The Smooth Muscle Cell-restricted KCNMB1 Ion Channel Subunit Is a Direct Transcriptional Target of Serum Response Factor and Myocardin*§

Received for publication, July 29, 2009, and in revised form, September 30, 2009. Published, JBC Papers in Press, October 1, 2009, DOI 10.1074/jbc.M109.050419

Xiaochun Long†, Darla L. Tharp†, Mary A. George†, Orazio J. Silvano†, Monica Y. Lee§, Brian R. Wamhoff§, Douglas K. Bowles§, and Joseph M. Miano†

From the †Aab Cardiovascular Research Institute, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, the ‡Department of Biomedical Sciences, University of Missouri, Columbia, Missouri 65211, and the §Department of Medicine, Cardiovascular Division, Biomedical Engineering, Robert M. Berne Cardiovascular Research Center, University of Virginia, Charlottesville, Virginia 22908

Large conductance calcium-activated potassium (MaxiK) channels play a pivotal role in maintaining normal arterial tone by regulating the excitation-contraction coupling process. MaxiK channels comprise α and β subunits encoded by Kcnma and the cell-restricted Kcmb genes, respectively. Although the functionality of MaxiK channel subunits has been well studied, the molecular regulation of their transcription and modulation in smooth muscle cells (SMCs) is incomplete. Using several model systems, we demonstrate down-regulation of Kcmb1 mRNA upon SMC phenotypic modulation in vitro and in vivo. As part of a broad effort to define all functional CArG elements in the genome (i.e. the CArGome), we discovered two conserved CArG boxes located in the proximal promoter and first intron of the human KCNMB1 gene. Gel shift and chromatin immunoprecipitation assays confirmed serum response factor (SRF) binding to both CArG elements. A luciferase assay showed myocardin (MYOCD)-mediated transactivation of the KCNMB1 promoter in a CArG element-dependent manner. In vivo analysis of the human KCNMB1 promoter disclosed activity in embryonic heart and aortic SMCs; mutation of both conserved CArG elements completely abolished in vivo promoter activity. Forced expression of MYOCD increased Kcmb1 expression in a variety of rodent and human non-SMC lines with no effect on expression of the Kcnma1 subunit. Conversely, knockdown of Srf resulted in decreases of endogenous Kcmb1. Functional studies demonstrated MYOCD-induced, iberiotoxin-sensitive potassium currents in porcine coronary SMCs. These results reveal the first ion channel subunit as a direct target of SRF-MYOCD transactivation, providing further insight into the role of MYOCD as a master regulator of the SMC contractile phenotype.

* This work was supported, in whole or in part, by National Institutes of Health Grants HL02572 and HL091168 (to J. M. M.), HL52490 and HL079934 (to D. K. B.), and HL001682 (to B. R. W.). This work was also supported by American Heart Association Scientist Development Grant 0535002N (to B. R. W.).

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–5.

‡ To whom correspondence should be addressed: Aab Cardiovascular Research Institute, Dept. of Medicine, Box CVRI, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave., Rochester, NY 14642. Tel.: 585-276-9789; Fax: 585-276-9830; E-mail: j.m.miano@rochester.edu.

§ The abbreviations used are: SMC, smooth muscle cell; ChIP, chromatin immunoprecipitation; Ad, adenosine; shEGFP, short hairpin-enhanced green fluorescent protein; shSRF, short hairpin RNA to SRF; HCA MSC, human coronary artery smooth muscle cell; Ibtx, iberiotoxin; MABT, maleic acid buffer; MaxiK, large conductance calcium-activated potassium; PCASMC, porcine coronary artery smooth muscle cell; PDGF, platelet-derived growth factor; RASMC, rat aortic smooth muscle cell; RT, reverse transcription; VISTA, visualization tools for alignments; I\textsubscript{p}, potassium current; MYOCD, myocardin.

2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–5.
for a synthetic SMC phenotype in blood vessels (12). A complementary ultrastructure study showed that MYOCD gain of function directs de novo SMC myofilament formation in cultured cells with no evidence of cardiac sarcomerogenesis (13).

In addition to SMC contractile filament genes, MYOCD and SRF orchestrate expression of regulatory genes involved with SMC contraction. The telokin promotor, which drives expression of a gene encoding the carboxyl-terminal end of SM myosin light chain kinase, contains a single CArG element that is responsive to both SRF and MYOCD (14, 15). The SM myosin light chain kinase gene itself was shown to harbor conserved CArG elements also reactive to SRF-MYOCD (16, 17). One of myosin light chain kinase's substrates, the 20-kDa myosin light chain, exhibits increased phosphorylation with MYOCD overexpression coincident with SMC-like contractile activity (13). Further, the calcium transport pump, sarcoplasmic reticulum calcium-ATPase, contains a conserved CArg box (18) and is induced upon forced expression of MYOCD (19). Thus, MYOCD triggers expression of both structural and regulatory genes to enable SMC contractile force generation.

