Reactive Oxygen Species-Induced Activation of p90 Ribosomal S6 Kinase Prolongs Cardiac Repolarization Through Inhibiting Outward K⁺ Channel Activity

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Abstract—p90 ribosomal S6 kinase (p90RSK) is activated in cardiomyopathies caused by conditions such as ischemia/reperfusion injury and diabetes mellitus in which prolongation of cardiac repolarization and frequent arrhythmias are common. Molecular mechanisms underlying the electric remodeling in cardiac diseases are largely unknown. In the present study, we determined the role of p90RSK activation in the modulation of voltage-gated K⁺ channel activity determining cardiac repolarization. Mice with increased cardiac p90RSK activity due to transgenic expression of p90RSK (p90RSK-Tg) had prolongation of QT intervals and of ventricular myocyte action potential durations. Fast transient outward K⁺ current (Iₒ), slow delayed outward K⁺ current (Iₛ,slow), and steady-state K⁺ current (Iₛ) were significantly decreased in p90RSK-Tg mouse ventricular myocytes. mRNA levels of Kv4.3, Kv4.2, Kv1.5, Kv2.1, and KChIP2 from ventricles between p90RSK-Tg and nontransgenic littermate control mice were similar, as assessed by quantitative reverse transcriptase–polymerase chain reaction, indicating that p90RSK regulates voltage-gated K⁺ channels through posttranslational modification. Kv4.3- and Kv1.5- rather than Kv4.2- and Kv2.1-encoded channels in HEK 293 cells were inhibited by p90RSK. In vitro phosphorylation analysis showed that Kv4.3 was phosphorylated by p90RSK at 2 conserved sites, Ser516 and Ser550. p90RSK expression significantly inhibited Kv4.3- and Kv4.3 and KChIP2-encoded channel activities in HEK 293 cells, whereas p90RSK’s effects were blocked by amino acid mutation(s) at phosphorylation site(s) in Kv4.3. Hydrogen peroxide, a mediator of induced cardiac p90RSK activation in ischemia/reperfusion injury and diabetes mellitus, had effects similar to those of p90RSK on Kv4.3- or Kv4.3- and KChIP2-encoded channels. Fluromethylketone, a specific p90RSK inhibitor, abolished hydrogen peroxide effects. These findings indicate that p90RSK activation is critical for reactive oxygen species-mediated inhibition of voltage-gated K⁺ channel activity and leads to prolongation of cardiac repolarization. (Circ Res. 2008;103:269-278.)

Key Words: arrhythmias ■ cardiac repolarization ■ hydrogen peroxide ■ p90RSK ■ phosphorylation ■ reactive oxygen species ■ voltage-gated outward K⁺ currents

Arrhythmia is a common clinical feature in cardiac abnormalities such as coronary artery atherosclerosis, cardiac hypertrophy and failure, and diabetic cardiomyopathy.1 Downregulation of fast transient outward K⁺ current (Iₒ) has been shown in hypertrophied, failing, ischemic, and diabetic hearts,2-10 and it is associated with prolongation of cardiac repolarization, prompting speculation that decreased Iₒ may play an important role in the increased incidence of cardiac arrhythmias in these cardiac diseases. Identification of the specific pathways involved in regulation of the channel (Iₒ) activity might lead to the development of innovative therapeutic strategies for preventing the onset of arrhythmias.

Molecular mechanisms underlying cardiac arrhythmias remain poorly understood, particularly in regard to posttranslational modulation of channel (Iₒ) activity through kinase-mediated phosphorylation. p90 ribosomal S6 kinase (p90RSK) is a serine/threonine kinase that contains 2 functional kinase domains.11 The N-terminal kinase domain belongs to the AGC group of kinases, which include protein kinase A and protein kinase C. The N-terminal kinase is responsible for phosphorylating RSK substrates and recognizes the basic consensus motif: (R/K)XRXX(S/T) or RRX(S/T).11,12 The C-terminal kinase belongs to the calcium/calmodulin-dependent kinase group of kinases, and the only known function of the C-terminal kinases is regulation of the N-terminal kinase activity. The C-terminal tail also contains a short docking motif, which is responsible for the specific association of p90RSK and ERK1/2.11,13,14 p90RSK activity...
is dramatically increased in failing human hearts,15 guinea pig hearts with ischemia or ischemia/reperfusion injuries,16 and hearts from mice with diabetes mellitus induced by streptozotocin.17

p90RSK activation was convincingly linked to cardiac dysfunctions in cardiomyopathies associated with electric remodeling. We tested the hypothesis that p90RSK is an important molecule modulating voltage-gated K+/H+ channel activity and its activation prolongs cardiac repolarization. The electric remodeling was determined in mice with cardiac-specific expression of p90RSK (p90RSK-Tg), and molecular mechanisms involved in the modulation of voltage-gated K+ channel activity by p90RSK were investigated in vitro.

Materials and Methods
Mice were handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals; all protocols were approved by the University of Rochester Animal Study Committee. The methods/protocols used in the present study are detailed in the online Data Supplement (http://circres.ahajournals.org).

Results
QT Intervals Are Prolonged in p90RSK Transgenic Mice
Surface electrocardiograms from lead II were recorded from the conscious unrestrained nontransgenic littermate control (NLC) and p90RSK-Tg adult (8 to 12 weeks) mice (Figure 1A–B). QT interval was determined manually by placing cursors at the beginning of the QRS signal and the end of the T-wave, and it was prolonged in p90RSK-Tg mice (Figure 1B; mean ± SEM). QT intervals were significantly longer (P < 0.01; Figure 1C; mean ± SEM). QTc intervals were 50.2 ± 0.9 ms (n = 9) and 61.2 ± 2.3 ms (n = 8) in NLC and p90RSK-Tg mice, respectively. Neither PR intervals nor heart rates (RR intervals) were affected by p90RSK expression (Figure 1C–E; mean ± SEM). RR intervals were 106.5 ± 2.5 ms (n = 9) and 108.9 ± 5.5 ms (n = 8), and PR intervals were 34.7 ± 1.3 ms (n = 9) and 36.6 ± 1.5 ms (n = 8) in NLC and transgenic mice, respectively. When QT intervals were corrected for heart rate, the differences between these 2 groups were also significant (P < 0.01; Figure 1C; mean ± SEM).

Figure 1. QT interval prolongation in p90RSK-Tg mice. Electrocardiograms were recorded from NLC (A) and p90RSK-Tg conscious unrestrained mice (B). QT and QTc intervals were significantly prolonged in p90RSK-Tg mice (C; **P < 0.01) compared with those in NLC. PR interval, RR interval, and heart rate were not different between nontransgenic and transgenic mice (C–E).

