Transcriptional control of KCNQ channel genes and the regulation of neuronal excitability

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Abstract
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Keywords
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Transcriptional Control of KCNQ Channel Genes and the Regulation of Neuronal Excitability

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Regulation of the resting membrane potential and the repolarization of neurons are important in regulating neuronal excitability. The potassium channel subunits Kv7.2 and Kv7.3 play a key role in stabilizing neuronal activity. Mutations in KCNQ2 and KCNQ3, the genes encoding Kv7.2 and Kv7.3, cause a neonatal form of epilepsy, and activators of these channels have been identified as novel antiepileptics and analgesics. Despite the observations that regulation of these subunits has profound effects on neuronal function, almost nothing is known about the mechanisms responsible for controlling appropriate expression levels. Here we identify two mechanisms responsible for regulating KCNQ2 and KCNQ3 mRNA levels. We show that the transcription factor Sp1 activates expression of both KCNQ2 and KCNQ3, whereas the transcriptional repressor REST (repressor element 1-silencing transcription factor) represses expression of both of these genes. Furthermore, we show that transcriptional regulation of KCNQ genes is mirrored by the correlated changes in M-current density and excitability of native sensory neurons. We propose that these mechanisms are important in the control of excitability of neurons and may have implications in seizure activity and pain.

Introduction

Regulation of the resting membrane potential and the repolarization of neurons are important in regulating neuronal excitability. One ionic current that plays a key role in stabilizing neuronal activity is the M-current (I_{M}), a slowly deactivating, non-inactivating potassium current first identified nearly 30 years ago as the one underlying the excitatory effect of acetylcholine (Brown and Adams, 1980). The M-current is produced by the action of Kv7 channels encoded by members of the KCNQ gene family KCNQ1–KCNQ5, each of which encodes an individual potassium channel subunit Kv7.1–Kv7.5 (Jentsch, 2000; Robbins, 2001).

Of the five KCNQ gene family members, KCNQ2 and KCNQ3 are particularly important for regulating neuronal activity because these subunits are expressed widely throughout the CNS with expression patterns that almost entirely overlap (Tinel et al., 1998; Yang et al., 1998). Kv7.2 and Kv7.3 form functional heteromultimers that are believed to represent a major M-channel isoform in the CNS and peripheral nervous system (Delmas and Brown, 2005). The importance of the appropriate functioning of Kv7.2 and Kv7.3 in the nervous system is highlighted by the fact that mutations in KCNQ2 and KCNQ3 are associated with benign familial neonatal convulsions, an autosomal dominant neonatal epilepsy (Charlier et al., 1998; Singh et al., 1998; Yang et al., 1998; Biervert and Steinlein, 1999). The M-current is also one of the key players in nociceptive transmission; inhibition of M-current leads to membrane depolarization and hyperexcitability of nociceptive neurons (Passmore et al., 2003; Crozier et al., 2007; Linley et al., 2008; Liu et al., 2010), an important determinant of many pain conditions. Pharmacological openers of Kv7.2/Kv7.3 channels therefore now represent important analgesic targets (Surti and Jan, 2005; Gribkoff, 2008; Wickenden and Naunton-Smith, 2009) because current therapies are not efficacious for the majority of inflammatory and chronic pain conditions.

Despite their importance, very little is known about the mechanisms responsible for regulating KCNQ2 and KCNQ3 expression. Here we use a functional assay to identify important regulatory regions within the KCNQ2 and KCNQ3 genes. We show that both KCNQ2 and KCNQ3 contain GC box motifs and provide evidence that their transcription is enhanced by the Sp1 transcription factor. We also show that expression of both KCNQ2 and KCNQ3 is repressed by REST (repressor element 1-silencing transcription factor) and that expression of REST in neurons is sufficient to repress KCNQ2 and KCNQ3 expression, inhibiting functional expression of the M-current and resulting in hyperexcitable neurons. We show that REST levels are increased in dorsal root ganglia (DRG) neurons in response to inflammatory mediators and that Kv7.2 levels and M-current density are reduced, suggesting a potential role in regulating inflammatory pain responses. Neuronal expression of REST is increased in response to sustained neuronal hyperactivity, i.e., in

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epileptic insults (Palm et al., 1998), cerebral ischemia (Calderone et al., 2003), and a model of neuropathic pain (Uchida et al.); we therefore suggest that, by repressing KCNQ2 and KCNQ3 expression, REST may contribute to chronic overexcitability of neuronal circuits seen in epilepsy and chronic pain.

Materials and Methods

Cell culture. SHSY-5Y and HEK293 cells were grown in DMEM/F-12 supplemented with 10% fetal calf serum (PAA Laboratories), 6 g/liter penicillin, 10 g/liter streptomycin, and 2 mM l-glutamine at 37°C and 5% CO2. DRG neurons were cultured as described previously (Crozier et al., 2007; Linley et al., 2006; Liu et al., 2010); briefly, ganglia were extracted from 7- to 10-d-old rats from all spinal levels. Ganglia were enzymatically dissociated in HBSS supplemented with collagenase type 1A (1.5 mM EC) and dispase (15 mg/ml; Invitrogen) at 37°C for 15–20 min. Cells were then mechanically triturated, washed twice by centrifugation (800 rpm for 5 min), resuspended in 800 μl of medium growth, and plated onto glass coverslips coated with poly-o-lysine and laminin. DRG neurons were cultured for 2–5 d in a humidified incubator (37°C, 5% CO2) in DMEM supplemented with Glutamax I (Invitrogen), 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). For the inflammatory conditions, cells were incubated with the following inflammatory mediators for 48 h: 1 μM bradykinin, 1 μM histamine, 1 μM ATP, 1 μM substance P, and 10 μM PAR-2 activating peptide. For patch-clamp experiments, cells were washed twice and media were replaced with fresh media that did not contain inflammatory mediators for at least 2 h before experiments, to remove any acute effects of inflammatory mediators on the M-current.