A critical prerequisite for muscle contraction is the proper expression and activity of ion channels, none of which have yet to be defined as direct targets of SRF-MYOCD. SMCs are known to express an array of ion channels, including the voltage-dependent L-type calcium channels and the MaxiK channels (20). MaxiK channels are composed of an α gene (Kcnmai) encoding a pore-forming subunit and one of four β genes (e.g. Kcnmb1) encoding cell-restricted modulatory subunits (21–23). KCNMB1 confers heightened channel sensitivity to calcium and voltage in vascular SMCs, which provides for efficient fine-tuning of vascular tone (24, 25). Although the functionality of KCNMB1 has been studied intensively, much less is known regarding its transcriptional regulation. Here, we show that transcription of the KCNMB1 gene is a function of SRF-MYOCD acting over two conserved CArG elements.

**EXPERIMENTAL PROCEDURES**

**Animals**—Tissues were rapidly harvested from 2-month-old C57BL/6 male mice or castrated male swine and rinsed in PBS prior to processing for RNA extraction and quantitative analysis of Kcnmb1 expression. All animal protocols were approved by local institutional animal care and use committees.

**In Situ Hybridization**—Mouse organs were fixed in 10% neutral buffered formalin, paraffin-embedded, and sectioned at 6-μm thickness under RNase-free conditions. A mouse Kcnmb1 riboprobe was PCR-amplified (see supplemental Table 1 for primers) and cloned into pBluescript. As a control, we also included a riboprobe to Sm22α (Tagln). Linearized antisense and sense ribopropes were purified and labeled with digoxigenin utilizing the MEGAscript Kit (Ambion Inc.). All slides were deparaffinized and rehydrated in RNase-free water. Slides were pretreated for 15 min at room temperature in 10 μg/ml proteinase K, washed in 0.2% glycine, postfixed for 10 min in 4% paraformaldehyde, and acetylated for 30 min in 0.1 M triethanolamine, 0.25% acetic anhydride. Slides were then incubated at 65 °C for 1.5 h in prehybe solution (diethylpyrocarbonate-treated water containing 50% formamide, 5× saline sodium citrate, 1% SDS, 50 μg/ml yeast tRNA, and 50 μg/ml heparin) and overnight in the same solution containing 10% dextran sulfate and 100 ng/μl digoxigenin-labeled probe. Washes were conducted at 65 °C with varying concentrations of saline sodium citrate, followed by a final wash in maleic acid buffer (MBAT). Slides were then blocked for 30 min in 1% milk in MBAT and incubated for 2 h in 1:1000 sheep anti-digoxigenin antibody/MBAT (Roche Applied Science), sequentially washed in MBAT and chromogenic buffer for 10 min each, and then stained from 1 to 5 days with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (Roche Applied Science) in alkaline phosphate buffer. Sections were counterstained with nuclear fast red and permanently covered for photomicroscopy.

**Vascular Injury Models**—10–12-week-old male C57BL/6 mice underwent left carotid artery ligation as described (26). Four days after ligation, animals were perfused with 3.0 ml of 0.9% NaCl to clear blood from major arteries. Injured arteries and control uninjured arteries were rapidly dissected for total RNA extraction. Balloon angioplasty (1.4× overinflation) was performed on the left circumflex and left anterior descending coronary arteries of castrated male swine as described (27). Coronary arteries were harvested at 2 h and 2 days following balloon catheter injury and quickly frozen in liquid nitrogen. Medial cells were isolated by laser capture microdissection (Arcturus, Pixcell Ile), and total RNA was isolated using a Pico-pure RNA isolation kit (Arcturus) for quantitative RT-PCR.

**Cell Culture**—10T1/2, COS7, HeLa, HEK293, rat aortic SMCs (RASMCs), and PAC1 SMCs were maintained in 10-cm plates containing Dulbecco’s modified Eagle’s medium (high glucose) and 10% fetal bovine serum without antibiotics or antimiycotics. Growing and differentiated BC3H1 cells were studied as described previously (28). Human coronary artery smooth muscle cells (HCASMCs) were maintained in growth medium 231 and supplements as provided by the manufacturer (Invitrogen). Porcine coronary artery smooth muscle cells (PCASMCs) were isolated and grown to postconfluence, serum-starved for 6 days to up-regulate expression of SMC-specific marker genes, and then treated with or without 10 ng/ml platelet-derived growth factor BB (+BB) for 24 h as described (29). RASMCs were growth-arrested at 40% confluence for 48 h before treatment with either vehicle or PDGF-BB (30 ng/ml; Upstate). For adenovirus studies, cells were seeded and allowed to adhere overnight and transduced the following day with either cytomegalovirus promoter-driven myocardin (Ad-Mycod; cardiac long form), cytomegalovirus promoter-driven myocardin-related transcription factor A (Ad-MRTF-A; a kind gift from Dr. Paul Herring), or U6 promoter-driven short hairpin RNA to SRF (Ad-shSRF). Equal amounts of controls were adenovirus carrying cytomegalovirus-driven lacZ (for Ad-Mycod or Ad-MRTF-A) or U6-driven short hairpin-enhanced green fluorescent protein (shEGFP; for Ad-shSRF). Cells were washed and refed new medium the next day, and protein or RNA was harvested 48–72 h later.