Action Potential Durations Are Prolonged in p90RSK-Tg Mouse Ventricular Myocytes
Action potentials (APs) recorded from p90RSK-Tg mouse ventricular myocytes were substantially broader than those obtained from NLC cells at physiologically relevant temperature (35°C) with a stimulation frequency of 8 Hz (Figure 2A; mean ± SEM). AP durations at 90%, 75%, 50%, and 25% repolarization (APD90, APD75, APD50, and APD25) were 12.4 ± 1.5 ms, 5.2 ± 0.5 ms, 4.0 ± 0.5 ms, and 3.0 ± 0.3 ms in NLC (n = 5), and 22.9 ± 1.6 ms, 5.6 ± 0.7 ms, 4.0 ± 0.5 ms and 3.0 ± 0.4 ms in p90RSK-Tg ventricular myocytes (n = 5), respectively; APD90 were significantly different (P < 0.01) between these 2 groups (Figure 2B). However, AP amplitudes and resting membrane potentials were not significantly different (Figure 2C–D).

Total Outward K+ Currents Are Attenuated in p90RSK-Tg Mouse Ventricular Myocytes
We next tested the hypothesis that p90RSK activation leads to the prolongation of cardiac repolarization through modulation of voltage-gated K+ channels. Outward K+ currents were recorded from myocytes isolated from ventricular-free walls of adult (8 to 12 weeks) mice at room temperature (24°C). After establishing whole-cell recording, the currents were recorded by depolarizing to −60 mV and hyperpolarizing −80 mV from a holding potential (HP), −70 mV, to determine cell capacitance. Then, the cells were depolarized from −40 mV to +60 mV with durations of 4.5 seconds or
500 ms and 10-mV interval. Cell capacitances were not different between NLC and p90RSK-Tg ventricular cells (136.0±10.6 pF, n=16 versus 117.2±7.0 pF, n=15; Figure 3E). Outward K⁺ currents at all test potentials were lower in cells from transgenic animals (Figure 3B, D) compared with those in NLC myocytes (Figure 3A, C). Mean±SEM peak outward current densities at +40 mV were 64.7±5.8 pA/pF (n=16) and 45.8±3.0 pA/pF (n=15) in NLC and p90RSK-Tg ventricular myocytes, respectively; these values were significantly different (P<0.01; Figure 3F).

Figure 2. AP prolongation in p90RSK-expressing ventricular myocytes. APs were recorded from mouse ventricular myocytes at 35°C with a stimulation frequency of 8 Hz. AP is prolonged in p90RSK-expressing ventricular myocyte compared with that in NLC (A). APD₉₀ is significantly (**P<0.01) prolonged in p90RSK-Tg ventricular cells compared with NLC (B). AP amplitude and resting membrane potential were not significantly different between these 2 groups (C–D).

Figure 3. Ventricular outward K⁺ currents are decreased in p90RSK-Tg mice. Outward K⁺ currents recorded from NLC (A, C) and p90RSK-Tg (B, D) mouse ventricular myocytes were evoked during 4.5 seconds (A–B) or 500 ms (C, D) depolarizing voltage steps to potentials between −40 and +60 mV from a HP of −70 mV. Each trial was preceded by a brief (20 ms) depolarization to −20 mV to eliminate voltage-gated Na⁺ currents not being blocked by tetrodotoxin. Outward K⁺ currents in NLC (A, C) and transgenic (B, D) myocytes were distinct; peak outward K⁺ current amplitudes are reduced in p90RSK-expressing cells (B, D) compared with those in NLC cells (A, C). The decay phases of K⁺ currents in A and B were analyzed to provide amplitudes of $I_{to,f}$, $I_{K,slow}$, and $I_{ss}$ in individual cells and normalized to cell capacitance to obtain the current densities. Time constants of fast and slow components were also obtained. Cell capacitance is unchanged by p90RSK expression (E). Mean±SEM current densities of $I_{peak}$, $I_{to,f}$, $I_{K,slow}$, and $I_{ss}$ are significantly decreased (∗P<0.05 or **P<0.01) in p90RSK-expressing ventricular myocytes (F). Both slow and fast time constants are unaltered by p90RSK expression (G).
transgenic mouse ventricular myocytes

6 ms and 1328 myocytes (n=15) were

tion at different (Figure 3G). As reported previously, neither time

We next determined if K\textsubscript{ChIP2} Are Unaltered in p90RSK

mRNAs of Kv4.3, Kv4.2, Kv2.1, Kv1.5, and

Result 1).

The current decay during 4.5 seconds depolarization in NLC

was applied to cells, and 4-AP sensitive currents were

significantly decreased in p90RSK-Tg ventricular cells com-

pared with NLC (Supplemental Figure 1 and Supplemental

Figure 5). 50 mol/L 4-amiopyridine (4-AP) blocking

I\textsubscript{to,f}, and I\textsubscript{SS} densities at +40 mV in NLC ventricular

cells (n=16) were 32.0±3.8 pA/pF, 21.0±2.0 pA/pF, and

9.7±0.7 pA/pF, respectively; mean±SEM densities of I\textsubscript{to,f},

I\textsubscript{slow}, and I\textsubscript{SS} at +40 mV were 23.1±2.2 pA/pF, 15.2±1.3

pA/pF, and 7.3±0.7 pA/pF in p90RSK-Tg ventricular

cells (n=15). They were significantly different (P<0.05; Figure

3F). 50 μmol/L 4-amiopyridine (4-AP) blocking I\textsubscript{slow} was

applied to cells, and 4-AP sensitive currents were

significantly decreased in p90RSK-Tg ventricular cells com-

pared with NLC (Supplemental Figure 1 and Supplemental

Result 1).

mRNAs of Kv4.3, Kv4.2, Kv2.1, Kv1.5, and

KChIP2 Are Unaltered in p90RSK-Tg

Mouse Ventricles

We next determined if K\textsuperscript{+} channel subunit transcripts are

affected by p90RSK. SYBR green quantitative reverse trans-

criptase–polymerase chain reaction was performed on ven-

tricles of NLC (n=5) and p90RSK-Tg mice (n=5) using Kv

α and KChIP2 subunit specific primers (Supplemental Table

1). The analysis revealed that there was no significant

difference in Kv4.3, Kv4.2, Kv2.1, Kv1.5, or KChIP2 expres-

sion between NLC and p90RSK-Tg ventricles (Figure 4).

These data indicate that downregulation of outward K\textsuperscript{+}

currents is likely due to posttranslational modulation of K\textsuperscript{+}

channel by p90RSK.

p90RSK Inhibits Kv4.3- and Kv1.5- Rather Than

Kv4.2- and Kv2.1-Encoded Channel Activities

To determine which Kv α channel is modulated by p90RSK, HEK

293 cells were transfected with plasmids encoding Kv4.3, Kv4.2, Kv2.1, or Kv1.5 (which were provided by Dr Jeanne M. Neronne’s laboratory at Washington University School of Medicine in St Louis) and green fluorescent protein and with plasmids lacking or having p90RSK sequence.