DNase I hypersensitivity assay. SHSY-5Y cells were washed twice with ice-cold PBS, resuspended in DNase I buffer (10 mM Tris, pH 8.0, 50 mM KCl, 5 mM MgCl2, 3 mM CaCl2, 1 mM EDTA, 0.1% Nonidet P-40, and 80 μM DTT) and incubated on ice for 10 min. Cells were counted and transferred to a Dounce homogenizer and lysed with 15 strokes of a type B pestle. Nuclei were divided into independent samples (~1.6 × 10^7 per reaction) and treated with 0, 1, 3, 5, and 10 U of DNase I (Sigma) at 37°C for 5 min. Reactions were stopped by addition of equal volume of stop/lysis buffer (20 mM EDTA, 1% SDS, and 0.1 mg/ml proteinase K) and incubated at 55°C overnight. DNA was extracted several times with phenol/chloroform and once with chloroform and then ethanol precipitated. DNA was resuspended in water, and the concentration was measured by spectrophotometry. Ten micrograms of DNA were digested overnight with the appropriate restriction enzymes in a final volume of 30 μl and electrophoresed through 40 μM Tris-acetate, 1 mM EDTA, pH 8, and 0.6% (w/v) agarose gel at 0.5 V/cm. DNA was transferred to Hybond XL membranes (GE Healthcare) using alkaline capillary transfer. DNA fragments were radiolabeled using Prime-It kit (Stratagene) and [α-32P]dATP. Hybridization to Hybond XL membranes was performed as per the instructions of the manufacturer.

Gel mobility shift assay. Nuclear extracts were prepared from SHSY-5Y and JTC-19 cells using the method described previously (Andrews and Faller, 1991). DNA probes were radioactively labeled by Klenow (New England Biolabs) fill in of a restriction-enzyme-generated overhang using [α-32P]dATP. Hybridization of Hybond XL membranes was performed as per the instructions of the manufacturer.

Plasmid and adenoviral delivery. The adenoviral constructs have been described a, 5′-AAAGATCTCATGCTGTCGCTGTTCCCGGCTCACTGGTCCACTGGAAG; mouse KCNQ2 RE1, 5′-GATCTGTCGTTCCAGGACAGGACATGTCAGAGCAGCCAC; rat KCNQ2 RE1, 5′-GATCTTGTGGCCAGGACAGGACATGTCAGAGCAGCCAC; dog KCNQ2 RE1, 5′-GATCTGTCGTTCCAGGACAGGACATGTCAGAGCAGCCAC; mouse KCNQ2 RE1, 5′-GATCTGTCGTTCCAGGACAGGACATGTCAGAGCAGCCAC; rat KCNQ2 RE1, 5′-GATCTTGTGGCCAGGACAGGACATGTCAGAGCAGCCAC; dog KCNQ1 RE1, 5′-GATCTGTCGTTCCAGGACAGGACATGTCAGAGCAGCCAC; human CHRM4; 5′-GATCTTGTGGCCAGGACAGGACATGTCAGAGCAGCCAC; and human KCNQ5, 5′-GATCTTGTGGCCAGGACAGGACATGTCAGAGCAGCCAC. Underlined nucleotides correspond to newly formed restriction sites. Additionally, we introduced mutations into the Sp1 binding site to prevent Sp1 binding using the primer for Sp1, 5′-AAGAGCTCAACGCGGAGGACAGGAGAGGCTGAG; and 5′-AACGCGGAGGACAGGAGAGGCTGAG, containing a substitution of GG with AA (in bold). Amplified fragments were digested with appropriate restriction enzymes and cloned into pGL3 Basic (Promega). Cells were transfected in 24-well plates with 250 ng of pGL3 plasmid and 1 ng of pRL cytoplasmic renilla luciferase (Promega) using 4 μl of Lipofectamine (Invitrogen). Each transfection was performed in triplicate for each experiment. Cells were harvested 48 h after transfection into passive lysis buffer, and luciferase and renilla luciferase expression were quantified using a Dual Luciferase Assay kit (Promega) on a Makers PHL luminometer.

Quantitative reverse transcriptase-PCR. Total RNA was extracted from dorsal root ganglia cells using Tri-reagent (Sigma). DNase-treated (Ambion) RNA was reverse-transcribed by using Moloney murine leukemia virus reverse transcriptase (Promega) and purified using QiAquick columns (Qiagen). Quantitative PCR was performed using SYBR Green incorporation (Bio-Rad) for duplicate samples in each experiment. The specificity of PCR was verified by melt curve analysis of products obtained from DNA as well as controls in which the reverse transcriptase was omitted. Levels of signal were normalized to levels of U6 small nuclear RNA, which were not different between any of the datasets. Significance was tested using Student’s t-test. Primers used were as follows: U6, 5′-CTCGGTTCGGCAGGTGACAGUCU, 5′-AAGCGGTCAGAATTGG GGCTG; KCNQ5, 5′-GTGTTGCTTACGCTACAGCAAGAAAAGATCTCATGCTGTCGCTGTTCCCGGCTCACTGGTCCACTGGAAG; KCNQ2, 5′-AGGTTGAACTGCTGGCTGCAG; KCNQ3, 5′-GCCCAAGACTGCTGACCTTGTACAG; KCNQ4, 5′-CCGTCCATGATTCCGTTTTCTGGG; RESTs, 5′-GAACTACACCAGAAAGCCAAGCT; RSTs, 5′-AGGGAGCCATATGACTTCAG; SCN2A, 5′-GCTGCTATGCTCTTCTTGTCG; and SCN2B, 5′-GACGAGATTTGTGCTAC.
previously (Wood et al., 2003). Green fluorescent protein was a marker of the efficiency of viral infection. Cells were cultured and infected with adenoviral particles for 48 h before harvesting and RNA or electrophysiological analysis.

