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

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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 (Ito), 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 Ito peak amplitude as detected in voltage-clamp measurements. We observed a 29% reduction of Kcn2d/Kv4.2 mRNA in CREB KO cardiomyocytes mice while the other Ito-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 Ito-dependent AP prolongation went along with an increase of Ifak and a decrease of ICa,L associated with an upregulation of Scn8a/Nav1.6 and downregulation of Cacna1c/Cav1.2 mRNA in CREB KO cardiomyocytes. Our results from mice with cardiomyocyte-specific inactivation of CREB definitively indicate that CREB critically regulates the AP shape and duration in the mouse ventricle, which might have an impact on ion channel remodeling in situations of altered cAMP-dependant signaling like heart failure.

ion channel remodeling; heart; transcriptional regulation; adenosine 3’,5’-cyclic monophosphate response element binding protein; transient outward current

THE CARDIAC ACTION POTENTIAL (AP) shape and duration are determined by the interplay of inward and outward ion currents. The rapid AP upstroke reflects inward Na+ current (Ito) mainly through the voltage-dependent Na+,L channel (28, 29). Depolarization then again activates Ca2+ current (ICa,L) through the L-type voltage-gated (Ca,L) Ca2+ channel (4). The major determinants of AP repolarization in the myocardi um are voltage-gated K+ channels. They can be divided in two functional groups, the rapidly activating and inactivating transient outward currents (Ito) and the delayed rectifiers (IK); Ref. 36). In ventricular cardiomyocytes, the activation and inactivation characteristics of Ito are particularly important for the modulation of the early phase of AP repolarization and of excitation-contraction coupling (29, 33, 42). Prominent Ito currents are present in ventricular cardiomyocytes of most mammalian species, including mouse, rat, rabbit, canine, and human. Two distinct Ito currents can be distinguished based on their biophysical properties leading to anatomical heterogeneities of the AP waveform. The Ito,fast (Ito,3) recovers rapidly, while Ito,slow (Ito,4) recovers slowly with time constants in the order of seconds (2, 36, 52); in the mouse, Ito,os is only expressed in septal myocytes (52). On the molecular level, Ito,f and Ito,os underlying channels are encoded by the potassium voltage-gated channel subfamily D member 2 (Kcn2d/Kv4.2) and member 3 (Kcn3d/Kv4.3), the Kv channel-interacting protein 2 (Kcnip2/KChIP2), and potassium voltage-gated channel, shaker-related subfamily, member 4 (Kcn4a/Kv1.4) subunits, respectively (for review, see Ref. 5). The knockdown of either Kcnd2 or Kcnd3 reduces Ito by 50% in rat ventricular cardiomyocytes (6) whereas the lack of KChIP2 leads to a complete loss of Ito in cardiomyocytes and a marked increase in AP duration (14). In mouse ventricle, the delayed rectifiers are represented by the potassium voltage-gated channel, shaker-related subfamily, member 4 (Kcn4a/Kv1.4)-based IKs(long) and the potassium voltage gated channel Shab-related subfamily member 1 (Kcnb1/Kv1.2)-based IKs(short) (53).

Ion channel remodeling, in particular a decreased Ito 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, TGACGTCA, 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 Crebl exon 10 resulting in a truncated CREB protein without DNA-binding and dimerization domains. Mice not expressing Cre recombinase (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 amphotericin B (38). Calcium-tolerant, rod-shaped, and clearly striated ventricular myocytes were selected randomly for recording. The blocked membranes were incubated for 2 h in the antibody-enzyme conjugate (1:5,000, ECL Mouse IgG, HRP-Linked) and washed four times with TBS-T. The corresponding secondary anti-rabbit antibody (1:20,000, ECL Mouse IgG, HRP-Linked) was applied for 1 h at room temperature in PBS-0.1% Tween-20. Signals were visualized and quantified 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-

**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 (CSQ), 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), CSQ (1:2,500, PAI-913; Thermo Fisher, Bonn, Germany), and KChIP2 (1:200, KChIP2 H-100, sc-256851; Santa Cruz Biotechnology, Heidelberg, Germany) membranes were treated in TBS containing 5.0% dry fat milk for 2 h and washed four times for 10 min in TBS-0.1% Tween-20 (TBS-T), incubated 2 h at room temperature or overnight at 4°C with the primary antibody in TBS, followed by being washed four times with TBS-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 TBS-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 antigens 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-

both experiments, the following solutions were used (in mM): pipette solution: 5 NaCl, 90 KCl, 35 KOH, 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 NaH2PO4, 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 (ΔΔ 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 (ΔΔ 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.
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: “Mismiring 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 pm). Reactions were incubated at 95°C for 5 min followed by 50 cycles at 95°C for 5 s, 60°C for 15 s, and 72°C for 10 s. Relative levels of particular cDNAs were determined by using the help of LightCycler software (version 3.5) with appropriate calibration curves.

