Regulation of an ERG $K^+$ Current by Src Tyrosine Kinase*

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The human “ether-a-go-go”-related gene (HERG) $K^+$ channel, and its homologues are present in heart, neuronal tissue, some cancer cells, and the MLS-9 rat microglia cell line (Zhou, W., Cayabyab, F. S., Pennefather, P. S., Schlichter, L. C., and DeCourcey, T. E. (1998) J. Gen. Physiol. 111, 781–794). Despite its importance, there are few studies of ERG modulation. In this first report on regulation by tyrosine phosphorylation, we show that MLS-9 cells express transcripts for r-erg1 (rat homologue of HERG) and r-erg2, and an immunoreactive doublet was identified using an anti-HERG antibody. The constitutive tyrosine phosphorylation of the ERG1 protein, detected by co-immunoprecipitation, was reduced by the protein-tyrosine kinase inhibitors, lavendustin A, herbimycin A, or genistein (but not daidzein). The whole cell ERG current was reduced by protein-tyrosine kinase inhibitors or the Src-selective inhibitory peptide, src40–58, but not by a scrambled peptide. Conversely, the current was increased by the Src-activating peptide, srcPY, but not by an inactive analogue. Activating endogenous Src or transfecting constitutively active v-Src altered the voltage dependence and deactivation kinetics to produce more current at negative potentials. Co-immunoprecipitation identified an association between the channel protein and Src. Thus, r-ERG1 and Src tyrosine kinase appear to exist in a signaling complex that is well positioned to modulate this $K^+$ channel and affect its contribution to cellular functions.

The potassium channel encoded by the human ether-a-go-go-related gene (HERG)$^1$ was first cloned from a hippocampal cDNA library (2). However, the role of HERG is best understood in the heart, where it contributes to the repolarizing current, $I_{Kr}$ (3–6). Mutations in HERG underlie “long QT” syndrome type 2, which can cause sudden death from ventricular arrhythmia (7). HERG, and the rat homologue, r-erg1, have now been identified in a wide variety of tissues (6); whereas, the other rat isoforms (r-erg2 and r-erg3) are thought to be restricted to neural tissue (5). Despite the presence of all three erg isoforms in neural tissue, the cellular distribution and roles of these channels are not well understood. Mutations in the Drosophila HERG homologue, sei (“seizure” locus) cause neuronal hyperexcitability and seizure-like behaviors (8, 9). HERG or ERG channels may help set the resting membrane potential in neurons and neuronal cell lines (10), mediate spike-frequency adaptation (11), modulate hormone secretion (12–14), and affect neuronal differentiation (15 and neurogenesis (10, 15, 16). HERG channels may also be important in proliferating cancerous and undifferentiated cells, where there is increased HERG or ERG compared with their normal cellular counterparts (10, 17).

After discovering that the major $K^+$ current in a highly proliferating rat microglia cell line, MLS-9, is very similar to HERG (1, 18), we became interested in biochemical processes that regulate the current, and thus, affect its potential contribution to microglial functions. Mechanisms that modulate HERG channel activity are now being elucidated, in particular, its regulation by serine/threonine phosphorylation (2, 12, 13, 19, 20). There is no information about HERG regulation by tyrosine phosphorylation, despite the presence in the channel of numerous consensus sites for tyrosine phosphorylation, as well as sequence motifs that often underlie complex formation with cytosolic protein-tyrosine kinases (PTKs). PTKs and tyrosine phosphatases regulate numerous cellular processes that include proliferation, differentiation, and apoptosis (21), so the possibility that tyrosine phosphorylation regulates native HERG channels is of broad interest. Microglia, for instance, express and are regulated by the cytosolic Src family PTKs, Src, Yes, Lyn (22–25), and the receptor-linked PTKs, c-Fms and c-Kit (26, 27).