**Electrophysiology.** In patch-clamp experiments, the standard bath solution contained the following (in mM): 160 NaCl, 2.5 KCl, 1 MgCl2, and 10 HEPES, pH adjusted to 7.4 with NaOH. For perforated-patch experiments, the patch pipette contained 90 mM K-acetate, 20 mM KCl, 1 mM CaCl2, 3 mM MgCl2, 3 mM EGTA, 40 mM HEPES, and 400 μg/ml amphotericin B, pH adjusted to 7.4 with NaOH. Currents were amplified using an EPC-10 patch-clamp amplifier (HEKA) and recorded using Patchmaster software (version 2.2; HEKA). The current signal was sampled at 1 kHz and filtered online at 500 Hz using a software-based Bessel filter. Patch pipettes were fabricated from borosilicate glass (Harvard Apparatus) using a horizontal puller (DMZ-universal puller; Zeitz-Instrumente GmbH) and heat polished to a resistance of 2–4 MΩ. Cells were mounted on an inverted microscope (TE-2000; Nikon) in a low-profile perfusion chamber fed by a gravity perfusion system flowing at ~2 ml/min, resulting in a bath exchange time of ~15 s. Series resistance was corrected online by up to 70% using the Patchmaster software, and liquid junction potentials were corrected. The magnitude of the neuronal M-current was measured from the deactivation current when stepping the membrane voltage from −30 to −60 mV and was calculated as the difference between current amplitude at 10 ms into the voltage pulse and that at the end of the pulse. This analysis method was designed to minimize any contribution from other K+ currents and capacitance artifacts. XE991 [10,10-bis(4-pyridinylmethyl)-9(10H)-antheracene dihydrochloride] was obtained from Tocris Bioscience. All analysis of patch-clamp data was conducted using Fitmaster software (version 2.11; HEKA).

**Statistics.** All data are given as mean ± SEM. Differences between groups were assessed by Student’s t test or one-way ANOVA. The differences were considered significant at p ≤ 0.05.

**Results**

**Identification of DNase I hypersensitive sites in the human KCNQ2 gene.** To identify regions of the KCNQ2 gene that are important for regulation of expression in vivo, we used a DNase I hypersensitivity assay. DNase I hypersensitive sites (HSS) correlate well with important transcriptional regulatory regions, such as promoters, enhancer, and repressor elements (Fritton et al., 1987; Steiner et al., 1987; Elgin, 1988; Wong et al., 1997), and map to regions that recruit transcription factors (Wong et al., 1995; Boyes and Felsenfeld, 1996). A DNase I HSS assay was performed in SHSY-5Y cells, which are known to express KCNQ2 and KCNQ3 (Wickenden et al., 2008) and identified two HSS within the KCNQ2 gene (Fig. 1A). We performed a bioinformatic analysis of the regions identified to look for transcription factor binding sites. An analysis of HSS1 identified a putative Sp1 transcription factor binding site (Fig. 1B) that is conserved within the promoters of the human, rat, and mouse KCNQ2 genes. It has been shown previously that regions of DNA that recruit Sp1 display hypersensitivity to DNase I (Philipson et al., 1990; Pruzina et al., 1991; Jiang et al., 1997; Sinha and Fuchs, 2001). An analysis of the region surrounding HSS2 identified a putative binding site for the transcriptional repressor REST that is conserved within multiple mammalian species (Fig. 1C). REST binding to DNA is known to be sufficient to produce a DNase I HSS (Wood et al., 2003).

**KCNQ2 and KCNQ3 genes contain similar regulatory elements.** To determine whether the potential Sp1 factor binding sites are important for KCNQ2 gene regulation, we used an in vitro DNA–protein interaction experiment to examine the recruitment of Sp1. A radiolabeled fragment of the KCNQ2 promoter containing the Sp1 site was incubated with nuclear protein extracts from SHSY-5Y cells. Incubation of this region of DNA with nuclear protein produced two slowly migrating bands (Fig. 1D, lane 1, Sp1:DNA, Non spec) that could be competed by excess of an unlabeled Sp1 consensus sequence (Fig. 1D, lane 2). The faster migrating DNA–protein complex could also be competed by an unrelated sequence (Fig. 1D, lane 3), indicating that this complex is the result of nonspecific protein–DNA interactions. The slower migrating protein–DNA complex could only be competed with a consensus Sp1 sequence and was further retarded by an anti-Sp1 antibody (Fig. 1D, lane 4, Sp1:Ab:DNA), indicating the presence of Sp1 protein within this complex. Binding of the radiolabeled probe was also competed by an excess of unlabeled wild-type Sp1 sequence from the KCNQ2 promoter (Sp1 WT) (Fig. 1D, lanes 5, 6) but not by a KCNQ2 sequence containing mutations predicted to destroy the Sp1 site (Sp1 mut) (Fig. 1D, lanes 7, 8). These data indicate that the Sp1 site within the KCNQ2 promoter can recruit Sp1 protein present in SHSY-5Y cells. Given the overlap of KCNQ2 and KCNQ3 expression and their shared evolutionary origin by duplication from a common ancestor gene (Hill et al., 2008), it is quite likely that these two genes would share at least some regulatory mechanisms. We therefore searched the KCNQ3 gene for potential Sp1 and RE1 sites. We identified a conserved Sp1 site in the KCNQ3 gene just upstream of the first exon, at an equivalent location to the Sp1 site in the KCNQ2 gene (Fig. 1E, F). We also identified an RE1 site in the KCNQ3 gene that is conserved across multiple mammalian species (Fig. 1E, G). Thus, the two regulatory elements that we identified within the KCNQ2 gene are also present in the KCNQ3 gene.