The reaction components were 2 μl undiluted cDNA, 2 μl 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 15 s, and 72°C for 10 s. Relative levels of particular cDNAs were determined by using 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

**Prime and Primer (10 pm). Reactions were incubated at 95°C for 5 min followed by 50 cycles at 95°C for 5 s, 60°C for 15 s, and 72°C for 10 s. Relative levels of particular cDNAs were determined by using 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 ratios using the ΔΔCT method and the relative expression software tool (REST Version 2.013; see refs. 18, 37, 46). Random statistical analysis was performed with 10,000 iterations, and hypoxanthine guanine phosphoribosyl transferase (Hprt) was used as a reference gene.

**Chromatin immunoprecipitation.** The chromatin immunoprecipitation (ChIP) was adapted from published protocols (31, 44). Mouse hearts were frozen in liquid nitrogen, crushed with a pestle, and mechanically homogenized (Polytron PT 3000; Kinematica, Luzern, Switzerland) in ice cold PBS with protease inhibitors (Complete Mini-protease inhibitor cocktail tablets; Roche). After a 5-min centrifugation at 5,000 × g, the supernatant was precleared with the addition of 80 μg of affinity-purified rabbit IgG antibody (anti-CREB 06–863; Millipore) or a rabbit IgG antibody (normal rabbit IgG 12–370; Millipore), and rotated at 4°C overnight. For immunoprecipitation, the probes were incubated with 80 μl blocked agarose beads for 2 h at room temperature and centrifuged at 150 × g, and the supernatant was discarded. Then, the beads were washed with 500 μl low-salt buffer (0.1% SDS, 1% Triton-X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, and 150 mM NaCl); high-salt buffer (0.1% SDS, 1% Triton-X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, and 500 mM NaCl); LiCl buffer [250 mM LiCl, 1% Nonident 40, 1% deoxycholic acid (sodium salt), 1 mM EDTA pH 8.0, and 10 mM Tris-HCl pH 8.0]; and two times with TE buffer (1 mM EDTA and 20 mM Tris-HCl pH 8.0). After each washing step, the slurry was centrifuged at 150 × g for 1 min and the supernatant was discarded. After being washed, the immune complexes were eluted by a 15-min incubation of the agarose beads with 150 μl fresh elution buffer (1% SDS and 0.1 M NaHCO3), centrifugation at 150 × g, and collection of the supernatant followed by a second incubation with 100 μl elution buffer and centrifugation. The collected supernatants were combined and crosslinks were reversed by the addition of 20 μl 5 M NaCl per sample and a subsequent incubation at 65°C for 6 h. Then, the samples were incubated with 2 μl proteinase K (10 mg/ml) and 2.5 μl RNase (10 mg/ml) for 1.5 h at 55°C. The DNA was extracted by a PCR-purification kit (Qiagen) and amplified by a whole genome amplification Kit [GenomePlex Complete Whole Genome Amplification (WGA2) Kit, Sigma-Aldrich, Munich, Germany] according to the manufacturer’s instructions. For real-time PCR analysis, the amplified probes were diluted 1:10 and 1 μl was used for analysis. Primers were for the glyceraldehyde-3-phosphate dehydrogenase (Gapdh) promoter F-5′-caaccctccattttcctccccgcttt-3′; and for the FBJ osteosarcoma oncogene (Fox) promoter F-5′-tgccacagcagttgaag-3′, R-5′-ccattcacegccagctcg-3′. To detect a possible enrichment of the precipitated DNA fragments from the potassium voltage-gated channel, Shal-related family, member 2 (Kcn2) gene promoter, two primer pairs were used: -2,131 to -1,940 bp (Kcn2-2,131); F-5′-ttagatatgggcagagatcgtc-3′, R-5′-tattaccgcctacactgctg-3′; and -1,052 to -874 bp (Kcn2-1,052); F-5′-tttgagaggtgcaacaggag-3′, R-5′-gggctaggctctacagaaga-3′; and -694 to -495 bp (Kcn2-694); F-5′-agagggctgctgctg-3′, R-5′-ccagggtgcttacaaaaga-3′. Numbers indicate the distance to the ATG start codon. Since precipitated DNA fragments have an approximate size of 500-700 bp by 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.