Our findings show the functional regulation of an endogenous ERG current by tyrosine phosphorylation, and the physical association of the protein-tyrosine kinase, Src, with ERG protein. MLS-9 cells express transcripts for r-erg1 and r-erg2, and the degree of tyrosine phosphorylation of the channel protein correlated with channel activity. That is, the ERG1 protein was constitutively tyrosine phosphorylated, and this was functionally significant since inhibiting PTKs (src in particular) reduced the current. Moreover, activating endogenous Src increased the current, altered the voltage dependence of channel activation, and slowed deactivation. Similar effects on voltage dependence and deactivation were seen after transfecting MLS-9 cells with the constitutively active v-src. Since we found that endogenous ERG protein associates with endogenous Src

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in MLS-9 cells, our results support the view that ERG exists within a signaling complex that up-regulates channel function.

**EXPERIMENTAL PROCEDURES**

**MLS-9 Cells and Transfection**—The MLS-9 cell line was derived by treating cultured microglia with colony-stimulating factor-1 for several weeks, then harvesting colonies (1, 25). Like culture-derived microglia, MLS-9 cells stain with isoelectric B4, and the antibodies, OX-42 and ED-1, and take up Di-acetylated LDL and Lucifer Yellow by pinocytosis. They are not labeled with antibodies against the glial fibrillary acidic protein (an astrocyte marker) or the fibroblast protein, fibronectin. For biochemical analyses, MLS-9 cultures were grown to near confluence in endotoxin-free minimal essential medium containing 5% horse serum, 5% fetal bovine serum, and 50 μg/ml gentamicin. They were harvested by two washes (10 min, 37 °C) with sterile phosphate-buffered saline (PBS), then with sodium citrate solution (130 mM NaCl, 15 mM Na citrate, 10 mM HEPES, 10 mM δ-glucose, pH 7.4). After adding an equal volume of minimal essential medium to the cell suspension, the cells were centrifuged at 700 rpm for 10 min, and resuspended in minimal essential medium. All cell culture reagents were from Invitrogen (Burlington, ON, Canada).

For electrophysiology, MLS-9 cells were plated at lower density (about 30% confluence) on glass coverslips in 35-mm dishes and allowed to adhere >6 h before transfection, as previously described (25). To study longer term ERG channel regulation by Src protein-tyrosine kinase, MLS-9 cells were transfected with 2 μg of constitutively active v-src (in pM5 h vector; Dr. R. Jove, University of Michigan Medical School, Ann Arbor, MI) or 2 μg of the vector with no cDNA (pBA-neo or pRSVγgal; gift from Dr. F. W. L. Tsui, Toronto Western Hospital Research Institute, Toronto, ON) using LipofectAMINE (Invitrogen). Cells were co-transfected with 1 μg of the marker, enhanced green fluorescent protein (CLONTECH, Palo Alto, CA) (25). All recordings were performed 24–48 h post-transfection.

**Electrophysiology**—Whole cell patch clamp recordings from MLS-9 cells were made with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), with on-line compensation for series resistance and capacitance. The signals were filtered at 5 kHz and analyzed using pCLAMP 6.0 software (Axon Instruments). Pipettes with resistance of 3–5 MΩ were made from thin-walled borosilicate glass capillaries (WPI, Sarasota, FL). Only isolated bipolar cells with relatively low series resistance (4–15 MΩ) and moderate-sized currents (300–1500 pA) were studied. After establishing a whole cell recording, the series resistance was compensated 50–70% to a final value <6 MΩ, thus the voltage error was <10 mV for the largest currents. We only analyzed cells that exhibited good voltage control, as judged by smoothly rising currents (e.g. see Fig. 1A), and well compensated capacitance transients. Cells were plated in minimal essential medium on sterile glass coverslips, allowed to adhere >3 h, then superfused during recordings with an extracellular solution containing (in mM): 130 K aspartate, 1 CaCl2, 1 MgCl2, 10 HEPES, 40 sucrose, and 5 δ-glucose (pH 7.4, 300 mosmol). The pipette solution contained (in mM) 130 K aspartate, 2 CaCl2, 1 MgCl2, 10 EGTA, 10 HEPES, 2 K2ATP, titrated with KOH to pH 7.2 (290 mosmol). Aspartate was used as the major anion to reduce contamination with NaCl, pH 7.5, 150 mM NaCl, 100 mM NaF, 5 mM EDTA, 1 mM Na3VO4, and the protease inhibitors leupeptin (2 μg/ml), aprotonin (2 μg/ml), and phenylmethylsulfonfluoride (1 μmol). The lysates were centrifuged at 15,000 × g (15 min, 4 °C) to remove cellular debris, then the supernatant was cleared by incubation with Protein A/G-agarose (3 mg/ml, 1 h) and then incubating for 3 h in Protein A/G-agarose, followed by centrifugation. The immunoprecipitates were washed three times with ice-cold solubilization buffer containing 0.1% Triton X-100, then eluted in 50 μl of gel-loading buffer for Western analysis. Protein concentrations were measured with the DC Protein Assay (Bio-Rad, Mississauga, ON, Canada), then proteins were
run on a 6.5% polyacrylamide gel and electrophoresed to nitrocellulose, blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBST). The membrane was incubated overnight at 4 °C with a polyclonal anti-HERG (human homologue of r-ERG1) antibody (1:160; Alomone Labs, Jerusalem). After four washes with PBST, the membranes were incubated (1 h, room temperature) with horseradish peroxidase-conjugated secondary antibody (1:3000; Cederlane Labs, Hornby, ON). Following another four washes with PBST, labeled proteins were visualized using enhanced chemiluminescence (ECL, Amersham Bioscience, Arlington Heights, IL) on XAR-2 film (Kodak, Rochester, NY) and the signals quantified by densitometry (Bio-Rad model GS-670).