**Sp1 regulates KCNQ2 and KCNQ3 expression.** To determine whether the Sp1 site is functionally important in regulating KCNQ2 and KCNQ3 transcription, we used a reporter gene assay with the KCNQ promoter regions. We cloned a region of the KCNQ2 promoter encompassing nucleotides −416/+151 bp relative to the transcription start site into a luciferase reporter vector such that luciferase expression is driven by KCNQ2 promoter sequences. This 568 bp region encompasses the Sp1 site and 137 bp of the first exon. We also produced a truncated region that lacks some of the sequence upstream of the Sp1 site (−64/+179), and we introduced mutations into the Sp1 site that prevent Sp1 binding (−64/+179 Sp1 mut) (Fig. 1D, lanes 7, 8). We transfected these DNA constructs into SHSY-5Y cells and measured the resulting luciferase activity. Luciferase activity from the −416/+151 bp promoter fragment was sixfold higher than the control plasmid (Fig. 2A, compare −416/+151 bp with pGL3 Basic), suggesting that this region of the KCNQ2 gene and the Sp1 site within it elevate expression of KCNQ2. Removal of the upstream region did not result in a significant loss of expression, suggesting that this region is not important in regulating transcription in SHSY-5Y cells (Fig. 2A, compare −416/+151 with −64/+179 bp). Introduction of mutations that prevent Sp1 binding (see Materials and Methods) (Fig. 1D), however, resulted in loss of >50% luciferase activity (Fig. 2A, compare −416/+179 with −64/+179 bp Sp1 mut), consistent with the hypothesis that KCNQ2 expression is regulated by Sp1. In our bioinformatic analysis, we noticed that the KCNQ2 gene contained a second Sp1 site that overlapped with the first although its score was less, suggestive of a lower affinity for Sp1 (supplemental Fig S1A, available at www.jneurosci.org as supplemental material). In gel mobility shift experiments, this second site did indeed bind to Sp1, showing a lower affinity than our originally identified site (supplemental Fig S1B, available at www.jneurosci.org as supplemental material). This second Sp1 site is unlikely to be bound by Sp1 in the wild-type promoter because the presence of Sp1 at the
high-affinity site, which overlaps with the low-affinity site, would presumably occlude it. Mutation of the high-affinity site, however, would uncover this second site, and the low level of promoter activity seen for the construct −64/+179 Sp1 mutant is likely to be attributable to low levels of Sp1 recruitment. To determine whether the KCNQ3 gene could also be regulated by Sp1, we conducted a bioinformatic search of the human KCNQ3 promoter region. We identified a sequence matching a consensus Sp1 site that was highly conserved between the human, mouse, and rat genomes (Fig. 1F). To determine whether this potential Sp1 site is functional, we cloned an 876 bp region of the human KCNQ3 gene encompassing nucleotides −540 to +336 relative to the transcription start site and including the Sp1 site into the same luciferase reporter vector as for the KCNQ2 promoter. We also cloned a region of the promoter encompassing nucleotides −169 to +336 that lacks the conserved Sp1 site. Luciferase activity from the −540/+336 promoter region drove high levels of luciferase expression in SHSY-5Y cells (Fig. 2B), which was reduced by ~80% when the region containing the Sp1 sites was removed (Fig. 2B). Together, these data suggest that Sp1 can regulate both KCNQ2 and KCNQ3 promoter activity.

Sp1 regulates the endogenous KCNQ2 and KCNQ3 genes

Having shown that Sp1 can be recruited to the KCNQ2 promoter in vitro and the Sp1 site within the promoter is important for driving reporter gene activity, we wanted to test the relevance of Sp1 transcription factor activity to endogenous KCNQ2 and KCNQ3 expression. To do this, we took advantage of mithramycin A, a compound that binds to Sp1 binding sites (GC box) and inhibits the function of Sp1 (Ray et al., 1989; Liu et al., 2002; Kim et al., 2006). Transfection of KCNQ2 and KCNQ3 luciferase reporter constructs into SHSY-5Y cells in the presence of mithramycin A, a competitor (No comp, lane 1), oligonucleotides containing a consensus Sp1 sequence (Sp1cons1, lane 2), an unrelated sequence (Non spec, lane 3), an anti-Sp1 antibody (Ab:Sp1:DNA), and resulting from nonspecific binding (Non spec) are shown on the left. A schematic representation of the Sp1 region of the human KCNQ2 gene with the equivalent regions of the mouse and rat KCNQ2 genes. The consensus Sp1 sequence is shown above the alignment (n, A, C, G, or T; y, C or T; m, A or C). B, DNA sequence alignment of the Sp1 sequence identified in the HSS1 region of the human KCNQ2 gene with the equivalent regions of the mouse and rat KCNQ2 genes. The consensus Sp1 sequence is shown above the alignment (n, A, C, G, or T; y, C or T; m, A or C). C, DNA sequence alignment of the RE1 sequence identified in the HSS2 region of the human KCNQ2 gene with the equivalent regions of the chimpanzee, rhesus, mouse, rat, dog, cow, and elephant KCNQ2 genes. The consensus RE1 sequence is shown above the alignment (n, A, C, or G; t, C or T; m, A or C, or G). D, Gel mobility shift assay of the Sp1 region of the KCNQ2 promoter. Labeled DNA was incubated with protein isolated from SHSY-5Y cells in the presence of no competitor (No comp, lane 1), oligonucleotides containing a consensus Sp1 sequence (Sp1cons1, lane 2), an unrelated sequence (Non spec, lane 3), an anti-Sp1 antibody (Ab:Sp1:DNA), and resulting from nonspecific binding (Non spec) are shown on the left. E, A schematic representation of the Sp1 promoter region in the human KCNQ2 gene with the equivalent regions of the mouse and rat KCNQ2 genes. The consensus Sp1 sequence is shown above the alignment (n, A, C, or G; t, C or T; m, A or C). F, DNA sequence alignment of the Sp1 sequence identified in the human KCNQ2 gene with the equivalent regions of the chimpanzee, rhesus, mouse, rat, dog, cow, and elephant KCNQ2 genes. The consensus RE1 sequence is shown above the alignment (n, A, C, or G; t, C or T; m, A or C). G, DNA sequence alignment of the RE1 sequence identified in the human KCNQ3 gene with the equivalent regions of the chimpanzee, rhesus, mouse, rat, dog, cow, and elephant KCNQ3 genes. The consensus RE1 sequence is shown above the alignment (n, A, C, or G; t, C or T; m, A or C).