**RT-PCR**—Total RNA was extracted from mouse tissues or different cells by TRIzol or Pico-pure RNA (for Fig, 2D) isolation kit and reverse transcribed to cDNA with a first strand cDNA synthesis kit (GE Healthcare) or the iScript cDNA synthesis kit (Bio-Rad; for Fig. 2C). Semiquantitative PCR or SYBR Green-based real-time PCR (MyIQ; Bio-Rad) was conducted to
measure mRNA expression levels across a variety of experimental conditions as indicated under “Results.” The primers used to amplify the various target genes are listed in supplemental Table 1.

**Bioinformatics**—Orthologous KCNMB1 genomic sequences from several vertebrate species were downloaded from the University of California Santa Cruz genome browser (30) and analyzed either with the FASTA algorithm for sequence homologies or the FINDPATTERNS algorithm that rapidly searches large genomic sequences for all 1,216 permutations of the CArG box using the Genetics Computer Group software package (Accelrys, San Diego CA). Comparative genomic analysis was done with the visualization tools for alignments (VISTA) algorithm (available from the VISTA web site) at a threshold of 75% homology over at least 100 base pairs of sequence. Sequence logos were generated with an on-line tool (available from the WebLogo web site). Detailed protocols for facile use of these bioinformatic tools are available upon request.

**Plasmid Construction and Mutagenesis**—Human short and long KCNMB1 promoters (Fig. 4A) encompassing CArG elements were PCR-amplified from genomic DNA derived from HeLa cells using high fidelity polymerase (Roche Applied Science) with restriction enzyme-clamped primers (supplemental Table 1) and cloned into XhoI-HindIII sites of the pGL3-Basic luciferase reporter plasmid (Promega). All CArG mutants were constructed through PCR-based mutagenesis as per the manufacturer’s directions (QuickChange; Stratagene). Big-dye sequencing was done by the Cornell University Life Sciences Core Laboratories Center to confirm nucleotide sequence fidelity.

**Western Blot**—Indicated cells were washed with phosphate-buffered saline, and protein was extracted in cold lysis buffer containing 1% protease inhibitor mixture (Sigma) as described (13). Protein concentration was determined by a detergent-compatible protein assay (Bio-Rad). Equal amounts of protein (50 μg) were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, blocked with 5% nonfat milk for 1 h, and then incubated with antibodies to KCNMB1 (sc33608, Santa Cruz Biotechnology, Inc.) overnight at 4 °C, followed by incubation with secondary antibody for 1 h. Sequence logos were generated with an on-line tool (available from the WebLogo web site). Detailed protocols for facile use of these bioinformatic tools are available upon request.

**Upstream Promoter Region**—We cloned a short and long form of human KCNMB1 upstream of a nuclear targeted lacZ reporter gene and tested activity in cultured PAC1 SMCs by staining for β-galactosidase. This analysis revealed higher activity from the long form of human KCNMB1 comprising the 5’ promoter and a fragment of the first intron (data not shown). The latter linearized plasmid was microinjected into fertilized oocytes (SIL/C57BL/6 hybrid) by the University of Rochester Transgenic Core Facility to generate embryonic day 12.5 founder mice. Dissected embryos were processed for lacZ staining and genotyped (see supplemental Table 1 for primers) as described (33). Embryos were either sectioned for microscopy or “cleared” through a graded series of methanol washes (25–100%) followed by overnight washing in 100% methanol and the addition of a 2:1 (v/v) solution of benzyl benzate and benzyl alcohol.

