Kv1.5 potassium channel gene regulation by Sp1 transcription factor and oxidative stress

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Over the past twenty years, many mammalian voltage-gated potassium (Kv) channel genes have been identified, functionally expressed, and ascribed physiological and pathological roles (30). A Kv channel attracting particular attention has been Kv1.5, which switches on in response to depolarization past about −40 mV, has moderate-to-slow inactivation, and is regulated by factors, including acid and hydrogen peroxide (9, 48). Kv1.5 is perhaps best known in relation to vascular smooth muscle and cardiac muscle (32, 33, 36, 37, 42), but it also occurs in GH3 pituitary cells, tumors, oligodendrocyte precursor cells, macrophages, and somato dendritic Purkinje cells of the cerebellum (12, 14, 38, 41, 52).

Along with other Kv1 subunits, Kv1.5 has emerged as a regulator of vascular tone, as a tonic hyperpolarizing influence against voltage-dependent Ca2+ entry (3, 4, 10, 11, 33). It shows differentially regulated expression in conduit compared with resistance arteries and in normotensive vs. hypertensive rats (16, 24). Overexpression of Kv1.5 induces apoptosis of pulmonary artery smooth muscle cells in culture and reduces pulmonary hypertension in rats (8, 40). Kv1.5 expression in vascular smooth muscle cells is inhibited by hypoxia or Bcl-2 (21, 39). In the heart, Kv1.5 underlies the ultrarapid delayed rectifier potassium current and has a primary role in controlling action potential repolarization in humans at physiological heart rates (22, 23). Block of Kv1.5 has been proposed as a treatment for atrial arrhythmias, and drug development programs are in progress (17). Ventricular Kv1.5 expression is dependent on thyroid hormone and suppressed after myocardial infarction (29, 46). Despite the importance of Kv1.5, there is little known of the mechanisms regulating expression of the underlying gene, Kcna5. In this study, we focused on mechanisms underlying expression, uncovering a regulatory role for the transcription factor Sp1, and identifying a potential mechanistic link for Kcna5 responses to oxidative stress. Such mechanisms are likely to play important roles in physiological regulation and cardiovascular disease.

Sp1 was identified as a protein factor required for transcription of the simian virus 40 promoter and was the first mammalian transcription factor to be cloned (7, 20). Sp1 clearly has functional importance, because gene disruption in mice is lethal at embryonic day E10.5–11.1 (31). It is a triple zinc finger protein that binds G/C-rich nucleotide sequences, including GC and CACCC boxes (5, 28). Identification of GC/CACCC boxes in many so-called housekeeping genes led to the initial hypothesis that Sp1 is a basal transcription factor. This diffuse concept has, however, been contradicted by subsequent and extensive studies suggesting that Sp1 plays multiple and sophisticated roles as a modulator of tissuespecific transcription (5, 7). Activity of Sp1 shows complex regulation by glycosylation, phosphorylation, oxidative stress, proteolytic cleavage, and other transcription factors, including NF-κB (1, 13).

METHODS

Source of blood vessels. Experiments were carried out on cell lines and on tissue dissected from animals only after euthanization, according to agreed national procedures within the University’s facility. Eight-week-old male C57/BL6 mice were euthanized by CO2 asphyxiation and cervical dislocation carried out in accordance with the Code of Practice UK Animals Scientific Procedures Act 1986, as approved by the University Ethical Review Committee. The thoracic aorta and mesenteric artery (−0.75 and 0.2 mm external diameter, respectively) were removed and placed in ice-cold Hanks’ solution. Fat was removed completely by dissection, and blood was flushed from the lumen with Hanks’ solution (24).

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DNA constructs and mutagenesis. A 2.5-kb insert containing 5′ flanking and UTR sequence was amplified by PCR from a murine BAC clone template using Pfu turbo (Strategene) and the following primers: forward 5′-TAAAGTACCCGAGGCTGACTAATGTGC-3′ and reverse 5′-GACTCTGCCGTCTCATGGA-3′ (the underlined sequence is a linker region with engineered KpnI site). The insert was subcloned into pGL3 basic luciferase vector (Promega) at KpnI/SmaI sites. The insert corresponds to positions −2,299 to +226 bp relative to the Kcna5 transcriptional start site denoted as +1. Truncation to position −1,982 bp was achieved by restriction of an endogenous Asel site followed by blunting and vector religation. Disruption of CACCC box motifs was achieved by triple mutation (CACCC to CAAA) using mutagenic primers and Quick-Change PCR (Stratagene). Oligonucleotide primers for truncation to insert to positions −1,797 and −1,925 bp and mutagenesis of CACCC box motifs are available on request. Rat Sp1 was in pcDNA3.1. To create Sp1DN (dominant-negative Sp1), Sp1 plasmid was restricted with BamHI followed by religation of the vector (18). All mutations were confirmed by sequencing (Lark).