Figure 1. DNase I hypersensitive sites present in the KCNQ2 gene. A, Top shows a schematic representation of the Sp1 gene. Filled boxes represent exons, and the transcription start site is marked by an arrow. The location of identified regulatory elements (Sp1 and RE1) are shown as open boxes. Below shows two DNase I hypersensitivity assays. Nuclei were isolated from SHSY-5Y cells and analyzed for DNase I hypersensitive sites. DNA was digested with HindIII (top) or AseI (bottom), and the Southern blot was hybridized with a consensus Sp1 sequence-specific probe. The positions of the hypersensitive sites (HSS1 and HSS2) are indicated on the diagram as is the position of the full-length DNA fragment (F). B, DNA sequence alignment of the Sp1 sequence identified in the HSS1 region of the human KCNQ2 gene with the equivalent regions of the mouse and rat KCNQ2 genes. The consensus Sp1 sequence is shown above the alignment (n, A, C, G, or T; y, C or T; m, A or C). C, DNA sequence alignment of the RE1 sequence identified in the HSS2 region of the human KCNQ2 gene with the equivalent regions of the chimpanzee, rhesus, mouse, rat, dog, cow, and elephant KCNQ2 genes. The consensus RE1 sequence is shown above the alignment (n, A, C, or G; t, C or T; m, A or C, or G). D, Gel mobility shift assay of the Sp1 region of the KCNQ2 promoter. Labeled DNA was incubated with protein isolated from SHSY-5Y cells in the presence of no competitor (No comp, lane 1), oligonucleotides containing a consensus Sp1 sequence (Sp1cons1, lane 2), an unrelated sequence (Non spec, lane 3), an anti-Sp1 antibody (Ab:Sp1:DNA), and resulting from nonspecific binding (Non spec) are shown on the left. E, A schematic representation of the Sp1 region of the KCNQ2 gene with the equivalent regions of the mouse and rat KCNQ2 genes. The consensus Sp1 sequence is shown above the alignment (n, A, C, or G; t, C or T; m, A or C). F, DNA sequence alignment of the Sp1 sequence identified in the human KCNQ2 gene with the equivalent regions of the chimpanzee, rhesus, mouse, rat, dog, cow, and elephant KCNQ2 genes. The consensus RE1 sequence is shown above the alignment (n, A, C, or G; t, C or T; m, A or C). G, DNA sequence alignment of the RE1 sequence identified in the human KCNQ3 gene with the equivalent regions of the chimpanzee, rhesus, mouse, rat, dog, cow, and elephant KCNQ3 genes. The consensus RE1 sequence is shown above the alignment (n, A, C, or G; t, C or T; m, A or C).
deactivating tail current at $-60$ mV sensitive to specific M channel blocker XE991 ($3 \mu M$ ) applied at the end of each recording. M-current density was significantly larger in Sp1-transfected compared with control neurons (Fig. 3 B, C) ($1.54 \pm 0.34 \, pA/pF$, $n = 8$ compared with $0.91 \pm 0.12 \, pA/pF$, $p < 0.05$). Consistently, the Kv7.2 immunoreactivity was greater in Sp1-transfected than in control neurons (Fig. 3 A, D) (relative pixel intensity of $9.53 \pm 2.15$, $n = 4$ compared with $5.49 \pm 0.85$, $n = 3$, $p < 0.05$). Together, these data implicate an important role for Sp1 in promoting both KCNQ2 and KCNQ3 gene expression and enhancing levels of the M-current in neurons.

**KCNQ genes are regulated by REST**

Bioinformatic analysis of the second DNase I hypersensitive site (Fig. 1 A, HSS2) in the KCNQ2 gene identified a putative RE1 binding site for REST. The sequence for this site is conserved between multiple mammalian species (Fig. 1C), suggesting that it is functionally important. REST was first identified as a repressor of neuronal gene expression (Chong et al., 1995; Schoenherr and Anderson, 1995) but also has roles in heart, blood vessels, and epithelial cells (Ooi and Wood, 2007). We used a gel mobility shift assay to determine whether the RE1 site identified in the KCNQ2 gene can recruit REST protein. Incubation of a radioactive, RE1-containing, SCNA2A promoter region with nuclear extracts from DNA–protein complexes (Fig. 4 A, lane 1). One of these (indicated with an arrow on Fig. 4A) was competed by an RE1 sequence from the CHRM4 gene but not by an unrelated sequence (Fig. 4A, lanes 2, 3). Furthermore, an anti-REST antibody produced an additional shift of this complex, indicating the presence of REST protein (Fig. 4A, lane 4). Oligonucleotides containing RE1 sequences from human, dog, mouse, and rat were all able to compete for REST binding, indicating that the KCNQ2 gene contains an evolutionary conserved, functional RE1 site (Fig. 4A, lanes 5–12).

To determine whether REST sites are present in other KCNQ genes, we used our position-specific scoring matrix (Johnson et al., 2006) to scan each of the five KCNQ genes for the best match to an RE1. High-scoring RE1s were identified in KCNQ2, KCNQ3, and KCNQ5, whereas the closest sequences in KCNQ1 and KCNQ4 to an RE1 site had low scores with our matrix, suggesting they would not bind REST. We tested each of these potential RE1 sequences in a gel mobility shift assay to determine which would bind REST with high affinity (Fig. 4B). The RE1 sequences from KCNQ2, KCNQ3, and KCNQ5 were each able to compete for REST binding, whereas the sequences in KCNQ1 and KCNQ4 could not (Fig. 4B). To determine whether REST could regulate the KCNQ2 and KCNQ3 promoters, we cloned the RE1 sequences upstream of the luciferase reporter constructs (Fig. 2 A) and transfected them into two cell lines, HEK293, which express robust levels of full-length REST natively, and SHSY-5Y, which express low levels of REST as well as a truncated version of REST resulting from an alternative splice variant (our unpublished observations). Inclusion of the RE1 site resulted in robust repression of luciferase activity for both KCNQ2 and KCNQ3 promoters in HEK293 (Fig. 4C), indicating that recruitment of REST to the RE1 site represses KCNQ2 and KCNQ3 expression. Consistent with the observation that SHSY-5Y cells express less REST protein, inclusion of the RE1 site resulted in only modest repression of luciferase activity for the KCNQ3 promoter in SHSY-5Y, whereas the KCNQ2 promoter did not show a significant reduction in activity (Fig. 4D). REST is normally expressed in high levels in non-neuronal cells and only at low levels in neurons (Sun et al., 2005; Olguin et al., 2006). Our luciferase data suggest that the absence or the low levels of REST in neurons may be important for permitting KCNQ2 and KCNQ3 expression. However, after epileptic seizures, global ischemia, or neuropathic injury, conditions characterized by periods of sustained neuronal hyperactivity, REST expression was seen in neurons (Palm et al., 1998; Calderone et al., 2003; Uchida et al., 2010). To determine whether such increased REST expression in neurons may affect KCNQ2 and KCNQ3 expression, we infected cultured DRG neurons with either GFP alone (control) or REST and GFP-expressing adenovirus (Ad) particles. Infection of DRG neurons with control adenovirus had no effect on KCNQ2 mRNA, whereas infection with adenovirus driving REST expression resulted in a significant reduction of KCNQ2 and KCNQ3 mRNA (Fig. 4E).