**Plasmid construction.** For reporter plasmid construction: a genomic fragment of the Kcn2 gene promoter was amplified by PCR using the following primers: (Kv4-2-Xho_For) F-5′-ggagagtggatttgaggaaggagagcagaggag-3′ and (Kv4-2-Hind_Rev) R-5′-atgaatcctggaggtgaagctcagagagagt-3′. After XhoI/HindIII digestion, this fragment was cloned into the luciferase gene containing vector pGL4.16 (Promega, Madison, WI), which was also cut by Xhol and HindIII (resulting plasmid: pKv4.2minLuc). A second fragment lying upstream of the first fragment was synthesized (Mr. Gene, Regensburg, Germany) and digested by KpnI and Xhol. This fragment was inserted into pKv4.2minLuc digested by KpnI and Xhol (resulting plasmid: pKv4.2Luc). Finally, the reporter gene construct contains a genomic region starting 2,541 bp upstream of the Kcn2 start codon.

**Reporter gene assay.** Human embryonic kidney 293 (HEK293) cells were maintained in DMEM supplemented with 10% (vol/vol) FCS, 100 U penicillin, and 1 mg streptomycin per ml, and 7 mM glutamine (all PAA, Pasching, Austria) at 37°C in an atmosphere of 5% CO2. HEK 293T cells were transfected using TurboFect in vitro transfection reagent (Fermentas, St. Leon-Roth, Germany) according to manufacturer’s protocol. For transfection, cells were maintained in 24-well plates (Corning, Hagen, Germany) and each well was cotransfected with 200 ng of the expression plasmid pCDNA3 (Life Technology) containing either the mCherry open reading frame (control) or the dominant negative CREB encoding ORF (45). Twenty-four hours posttransfection cells were lysed and assayed using the Dual-Luciferase Reporter Assay System (Promega) as described in the manufacturer’s protocol. Luciferase activities were determined by a Mithras LB 990 Microplate Analyser (Berthold
two groups, a Mann-Whitney-rank sum test was conducted. Statistical
revealed significant differences between groups. For comparison of
hoc testing was performed only if the preceding pro-omnibus test
two-way ANOVA with post hoc testing according to Bonferroni. Post
independent experiments. Statistical evaluation was performed using
experiments are reported as

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<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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Calculation of relative expression ratios using hypoxanthine guanine phosphoribosyl transferase (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, Cacna1c, and Crem ICER and the upregulation of Scn8a and Scn 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

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<td>Scn5a (Nav1.5)</td>
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<td>1.01</td>
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<td>0.59–1.81</td>
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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.
unaltered 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 resting membrane potential (RMP) was unaltered between groups (C), while AP amplitude was enhanced in CREB KO cardiomyocytes (B). Genes of other regulatory subunits of the I$_{K,slow}$ 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, A and B, and Table 1). Moreover, no changes in the expression of Kcnad4/Kv1.4, Kcnad5/Kv1.5 and Kcnb1/Kv2.1, the underlying subunits of I$_{\text{to,s}}$, I$_{\text{K,slow1}}$ and I$_{\text{K,slow2}}$, 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 Kcnad2 promoter by CREB was not detected. Downregulation of Kcnad2 mRNA and protein levels raised the question if CREB directly interacts with the Kcnad2 promoter and regulates its activity. The Kcnad2 promoter exhibits no conserved full or half site CRE elements. ChIP with a CREB antibody revealed no enrichment of DNA Kcnad2-promoter fragments in the mouse ventricle, using primer pairs covering the Kcnad2 promoter (Kcnad2–694 1.2 ± 0.6, Kcnad2–1053 1.4 ± 0.7, and Kcnad2–2131 1.3 ± 0.7; n = 4; Fig. 4A; for details, see MATERIALS AND METHODS), whereas a promoter region carrying a CREB binding CRE site from the Fos gene as a positive control showed a strong enrichment 183 ± 92 fold.
Fig. 2. A: representative traces and current-voltage (I-V) curves of inward Na\(^+\) current (\(I_{\text{Na}}\)). \(I_{\text{Na}}\) 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 (\(I_{\text{Ca,L}}\)). \(I_{\text{Ca,L}}\) peak amplitude was reduced in CREB KO cardiomyocytes by \(-45\%\) (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 (\(I_{\text{to}}\)). A classic step protocol (-40 to +60 mV, \(\Delta I = 10\) 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 \(I_{\text{to}}\). E: I-V curves of total peak potassium outward current (\(I_{\text{K}}\)) illustrate a reduction of total peak potassium outward current in CREB KO cardiomyocytes. F: I-V curves of the separated \(I_{\text{to}}\) peak amplitude. \(I_{\text{to}}\) 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.
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 Kcn2d/Kv4.2, Kcn3d/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. C: immunoblotting against Kv4.2, Kv4.3, KChIP2, cardiac calsequestrin (CSQ), and GAPDH from ventricular homogenates of CTR and CREB KO (KO) mice. D: 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.