Co-immunoprecipitation was used to examine interactions between native r-ERG1 protein and Src tyrosine kinase. MLS-9 cells were washed in PBS, then lysed in 1 ml of ice-cold modified RIPA buffer containing 1% Nonidet P-40, 50 mM Tris at pH 8.0, 150 mM NaCl, 1 mM EDTA, aprotinin (1 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 1 mM NaF, and complete protease inhibitor mixture tablets (2 tablets/100 ml; Roche Molecular Biochemicals). Following a 20-min incubation on ice, the lysates were centrifuged at 14,000 × g for 20 min at 4 °C. About 500 μl of the cleared lysate (~500 μg of solubilized protein) was incubated overnight at 4 °C with the polyclonal anti-HERG (r-ERG1) antibody (1:83) or a monoclonal anti-Src antibody (1:125; Upstate Biotechnology, Lake Placid, NY). The r-ERG1 or Src immunoprecipitates were incubated with 50 μl of a 50% slurry of anti-rabbit or anti-mouse agarose beads, respectively, and the mixtures rotated for 3 h or overnight at 4 °C. The immunoprecipitates were washed three times with modified RIPA buffer containing 0.1% Nonidet P-40, eluted in 50 μl of SDS loading buffer, and separated by SDS-PAGE. They were analyzed by immunoblotting with either anti-HERG (1:160) or anti-Src antibody (1:250), with the appropriate secondary antibody (horseradish peroxidase-conjugated, goat anti-rabbit, or anti-mouse IgG) and visualized by ECL. A431, an epidermal growth factor-stimulated human carcinoma cell line (Upstate Biotechnology Inc.) that expresses abundant Src, was used as a positive control. Reagents were from Sigma, unless otherwise indicated.

**Statistical Analysis**—Data are expressed as mean ± S.E. We used either a two-tailed Student's paired t test or analysis of variance (ANOVA) with Bonferroni corrected multiple-comparison post-test (INSTAT2 software, version 2.04; GraphPad Instat Software, Sunnyvale, CA). In either case, p < 0.05 was taken as statistically significant.