**Figure 2.** The KCNQ2 and KCNQ3 promoters contain functional Sp1 sequences. A, Regions of the KCNQ2 promoter were cloned upstream of luciferase and transfected into SHSY-5Y cells. Shown are normalized luciferase values expressed relative to empty vector, pGL3 Basic (mean $\pm$ SEM; $n = 3$, $p < 0.05$). Base pair numbers are expressed relative to transcription start sites. B, Regions of the KCNQ3 promoter were cloned upstream of luciferase and transfected into SHSY-5Y cells. Shown are normalized luciferase values expressed relative to empty vector (mean $\pm$ SEM; $n = 3$, $p < 0.05$). C, Luciferase reporter vector containing the KCNQ2 or KCNQ3 promoter regions or empty vector, pGL3 Basic (Basic), were transfected into SHSY-5Y cells that were subsequently treated with either 100 nM mithramycin A or water control. D, Reverse transcriptase-PCR analysis of KCNQ2, KCNQ3, and SCN2A mRNA levels in control and mithramycin A-treated cultures of rat dorsal root ganglia cells. Levels are normalized to U6 gene (mean $\pm$ SEM; $n = 3$, $p < 0.05$). E, Antibodies to Kv7.2 and $\beta$-actin were used to analyze respective protein levels in extracts from control or mithramycin A (Mith, 100 nM)-treated SHSY-5Y cells.
Overexpression of REST reduces M-current density and changes firing properties of DRG neurons

KCNQ2 and KCNQ3 encode the two subunits Kv7.2 and Kv7.3, which together form a heterotetrameric potassium channel that is believed to be the most abundant M-channel isoform in the CNS and peripheral nervous system (Delmas and Brown, 2005). I\(_\text{M}^{\text{rest}}\) is a slowly activating and deactivating potassium current that provides a brake for repetitive action potential (AP) firing (Wang et al., 1998). To determine whether increased neuronal REST expression could downregulate the endogenous M-current, we infected cultured DRG neurons with REST-expressing adenovirus and examined the electrophysiological properties of these neurons. DRG neurons significantly differ in their function and channel expression profile; thus, to restrict our study to a more homogeneous population of neurons, we applied the TRPV1 agonist capsaicin (1 \(\mu\)M) at the end of each recording and only considered those neurons that were TRPV1 positive (nociceptive neurons). Whole-cell currents were measured from small (whole-cell capacitance of 23.7 ± 2.8 pF, \(n = 28\)) DRG neurons using a standard voltage protocol for M-current (Fig. 5A, inset). In control cells infected with adenoviral construct coding for GFP alone, the hyperpolarizing test pulse resulted in a slowly deactivating whole-cell current, characteristic of M-current, which was sensitive to the specific M-channel blocker XE991 (3 \(\mu\)M; 62 ± 4% inhibition of deactivation current, \(n = 8\)). In neurons infected with AdREST, I\(_\text{M}^{\text{rest}}\) was dramatically reduced to 14% of the value of control Ad neurons (\(p < 0.01\), \(n = 9\) for GFP and \(n = 11\) for GFP + REST-expressing neurons) (Fig. 5C). We also compared responses of virally infected neurons with 1 \(\mu\)M of the TRPV1 agonist capsaicin (measured as the inward current at −60 mV). Capsaicin responses were unaltered by overexpression of M-current (control, −21.8 ± 8.9 pA/pF, \(n = 9\); REST, −26.6 ± 7.5 pA/pF, \(n = 11\); ANOVA, \(p = NS\)) (data not shown), suggesting that the effect of REST was specific to its target genes. To further confirm that viral infection did not affect I\(_\text{M}^{\text{rest}}\) nonspecifically, whole-cell currents were measured from non-infected neurons from AdREST-exposed neuronal cultures. In these cells, I\(_\text{M}^{\text{rest}}\) was not significantly different from Ad controls (2.21 ± 0.26 vs 2.37 ± 0.48 pA/pF, \(n = 8\); \(p < 0.05\)) but was significantly larger than REST-infected cells (0.31 ± 0.10 pA/pF, \(n = 8\); ANOVA, \(p < 0.01\)) (Fig. 5C). Thus, increasing REST expression (Fig. 5A, AdREST) resulted in almost a complete absence of the M-current when compared with uninfected neurons from the same dish [Fig. 5A, AdREST (non-infected)] or neurons infected with a control adenovirus (Fig. 5A, Ad).

M-current is known to contribute to the resting membrane potential and acts as a brake on AP firing of DRG neurons (Passmore et al., 2003; Linley et al., 2008). We therefore tested whether increasing REST expression affected the AP firing properties and the resting membrane potential of DRG neurons. Using whole-cell current clamp, the membrane potential was adjusted to −65 mV by injection of current. Injection of +400 pA produced one AP in the majority (seven of nine) of Ad-infected cells and two action potentials in the remainder (two of nine) of cells with a similar pattern of firing observed in non-infected AdREST cells (five of six cells firing one AP). In contrast in REST overexpressing cells, 6 of 11 cells fired multiple action potentials (mean AP number of 6.5), which did not show accommodation (Fig. 5B), indicating that the brake on neuronal firing had been removed. REST-overexpressing neurons also were significantly depolarized compared with controls (Fig. 5D: AdREST, −60.5 ± 2.4 mV; Ad, −67.6 ± 2.6 mV; Non-infected, −68.6 ± 2.3 mV; \(n = 8\); ANOVA, \(p < 0.01\)), consistent with a reduction in M-current in these cells. REST has many gene targets (Bruce et al., 2004; Johnsson et al., 2009) in addition to the KCNQ genes, and the change in the excitability profile of a DRG neuron after REST overexpression is likely to reflect multiple changes in the expression of ion channels at the neuronal membrane. However, experimental data (Gamper et al., 2006) and modeling (Zaika et al., 2006) suggest that inhibition of M-current alone is sufficient to explain the increase in DRG neuron firing rate observed here. In additional support to this assumption, the selective Kv7 blocker XE991 produced an increase in AP firing when acutely applied to a nontransfected DRG neuron (Fig. 5E), the increase in excitability was similar to that produced by REST overexpression. These data suggest that the regulation of KCNQ gene expression levels underlies the increased neuronal excitability resulting from expression of REST.