**Smooth Muscle Whole-cell Voltage Clamp**—Whole-cell potassium current (I_K) was measured as previously described (29). Cultured PCASMCs, transduced with either Ad-Myocd or a control empty virus, were trypsinized, suspended in serum-free medium, and stored at 4 °C until use (0–4 h). Cells were superfused at room temperature (22–25 °C) under gravity flow with low Ca^2+^ physiologic saline solution containing 0.1 mM CaCl_2_, 10 mM glucose, 10 mM HEPES, 5 mM KCl, 1 mM MgCl_2_, 0.1 mM CdCl_2_, and 140 mM NaCl, pH 7.4. Pipettes (2–6 megaohms) were filled with solution containing 100 mM KCl, 10 mM NaCl, 1 mM MgCl_2_, 10 mM HEPES, 10 mM EGTA, and 5.77 mM CaCl_2_ (500 nm free Ca^2+_), pH 7.1. Ionic currents were amplified with an Axopatch 200B patch clamp amplifier (Axon Instruments). Currents were elicited by 500-ms step depolarizations to potentials ranging from −60 to +120 mV (in 20-mV increments) from a holding potential of −80 mV.
Expression of Kcnmb1 is modulated in a manner similar to SMC cytocontractile genes during phenotypic modulation, we examined its expression in four distinct model systems. We first used the BC\textsubscript{1}H1 cell line, which was previously shown to exhibit a reversible SMC-like phenotype (28). When BC\textsubscript{1}H1 actively grow, Kcnmb1 and other SMC markers (e.g. Myocd) are expressed (Fig. 2A) (35). Upon serum withdrawal, BC\textsubscript{1}H1 display lower expression of Kcnmb1 and other SMC markers as levels of the skeletal myogenic regulatory factor Myog (myogenin) increase (Fig. 2A). In the second model, we tested the effect of PDGF-BB on Kcnmb1 expression in cultured RASMCs and PCASMCs because this growth factor is known to promote a less contractile SMC phenotype while increasing proliferation and migration (36). Kcnmb1 mRNA decreased in a time-dependent manner along with Myocd and Myh11, whereas Map2k3 showed increases in expression (Fig. 2B). A similar decline in Kcnmb1 mRNA (but not Kcnma1 mRNA) was evident in cultured PCASMCs treated with PDGF-BB (supplemental Fig. 2). In the third system, we used the mouse carotid artery ligation model to examine whether expression of Kcnmb1 mRNA was sensitive to an in vivo condition of SMC phenotypic modulation. Consistent with PDGF stimulation, which is known to occur in the vessel wall following arterial insult (37), Kcnmb1 transcript levels were down-regulated 4 days after ligation injury. In contrast, Kcnma1 mRNA showed no decrease in expression after ligation injury (Fig. 2C). To extend these in vivo findings to a larger mammalian model of vascular injury, we performed balloon angioplasty of the left anterior descending and left circumflex coronary arteries of male swine. Significant decreases in Kcnmb1 mRNA were observed as early as 2 h postangioplasty and persisted for an additional 2 days (Fig. 2D). Taken together, these results demonstrate that Kcnmb1 is part of the SMC genetic program subject to down-regulation following growth factor stimulation or vascular injury.

**Comparative Genomic Analysis Reveals Multiple CArG Elements in the Human KCNMB1 Gene**—The restricted expression of Kcnmb1 mRNA and its down-regulation in models of SMC phenotypic modulation, suggested that Kcnmb1 transcription may be under the control of the SRF-MYOCD transcriptional switch. To begin exploring this possibility, we carefully examined orthologous species of Kcnmb1 through a bioinformatics approach used previously to further define the mammalian CArGome (38). Interestingly, the ~11.5-kb human KCNMB1 gene is found within the 370-kb intron 1 of KCNIP1, which encodes for a neuronal calcium-binding protein that alters type A potassium channels (39) (Fig. 3A). VISTA analysis reveals high homology across the four exons of Kcnmb1.
**SRF-Myocardin Regulation of KCNMB1**

**KCNMB1** between human, monkey, dog, mouse, and rat (Fig. 3B). We found a total of five CArG boxes within 2 kb of the annotated transcription start site of human **KCNMB1** (supplemental Fig. 3). Three of the CArG elements (C1, C2, and C4) are not conserved in the orthologue mouse gene. Within the first untranslated exon is a non-consensus CArG element that is 100% conserved across human, monkey, horse, dog, rat, mouse, and armadillo orthologs of **KCNMB1**; we shall refer to this CArG box as C3 (Fig. 3C and supplemental Fig. 3). In addition, we discovered another non-consensus CArG element within the first intron of **KCNMB1** that is conserved in mouse but shows some sequence divergence across species; we shall hereafter refer to this CArG box as C5, (Fig. 3C and supplemental Fig. 3). The single nucleotide polymorphism tracker at the UCSC Genome Browser (available at the UCSC web site) revealed no annotated single nucleotide polymorphisms within 50 bp of either C3 or C5 of the human **KCNMB1** gene. This bioinformatic analysis provided good evidence for a CArG-dependent ion channel subunit gene.

**KCNMB1 Contains Functional CArG Elements Responsive to SRF and MYOC—in**—Having identified five CArG elements in close proximity to the regulatory region of **KCNMB1**, we tested whether **KCNMB1** promoter activity was elevated in cultured SMCs and responsive to SRF/MYOC. To this end, we PCR-cloned into a luciferase reporter the human **KCNMB1** promoter and 5'-untranslated exon encompassing the first four CArG elements (designated short) or the same promoter region plus a portion of the first intron containing C5 (designated long; Fig. 4A). The short **KCNMB1** reporter showed ~4-fold increase in basal luciferase activity in two distinct SMC lines (PAC1 and A7r5), whereas little to no activity was found in several non-SMC lines (Fig. 4B). Both long and short versions of the **KCNMB1** promoter were activated by SRF-VP16 and MYOC, providing evidence for functional CArG elements around the **KCNMB1** locus (Fig. 4, C–E).