Cell culture and transfection. Embryonic rat aortic smooth muscle cells (A7r5, ATCC) and primary unpassaged murine aortic smooth muscle cells were cultured in 10% (vol/vol) FCS DMEM containing 1% (vol/vol) penicillin-streptomycin in a humidified 5% CO2 incubator at 37°C. A7r5 cells were used in passages 2–10. Primary aortic smooth muscle cells were enzymatically isolated from strips of aortic medial layer (24) and cultured for 14 days in six-well plates. Smooth muscle purity was confirmed by immunoreactivity with anti-smooth muscle α-actin. Cells were growth arrested before transfection by complete removal of serum for 24 h. Plasmid DNA was transfected using FuGENE 6 reagent (Roche). One-microgram Kcna5 luciferase construct or empty vector plus 10 ng pCMV-RhL renilla vector (Promega) were transfected per well. Where Sp1 constructs were overexpressed, Sp1 DNA was titrated against empty pcDNA3.1 vector to keep total DNA per transfection constant. Mithramycin A was administered 6 h posttransfection.

Luciferase assay. Luciferase activity was measured in total cell lysates 48 h posttransfection using PhL Mediators Luminometer and Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was normalized to renilla activity or to total protein, as determined by the Dual-Luciferase Reporter Assay. Data analysis. Quantified data are means ± SE from three to four independent transfections or cell samples. Statistical analysis was performed by ANOVA and Student’s t-test, and differences are indicated for P < 0.05 and P < 0.01. Transcription factor binding sites in nucleotide sequence were predicted using AliBaba2 software and the TRANSFAC database.

RESULTS

Kcna5 promoter activity in vascular smooth muscle cells. An approximate 2.5-kb section upstream of the mouse Kcna5 gene coding region was isolated. To test for promoter activity in vascular smooth muscle cells, the section was cloned upstream of a firefly luciferase reporter gene and expressed in A7r5 or mouse primary aorta smooth muscle cells. Cells expressing luciferase reporter containing the Kcna5 insert showed four- to fivefold higher luciferase activity compared with cells expressing empty reporter (Fig. 1A), indicating the insert had promoter activity in vascular smooth muscle cells. To ascertain the sections of the promoter important for constitutive activity, a series of truncated promoters were generated. Deletion mutants of the region −2,299 to −1,797 bp caused significant decreases in promoter activity, with removal of the region −2,299 to −1,796 bp causing complete loss of promoter activity in A7r5 and primary smooth muscle cells (Fig. 1B).

Identification of transcription factor binding sites. In a bioinformatics search for potential transcription factor binding sites, we identified three CACC box motifs within this critical region at positions −2,105 (site 1), −1,926 (site 2), and −1,826 bp (site 3) (Fig. 2). CACC boxes are also evident in this region of human and rat Kcna5 (data not shown). To test
whether the CACCC boxes in the upstream region contributed to promoter activity, we systematically introduced mutations (CACCC to CAAAA) to disrupt each motif. Disruption of individual motifs lead to significant decreases in promoter activity (Fig. 3); furthermore, disruption of all three CACCC boxes abolished promoter activity in A7r5 cells (see Fig. 6D). These data suggest the presence of all three motifs is required for \textit{Kcna5} full promoter activity in vascular smooth muscle cells.

\textit{Sp1} binds CACCC boxes in \textit{Kcna5} promoter. To address whether \textit{Sp1} bound to CACCC boxes underlies the \textit{Kcna5} promoter activity observed in vascular smooth muscle cells, we first tested whether \textit{Sp1} could bind the \textit{Kcna5} promoter. \textit{Sp1} mRNA expression was detectable in A7r5 and native blood vessels by RT-PCR (Fig. 4A). Endogenous \textit{Sp1}, from A7r5 nuclear extracts, bound a radioactively labeled DNA probe containing a generic \textit{Sp1}-binding motif, and the \textit{Sp1}-probe interaction was confirmed by using anti-\textit{Sp1} to supershift the complex (Fig. 4B). The interaction was abolished by competition with unlabeled DNA probe and by probes containing each \textit{Kcna5} promoter CACCC box site, revealing an apparent rank order of affinity (site 3 > site 2 > site 1). These data suggest endogenous \textit{Sp1} is able to bind the CACCC motifs in the \textit{Kcna5} promoter. In addition to this, chromatin immunoprecipitation performed on smooth muscle cells freshly isolated from mouse aorta revealed enrichment of site 3 CACCC box with anti-\textit{Sp1} antibody (Fig. 4C), demonstrating that \textit{Sp1} is bound at this site in the \textit{Kcna5} promoter in aorta under physiological conditions in vivo.