Green fluorescent protein expression allowed us to visualize

cells for whole-cell recording. Kv4.3-expressed K\textsuperscript{+} currents were reduced by p90RSK (Figure 5Ab). Kv4.3-encoded channel inactivation was accelerated (Figure 5Ab), and peak current densities were significantly reduced (P<0.05) by p90RSK at +20 to +40 mV (Figure 5Ac). Analysis of the decay phases of outward K\textsuperscript{+} currents evoked during 4.5-second depolarization revealed that the current decay was well described by the sum of 2 exponentials, and fast and slow inactivation time constants analyzed from Kv4.3-encoded currents with or without p90RSK were voltage-independent and p90RSK significantly accelerated Kv4.3 channel inactivation at +20 to +40 mV (Figure 5Ad). Peak outward Kv1.5 currents were decreased by p90RSK (Figure 5Bb). Kv1.5 current densities were significantly reduced (P<0.01) by p90RSK at −30 to +60 mV (Figure 5Bc). Analysis of Kv4.3 and Kv1.5 K\textsuperscript{+} current activation phases revealed that activation of currents was well described by one exponential. Activation time constants of Kv4.3 or Kv1.5 currents were unchanged at +40 mV by p90RSK (Kv4.3:1.2±0.1 ms, n=11 versus Kv4.3+p90RSK:1.3±0.2 ms, n=11; Kv1.5: 2.1±0.2 ms, n=8 versus Kv1.5+p90RSK: 2.0±0.2 ms, n=9). p90RSK did not shift the voltage
Figure 5. Kv4.3- and Kv1.5-encoded channel activities are inhibited by p90RSK expression. A, Outward K⁺ currents were evoked during 4.5-second depolarizing voltage steps to potentials between −60 and +60 mV from a HP of −70 mV in HEK 293 cells expressing Kv4.3 (a) or Kv4.3 and p90RSK (b). Kv4.3-expressed K⁺ currents are markedly reduced by p90RSK (b). Kv4.3 current inactivation is accelerated by p90RSK (b). Mean±SEM Kv4.3 current densities in cells expressing Kv4.3 (open circles; n=11) and Kv4.3 and p90RSK (filled circles; n=11) were plotted as a function of test potentials and they are significantly different (*P<0.05) at voltage of −20 to −40 mV (c). Mean±SEM fast and slow inactivation time constants (τ values) analyzed from currents recorded from cells expressing Kv4.3 or Kv4.3 and p90RSK were plotted as a function of test potentials (d). They are voltage-independent and they are significantly different at 0 to +40 mV (**P<0.01) between these 2 groups (open circle and square: Kv4.3; filled circle and square: Kv4.3+p90RSK) (d). B, Outward K⁺ currents were evoked during 100-second depolarizing voltage steps to potentials between −40 and +60 mV from a HP of −70 mV in HEK 293 cells expressing Kv1.5 (a) or Kv1.5 and p90RSK (b). Kv1.5-expressed K⁺ currents are markedly reduced (b). Mean±SEM Kv1.5-(open circles; n=8) and Kv1.5- and p90RSK-expressed current densities (filled circles; n=9) were plotted as a function of test potentials; they are significantly different (**P<0.01) at voltages of −30 to +60 mV.
dependence of activation of Kv4.3 or Kv1.5 currents (data not shown). Kv4.2- or Kv2.1-expressed currents were unaltered by p90RSK (Supplemental Figure 2 and Supplemental Result 2).

p90RSK Phosphorylates Kv4.3

Kv4.3 protein has 2 conserved phosphorylation sequences, RSRPLS10 and RLRS550 that are located at the C-terminus. To confirm that p90RSK can phosphorylate these 2 serine sites, 4 constructs encoding GST-Kv4.3-C-terminus fusion proteins were generated. Results in Supplemental Figure 3 showed that wild-type GST-Kv4.3-CT fusion protein at 40 kDa was phosphorylated (lane 1), and phosphorylation was largely abolished by double mutations (Ala516 and Ala550; lane 7).

These results indicate that p90RSK phosphorylates Kv4.3 at Ser516 and Ser550. Unexpectedly, phosphorylation intensity of wild-type Kv4.3-CT was slightly increased by single amino acid mutation Ala516 (lane 3) or Ala550 (lane 5). These could be due to one mutation enhancing phosphorylation intensity in other site(s). Three same experiments were performed to show similar results.

p90RSK Inhibits Kv4.3-encoded Channel Activity But Phosphorylation Site Mutation(s) Block Its Effects

We next determined the effects of p90RSK-mediated phosphorylation of the Kv4.3 channel. Kv4.3 mutants with single Ala516 or Ala550 or double Ala516 and Ala550 mutations were generated in the rat short form Kv4.3-pcDNA3.1. These findings indicate these 2 serine amino acids are also important for channel activity.

Kv4.3 and KChIP2-Encoded Channel Activity Is Reduced But Phosphorylation Site Mutations Block p90RSK’s Effects

As expected, Kv4.3- and KChIP2-expressed currents in HEK 293 cells were reduced by p90RSK expression (Figure 6).
accelerated in cells treated with 30 μmol/L H₂O₂ for 2 hours (Figure 7B) compared with the control group (Figure 7A). Peak outward current densities were decreased by H₂O₂, but there was no significant difference (Figure 7E). Analysis of the current decay phases revealed that the inactivation time constant of Kv4.3 fast component was significantly decreased (*P<0.05) at +40 mV (Figure 7F), whereas inactivation of the slow component was not affected (n=7; G). fmk does not affect Kv4.3 expressed currents (C) in 1 μmol/L concentration (n=18), but it prevents H₂O₂’s effects (n=5; D). Acceleration of Kv4.3 fast component inactivation by H₂O₂ is abolished by fmk (n=5; F).

Fluoromethylketone Does Not Affect Nontransgenic Littermate Control Ventricular Outward K⁺ Currents and Action Potential But It Rescues Them Inhibited or Prolonged by p90RSK

We next focused on studies of fmk effects on outward K⁺ currents in NLC and p90RSK-Tg ventricular cells. Ventricular myocytes were incubated with fmk for 2 hours, and outward K⁺ currents and action potentials were recorded at room temperature (24°C). As illustrated in Figure 8A, 3 μmol/L fmk did not affect NLC outward K⁺ currents (Figure 8Ab), but it rescued the K⁺ current inhibition by p90RSK (Figure 8Ad; mean±SEM). I_peak, I_in, I_K,slow, and I_SS densities at +40 mV obtained from NLC +3 μmol/L fmk (n=4) and NLC (n=16) cells were 54.9±12.6, 28.1±7.8 pA/pF, 19.0±4.7 pA/pF, and 7.3±0.3 pA/pF, and 64.7±5.8 pA/pF, 32.0±3.8 pA/pF, 21.0±2.0 pA/pF, and 9.7±0.7 pA/pF, respectively; and they were not significantly different (mean±SEM). I_peak, I_in, I_K,slow, and I_SS densities at +40 mV obtained from p90RSK-Tg +3 μmol/L fmk (n=5) and p90RSK-Tg (n=15) cells were 45.8±3.0 pA/pF, 23.1±2.2 pA/pF, 15.2±1.3 pA/pF, and 7.3±0.7 pA/pF, and 59.0±5.7 pA/pF, 33.7±4.4 pA/pF, 15.3±2.6 pA/pF,
and 8.1±1.3, respectively; and \(I_{\text{Peak}}\) and \(I_{\text{out}}\) densities were significantly different \((P<0.05)\) in these 2 groups. Mean±SEM fast and slow decay time constants of the currents recorded from p90RSK-Tg or NLC cells in the presence of 3 \(\mu\)mol/L fmk were not significantly different from those in NLC cells (data not shown). One micron fmk did not affect NLC and p90RSK-Tg ventricular outward \(K^+\) currents (data not shown). APs recorded from p90RSK-Tg ventricular cells with a stimulation frequency of 1 Hz were substantially broader than those in NLC cells (Figure 8Bc; mean±SEM). APD90, APD75, APD50, and APD25 were 19.0±2.6 ms, 9.0±0.9 ms, 4.9±0.3 ms, and 3.2±0.3 ms in NLC cells \((n=10)\), and 44.2±7.2 ms, 14.9±2.2 ms, 7.1±0.8 ms, and 3.2±0.3 ms in p90RSK-Tg cells \((n=9)\), respectively. APD90, APD75, and APD50 in these 2 groups were significantly different \((P<0.01 \text{ or } P<0.05)\).