Although its levels are normally low, neuronal REST expression was shown to increase after extended periods of neuronal overactivity seen in seizures, ischemia, and neuropathic pain (Palm et al., 1998; Calderone et al., 2003; Uchida et al., 2010).
Having shown that REST can regulate the expression of KCNQ2 and KCNQ3 in DRG neurons, we sought to determine whether REST levels in DRG neurons may be increased physiologically. We recently reported that inflammatory mediators increase the excitability of nociceptive neurons via inhibition of the M-current mediated through short-term G-protein signaling (Linley et al., 2008; Liu et al., 2010). Long-term changes in nociceptive neurons may be increased physiologically. REST levels in nociceptive neurons were very low, but they increased significantly in response to nerve injury (Fig. 6A–C). In control conditions, REST levels in nociceptive neurons were very low, but they increased significantly in response to inflammatory mediators (Fig. 6A,C). The levels of TRPV1 in nociceptive neurons did not change significantly (Fig. 6C) (TRPV1 is not a predicted target gene of REST). TRPV1-positive neurons cultured in inflammatory conditions showed lower Kv7.2 immunoreactivity than those in control conditions (Fig. 6B,C). We also analyzed M-current density in capsaicin-responsive neurons. For patch clamp, inflammatory mediators were washed out for at least 2 h before experiments to remove any acute effects on $I_M$. Inflammatory conditions dramatically reduced $I_M$ compared with control ($0.25 \pm 0.04$ compared with $0.95 \pm 0.16 \text{ pA/pF}$; $p = 0.01; n = 9$ for control and $n = 5$ for inflammatory), consistent with a downregulation of KCNQ2 and KCNQ3. These data are consistent with a mechanism whereby REST levels are increased in nociceptive neurons in response to inflammatory signals, resulting in an increase in excitability brought about by the reduced expression of KCNQ2 and KCNQ3. Such a mechanism could contribute to a long-term peripheral sensitization of inflamed sensory fibers.

**Discussion**

In this work, we have identified two common mechanisms that regulate expression of KCNQ2 and KCNQ3. We have identified consensus sequences for the Sp1 transcription factor in each of the promoter regions. These sequences are evolutionarily conserved, and we show that Sp1 directly interacts with the KCNQ2 promoter in vitro and that removal or mutation of these sequences results in reduced activity of the promoters. Furthermore, chemical inhibition of Sp1–DNA interactions result in reduced KCNQ2 and KCNQ3 mRNA expression, whereas ectopic Sp1 results in increased M-current and Kv7.2 expression in cultured neurons. Our data implicating Sp1 as an important positive-acting transcription factor that regulates KCNQ2 expression are consistent with a previous report on the human KCNQ2 promoter (Xiao et al., 2001). Xiao et al. used a bioinformatic analysis to identify GC boxes in the proximal promoter of KCNQ2. Our new data show that these GC box regions are evolutionarily conserved and are also present in the KCNQ3 promoter. Furthermore, Sp1 does in fact interact with these GC boxes, and this interaction is important for expression of the endogenous KCNQ2 and KCNQ3 mRNA in neurons. The physiological relevance of Sp1 regulation of KCNQ2 and KCNQ3 remains to be determined, and, although Sp1 is often thought of as a constitutive transcriptional activator, recent evidence suggests that Sp1 activity is important for mediating changes in neuronal gene expression in response to developmental or disease stimuli. For example, induced expression of the reelin gene during neuronal differentiation is dependent on Sp1, and mutation in the binding site for Sp1 within the reelin promoter is sufficient to prevent increased reelin expression (Chen et al., 2007). Sp1 is also important for the increased expression of the damage-induced neuronal endopeptidase (DINE) gene in response to nerve injury (Kiryu-Seo et al., 2008). In this context, Sp1 acts as a platform to which other transcription factors are recruited in response to nerve injury and activate expression of DINE (Kiryu-Seo et al., 2008).

Our data also highlight a second conserved mechanism for the regulation of KCNQ2 and KCNQ3: repression by REST. Both
KCNQ2 and KCNQ3 contain evolutionarily conserved REST binding sites that can interact with REST in vitro, and overexpression of REST in neurons results in reduced KCNQ2 and KCNQ3 mRNA and loss of M-current. Interestingly, we also identified a functional binding site for REST in the KCNQ5 gene, suggesting that KCNQ5 may also be repressed by REST. Like KCNQ2 and KCNQ3, KCNQ5 is widely expressed throughout the nervous system, and it has been suggested that Kv7.5 subunits may contribute to the M-current in vivo either on their own or in complex with Kv7.3 (Lerche et al., 2000; Schroeder et al., 2000). In a whole genome chromatin immunoprecipitation analysis to look at REST binding in human Jurkat cells, REST is enriched at the RE1 sites for KCNQ2 and KCNQ3 (Johnson et al., 2007). In that study, REST was not found to be present at the KCNQ5 RE1 site we identified, but this may be a cell-specific effect because enrichment of REST at the KCNQ5 RE1 site has been identified in several other human cell lines (Bruce et al., 2009). Although REST was originally proposed to be important in silencing neuronal-specific genes in non-neuronal cells (Chong et al., 1995; Schoenherr and Anderson, 1995), it clearly has a functional role within the nervous system. REST is expressed at low levels in neurons and regulates some target genes (Wood et al., 2003). Neuronal REST expression is upregulated in response to epileptic insults and cerebral ischemia resulting in reduced expression of brain derived neurotrophic factor (BDNF) and the glutamate receptor subunit gene GRIA2 and increased expression of the substance P gene PPT-A (Palm et al., 1998; Calderone et al., 2003; Garriga-Canut et al., 2006; Spencer et al., 2006). In addition, nuclear levels of REST protein are elevated in neurons in Huntington’s disease, leading to reduced BDNF expression (Zuccato et al., 2003, 2007).