**RESULTS**

**The ERG Current in MLS-9 Cells**—Fig. 1 illustrates voltage protocols and several important features of the ERG-like K+ current in MLS-9 cells (see Refs. 1 and 18 for further biophysical characterization). We previously used the terminology from the cardiac literature that described HERG as an inward-rectifying K+ current, but have now adopted the terminology that followed its cloning and expression. That is, HERG is a depolarization-activated channel that has unusual kinetics: it inactivates faster than it activates at most voltages, and thus displays little or no outward current (7, 30–33). Since HERG currents increase dramatically in high external K+ solutions, we used 130 mM extracellular K+ (Nernst potential, 0 mV) throughout this study. When a depolarized holding potential was used to open the channels (Fig. 1A), subsequent hyperpolarizing steps elicited large inward currents. As previously shown (1), hyperpolarization relieves the inactivation that occurs rapidly at depolarized potentials, thus the inward current amplitude depends on both the driving force and the degree to which inactivation is relieved at each test potential. In MLS-9 cells, the current-versus-voltage relation of the open channels is linear, unlike other ionic conductances; i.e., the inward-rectifying current is a linear function of the test potential (1). At negative potentials (below about −40 mV in Fig. 1A) the characteristic slow channel closing (deactivation) is seen (1). We used the voltage protocol in Fig. 1A to calculate the time constants for deactivation by fitting an exponential function to the decay phase (see below). The protocol in Fig. 1B demonstrates voltage-dependent channel activation. Depolarizing pre-pulses were applied to different voltages from a very negative holding potential (−80 mV) to activate and inactivate the channels. Then, during each test pulse to −120 mV, which fully relieves inactivation, the inward current amplitude was proportional to the number of channels opened during the pre-pulse.

The current in MLS-9 cells, like HERG in the heart and in expression systems, was blocked by the class III anti-arrhythmic drug, E-4031, thus a subtraction procedure was used to isolate the current. Although there was no Ba2+-sensitive inward-rectifying current in MLS-9 cells, we confirmed the specificity of E-4031 since primary-cultured rat microglia express mRNA for Kir2.1 (34). The inward-rectifier current in HEK 293 cells stably expressing human IRK1 (Kir2.1) channels was not inhibited by 1–10 μM E-4031, whereas it was reversibly abolished by 50 μM Cs+ (not shown). Thus, the block of current by E-4031 in rat MLS-9 cells was specific. For simplicity, we will call the E-4031-sensitive current, “ERG.” The lower panels in Fig. 1, A and B, show full block of the inward current by 1 μM E-4031. Consistent with the previously described open-channel block by E-4031, the block in MLS-9 cells was somewhat voltage dependent (Fig. 1C) with an IC50 of 37 nM when the holding potential was 0 mV, and 66 nM when it was −80 mV (n = 5, p < 0.01). Except where indicated, the ERG current was calculated as the E-4031-sensitive component. The remaining current in the presence of 1–3 μM E-4031 was outward, time-independent, and present only at very positive potentials (see traces at +60 and +80 mV, Fig. 1B), thus it did not affect measurements of ERG current. These E-4031-insensitive outward currents were abolished by the chloride-channel blocker 5-nitro-2-(3-phenylpropylamino)benzoic acid (200 μM) (not shown).

An activation curve was obtained by normalizing the peak tail current at −120 mV (Fig. 1B) to that obtained following a pre-pulse to +80 mV, and plotting these values against the
Src Regulation of ERG Current

Pre-pulse potential, as is commonly done (12, 20, 35). After fitting the experimental points to a Boltzmann equation, the voltage for half-activation \( V_{1/2} \) and slope factor \( k \) were calculated. The \( V_{1/2} \) for activation was \( +32.7 \pm 0.1 \) mV and \( k \) was \( 15.1 \pm 0.3 \) mV (n = 12). As we previously reported for MLS-9 cells (1) and as observed in heterologous expression systems (7, 17, 35, 36), the \( V_{1/2} \) is variable and depends on the voltage protocol used. The values in Fig. 1 are shifted positive, owing to the shorter conditioning pre-pulses (300 ms) and hyperpolarized holding potential (−80 mV), which were used to monitor changes in voltage dependence of the current throughout experiments that used a peptide activator or inhibitor of Src (see Figs. 4 and 5). When 20-s long conditioning pre-pulses were used to assess the effects of v-Src transfection on steady-state activation (see Fig. 6), the \( V_{1/2} \) for activation was much more negative and the slope was steeper.