\textit{Sp1} drives \textit{Kcna5} promoter activity. We next tested the effect of manipulating \textit{Sp1} binding on \textit{Kcna5} promoter activity in vascular smooth muscle cells. Mithramycin A, an inhibitor of transcription factor binding to G and/or C-rich motifs, had a concentration-dependent inhibitory effect on \textit{Kcna5} promoter activity (IC50 47.2 ± 2.5 nM, \(n = 4\); Fig. 5A). The slope of the mithramycin A concentration-response curve of 3.2 suggested positive cooperativity, consistent with multiple \textit{Sp1} binding
sites. Furthermore, promoter activity was inhibited by overexpression of Sp1DN, containing the DNA binding but not transactivation domain (Fig. 5B). Overexpression of wild-type Sp1 enhanced promoter activity in a dose-dependent fashion in A7r5 cells (Fig. 6A). Consistent with the mithramycin effect on constitutive promoter activity, mithramycin A also inhibited promoter activity induced by overexpression of wild-type Sp1 (Fig. 6B); methanol vehicle was without effect (data not shown). Sp1-evoked activity was also inhibited by overexpression of Sp1DN (Fig. 6C). Hence, both constitutive and over-

Fig. 5. Sp1 drives constitutive Kcna5 promoter activity in A7r5 cells. A: concentration-dependent inhibition of full-length, wild-type constitutive Kcna5 promoter activity by mithramycin A. Luciferase activity was measured following mithramycin A treatment and compared with control cells without treatment. Curve was fitted with Hill equation with an IC_{50} of 47.2 ± 2.5 nM and slope of 3.19 (n = 4). B: effect of dominant-negative Sp1 (Sp1DN) overexpression on constitutive promoter activity. Cells were transfected with full-length, wild-type constitutive Kcna5 promoter construct, with or without 10, 50, or 100 ng Sp1DN (n = 4). ***P < 0.01 vs. empty vector.

Fig. 6. Exogenous Sp1 enhances Kcna5 promoter activity via CACCC boxes. A: Luciferase activity measured in A7r5 cells transfected with full-length, wild-type Kcna5 genomic insert luciferase construct alone or cotransfected with 10, 50, or 100 ng Sp1 construct (n = 4). Luciferase activity was normalized to promoter activity in the absence of exogenous Sp1. B: mithramycin A inhibits Sp1-evoked promoter activity. Luciferase activity was measured in A7r5 cells cotransfected with wild-type Kcna5 luciferase and 100 ng Sp1 expression plasmid, with or without 100 nM mithramycin A (n = 4). Luciferase was normalized to activity in the absence of mithramycin A and exogenous Sp1. C: overexpression of Sp1DN inhibits Sp1-evoked promoter activity in A7r5 cells. Luciferase activity was measured in cells cotransfected with wild-type Kcna5 genomic insert luciferase construct, 100 ng Sp1, and 10, 50, or 100 ng Sp1DN (n = 4). Luciferase activity was normalized to cells in the absence of Sp1DN. D: effect of triple CACCC box disruption on Sp1-evoked promoter activity. Luciferase activity was measured in cells cotransfected with wild-type promoter, with or without 100 ng Sp1, and triple CACCC box mutant promoter with 100 ng Sp1 (n = 4). Luciferase was normalized to activity of wild-type promoter in absence of exogenous Sp1. ***P < 0.01 vs. empty vector (A and C), vs. without drug (B), vs. wild-type vector cotransfection (D).
expressed Sp1-evoked promoter activity share the same promoter activity and share the same pharmacology, suggesting that constitutive Kcna5 promoter activity in A7r5 cells is driven by Sp1. Most strikingly, Sp1 failed to evoke activity in the CACCC box null promoter, indicating the CACCC boxes are required for Sp1-dependent activity (Fig. 6D).

Oxidative stress attenuates Kcna5 promoter activity. Finally, we examined the effect of exogenous stimuli of known vascular importance on Sp1-dependent constitutive Kcna5 promoter activity in vascular smooth muscle. The activity of Sp1 is sensitive to oxidative stress, a factor important for vascular physiology. To this end, we tested the effect of hydrogen peroxide on constitutive promoter activity in A7r5 cells. Luciferase activity measured in A7r5 cells expressing the Kcna5 promoter construct was reduced by ~50% following treatment with hydrogen peroxide (200 μM, 24 h; n = 4; Fig. 7). Combined with the observation that Sp1 is bound in vivo to Kcna5 promoter, oxidative stress may regulate expression of Kcna5 in native arteries.

