ventricle (A) and in isolated ventricular cardiomyocytes (B) of CREB KO and CTR mice ($n=10$ for each genotype). Data are shown as relative mRNA amount in relation to the housekeeping gene $Hprt$ and normalized to the mean of the CTR animals. A: immunoblotting against Kv4.2, Kv4.3, KChIP2, cardiac calsequestrin (CSQ), and GAPDH from ventricular homogenates of CTR and CREB KO (KO) mice. C: quantification of immunoblotting. Protein content of Kv4.2 and KChIP2 was standardized to CSQ, and protein content of Kv4.3 was standardized to GAPDH and shown as relative protein content normalized to the mean of the CTR animals ($n=12–13$ for Kv4.2; $n=9$ for Kv4.3; and $n=12$ for KChIP2 for each group). Note the significant downregulation of Kv4.2 mRNA and protein content in CREB KO mice compared with CTR. RLU, relative light units. *$P<0.05$ vs. CTR.

The $Kcnd2$ promoter activity by CREB could be possible via nonconserved binding sites, we generated a reporter gene construct containing 2,541 bp of the $Kcnd2$ promoter (5'-UTR) and examined whether overexpression of a dominant negative CREB (dnCREB) isoform has an impact on the promoter activity. The dnCREB contains a Ser-133 to Ala substitution and functions as a potent dominant negative repression of CREB-dependent gene expression both in vitro and in vivo (5, 45). Overexpression of the dominant-negative CREB isoform dnCREB has no impact on the reporter activity ($n=24/4$).

Alternative regulation of the cAMP transcription factor family members. To evaluate a possible compensatory role of cAMP transcription factor family members, namely CREM and ATF1, mRNA levels of Crem splice variants and Atf1 were determined (Table 1). A specific primer pair (Crem ICER) for the CREM-inducible cAMP early repressor splice variants Icer I and Icer II revealed a downregulation of mRNA of 29% in CREB KO cardiomyocytes. The mRNA level was quantified by a primer pair (Crem 3'-UTR) recognizing several other transcript variants of Crem (e.g., Crema) was upregulated twofold, whereas Atf1 transcripts were not altered.

**DISCUSSION**

Our results show that the inactivation of transcription factor CREB leads to prolongation of the early phase of the AP by the regulation of underlying currents. The AP prolongation is mainly caused by a downregulation of the $I_{Na}$ underlying channel subunit Kv4.2 and a subsequent reduction of the transient outward current ($I_{To}$) in adult mouse ventricular cardiomyocytes but also accompanied by an increase of $I_{Na}$ along with an upregulation of $Scn8a/Nav1.6$ and decrease of $I_{Ca,L}$ along with a downregulation of $Scn8a/Nav1.6$. This indicates CREB as an important regulator of AP shape and duration in the mouse heart.

In contrast to the human cardiac ventricular AP, the mouse ventricular AP is rather short lacking a marked plateau phase and is characterized by a rapid repolarization phase mainly determined by the transient outward current $I_{To}$ and a less marked L-type Ca$^{2+}$ current (28). $I_{To}$ activates and inactivates fast, which made this current the most important candidate for explaining the observed AP prolongation in CREB KO cardiomyocytes; other major repolarizing K currents like the delayed rectifiers $I_{K_{\text{slow}1}}$ and $I_{K_{\text{slow}2}}$ rather determine the later repolarization phase of the AP (16). After measuring a peak amplitude reduction of total potassium outward current in CREB KO, we indeed detected a marked reduction of the $I_{To}$ peak amplitude in CREB KO cardiomyocytes along with the observed AP prolongation. The reduced $I_{To}$ together with the detected increase in $I_{Na}$ can also well explain the increased AP amplitude in CREB KO cardiomyocytes since the amplitude of the AP is mainly determined by the influx of Na$^{+}$ ions and the countering $I_{Na}$. Furthermore, the increased $I_{Na}$ may contribute to the AP prolongation itself. Interestingly, we detected an upregulation of $Scn8a$ mRNA encoding the brain type Na$^{+}$ channel subunit Na$_{1.6}$ while the level of $Scn8a$ encoding the predominant cardiac Na$^{+}$ channel subunit Na$_{1.5}$ remained unchanged. Recent studies describe the importance of Na$_{1.6}$ in the mouse heart for AP propagation, excitation-contraction coupling (30) and its upregulation in a rat pressure-overload model (50). Unexpected with regard to the extended AP duration was the observed $I_{Ca,L}$ reduction in CREB KO car-
diomyocytes along with a reduction of the Ca_{1.2} encoding mRNA Cacnalc. One may speculate that the AP prolongation would have been more pronounced with an unaltered I_{Ca,L} amplitude in CREB KO. The electrophysiological data are strongly supported by the data concerning Kcnd2/Kv4.2 mRNA and protein levels. In mice, I_{Ca,L} is composed of Kv4.2 and Kv4.3 α-subunits and the accessory subunit KChIP2, whereas Kv4.2 is the major determinant of regional heterogeneities of I_{Ca,L} expression in the adult mouse ventricle (10). CREB-deficient mice showed a significant decline in cardiac Kcnd2 mRNA and protein content compared with control animals. In line with our findings, a deletion of the Kcnd2 gene in the mouse eliminates the ventricular I_{Ca,L} underlying the critical role of Kv4.2 in the generation of the I_{Ca,L} in rodents (9). Other publications reported that in large mammals like dogs and humans Kv4.3 seems to play the major role for I_{Ca,L} generation (13, 47). On the other side, an absence of KChIP2 can lead to a complete loss of I_{Ca,L} in mouse cardiomyocytes and a marked increase in AP-duration, while RNA and protein levels of Kcnd2/Kv4.2 and Kcnd3/Kv4.3 remain unchanged (14). However, we found no altered mRNA- and protein levels of other I_{Ca,L} subunits namely Kcnd3/Kv4.3 and Kcnip2/KChIP2, suggesting that the downregulation of Kcnd2/Kv4.2 mRNA and protein level was exclusively responsible for the decreased I_{Ca,L} and the enhanced AP duration in CREB KO mice. Previous studies (34, 35) indirectly linked CREB to the regulation of I_{Ca,L} and I_{Ca,L} underlying subunits: a downregulation of the Kcnd2 and a decrease of Kv4.3 and KChIP2 went along with reduced CREB protein levels after ventricular pacing in the canine heart. Moreover, a decreased phosphorylation of the extracellular signal-regulated kinase (ERK) and CREB was associated with a decrease in I_{Ca,L} and a decline of Kv4.2 and KChIP2 protein content (39) in a model of pharmacologically induced nerve sprouting in rats. However, we were not able to detect direct CREB binding to the promoter of Kcnd2/Kv4.2 in the mouse heart with ChIP nor a reduction of the Kcnd2/Kv4.2 promoter activity in HEK cells after overexpression of a dominant negative CREB mutant (dnCREB; Ref. 5), which suppresses CRE-mediated transcriptional activation. This implies an indirect regulation of Kv4.2 and hence I_{Ca,L} by CREB in mice.