Reduction of ERG Current by Tyrosine Kinase Inhibitors—Spontaneous rundown of currents after establishing whole cell recordings is often caused by a change in the phosphorylation state of the channel. The first indication that the ERG current in MLS-9 cells was post-insertionally regulated was the ability of intracellular ATP to reduce its spontaneous rundown during whole cell recordings (Fig. 2). A large depolarizing pre-pulse (to +80 mV, 300 ms) was used to activate the available channels, then test pulses to −120 mV were applied to monitor the current amplitude as a function of time after establishing a whole cell recording. Without ATP in the pipette solution, considerable rundown occurred, with −50% decrease during 45 min of recording. The decline was significantly reduced (to 10−15%) when ATP was present. In addition to the current rundown, the closing rate in Fig. 2A became slower as the recording time increased. This slowing, which occurred with or without ATP, will be addressed below. The ERG contribution to the inward current was confirmed at the end of each experiment by perfusing in the blocker, E-4031 (1–3 \( \mu M \)).

First, we used membrane-permeant inhibitors of protein-tyrosine kinases to assess whether tyrosine phosphorylation affects the ERG current (Fig. 3). Broad-spectrum PTK inhibitors, either genistein or lavendustin A, were bath-applied 5–10 min after establishing a whole cell recording. During the subsequent 15–20 min, the inhibitory effects of these drugs reached a plateau. Only −10% decline is expected from spontaneous current rundown after 20–30 min recording (see Fig. 2B). Lavendustin A reduced the current amplitude by −35%, genistein reduced it by −60%, and its inactive analogue, daidzein, did not significantly affect the amplitude. Both the spontaneous rundown without ATP and the effect of PTK inhibitors suggest that dephosphorylation can exceed phosphorylation during whole cell recordings, and implies that there is an active tyrosine phosphatase. Presumably, ATP helps maintain the phosphorylation. In separate experiments, <12-h treatment of intact cells with the more Src-selective PTK inhibitor, herbimycin A, decreased the current by −70% (Me2SO alone had no effect), we next asked whether endogenous Src regulates the ERG current.

The function of endogenous Src was next manipulated by several selective peptides added to the pipette solution. Since the peptides require time to diffuse into the cell and act on their targets, the maximal amplitude during the first 5 min of whole cell recording was taken as the control current for each cell. All pipette solutions contained ATP, which reduced current rundown in control cells to <15% (non-significant) during the longest recordings used (50 min; see Fig. 2). At the end of each recording, E-4031 (1–3 \( \mu M \)) was added to block the ERG channels, then the background current, which was very small after −120 mV, was subtracted to calculate the ERG current amplitude. The unique domain of Src tyrosine kinase binds to a specific amino acid sequence in target proteins, thus a peptide (src40–58) that binds to this site has been used to disrupt Src function during whole cell recordings (28). With this Src-inhibiting peptide in the pipette (Fig. 4A) there was a time-dependent decrease in current, by an average of −45% (Fig. 4C; \( p < 0.05 \)) compared with the −10% rundown in time-matched control recordings (Fig. 2). The inhibition by src40–58 was specific, since the current was not reduced by a scrambled peptide containing the same amino acids (src40–58s) (Fig. 4, A and C).

Activating Src Kinase Increases ERG Current and Alters Its Voltage Dependence—We used a tyrosine-phosphorylated de-
The 30-min time point stabilized (about 20 min). * indicates summary of the peak current at 30 min (or 50 min for genistein).

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cap peptide, EPQYEEIPIA, which binds to the SH2 domains of src, the non-phosphorylatable peptide, EPQYEEIPIA (0.1 mg/ml) was used in the pipette solution. Right, the scrambled peptide, src40–58s (0.1 mg/ml) was used as a negative control. B, left: the Src-activating peptide, phosphorylated EPQ(pY)EEIPIA (srcpY; 1 mM), was included in the pipette solution. After 30 min recording, the PTK inhibitor, genistein (50 μM) was added to the bath (arrow; 20 min later). Middle, the inactive non-phosphorylated peptide, EPQYEEIPIA (src; 1 mM) was used as a negative control. Right, control recording with no peptide in the pipette. Genistein (50 μM) was added to the bath after 30 min (arrow; 20 min later). C, summary of the peak current at 30 min (or 50 min for genistein), normalized to the maximal current in the first 5 min of recording. Values are mean ± S.E. for the number of cells indicated. After adding genistein at 50 min, the current was re-measured when the effect had stabilized (about 20 min). * indicates p < 0.05 for src40–58 or srcpY at the 30-min time point versus the first 5 min (control), and src40–58 versus src40–58s at the 30-min time point. # indicates p < 0.01 for srcpY versus srcpY plus genistein. ++ indicates p < 0.01 for srcpY versus srcpY. % indicates p < 0.05 for no peptide versus srcpY. ## indicates p < 0.01 for no peptide versus non-peptide plus genistein.