As shown in Figure 8Bd, AP recorded from ventricular myocyte expressing p90RSK treated with 3 \(\mu\)mol/L fmk for 2 hours was similar to that in NLC cells (Figure 8Ba) but it was shorter than that in p90RSK-Tg cell (Figure 8Bc). NLC AP was unaltered by fmk (Figure 8Bb; mean±SEM). APD90, APD75, APD50, and APD25 were 11.1±0.7 ms, 8.2±0.7 ms, 6.2±1.0 ms, and 4.3±0.5 ms in NLC+fmk cells \((n=4)\), and 15.6±0.8 ms, 8.8±0.4 ms, 5.8±0.3 ms, and 4.0±0.1 ms in p90RSK-Tg+fmk cells \((n=6)\); and they were not significantly different. APD values were not significantly different either between NLC and p90RSK-Tg+fmk groups. AP amplitudes and resting membrane potentials among these 4 groups were not significantly different (data not shown).

Discussion

Prolongation of Cardiac Repolarization by Activation of p90RSK

The results presented here reveal that activation of p90RSK inhibited \(I_{\text{out}}\), \(I_{\text{K,slow}}\), and \(I_{\text{SS}}\) and led to the prolongation of cardiac repolarization without affecting mRNA levels of Kv subunits. \(I_{\text{out}}\) decrease was caused by p90RSK phosphorylating Kv4.3 subunit and reduction of \(I_{\text{K,slow}}\) was due to p90RSK inhibiting Kv1.5-encoded channel activity. \(H_2O_2\), a mediator of induced cardiac p90RSK activation in ischemia/reperfusion injury and diabetes mellitus, had effects similar to p90RSK in the modulation of Kv4.3 or Kv4.3 and KChIP2-encoded channel activity. Importantly, a p90RSK-specific inhibitor, fmk,23 was able to abolish \(H_2O_2\)'s effects on Kv4.3 channel activity and rescued the mouse ventricular \(K^+\) channel activity inhibited by p90RSK. To our knowledge, this is the first report to document that activation of p90RSK leads to the prolongation of cardiac repolarization by inhibiting voltage-gated outward \(K^+\) currents.

Downregulation of outward \(K^+\) currents \((I_{\text{out}})\) has been observed in a variety of cardiac abnormalities associated with prolongation of cardiac repolarization,2–10 which predisposes to cardiac arrhythmias. Both transcriptional and posttranslational modulations of \(K^+\) channels are able to alter the channel activities. Phosphorylation is a ubiquitous means of modulating protein function; a number of kinases, including protein kinase A, protein kinase C, and calcium/calmodulin-dependent kinase II, have been shown to modulate the function of at least one type of \(K^+\) channel, including Kv4.2 and Kv4.3.24–29 p90RSK activity is dramatically increased in

Figure 8. fmk did not affect ventricular outward \(K^+\) currents and action potential in NLC mice, but it rescued them, which was inhibited or prolonged by p90RSK. A, Outward \(K^+\) currents were recorded from NLC (a) and p90RSK-Tg ventricular myocyte (c). Three microns fmk does not affect NLC \(K^+\) currents (b), but it rescues them from p90RSK inhibition (d). B, AP was recorded from NLC (a) and p90RSK-Tg ventricular myocyte (c) with a stimulation frequency of 1 Hz. AP is prolonged in p90RSK-expressing ventricular myocyte (c) compared with that in NLC (a). Three microns fmk does not affect NLC AP (b), but it rescues broader p90RSK-Tg AP (c) to normal waveforms (d).
failing human hearts,15 guinea pig hearts with ischemia or ischemia/reperfusion,16 and hearts from mice with diabetes mellitus induced by streptozotocin,17 in which \( I_{\text{off}} \) was consistently reported to be downregulated.8–10 It has been reported that p90RSK can phosphorylate Na+/H+ exchanger and troponin I, and its activation provokes cardiac dysfunction as seen in cardiomyopathies caused by conditions such as ischemia/reperfusion injury16 and diabetes mellitus.17 \( I_{\text{off}} \) is encoded by Kv4.2, Kv4.3, and KChIP2.30 Kv4.3 expresses in both rodent animals30 and humans31 and has 2 p90RSK conserved phosphorylation sequences. Our findings indicate that p90RSK is another kinase involved in the downregulation of \( I_{\text{off}} \) through phosphorylation of Kv4.3. p90RSK accelerates Kv4.3 alone-encoded channel inactivation, but it did not change the inactivation of Kv4.3 and KChIP2-encoded channel or \( I_{\text{on}} \). KChIP2 interacts with Kv4.3 (Kv4.2) and decelerates the inactivation of Kv4.3 (Kv4.2) channel and increases Kv4.3 (Kv4.2) channel expression.30 KChIP2 may play an important role in the p90RSK regulation of \( I_{\text{off}} \) complex channel. Kv4.3 with double mutation (Ala516 and Ala550)-expressed currents were significantly decreased, indicating these 2 amino acids are also important for channel activity. p90RSK inhibits \( I_{\text{K,slow}} \) (Kv1.5) channel activity, but the mechanism remains to be investigated. Although studies of p90RSK’s effects on \( I_{\text{K}} \) and \( I_{\text{Ks}} \) were not performed, analysis of these 2 channel proteins revealed that there is one conserved p90RSK phosphorylation sequence, RRGS27 in \( I_{\text{K}} \) channel, and 4 conserved sequences, RRA283, RQRKRRLLS890, RRRT895, and RRLS1137, in the \( I_{\text{Ks}} \) channel, all of which were reported to be protein kinase A phosphorylation sites.32,33 Phosphorylation of these sites affects both channel activities.32,33 Studies of these 2 channel modulations by p90RSK are warranted.