It has been shown that activation of the nuclear factor of activated T-cells (NFAT) pathway leads to downregulation of Kv4.2 in the mouse heart (51). In the literature, CREB and NFAT are regarded as opponents, e.g., for cell cycle quiescence vs. hypertrophic growth and cell cycle progression (8, 17). There are several proteins (7, 32, 43, 49) interacting with both CREB and NFAT, e.g., the phosphatase calcineurin (Ppp3ca), which activates NFAT and inactivates CREB by dephosphorylation (8). Therefore, the loss of CREB might influence the balance between CREB and NFAT signaling resulting in preference of the NFAT pathway in our model. Furthermore, the inactivation of CREB might alter the expression of proteins involved in the regulation of calcium signaling, thereby activating the NFAT pathway. According to the literature, a prolongation of the early phase of the AP may also lead to an activation of calcineurin/NFATc3. In detail, cardiomyocytes from mice expressing a truncated Kv4.2 protein showed AP prolongation resulting in an elevated total Ca^{2+} entry and increased Ca^{2+} transient amplitude (41). The hearts of these mice developed cardiac hypertrophy with chamber dilatation by 13 to 15 wk of age (48), which could be prevented by calcineurin inhibition or Ca^{2+} channel blockade. These findings were also supported by a study on neonatal rat cardiomyocytes investigating the role of Kv4.2-based I_{Ca,L} reduction for hypertrophy (11).

The observed I_{Ca,L} reduction in CREB KO may serve as an explanation for two findings 1) the unaltered hemodynamic comparing CREB KO and CTR mice, and 2) the missing of hypertrophy in the heart of CREB KO mice up to an age of 24 wk (22). Bearing these studies in mind, one may speculate that CREB KO mice are protected by the reduction of I_{Ca,L} from detrimental effects mediated by an activation of the calcineurin/NFATc3 pathway due to the observed AP prolongation.

Interpreting the results of our study, one has to keep in mind that the inactivation of CREB might be compensated by its family members ATF1, CREM, and derived splice variants (21). The mRNA encoding the repressory CREM splice variant Icer was downregulated in CREB KO cardiomyocytes, whereas the mRNA level quantified by a primer pair recognizing several other CREM splice variants (e.g., Crema) was elevated in CREB KO. Therefore, it cannot be excluded that the observed alterations in CREB KO cardiomyocytes are influenced or partly compensated by other members of the cAMP-dependent transcription factor family.

In conclusion, our results from mice with cardiomyocyte-specific inactivation of CREB definitively indicate that CREB is a modulator of the AP shape and duration. CREB regulates the I_{Ca,L} amplitude maintaining Kcnd2 gene expression in the adult mouse ventricle, however, likely by an indirect mechanism. Furthermore, CREB inactivation leads to an increase of I_{Na} and decrease of I_{Ca,L}, which might limit myocyte contractility and protect from a hypertrophic response in presence of an AP prolongation.
Further work is needed to elucidate the mechanisms in detail leading to the complex electrophysiological phenotype in CREB KO mice and whether these mechanisms are relevant in the context of heart failure-associated ion channel remodeling.

ACKNOWLEDGMENTS

We thank G. Schütz (DKFZ, Heidelberg, Germany) for kindly providing CrebFlox/CrebFlox mice, and we thank Maria Schulk, Melanie Voß, and Andrea Walter for excellent technical assistance.

GRANTS

This study was funded by the Interdisciplinary Center of Clinical Research, Münster (IZKF, Mu1/014/11 to F. U. Müller and W. Schmitz) and the Deutsche Forschungsgemeinschaft (DFG Mu1376/10-3) and (DFG Mu1376/11-1 to F. U. Müller and W. Schmitz).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