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cap peptide, EPQ(pY)EEIPIA, which binds to the SH2 domains of Src and has previously been used to activate endogenous kinases and study membrane currents (25, 28). When this Src-activating peptide, srcpY, was included in the pipette solution, the ERG current increased dramatically during the first 20–35 min of whole cell recording (Fig. 4B). After the ERG amplitude was increased by srcpY, subsequent bath addition of the broad-spectrum PTK inhibitor, genistein, reduced the current by ~50% (example in Fig. 4B, summarized in Fig. 4C; p < 0.01). The percent inhibition by genistein was similar with or without a peptide in the pipette (Figs. 3 and 4C). The effect of the Src-activating peptide was specific since the non-phosphorylated inactive peptide, EPQYEEIPIA (srcY) did not change the current amplitude (Fig. 4C; p > 0.05). The small decrease observed with srcY was the same as for control cells without peptide (Fig. 4C) and is expected from normal time-dependent rundown (Fig. 2). Compared with the inactive srcY peptide, the Src-activating peptide (srcpY) increased the current more than 2-fold (Fig. 4C, p < 0.01).

srcpY increased the ERG current at all voltages, whether a holding potential of +20 mV was used to fully activate the channels (Fig. 5A) or pre-pulses to a variety of test potentials were used to elicit voltage-dependent activation (Fig. 5B). The activation curves deviate from the steady state because it was necessary to use shorter pre-pulses than the ideal seconds-long pulses; however, qualitatively similar results were obtained with steady-state protocols (see below). Long pulses activated an unidentified inward current that confounded the E-4031-subtraction procedure used to isolate the ERG current. With this protocol, srcpY caused a −16.5 mV shift in midpoint of the activation curve (V1/2) from +32.0 ± 0.5 to 15.5 ± 1.2 mV (p < 0.0001, n = 7, Fig. 5C), and a decrease in voltage sensitivity of the current, seen as a change in the activation slope factor (k) from 14.3 ± 0.4 to 18.6 ± 1.0 mV (p < 0.01, n = 7).

srcpY also appeared to slow the ERG current deactivation, seen as a slower relaxation of inward current in Figs. 4B and 5A. From a monoexponential fit to the time course at −120 mV, the time constant increased from 174 ms (control cells) to 685 ms in the presence of srcpY (n = 4, p < 0.001). The kinetics of channel closing were particularly labile; i.e. the deactivation time constant increased from 173 to 407 ms (n = 4, p < 0.01) when the inactive peptide (srcY) was used, and also increased during prolonged control recordings (Fig. 2). However, the greater degree of slowing in the presence of srcpY is likely attributable to Src-dependent phosphorylation.

We next examined effects of Src activation on ERG channel function by overexpressing the constitutively active tyrosine kinase, v-Src. This experiment examined longer-term effects and allowed steady-state activation to be examined. By using shorter recording periods (5–10 min after establishing whole cell recordings) this approach also reduced the spontaneous changes that occurred during prolonged recordings with srcpY peptide. Fig. 6, A and B, show a negative shift in steady-state activation (V1/2) from −23.6 ± 1.1 mV (n = 8) for the vector-transfected controls to −37.2 ± 1.5 mV for the v-Src transfectants (n = 5; p < 0.0001), as well as an increase in the activation slope factor (k) from 9.1 ± 1.0 to 17.5 ± 1.5 mV (p < 0.001). As expected, the steady-state activation curves (Fig. 6B) were shifted to much more negative potentials than those measured with shorter pre-pulses (e.g. Figs. 1D and 5C). Nevertheless, in either case the biophysical changes produced by Src activation are expected to produce more ERG current at the negative membrane potentials of non-excitable cells like microglia. Moreover, a significant slowing of deactivation induced by v-Src transfection (Fig. 6, C and D) mimicked the slowing seen with Src activation by srcpY. Thus, short-term and longer-term regulation of ERG channels by Src produced similar changes in channel function.