**FIGURE 2. Phenotypic modulation effects on Kcnmb1 mRNA expression.** A, RT-PCR analysis of SMC markers and the skeletal muscle myogenic factor, Myog, in growth versus differentiated BC1;H1 cells. Data are representative of four independent experiments. B, quantitative RT-PCR of RASMCs treated with PDGF-BB (30 ng/ml) over a 24-h duration. Note the concurrent decrease in y axis is plotted on a logarithmic scale. C, 10–12-week-old male C57BL/6 mice 4 days following left carotid artery ligation (paired t test, p = 0.030). D, quantitative RT-PCR of RASMCs treated with PDGF-BB (30 ng/ml) or balloon catheter injury (BCI) of porcine coronary arteries. *, p < 0.05 versus respective control.
SRF-Myocardin Regulation of KCNMB1

FIGURE 3. Comparative genomics of the human KCNMB1 gene. A, schematic of human KCNMB1 (denoted as small—10-kb horizontal bar at the far right) within the −370-kb first intron (broken with diagonal lines) of the 8 exon (black vertical lines) human KCNIP1 locus. The bent arrows denote start sites of transcription of the two divergently transcribed genes. B, four-way VISTA nucleotide sequence homology plot of the four-exon human (Hsa) KCNMB1 gene amplified from the schematic in A. The x axis represents the genomic distance (kb) relative to the annotated transcription start site of KCNMB1 (bent arrow at the top). The y axis denotes the percentage homology of exons (light blue peaks, untranslated; darker blue peaks, protein coding) and non-protein coding sequences (pink peaks with at least 75% homology, middle line of each VISTA plot) over 100 bp in intergenic and intronic regions between human KCNMB1 and the orthologous genes from rhesus monkey (Mmul), dog (Cfa), mouse (Mmu), and rat (Rno). C, sequence logos and location within locus (denoted by arrows) of the two conserved CArG elements in human, monkey, dog, mouse, and rat KCNMB1 genes. The logos represent the frequency of each nucleotide across the five species’ CArG elements as described (69). C3, CArG element number 3 located at +251 (from annotated transcription start site of human KCNMB1; see supplemental Fig. 3) within the 5 untranslated region. C5, CArG element number 5 located at +1042 within the first intron.

in a CArG-dependent manner (Fig. 7). Taken together, these results validate the in vitro importance of two CArG boxes (C3 and C5) in KCNMB1 that bind SRF and respond to MYOCD transactivation.

CArG Element-dependent KCNMB1 Promoter Activity in Vivo—To find out whether the human KCNMB1 promoter confers transcriptional activity in a living animal, we generated transgenic mouse founder embryos (embryonic day 12.5) carrying the long form of the KCNMB1 promoter with wild type or mutant C3 and C5 sequences linked to a lacZ reporter gene. In five independent wild type founders, we observed lacZ staining throughout the myocardium (Fig. 8, A and D, and supplemental Fig. 4), consistent with our in situ hybridization data (supplemental Fig. 1) and a previous report documenting expression of the endogenous Kcnmb1 transcript in the developing mouse heart (40). We further observed in three independent founders, KCNMB1 promoter activity in the dorsal aorta (Fig. 8, A and E) (data not shown), where endogenous Kcnmb1 mRNA levels could also be seen (supplemental Fig. 1). Interestingly, KCNMB1 promoter activity was also seen in the developing neural tube, but no detectable staining was ever seen in the developing gut region (Fig. 8, A and C). In contrast to lacZ staining with the wild type KCNMB1 promoter, mutating both conserved CArG elements (C3 and C5) resulted in no detectable lacZ staining in any of 22 independent transgenic founder embryos (Fig. 8B) (data not shown). These findings complement our in vitro studies by validating the necessity of C3 and C5 in directing restricted KCNMB1 promoter activity in embryonic mice.

Endogenous KCNMB1 Expression Control through SRF-MYOCD—Results thus far establish a critical role for two SRF-binding CArG elements in directing the activity of the KCNMB1 promoter in vitro and in vivo as well as MYOCD-dependent transactivation of the same promoter in vitro. To ascertain whether SRF-MYOCD is important in driving endogenous expression of the KCNMB1 gene and protein, we performed in vitro gain and loss of function studies. We first transduced Ad-Mycod in different cell lines and examined Kcnmb1 mRNA expression by semiquantitative RT-PCR. MYOCD increased Kcnmb1 mRNA expression in 10T1/2 fibroblasts, BC3H1 cells, HeLa cells, HEK-293 cells, and HCASMCs (Fig. 9A). Similarly, MRTF-A dose-dependently increased transcript levels of Kcnmb1 and the gold standard marker for SMC lineages, Myh11 (41) (Fig. 9B). Consistent with the RT-PCR data, KCNMB1 protein was elevated upon graded overexpression of MYOCD in PAC1 SMCs and BC3H1 cells (Fig. 9C). MYOCD had no effect on Kcnma1 mRNA, suggesting differential regu-
SRF-Myocardin Regulation of KCNMB1

The empty adenovirus control cells; therefore, data were combined into one control group. Fig. 10, A–D, displays representative traces from Ad-Myocd and control cells and induction of $I_K$ by Ad-Myocd. Fig. 10E reveals current-voltage (I-V) relationships of Ibtx-sensitive current (i.e. the MaxiK channel component of $I_K$, in both Ad-Myocd and control cells). Both representative traces and I-V relationships document increased Ibtx-sensitive current in Ad-Myocd-transduced cells compared with controls. These results provide evidence for a functional role of MYOCD in eliciting strong potassium currents in cultured SMCs.