Individuals who suffer an epileptic seizure become more susceptible to additional seizure activity (Hauser and Lee, 2002). The molecular mechanisms underlying this long-term change are not known, although, like other features of the brain that show long-lasting changes, e.g., long-term potentiation, they most likely involve changes in gene expression. The expression of several transcription factors has been shown to be increased in response to seizure activity in rodents. These include c-Fos (Morgan et al., 1987; Hiscock et al., 2001), nuclear factor-κB, Ap-1 (Rong and Baudry, 1996), DREAM

**Figure 5.** REST expression inhibits M-current and increases excitability in small-diameter DRG neurons. DRG neurons were infected with an adenoviral construct expressing GFP only (Ad) or REST and GFP (AdREST) and assessed functionally using whole-cell patch clamp. In cultures infected with REST and GFP, both green (AdREST) and non-green (AdREST non-infected) cells were tested. Only small-diameter neurons (18–35 pF) responsive to the TRPV1 agonist capsaicin (1 μM) were investigated. A, Whole-cell voltage-clamp traces obtained by 500 ms voltage pulses from -30 to -60 mV as indicated by the voltage pulse protocol (inset). Black trace represents steady-state basal current; red trace represents current in the presence of the specific M-channel blocker XE991 (3 μM). Dotted line indicates zero current.

B, Whole-cell current-clamp traces in which voltage was adjusted to -65 mV by current injection and 4 s square current pulses to different test currents were applied (inset). Black trace represents steady-state basal current; red trace represents current in the presence of the specific M-channel blocker XE991 (3 μM). Dotted line indicates zero current. C, Whole-cell current-clamp traces in which voltage was adjusted to -65 mV by current injection and 4 s square current pulses to different test currents were applied (inset). **, Bars show pooled current density data determined from the experiments shown in A. Magnitude of M-current was calculated as the XE991-sensitive deactivation current when stepping from -30 to -60 mV and normalized to cell capacitance (I_M density). Number of experiments is indicated within bars (**p < 0.01 compared to Ad and AdRes (noninfected)). D, Bars show mean resting membrane potentials of GFP, REST, and non-infected neurons. Membrane potential was measured in current-clamp mode (0 pA) (**p < 0.01 compared to Ad and AdREST (noninfected)). E, Exemplary current-clamp experiment showing excitatory effect of 3 μM XE991. Current injections at 400 pA (inset) were applied to whole-cell current-clamped DRG neurons before and during XE991 application. No voltage adjustment was performed.
downstream regulatory element antagonist modulator) (Matsura et al., 2002), and REST (Palm et al., 1998). One report has also shown that Sp1 levels increase after seizure (Feng et al., 1999), although another found no changes in levels of Sp1 (Rong and Baudry, 1996). Given our findings, an increase in expression of the transcriptional REST would result in a decrease in KCNQ2 and KCNQ3 expression, resulting in an increase in the excitability of the affected neurons. REST represses gene expression by recruiting protein complexes that modify the posttranslational marks within chromatin (Ooi and Wood, 2007). Thus, any changes induced on REST recruitment have the potential to be stably maintained even if REST expression is subsequently reduced, providing a prospective mechanism for long-term changes in gene expression levels. In addition to repressing transcription by modifying chromatin, REST has also been shown to interact with Sp1 and inhibit the ability of Sp1 to enhance transcription (Plaisance et al., 2005). Such a model is consistent with our data in which the ability of REST to repress KCNQ2 and KCNQ3 appears to be dominant over the ability of Sp1 to activate these genes and suggests that interaction between REST and Sp1 may be important for regulating the expression of many neuronal-specific genes. Consistent with such a hypothesis is the observation that, in addition to KCNQ2 and KCNQ3, many REST-regulated genes are also known to be regulated by Sp1. For example, Chrm4 (Wood et al., 1996), Nmdar1 (Bai et al., 1998), Gria2 (Myers et al., 1998), synaptophysin (Lietz et al., 2003), and Bsx (Park et al., 2007) genes are all regulated by both REST and Sp1, whereas regulation of the µ-opioid receptor gene involves an interaction with REST and the Sp1 family member Sp3 (Kim et al., 2006).

Recently, much interest has been placed on Kv7 channels as a potential target for analgesics in pain (Passmore et al., 2003; Linley et al., 2008; Brown and Passmore, 2009; Wickenden and McNaughton-Smith, 2009). Activators of Kv7 channels had an analgesic effect in a hindpaw model of chronic pain (Passmore et al., 2003), inhibited ectopic firing of axotomized sensory fibers (Roza and Lopez-Garcia, 2008), and reduced behavior associated with visceral pain (Hirano et al., 2007). Furthermore, pharmacological inhibition of M-channels at peripheral nerve terminals by acute injection of M-channel blocker XE991 produced spontaneous pain (Linley et al., 2008; Liu et al., 2010). Our data suggest that REST expression increases whereas Kv7.2 expression and M-current density decreases in DRG neurons under chronic inflammatory treatment. All these findings suggest that transcriptional downregulation of M-channel expression in nociceptive pathways may result in painful phenotype and contribute to chronic pain conditions. Moreover, peripheral axotomization (downstream regulatory element antagonist modulator) (Matsura et al., 2002), and REST (Palm et al., 1998). One report has also shown that Sp1 levels increase after seizure (Feng et al., 1999), although another found no changes in levels of Sp1 (Rong and Baudry, 1996). Given our findings, an increase in expression of the transcriptional REST would result in a decrease in KCNQ2 and KCNQ3 expression, resulting in an increase in the excitability of the affected neurons. REST represses gene expression by recruiting protein complexes that modify the posttranslational marks within chromatin (Ooi and Wood, 2007). Thus, any changes induced on REST recruitment have the potential to be stably maintained even if REST expression is subsequently reduced, providing a prospective mechanism for long-term changes in gene expression levels. In addition to repressing transcription by modifying chromatin, REST has also been shown to interact with Sp1 and inhibit the ability of Sp1 to enhance transcription in a rat model for neuropathic pain resulted in changes in the gene expression profile of DRGs (Xiao et al., 2002). Levels of neither KCNQ nor REST genes were reported in this study, but two well known REST target genes, chromagranin A and SNAP25 (synaptosomal-associated protein 25), showed reduced expression in axotomized DRGs (Xiao et al., 2002). Recently, expression of REST was shown to be upregulated after neuropathic injury (Uchida et al., 2010), thus it is tempting to speculate that the KCNQ–REST pathway discovered here may contribute to neuropathic pain pathophysiology.

Here we identify two transcription factors, Sp1 and REST, that are important for regulating expression of the potassium channel genes KCNQ2 and KCNQ3. Regulation of KCNQ2 and KCNQ3 by these transcription factors may be important for long-term changes in expression levels in response to epileptic seizure activity and during chronic pain syndromes.
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