r-ERG1 Protein Interacts with, and Is Tyrosine Phosphorylated by Src Kinase—Using RT-PCR in MLS-9 cells, we detected mRNA for r-erg1 and r-erg2, but not r-erg3 (Fig. 7). Since the level of r-erg2 in the positive-control tissue (adult cortex) was low, we amplified the signal from each tissue by a second round of RT-PCR (lanes 1–5). r-erg1 is homologous to the human erg (HERG) gene and, as previously reported (5), we found that it was abundant in brain and heart. r-erg3 was present in brain but not in heart or bladder, and r-erg2 was not detected in heart, also as previously reported (5).

We next examined the expression of ERG protein in MLS-9 cells, using immunoprecipitation and Western analysis (Fig. 8). The anti-HERG antibody labeled two prominent bands at about 130 and 145 kDa in the immunoprecipitated protein sample and in cell lysates, and both bands were eliminated when the antibody was preincubated with the antigenic peptide. Thus, the anti-HERG antibody appears to recognize the homologous rat protein, r-ERG1. r-ERG1 has a predicted molecular weight of 127,000 (2, 3); however, previous studies of HERG have identified two similar-sized bands (37, 38). These bands have been attributed to a lower molecular weight coreglycosylated immature form, located in the endoplasmic reticulum, and a higher molecular weight mature protein expressed on the cell surface (37).

Having observed Src-dependent regulation of the ERG current in MLS-9 cells, we next used co-immunoprecipitation to
determine whether r-ERG1 protein is tyrosine phosphorylated. MLS-9 proteins were immunoprecipitated with anti-phosphotyrosine antibody, then Western blots were prepared and probed with anti-HERG (r-ERG1) antibody. The antibody labeled two bands at about 130 and 145 kDa in immunoprecipitates and lysates from MLS-9 cells (Fig. 9A), showing that native r-ERG1 protein is constitutively tyrosine phosphorylated. This phosphorylation was reduced by PTK inhibitors that

FIG. 5. Biophysical changes in ERG current induced by Src activation. ERG currents were assessed within 5 min (control) or 20–30 min after whole cell recording with 1 mM srpY in the pipette solution. A, voltage steps were applied between −160 and +20 mV from a holding potential (V_H) of +20 mV. The ERG component was calculated by applying 3 μM E-4031 at the end of each recording, then subtracting the small remaining current. B, from V_H −80 mV, 300-ms long depolarizing steps between −160 and +80 mV were followed by steps to −120 mV. The background current was not subtracted. C, activation curves were fitted to Boltzmann equations (as in Fig. 1D) for control currents recorded in the first 5 min (filled circles) and 20–30 min after recording with srpY in the pipette (open circles).

FIG. 6. Biophysical changes in ERG current induced by transfecting the constitutively active v-Src. Voltage protocols (schematics at top) used to assess steady-state activation (A) and the deactivation kinetics (C). ERG currents in vector control (A, C, middle panels) and v-Src transfectants (A, C, bottom panels) were recorded within 10 min of establishing the whole cell configuration. A, steady-state channel activation was obtained by 20-s long voltage pre-pulses between +40 and −100 mV (progressively more negative), followed by steps to a test potential of −120 mV. B, activation curves for data like those in part A were fitted to Boltzmann functions (see “Experimental Procedures”). C, deactivation of ERG currents was assessed from the tail currents elicited by steps between −100 and −160 mV, after a 300-ms depolarization to +80 mV (holding potential, −80 mV). D, the time constants of deactivation were calculated from mono-exponential fits to tail currents like those in part C.