DISCUSSION

SRF and MYOCD constitute a molecular switch for the biochemical, structural, and physiological SMC differentiated phenotype. Although many SRF-MYOCD target genes encoding elements of the SMC contractile apparatus exist, there have been no reports of ion channel subunit genes directly regulated by this transcriptional complex. Here, we have extended the mammalian CArGome to the SMC-restricted KCNMB1 subunit of the MaxiK ion channel and find that this gene is down-regulated similarly to other SMC-specific genes in various models of SMC phenotypic modulation. We show that two conserved CArG elements (C3 and C5) located in the first exon and intron of KCNMB1 bind SRF and are vital for MYOCD-dependent transactivation. Transgenic mouse studies verify the necessity of these two CArG elements in mediating KCNMB1 promoter activity in embryonic cardiomyocytes and vascular SMCs. We further demonstrate endogenous Kcnmb1 mRNA induction upon forced expression of MYOCD in various non-muscle cell lines. Conversely, reducing levels of Srf elicits decreases in expression of Kcnmb1. Finally, MYOCD gain-of-function studies disclose increases in iberiotoxin-sensitive potassium currents in cultured SMCs.

The results reported here showing reductions in Kcnmb1 mRNA expression following growth factor stimulation and arterial injury are consistent with this ion channel subunit being altered in other instances of SMC phenotypic modulation. For example, in an angiotensin II-infused rat model of hypertension, MaxiK currents were reduced concomitantly with Kcnmb1 mRNA expression (44). The latter results are in line with the hypertensive phenotype observed in Kcnmb1 knock-out mice (34). SMCs derived from coronary vessels of...
aged Fisher 344 rats display attenuated MaxiK currents and Kcnmb1 mRNA expression (45). Levels of KCNMB1 protein are also depressed in retinal microvascular SMCs of streptozo-tocin-induced diabetic rats (46). In contrast to lower levels of Kcnmb1 reported here and elsewhere (29, 44, 46), we did not observe obvious changes in expression of the Kcnma1 subunit during SMC phenotypic modulation, suggesting that each MaxiK channel subunit is under distinct transcriptional control mechanisms (see below).

Altered Kcnmb1 expression appears to coincide with changes in levels of another SMC-restricted ion channel subunit, the L type voltage-gated calcium channel (Cacna1c), whose expression is reduced in cultured SMC and within the arterial wall following balloon angioplasty (47, 48). Prior work has delineated distinct regions of the Cacna1c gene that direct basal SMC promoter activity in vitro, although no muscle-restricted transcription factor binding sites have yet been identified and experimentally validated (49, 50). Interestingly, calcium current through the CACNA1C channel induces mRNA expression of Myocd and several contractile genes in cultured SMCs (51). The latter findings support the emerging concept of excitation-transcription coupling within vascular SMC (52, 53). It will be important to define the transcriptional code underlying current-induced alterations in Myocd expression (54) and explore the relationship between calcium current and expression of the Kcnmb1 subunit.

Surprisingly little information exists with respect to muscle-restricted ion channel genes and their transcriptional control through myogenic regulatory factors. The skeletal muscle type 1 sodium channel harbors a myogenin-binding E-box that directs expression of this ion channel by relieving an upstream repressor (55). Recently, the cardiac muscle-specific channel, hyperpolarization-activated cyclic nucleotide-gated potassium channel, was shown to be a direct target of the MEF2 (myocyte-specific, enhancer-binding factor 2) transcription factor (56). In Drosophila, the Kcnma1 gene (slowpoke) is controlled by multiple promoters, including one directing expression of this channel in muscle fibers and tracheal cells through sequences containing MEF2 and E-box binding sites (57). The orthologous mouse Kcnma1 gene is also under the control of several promoters that contain E-boxes, MEF2 sites, and putative CARG boxes (58), some of which are conserved in the human ortholog (59). However, as yet, there are no data supporting a critical role for any of these binding sites or their DNA-binding transcription factors in the regulation of Kcnma1 expression. Indeed, MYOCD overexpression studies performed in this report showed no effect on mRNA expression of KCNMA1 in human coronary artery SMCs, consistent
with the absence of conserved CArG boxes in the immediate vicinity of this ion channel subunit’s promoter. Further, we did not observe appreciable changes in Kcnma1 mRNA expression in several models of SMC phenotypic modulation. Although these negative results are consistent with independent models of SMC phenotypic change (44, 46), other studies have demonstrated attenuated expression of Kcnma1 in vitro and in vivo (60 – 62). We suspect that different time points of analysis and distinct model systems underlie these disparate findings.