Our studies showed that p90RSK activation inhibited cardiac \( I_{\text{off}}, I_{\text{K,slow}}, \) and \( I_{\text{Ks}} \) channel activities and led to prolongation of cardiac repolarization in p90RSK-Tg mice at the age of 8 to 12 weeks. This electric remodeling could be caused by cardiac structural abnormality induced by activation of p90RSK. However, cell capacitances representative of cell sizes were not different between NLC and p90RSK-Tg groups at this age. In addition, previous studies showed that p90RSK-Tg mice did not have any phenotypic changes to suggest cardiac hypertrophy and dysfunction at the age of 8 to 12 weeks as assessed by gross morphometric, histological, and noninvasive echocardiographic measurements.17 They only exhibited cardiac dysfunction after 6 to 8 months of age.17 All these findings support that p90RSK directly affects voltage-gated outward K+ channel activity.

**Novelty and Potential Significance**

p90RSK has been shown to be activated in different cardiac diseases to potentially modulate cardiac function.15–17,22,34 Our study is first to show that activation of p90RSK prolongs the QT interval through inhibiting outward K+ channel activity. Our results are relevant to the understanding of the molecular determinants of prolongation of cardiac repolarization predisposing to cardiac arrhythmias and potentially to the development of new therapeutic approaches. The present study demonstrates targeted inhibition of p90RSK and subsequent prevention of Kv channel activity reduction in response to agonists such as H2O2 produced in different types of cardiac abnormalities. Further studies of perturbing p90RSK signaling in animal models by the specific inhibitor or dominant negative p90RSK22 will be very important to address whether p90RSK inhibitor could be a drug for potentially preventing or treating cardiac arrhythmias.

**Potential Limitations**

We recorded surface electrocardiograms exhibiting prolongation of QT intervals and observed no ventricular arrhythmias in p90RSK transgenic mice. Further studies including increase in heart rate by a β-receptor agonist or intracardiac pacing may induce ventricular tachycardia or fibrillation in these transgenic mice and provide a useful animal model for antiarrhythmic drug selection. In this study, we focused on voltage-gated outward K+ currents altered by p90RSK, but effects of p90RSK on other ionic currents, including inward-rectifier K+ currents, L-type Ca2+ currents, and Na+ currents, participating in activation of p90RSK-induced prolongation of cardiac repolarization, are also likely. However, the extensive additional experiments required to address these issues as discussed go beyond the context of the present study.

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**Disclosures**

None.

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**Index Terms**

- Cardiac arrhythmia
-atrial fibrillation
-chronic heart failure
- myocardial remodeling
- potassium currents
- cardiac repolarization
- p90RSK
- Kv channel
- cardiac hypertrophy
- hypertrophied rabbit hearts.


Reactive Oxygen Species-Induced Activation of p90 Ribosomal S6 Kinase Prolongs Cardiac Repolarization Through Inhibiting Outward K+ Channel Activity

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Correction

In the article by Lu et al (Circ Res. 2008;103:269–278), in the legend for Figure 8, μM was mistakenly changed to microns during the copyediting of the article. It should read μmol/L. The corrected legend is provided below, and the corrected article can be found online at http://circres.ahajournals.org. The publisher regrets the error.

Figure 8. fmk did not affect ventricular outward K+ currents and action potential in NLC mice, but it rescued them, which was inhibited or prolonged by p90RSK. A, Outward K+ currents were recorded from NLC (a) and p90RSK-Tg ventricular myocyte (c). Three μmol/L fmk does not affect NLC K+ currents (b), but it rescues them from p90RSK inhibition (d). B, AP was recorded from NLC (a) and p90RSK-Tg ventricular myocyte (c) with a stimulation frequency of 1 Hz. AP is prolonged in p90RSK-expressing ventricular myocyte (c) compared with that in NLC (a). Three μmol/L fmk does not affect NLC AP (b), but it rescues broader p90RSK-Tg AP (c) to normal waveforms (d).
Online Supplement

Material and Methods

Recording of Mouse Electrocardiograms

Surface electrocardiograms (ECGs) from lead II were recorded from adult (8-12 weeks) conscious unrestrained FVB non-transgenic littermate control (NLC) and p90RSK-Tg mice. The hair of the mouse chest was shaved. Two electrodes were placed on the right anterior and superior, and left anterior and inferior chest surfaces, respectively, and they were connected to a Bioamplifier (AD instrument, CO) through the electrical swivel 6 channel (Promed-Tec Inc. Bellingham, MA). The mice were able to freely walk around in the cage. ECG recordings were obtained at a frequency response of 0.05 to 500 Hz. Signals were digitized at 2 kHz and recorded on a personal computer.

Cell Culture

Mouse ventricular myocytes, isolated from 8-12 week-old FVB mice were used for all electrophysiological experiments. Single cells were isolated as previously described, \(^1,2\) plated on laminin-coated coverslips and maintained in serum-free medium-199 (M-199) for up to 12 hours.

HEK-293 cells, obtained from the American Tissue Culture Collection, were maintained in MEM (Gibco) supplemented 10% heat-inactivated fetal bovine serum (FBS) and 1 unit/ml penicillin-streptomycin. Cells were passaged at confluence (every 3-4 days) by brief trypsinization.

Electrophysiological Recordings

Action potentials (APs) were recorded at room temperature (24°C) with a stimulation frequency of 1 Hz or physiologically relevant temperature (35°C) with a
stimulation frequency of 8 Hz. Total outward $\text{K}^+$ currents from mouse ventricular myocytes and currents from HEK 293 cells expressing interested genes included in our study were recorded using whole-cell patch-clamp techniques at room temperature (24°C). Experiments were conducted using a pClamp 200 amplifier (Axon) interfaced to a Dell computer equipped with a Digidata 3200 Series analog/digital interface (Axon) and pClamp 9 software (Axon). The bath solution for recording of APs contained (in mM): 136 NaCl, 4 KCl, 1 CaCl$_2$, 2 MgCl$_2$, 10 HEPES, and 10 glucose, pH 7.4. For recording voltage-gated outward $\text{K}^+$ currents from ventricular myocytes, 0.02 mM tetrodotoxin and 5 mM CoCl$_2$ were added into the bath solution to block sodium and calcium currents, respectively. The resistance of the glass pipette was 2–4 M$\Omega$ after filling with a recording pipette solution contained (in mM): 135 KCl, 1 MgCl$_2$, 10 EGTA, 10 HEPES and 5 glucose, pH 7.2. Whole-cell membrane series resistances were routinely compensated electronically ($\geq 85\%$); voltage errors resulting from the uncompensated series resistance were not corrected. Only data obtained from cells with input resistances $\geq 0.7 \text{ G}\Omega$ were analyzed. From a holding potential of -70 mV, voltage-gated outward $\text{K}^+$ currents were evoked during 100 ms, 500 ms, 800 ms or 4.5 s depolarizing voltage steps to potentials between –40 or -60 mM and +60 mV in 10 mV increments. The voltage steps were applied at 10 s intervals. Experimental data were acquired at variable sampling frequencies and the current signals were filtered on-line at 5 kHz before digitization and storage.