DISCUSSION

Understanding mechanisms that regulate KV1.5 expression in the vasculature is important, because KV1.5 influences smooth muscle tone, has heightened expression in resistance arteries, and is regulated by physiological factors such as oxygen tension (35, 43, 51) and differential expression of KV mRNAs in normotensive vs. hypertensive arteries (15). Experimental manipulation of KV channel expression can also have significant effects on blood vessel physiology (40). These data presented here identify promoter/transcription factor interactions important in regulation of the KV1.5 potassium channel gene Kcna5. Combined deletion, site-directed mutagenesis, and pharmacological approaches lead us to conclude that a CACCC box cluster is a critical element driving Kcna5 expression. We show the cluster is a target for Sp1 transcription factor, which enhances the promoter activity in a CACCC box-dependent manner. Furthermore, a Sp1DN mutant inhibits constitutive activity, and endogenous Sp1 binds the Kcna5 CACCC box cluster. Therefore, a molecular component underlying constitutive Kcna5 gene expression is suggested. Furthermore, the interaction may be a mechanism for regulated expression, because constitutive activity is modest but enhanced by addition of exogenous Sp1. Sp1 is bound to Kcna5 physiologically, and promoter activity is inhibited by oxidative stress evoked by hydrogen peroxide.

Our mutagenesis data indicate all three CACCC boxes within the cluster contribute to constitutive promoter activity, a conclusion strengthened by the Hill coefficient of >1 in the mithramycin A concentration-response curve. Nevertheless, gradation of binding efficiency is also evident, with the site 3 CACCC box having the strongest association. Therefore, we hypothesize that elevation of Sp1 activity will progressively increase binding to all three boxes, further increasing expression of Kcna5. This raises the possibility that Sp1 regulation of Kcna5 is, physiologically, in a constitutive but not maximal state of activation, i.e., it is in a state suitable for dynamic regulation. Activity of Sp1 is regulated by a range of factors (1, 13), and a clear candidate for regulation of the Sp1-dependent promoter of Kcna5 is oxidative stress, because promoter activity is sensitive to hydrogen peroxide. Hydrogen peroxide is a physiological vasodilator that enhances KV currents in both conduit and resistance arteries (25, 44). It is, therefore, interesting that hydrogen peroxide can also regulate KV activity at the transcriptional level, which may have vascular implications during prolonged states of oxidative stress, e.g., hypoxia. The concentration of hydrogen peroxide used in this study (200 μM) reflects the concentration required to produce maximal vasodilation in several arterial preparations (19, 27, 45). Unfortunately, we were unable to test the effects of oxidative stress on Kcna5 expression in the aorta, because there is rapid, spontaneous downregulation of Kcna5 expression once the vessel is dissected from the animal (J. Li and D. J. Beech, unpublished observations). It should be recognized that there are other potential regulators of Kcna5 expression. KV1.5 mRNA abundance is known to be regulated by thyrotropin-releasing hormone, insulin-like growth factor-1, Bcl-2 oncoprotein, c-Jun immediate early gene, chronic hypoxia, cyclic AMP, and glucocorticoids (21, 34, 39; for further references, see Ref. 24).

We have focused on Sp1, but it is premature to exclude the possibility that other related factors also interact with the CACCC boxes of the Kcna5 promoter. Sp1 is part of a large family of proteins, including other Sp factors and the extensive set of Kruppel-like factors (5, 7, 47, 49). Some of these other factors, Kruppel-like factor 5, for example, are expressed and functionally important in the cardiovascular system (47). These factors might have a greater influence than Sp1 on Kcna5 CACCC boxes, either as coactivators, or dominant-negative competitors with Sp1. To the best of our knowledge, identification of the CACCC box motif as a functional determinant of ion channel expression is novel in the field of ion channel research and should provide a foundation for future studies with a wider scope involving other Sp-like factors.

A few studies have previously explored Kcna5 gene regulation in cardiac myocytes and GH3 pituitary cells, showing...
that expression also involves a novel silencing element and binding factor (S0). This site is distinct and downstream from the CACCC box cluster. Kcna5 mRNA levels are also known to be regulated by cyclic AMP and tyrosine phosphorylation (26, 34).

In summary, we propose a mechanism whereby endogenous Sp1 factor is functionally involved in conferring constitutive expression of Kcna5 gene. Sp1 was once regarded as a simple basal transcription factor, underlying constitutive expression of many genes. However, it is emerging as a factor with intrinsic specificity, as well as highly dynamic regulation. It has the potential to be a focal point for understanding the physiological control of Kv1.5 potassium channel gene expression in the cardiovascular system.

GRANTS

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REFERENCES