(n = 4; P < 0.05 vs. CTR), proving the functionality of the ChIP with the CREB antibody.

Since regulation of the Kcn2d promoter activity by CREB could be possible via nonconserved binding sites, we generated a reporter gene construct containing 2,541 bp of the Kcn2d 3′-UTR and tested 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 repressor 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).

Altered 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., Cremα) 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 carried 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 Caenal1/Cav1.2. 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,slow1} and I_{K,slow2} 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 counteracting I_{to}. 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 Scn3a 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\textsubscript{1.2} encoding mRNA \textit{Cacna1c}. One may speculate that the AP prolongation would have been more pronounced with an unaltered \( I_{\text{Ca,L}} \) amplitude in CREB KO. The electrophysiological data are strongly supported by the data concerning \textit{Kcnd2}/\textit{Kv4.2} mRNA and protein levels. In mice, \( I_{\text{o,L}} \) is composed of \( \text{Kv4.2} \) and \( \text{Kv4.3} \) \( \alpha \)-subunits and the accessory subunit \textit{KChIP2}, whereas \( \text{Kv4.2} \) is the major determinant of regional heterogeneities of \( I_{\text{o,L}} \) expression in the adult mouse ventricle (10). CREB-deficient mice showed a significant decline in cardiac \textit{Kcnd2} mRNA and protein content compared with control animals. In line with our findings, a deletion of the \textit{Kcnd2} gene in the mouse eliminates the ventricular \( I_{\text{o,L}} \) underlining the critical role of \( \text{Kv4.2} \) in the generation of the \( I_{\text{o,L}} \) in rodents (9). Other publications reported that in large mammals like dogs and humans \( \text{Kv4.3} \) seems to play the major role for \( I_{\text{o,L}} \) generation (13, 47). On the other side, an absence of \textit{KChIP2} can lead to a complete loss of \( I_{\text{o,L}} \) in mouse cardiomyocytes and a marked increase in AP-duration, while RNA and protein levels of \textit{Kcnd2}/\textit{Kv4.2} and \textit{Kcnd3}/\textit{Kv4.3} remain unchanged (14). However, we found no altered mRNA- and protein levels of other \( I_{\text{o,L}} \) subunits namely \textit{Kcnd3}/\textit{Kv4.3} and \textit{Kcnip2}/\textit{KChIP2}, suggesting that the downregulation of \textit{Kcnd2}/\textit{Kv4.2} mRNA and protein level was exclusively responsible for the decreased \( I_{\text{o,L}} \) and the enhanced AP duration in CREB KO mice. Previous studies (34, 35) indirectly linked CREB to the regulation of \( I_{\text{o,L}} \) and \( I_{\text{o}} \) underlying subunits: a downregulation of the \( I_{\text{o,L}} \) and a decrease of \textit{Kv4.3} and \textit{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 \( I_{\text{o}} \) and a decline of \textit{Kv4.2} and \textit{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 \textit{Kcnd2}/\textit{Kv4.2} in the mouse heart with ChIP nor a reduction of the \textit{Kcnd2}/\textit{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 \textit{Kv4.2} and hence \( I_{\text{o,L}} \) by CREB in mice.

It has been shown that activation of the nuclear factor of activated T-cells (NFAT) pathway leads to downregulation of \textit{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 \textit{Kv4.2} protein showed AP prolongation resulting in an elevated total \( \text{Ca}^{2+} \) entry and increased \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) channel blockade. These findings were also supported by a study on neonatal rat cardiomyocytes investigating the role of \textit{Kv4.2}-based \( I_{\text{o}} \) reduction for hypertrophy (11).

The observed \( I_{\text{Ca,L}} \) 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 \( I_{\text{Ca,L}} \) 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 \textit{Icer} was downregulated in CREB KO cardiomyocytes, whereas the mRNA level quantified by a primer pair recognizing several other CREM splice variants (e.g., \textit{Cremt}) 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 \( I_{\text{o,L}} \) amplitude maintaining \textit{Kcnd2} gene expression in the adult mouse ventricle, however, likely by an indirect mechanism. Furthermore, CREB inactivation leads to an increase of \( I_{\text{Na}} \) and decrease of \( I_{\text{Ca,L}} \), 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 leading to the complex electrophysiological phenotype in H2006 CREB REGULATES

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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