**RNA Preparation and Quantitative Real Time RT-PCR**

Hearts were excised from 10-week-old WT ($n = 5$) and p90RSK-Tg ($n = 5$) mice. All animals were sacrificed by cervical dislocation. Ventricles were dissected from each
heart and flash-frozen in liquid nitrogen for RNA isolation. Total RNA was isolated and DNase treated using the RNeasy Fibrous Tissue Mini Kit (Qiagen). Genomic DNA contamination was assessed by PCR amplification of total RNA samples without prior cDNA synthesis; no genomic DNA was detected.

cDNA synthesis was performed Quantitative PCR amplification was performed with gene-specific primers for Kv1.5, Kv2.1, Kv4.2, Kv4.3, KChIP2 and β-actin was determined by quantitative real-time using an iCycler real-time PCR machine using iQ SYBR Green super-mix (both from Bio-Rad Laboratories) according to the manufacturer's instructions. The primer sequences are seen in table 1. The quantity of Kv α and β subunit mRNAs was then obtained by division of each value by the actin value. The relative expression levels were calculated.

**Generation of Kv4.3 mutants**

To determine if activation of p90RSK is via phosphorylation of Kv4.3 to modulate I_{to,f}, the mutants of Kv4.3 short isoform S516A, and S550A, and the double-mutant S516A and S550A of rat K_v4.3 were prepared by performing PCR-based site-directed mutagenesis using the Quick-Change Multi site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The following primers were used for PCR: rK_v4.3S516A:

5'CAGAACTACCATCCACCAGAAGCCCTGCTCTGTCCAGCCACTCGG-GCC-3' (nt 1,539–1,587); rK_v4.3S550A:

5'CTCCAACCTGCGCGCCACCCGCCTGCAGCGCGCATGCAAGAGGCTCAGCACC-3' (nt 1,640–1,688). Underlined characters show the mutation sites. Each sequence of rK_v4.3
mutants (rKv4.3S516A, rKv4.3S550A, and rKv4.3 S516A and S550A) was confirmed by DNA sequencing.

**GST–Kv4.3-CT fusion protein construction and purification**

The DNA segment coding the cytoplasmic, carboxyl-terminal tail of Kv4.3 (aa 505-628) was generated by polymerase chain reaction amplification of nucleotides (1533-1904) of the rat short form Kv4.3-pcDNA3.1 plasmid and cloned into the BamHI/EcoRI sites of the pGEX-KG expression vector. This construct is referred to as wild type (WT) GST-Kv4.3-CT. Two serine site single or double mutants of GST-Kv4.3-CT were prepared from WT GST-Kv4.3-CT as described above. The fidelity of all GST-Kv4.3-CT mutants was confirmed by DNA sequencing. GST fusion proteins were expressed in *Escherichia coli* (BL21) by induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 3 hours at 37°C. Cells were lysed by brief sonication on ice in lysis buffer containing 50 mM Tris, pH 8.0 with 1 mM EDTA, 100 mM NaCl, and 2.5 μl of Sigma proteinase inhibitor cocktail and then solubilized with 1% Triton X-100. GST fusion proteins were affinity purified from clarified cell lysates by overnight incubation at 4°C with glutathione-Sepharose™ beads (Amersham Pharmacia Biotech AB) followed by extensive washes with lysis buffer.

**p90RSK in vitro Kinase Assay**

Washed GST-Kv4.3-CT fusion protein beads were incubated in 50 μl of reaction buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 10 mM MnCl₂), 5 ng RSK1 (Upstate), 10 μCi of [γ−32P]ATP (Amersham Bioscience), 100 μM of ATP for 30 min at 30°C. The reaction was terminated by adding appropriate amount of sample loading buffer. After boiling for 10 minutes, samples were subjected to SDS-polyacrylamide gel
electrophoresis, and proteins were transferred to nitrocellulose membranes. Immunoreactive bands were visualized by exposing the membrane to a film (Kodak).

**Data Analysis**

Analysis of electrophysiological data was completed using *Clampfit* 9.0 (Axon). Whole-cell membrane capacitances were calculated by integrating the capacitive transients elicited during ± 10 mV voltage steps from the holding potential of -70 mV. Peak outward K⁺ currents were measured as the difference between the maximal outward current amplitudes and the zero current level. The decay phases of the outward K⁺ currents evoked during 800 ms or 4.5 s depolarizing voltage steps in HEK-293 cells and mouse ventricular cells were fitted by single or double exponential functions, respectively, using one of the following equations: $y(t) = A_1\exp(-t/\tau_1) + B$ or $y(t) = A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2) + B$, where t is time, $A_1$ and $A_2$ are the amplitudes, and $\tau_1$ and $\tau_2$ are the time constants of decay, of the inactivating K⁺ current components. In most mouse ventricular myocytes, two prominent components of inactivation, reflecting the expression of the fast transient outward K⁺ current, $I_{o,t}$, and the slowly inactivating outward K⁺ current, $I_{K,s}$, Mean ± S.E.M. decay time constants ($\tau_{\text{decay}}$) reported here were obtained from recordings at +40 mV. Resting membrane potentials, action potential amplitudes and action potential durations at 25% (APD₂₅), 50% (APD₅₀), 75% (APD₇₅) and 90% (APD₉₀) repolarization were measured using *Clampfit* (Axon). For analysis of ECGs, the onsets and offsets of the P, Q, R, S, and T waves were determined by measuring the earliest (onset) and the latest (offset) times from II lead. Measured QT intervals were also corrected for differences in heart rate using the formula $QTc=QT0/(RR/100)^{1/2}$, as previously described.⁴ All electrophysiological and molecular
data were presented as means±S.E.M. Statistical analysis of gene expression data and electrophysiological data were performed using the Student’s *t* test. In all cases, *P* values determined were presented in the text; *P* values < 0.05 were considered statistically significant.

**Supplemental Result 1:**

We used a pharmacological approach to confirm that I_{K,slow} were decreased in p90RSK-Tg ventricular myocytes. 50 µM 4-aminopyridine (4-AP) was applied to NLC and p90RSK-Tg mouse ventricular myocytes (Supplemental Figure 1B and E), respectively, to predominantly block I_{K,slow} encoded by Kv1.5.\(^3\),\(^4\) Subtraction currents inhibited by this drug revealed that 4-AP-sensitive peak currents were decreased in transgenic mouse ventricular cells (Supplemental Figure 1F), compared with NLC (Supplemental Figure 1C). The density of 4-AP sensitive peak current at +40 mV was significantly decreased (*p*<0.05) from 14.0 ± 1.5 pA/pF in NLC (n = 4) to 9.6 ± 0.4 pA/pF in p90RSK-Tg ventricular cells (n = 4).