In contrast to the multiple promoters proposed to regulate expression of KCNMA1 in both invertebrates and vertebrates, the human KCNMB1 gene contains a single promoter with one major transcription start site (22). Despite the reporting of several MEF2 and E-box elements in the KCNMB1 promoter (22), there has been virtually no information as to how this SMC-restricted gene is regulated transcriptionally either in vitro or in vivo. In fact, the only published report of authentic KCNMB1 promoter activity is a recent one suggesting a role for Sp1 in the regulation of rabbit Kcnmb1 (63). Comparative sequence analysis, however, proves that the Sp1 binding site is not conserved in human and mouse orthologous promoters. Thus, it is unclear at this time whether Sp1-mediated expression of Kcnmb1 is unique to the rabbit gene or more broadly applicable to other mammalian orthologs of this ion channel subunit.

Several lines of evidence provided here support an essential role for SRF in the SMC-restricted regulation of KCNMB1 both in vitro and in vivo. First, quantitative RT-PCR and in situ
hybridization assays show the highest expression of \textit{Kcnmb1} mRNA in mouse vascular and visceral SMC tissues, where SRF protein is abundantly expressed. Second, bioinformatic analyses demonstrate the existence of two conserved CArG boxes, C3 in the untranslated first exon and C5 in the first intron, which bind SRF and are requisite for promoter activity in cultured SMCs as well as dorsal aortic SMCs of the mouse embryo. Additional non-conserved CArG boxes have modest effects on \textit{KCNMB1} promoter activity in vitro but appear to be insufficient to drive promoter activity in transgenic mice. Finally, \textit{Srf} knockdown in an SMC-like cell line elicits corresponding decreases in expression of \textit{Kcnmb1} mRNA. The virtual absence of \textit{Kcnmb1} mRNA expression in the adult heart suggests that its expression and promoter activity in embryonic heart is ephemeral. This is consistent with a number of other SMC-specific genes that display transient expression and promoter activity in the developing myocardium, including the SMC isoforms of calponin and \(\alpha\)-actin, the smoothelin A isoform, SM22 \(\alpha\), dystrophin, and the cysteine-rich protein 1 gene, all of which contain conserved, SRF-binding CArG elements in the vicinity of their promoter regions (7). Given the expression of such a repertoire of SMC-specific genes in early cardiomyogenesis, it is intriguing to consider the possibility that early cardiomyocytes exhibit contractile activity more closely resembling adult SMCs than adult cardiac muscle. This hypothesis will require a comprehensive analysis of the functional characteristics of embryonic car-

**FIGURE 9.** SRF-MYOCARD-regulated expression of endogenous \textit{KCNMB1} mRNA and protein. A, the indicated cell lines were transduced with equal amounts (multiplicity of infection 50) of adenovirus harboring either \textit{Myocd} (\(+\)), \textit{lacZ} control (\(-\)), or increasing amounts of Ad-\textit{Myocd} (multiplicity of infection 50–150 in HCASMCs) for 72 h, and endogenous \textit{Kcnmb1} mRNA expression was assessed by RT-PCR. B, cells were transduced with Ad-\textit{lacZ} (\(-\)), a lower titer (multiplicity of infection \(\sim 10\)) of Ad-\textit{Myocd} (\(+\)), or increasing amounts of Ad-\textit{MRTF-A} for 48 h, and endogenous \textit{Kcnmb1} and \textit{Myh11} mRNA expression was assayed by RT-PCR. C, Western blots of endogenous MYOC and KCNMB1 protein in the indicated cell lines following exposure to increasing amounts of Ad-\textit{Myocd}. D, the indicated cell lines were transduced with Ad-\textit{Myocd} as in A, and levels of \textit{Kcnma1} and \textit{Kcnmb1} mRNA were measured by RT-PCR; brain and bladder mRNA samples were included as a positive control for \textit{Kcnma1} expression. BC\(3\)H1 cells were transduced with Ad-\textit{shSRF} (\(+\)) or Ad-\textit{shEGFP} (\(-\)) for 72 h and subjected to semiquantitative RT-PCR (E) or quantitative RT-PCR (F) for the indicated target genes. The knockdown of \textit{Kcnmb1} in F is presented as the percentage decrease from shEGFP control set to 1. All expression data are representative of two or more independent experiments.
diomyocytes. Alternatively, expression of SMC-specific transcripts in the developing heart may merely reflect the abundant expression of SRF during early cardiomyogenesis (65) and its ability to override negative signaling and/or repressors that otherwise are active in the normal adult heart to block SMC-restricted gene expression.