**Supplemental Result 2:**

Kv4.2 or Kv2.1-encoded channel activities were unaltered by expression of p90RSK in HEK 293 cells (Supplemental Figure 2). For example, mean±SEM densities of Kv4.2 peak currents at +40 mV in cells with or without co-expression of p90RSK were 226.6±68.6 (n=5) and 213.7±43.3 (n=8), and they were not different; Analysis of the decay phases of the outward Kv4.2 currents evoked during long (800 ms) depolarizations revealed that the current decay was well described by the sum of two exponentials. Time constants of fast and slow components were unchanged by p90RSK. For example, the mean±SEM time constants for the fast and slow components of
inactivation analyzed from the cells expressing Kv4.2 at +40 mV were 44.5±4.0 ms (n=8) and 253.9±21.6 ms (n=8); Mean±SEM time constants analyzed from the currents recorded from the cells expressing Kv4.2 and p90RSK were 44.8±7.3 ms and 264.8±45.1 ms (n=5). These values were not significantly different. K2.1-expressed peak current densities at +40 mV in cells with or without co-expression of p90RSK were 241.7±34.0 (n=8) and 223.8±24.0 (n=8), and they were not different. Analysis of the activation phases of both Kv4.2- and Kv2.1- currents revealed that the activation of these channels was well described by one exponential. Activation time constants of Kv4.2 or Kv2.1-expressed currents were not altered by p90RSK. For example, mean±SEM activation time constants analyzed from the cells expressing Kv4.2 alone and coexpressing Kv4.2 with p90RSK at +40 mV were 2.6±0.3 ms (n=8) and 2.3±0.4 ms (n=5); activation time constants of Kv2.1-encoded currents with or without expression of p90RSK were 6.1±0.6 ms (n=8) and 7.2±1.1 ms (n=9), respectively.

Supplemental Result 3:

In order to determine p90RSK effects on Kv4.3 and KChIP2-encoded currents via phosphorylation, voltage-clamp recordings were performed on cells expressing Kv4.3S516A+KChIP2 (Kv4.3S550A or Kv4.3S516A,S550A + KChIP2) or co-expressing them with p90RSK. Outward currents in Supplemental Figure 5A-H recorded from HEK 293 cells co-expressing Kv4.3 single or double mutants with KChIP2 were unaltered by expression of p90RSK. Mean±SEM current densities, activation and inactivation time constants of currents at +40 mV from cells co-expressing Kv4.3S516A or Kv4.3S550A or Kv4.3S516A, S550A with KChIP2 in the absence (n=9, 12, and 6) or presence (n=10, 15 and 6) of p90RSK were not significantly different (Supplemental
Figure 5G, H and I. These findings indicate that phosphorylation site mutation(s) of Kv4.3 abolished p90RSK effects on these channel activities. Interestingly, Kv4.3 mutant(s) and KChIP2-encoded currents (n=9, 12, and 6) (Supplemental Figure 5A, C and G) were decreased in comparison with those encoded by wild type Kv4.3 and KChIP2 (n=14) (Figure 6A). Mean±SEM current densities at +40 mV obtained from HEK 293 cells co-expressing Kv4.3S516A or Kv4.3S550A or Kv4.3S516A, S550A with KChIP2 (n=9, 12, and 6) were significantly decreased (P<0.01) compared with those obtained from wild type Kv4.3 and KChIP2-encoded currents (n=14) (Supplemental Figure 5G) but activation or inactivation time constants of Kv4.3 mutant(s) and KChIP2-encoded currents were unchanged (Supplemental Figure 5H and I). These findings indicate these two phosphorylation sites are also important for Kv4.3 and KChIP2-encoded channel activity.

**Supplemental Result 4:**

We focused on the effects of H2O2 on Kv4.3 and KChIP2-encoded channel activity. As we expected, Kv4.3 and KChIP2-encoded currents recorded from HEK 293 cells were decreased at different voltage levels after two hour incubation with 30 µM H2O2 (Supplemental Figure 6B) compared with control (Supplemental figure 6A). H2O2 significantly reduced the current density (P<0.01) at +40 mV, compared with the control group (Supplemental figure 6E), while analysis of the decay phases of the currents revealed that inactivation time constant was unaltered by H2O2 (Supplemental figure 6F). As expected, fmk blocked H2O2’s effects on Kv4.3 and KChIP2-encoded channel activity. The currents (Supplemental Figure 6D) recorded from HEK 293 cells-expressing Kv4.3 and KChIP2 pretreated with 1 µM fmk and 30 µM H2O2 for 2 hours were not
different from those (Supplemental Figure 6A) seen in control group. Peak outward current densities and inactivation time constants of currents recorded in cells expressing Kv4.3 and KChIP2 at +40 mV, treated with H$_2$O$_2$ in the presence of fmk were not significantly different (Supplemental Figure 6E and F).

**Supplemental Figure Legends:**

**Supplemental Figure 1. Downregulation of $I_{K,\text{slow1}}$ encoded by Kv1.5 in p90RSK ventricular myocytes.** A: current waveforms from a representative NLC control and a p90RSK-Tg ventricular myocyte before (A, D) and after the administration of 50 µM 4-aminopyridine (4-AP) (B, E). The voltage protocol was the same as described in Figure 3A. Digital subtraction ($A-B$; D-E) was performed off-line to demonstrate the 4-AP-sensitive currents (C, F).

**Supplemental Figure 2. Kv4.2- and Kv2.1-encoded currents are not altered by expression of p90RSK in HEK 293 cells.** A. a and b, whole-cell outward K$^+$ currents recorded from transiently transfected HEK-293 cells expressing Kv4.2. Currents were evoked during 800 ms depolarizing voltage steps to potentials between -60 and +60 mV from a HP of -70 mV. Kv 4.2 expressed outward K$^+$ currents were not affected by expression of p90RSK (b). B. a and b, whole-cell outward K$^+$ currents recorded from transiently transfected HEK-293 cells expressing Kv2.1. Currents were evoked during 100 ms depolarizing voltage steps to potentials between -40 and +60 mV from a HP of -70 mV. Kv2.1 expressed outward K$^+$ currents were not affected by (d), .

**Supplemental Figure 3. p90RSK directly phosphorylates Ser516 and Ser550 at the C-terminus of Kv4.3.** In vitro p90RSK phosphorylation assay revealed that WT-GST-Kv4.3-CT was phosphorylated at 40 kD (upper panel lane 1); phosphorylation was
largely deceased in GST-Kv4.3-CT with double mutations replacing Ser516 and Ser550 with Ala (upper panel, lane 7). Phosphorylation intensity of wild type Kv4.3-CT was slightly increased by single amino acid mutation, Ala516 (lane 3) or Ala550 (lane 5). In the lower panel, the gel stained with Coomassie Blue dye showed that WT GST-Kv4.3-CT and different GST-Kv4.3-CT mutated proteins were equally loaded. Three same experiments were performed to show the similar results.

**Supplemental Figure 4. Effects of mutagenesis on p90RSK putative consensus sequences on K,4.3 currents.** Representative current traces were evoked by stepping from a holding potential of -70 mV to test potentials from -60 to +60 mV for durations of 4.5 s from HEK 293 cells expressing Kv4.3S516A, Kv4.3S550A and Kv4.3S516AS550A (A, C, and E) and different Kv4.3 mutants with p90RSK (B, D, and F). Inactivation time constants of fast and slow components in individual cells were determined from two exponential fits to the decay phases of the total outward currents evoked at potential +40 mV from an HP of -70 mV while Activation time constants in individual cells were determined from one exponential fit to the activating phases. Peak current densities at +40 mV were obtained from current amplitudes divided by individual cell capacitance (G). Kv4.3 expressed current density at +40 mV (n=11) was significantly reduced (*P<0.05) by expression of p90RSK (n=11) but Kv4.3 mutant(s) encoded current densities (n=13, 11 and 16) were not affected by p90RSK (n=13, 11 and 16) (G). Kv4.3 mutants (s)-expressed current densities at +40 mV was significantly reduced (***P<0.01) compared with those determined by wild type Kv.43 (G). Fast and slow time constants of inactivation were also obtained (H and I). The mean±SEM values of fast and slow activation time constants analyzed from Kv4.3 and p90RSK-expressed currents (n=11)
were significantly smaller (*P<0.05 or **P<0.01) than those of Kv4.3 currents (n=11) (H and I). The mean±SEM fast inactivation time constant of Kv4.3-Ala516 mutant and p90RSK-expressed current (n=13) was not significantly different from that of Kv4.3-Ala516 mutant current (n=13) but the mean±SEM slow inactivation time constant of Kv4.3-Ala516 mutant and p90RSK-expressed current (n=13) was smaller (#P<0.05); The mean±SEM fast inactivation time constant of Kv4.3-Ala550 mutant and p90RSK-expressed currents (n=11) was significantly increased (##P<0.01) that of Kv4.3-Ala550 mutant currents (n=11) but the mean±SEM slow inactivation time constant of Kv4.3-Ala550 mutant and p90RSK-expressed currents (n=11) was not changed. The fast and slow inactivation time constants of Kv4.3-Ala516 and Ala550 double mutants and p90RSK-expressed currents (n=16) were not different from those of Kv4.3-Ala516 and Ala550 double mutants-expressed currents (n=16). Activation time constants were not significantly different between Kv4.3 or Kv4.3 mutant(s) and K4.3+p90RSK or Kv4.3 mutant(s)+p90RSK groups (J).

**Supplemental Figure 5. Effects of mutagenesis on p90RSK putative consensus sequences on Kv4.3 and KChIP2-encoded currents.** Representative current traces were evoked by stepping from a holding potential of –70 mV to test potentials from -60 to +60 mV for durations of 800 ms from HEK 293 cells expressing Kv4.3S516A, Kv4.3S550A, and Kv4.3S516AS550A ( A, C, and E) with KChIP2 and different Kv4.3 mutants with KChIP2 and p90RSK (B, D, and F). All mutations of Kv4.3 blocked downregulation of Kv4.3 + KChIP2-encoded currents by p90RSK. The peak current densities evoked at potential +40 mV from an HP of -70mV were obtained from the current amplitudes divided by individual cell capacitance, and they were no significantly different between
Kv4.3 mutants + KChIP2 (n=9, 12 and 3) and these mutants + KChIP2 + p90RSK (n=10, 15 and 3) groups (G), *p<0.05 in comparison of Kv4.3 and KChIP2 expressed current densities at +40 mV (n=17) with those obtained from currents recorded in the presence of p90RSK (n=12); **p<0.01 in comparison of Kv4.3 mutant(s) and KChIP2-encoded current densities at +40 mV (n=9, 12 and 3) with those Kv4.3 and KChIP2 expressed current densities at +40 mV (n=14) with those obtained from Inactivation and activation time constants of the currents recorded from individual cells evoked at potential +40 mV from an HP of -70 mV were determined from one exponential fit. Both activation and inactivation time constants were not different in the currents from cells expressing wild type Kv4.3 and KChIP2 (n=14) or mutant(s) and KChIP2 (n=9, 12 and 3) or coexpressing mutant(s) and KChIP2 with p90RSK (n=10, 15 and 3) (H and I).

Supplemental Figure 6. A specific p90RSK inhibitor, fmk, blocks H$_2$O$_2$’s effects on Kv4.3 and KChIP2-encoded channel activity. Currents were recorded from cells expressing Kv4.3 and KChIP2 in culture medium with or without H$_2$O$_2$ for two hours and evoked during 800 ms depolarizing voltage steps to potentials between -60 and +60 mV from a HP of -70 mV (A and B). Kv4.3 and KChIP2-expressed currents (A) were decreased by 30 µM H$_2$O$_2$ (B). Peak outward current density was significantly decreased (***p<0.01) at +40 mV by H$_2$O$_2$ (E) (Kv4.3+KChIP2: n=22 and Kv4.3+KChIP2+H$_2$O$_2$: n=10). Analysis of current rising and decay phases showed H$_2$O$_2$ did not affect activation and inactivation time constants (F and G). A p90RSK specific inhibitor, 1 µM fmk, which didn’t affect Kv4.3 and KChIP2 expressed currents (C), but it prevented H$_2$O$_2$’s effects on the currents recorded from cells pretreated with 1 µM fmk for 2 hours (D) (Kv4.2+KChIP2+fmk: n=5 and Kv4.2+KChIP2+fmk+H$_2$O$_2$: n=6). The current
waveforms were not different between cells incubated with 1 µM fmk alone (C) and with 1 µM fmk and 30 µM H₂O₂ (D). Activation and inactivation time constants were not changed by fmk (F and G).

Supplemental References:


Supplemental Figure 2

A Kv4.2

a Control  
b + p90RSK

B Kv2.1

a Control  
b + p90RSK
Supplemental Figure 3

p90RSK in vitro kinase assay

Comassie blue stain
Supplemental Figure 5

A  Kv4.3S516A + KChIP2

B  Kv4.3S516A + KChIP2 + p90RSK

C  Kv4.3S550A + KChIP2

D  Kv4.3S550A + KChIP2 + p90RSK

E  Kv4.3S516A,S550A + KChIP2

F  Kv4.3S516A,S550A + KChIP2 + p90RSK
Continuous Supplemental Figure 5

**G**
Peak Current Density (pA/pF)

- Kv4.3+KChIP2
- Kv4.3+KChIP2+p90RSK
- Kv4.3516A+KChIP2+p90RSK
- Kv4.3516A+KChIP2+550A+KChIP2+p90RSK

**H**
Inactivation Time Constant (ms)

- Kv4.3+KChIP2
- Kv4.3+KChIP2+p90RSK
- Kv4.3516A+KChIP2+p90RSK
- Kv4.3516A+KChIP2+550A+KChIP2+p90RSK

**I**
Activation Time Constant (ms)

- Kv4.3+KChIP2
- Kv4.3+KChIP2+p90RSK
- Kv4.3516A+KChIP2+p90RSK
- Kv4.3516A+KChIP2+550A+KChIP2+p90RSK
- Kv4.3516A+KChIP2+550A+KChIP2+p90RSK
Supplemental Figure 6

A) Kv4.3+KChIP2  
B) + H₂O₂ 30 μM

C) + fmk 1μM  
D) + fmk 1μM + H₂O₂ 30μM

E) Peak Current Density (pA/pF)  
F) Inactivation Time Constant (ms)  
G) Activation Time Constant (ms)

Control  H₂O₂  fmk + H₂O₂  fmk  Control  H₂O₂  fmk + H₂O₂  fmk  Control  H₂O₂  fmk + H₂O₂  fmk

**
Table 1. Primers Used for SYBR Green Quantitative Real Time RT-PCR

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<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
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<td>5’-aaggeaccaatagtacatccag</td>
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