In order for SMCs to contract appropriately, there must be tight control of both the expression and activity of ion channels that integrate electrical current with the cytocontractile machinery. We recently reported that forced expression of MYOCD was sufficient to orchestrate the assembly of SMC myofilaments leading to agonist-induced contraction in a cell type that normally does not display such phenotypes (13). The results of the present study provide the first evidence for MYOCD directly regulating expression of an ion channel subunit critical for normal SMC contractile activity. Moreover, patch clamp studies indicate that MYOCD can induce iberiotoxin-sensitive potassium currents in cultured coronary artery SMC that otherwise are essentially devoid of such current activity. The latter results are consistent with those of Jiang et al. (22), who showed that ectopic expression of KCNMB1 augmented the effects of the pore-forming α subunit (KCNMA1) on calcium/voltage sensitivities. Given the direct activation of Kcnmb1 by MYOCD and the former’s compromised expression in various disease processes (this report) (44–46), it will be instructive to establish whether MYOCD expression is similarly attenuated in such vascular diseases as hypertension and diabetic retinopathy. Based on published reports showing reductions in Myocd mRNA expression following arterial injury (66) or diet-induced atherosclerosis (29), it is possible that restoring MYOCD expression will normalize Kcnmb1 expression and MaxiK currents, thus minimizing disease progression. One potentially exciting way of normalizing MYOCD levels in the perturbed arterial wall will be to overexpress microRNA-145 which was recently shown to be a positive regulator of Myocd expression in vascular SMCs and stem cells with the potential to differentiate into SMCs (67, 68).

In summary, results of this report offer the first example of an SRF-MYOCARD target gene encoding an ion channel subunit. The molecular insight into the transcriptional basis for Kcnmb1 expression documented here provides a foundation to begin developing novel therapies for the potential treatment of hypertension, vascular occlusive disease, and diabetic retinopathy, all of which are characterized by aberrant expression and activity of this MaxiK channel subunit.

REFERENCES

1. Owens, G. K., Kumar, M. S., and Wamhoff, B. R. (2004) Physiol. Rev. 84, 767–801
2. Larsson, E., McLean, S. E., Mecham, R. P., Lindahl, P., and Nelder, S. (2008) Mol. Genet. Genomics 280, 127–137
3. Campbell, G. R., and Campbell, J. H. (1985) Exp. Mol. Pathol. 42, 139–162
4. Chow, N., Bell, R. D., Deane, R., Streb, J. W., Chen, J., Brooks, A., Van Nostrand, W., Miano, J. M., and Zlokovic, B. V. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 823–828
5. Woodman, L., Siddiqui, S., Cruse, G., Sutcliffe, A., Saunders, R., Kaur, D., Bradding, P., and Brightling, C. (2008) J. Immunol. 181, 5001–5007
6. Norman, C., Runswick, M., Pollock, R., and Treisram, R. (1988) Cell 55, 989–1003
7. Miano, J. M. (2003) J. Mol. Cell. Cardiol. 35, 577–593
8. Wang, D., Chang, P. S., Wang, Z., Sutherland, L., Richardson, J. A., Small, E., Krieg, P. A., and Olson, E. N. (2001) Cell 105, 851–862
9. Chen, J., Kitchen, C. M., Streb, J. W., and Miano, J. M. (2002) J. Mol. Cell. Cardiol. 34, 1345–1356
10. Cao, D., Wang, Z., Zhang, C. L., Oh, J., Xing, W., Li, S., Richardson, J. A., Wang, D. Z., and Olson, E. N. (2005) Mol. Cell. Biol. 25, 364–376
11. Li, S., Wang, D. Z., Wang, Z., Richardson, J. A., and Olson, E. N. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 9366–9370
12. Huang, J., Cheng, L., Li, J., Chen, M., Zhou, D., Lu, M. M., Proweller, A., Epstein, J. A., and Parmacek, M. S. (2008) J. Clin. Invest. 118, 515–525
13. Long, X., Bell, R. D., Gerthoffer, W. T., Zlokovic, B. V., and Miano, J. M. (2008) Arterioscler. Thromb. Vasc. Biol. 28, 1505–1510
14. Yoshida, T., Kawai-Kowase, K., and Owens, G. K. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 1596–1601
15. Zhou, J., and Herring, B. P. (2005) J. Biol. Chem. 280, 10861–10869
16. Wang, Z., Wang, D. Z., Pipes, G. C., and Olson, E. N. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 7129–7134
17. Yin, F., Hoggatt, A. M., Zhou, J., and Herring, B. P. (2006) Am. J. Physiol. Cell Physiol. 290, C1599–C1609
18. Baker, D. L., Dave, V., Reed, T., Misra, S., and Periasamy, M. (1998) Nucleic Acids Res. 26, 1092–1098
19. van Tuyn, J., Knaan-Shanzer, S., van de Watering, M. J., de Graaf, M., van der Laarse, A., Schalij, M. J., van der Wall, E. E., de Vries, A. A., and Atsma, D. E. (2005) Cardiovasc. Res. 67, 245–255
20. Thorneloe, K. S., and Nelson, M. T. (2005) Can. J. Physiol. Pharmacol. 83, 215–242