FIG. 7. Expression of rat erg transcripts. RT-PCR was used to examine expression of r-erg1, r-erg2, and r-erg3 mRNA in MLS-9 cells and several control rat tissues (PN2, post-natal day 2). Identities of products for MLS-9 and primary microglia were confirmed by restriction enzyme digestion or sequencing. For r-erg2, the control band from cortex was faint (lane 6), thus to further amplify the product a second round of RT-PCR was performed with a different primer pair (lanes 1–5). For details, see “Experimental Procedures” and Table I.
Regulation of ERG Current—In this study, we observed that inhibiting tyrosine phosphorylation reduced the ERG current amplitude in MLS-9 cells and conversely, stimulating tyrosine phosphorylation increased the current. This short-term regulation very likely reflects post-insertional modification, rather than changes in channel expression, since the full effects were seen within minutes when Src-activating or inhibiting peptides diffused from the pipette during whole cell recordings. Most significantly, use of these peptides demonstrates the ability of endogenous Src to regulate ERG, an interaction that may depend on the physical association we observed between native Src and ERG proteins. In principle, several biophysical mechanisms could contribute to the increase in ERG current after Src activation. Perhaps most important for non-excitable cells (e.g. microglia) which do not experience depolarizing action potentials, Src activation (by srcP or transfecting v-Src) produced a leftward shift in the activation curve and slowed deactivation, both of which are expected to contribute to a greater K+ conductance at negative membrane potentials. In addition to these changes, an increase in maximal current may reflect increases in the open-channel probability and/or number of active channels, but deciphering such changes will require single-channel analysis.

Numerous structure-function studies have led to models describing the kinetic behavior of HERG channels (1, 30, 33). The inactivation process is reminiscent of C-type inactivation (32, 36, 39, 40): it depends on amino acids in the outer pore, and is thus unlikely to involve intracellular tyrosine phosphorylation. Cytoplasmic channel regions are thought to control opening and closing. Within the N terminus, the first 16 amino acids slow HERG-channel closing (41) and there is a Per-Arnt-Sim domain which may interact with the cytoplasmic S4–S5 linker of the same channel (42, 43). Channel closing appears to involve interactions between the N terminus and the cytoplasmic S4–S5 linker, since deletions or mutations that disrupt this interaction accelerate closing and shift the conductance-versus-voltage (g-V) relation to more positive potentials (4, 31, 41, 42, 44, 45). Acidic residues in the S4–S5 linker are thought to contribute to the slow closing, which may explain why adding negative charges through phosphorylation by PKA accelerates channel closing and produces a positive shift in the g-V curve, thereby decreasing the current (46). The specific site(s) of phosphorylation have not been determined, but mutating all four PKA sites (1 in the N terminus, 3 in the C terminus) prevented the effects of PKA (20). Some effects of tyrosine phosphorylation that we observed were opposite to those of PKA; i.e. either Src activation with srcP or v-Src transfection induced a negative shift in the g-V curve which should increase the current at negative potentials. In addition, closing appeared to be slower. r-ERG1 has numerous potential sites for phosphorylation by tyrosine kinases, and our results might indicate an increase in interaction between the N terminus and S4–S5 loop. Further analysis will require site-directed mutagenesis.

MLS-9 cells expressed transcripts for r-erg1 and r-erg2, but not r-erg3. Some of the HERG-immunoreactive protein was
constitutively tyrosine phosphorylated, and tyrosine kinase inhibitors reduced this level. The site(s) of tyrosine phosphorylation was not determined; however, the r-ERG1 protein contains potential sites for tyrosine phosphorylation in the S4–S5 linker, the N terminus and the C terminus. One site, which is present in r-ERG1 but not in r-ERG2, is located within the N-terminal Per-Arnt-Sim domain, which acts as a Src-binding domain in other proteins (42, 43). If one or more N-terminal tyrosine residue undergoes Src-mediated phosphorylation, the addition of negative charges might stabilize the coupling between the N terminus and S4–S5 linker. Consistent with this model, we observed slower channel closing and a shift of the g-V curve to more negative potentials.

**ERG as Part of a Multimolecular Signaling Complex**—This is the first report of ERG interacting with signaling molecules or being regulated by tyrosine phosphorylation. Stable signaling complexes exist between other ion channels and protein kinases and phosphatases (25, 28, 29, 47, 48). Our finding that the ERG current is similarly modulated by activating endogenous Src kinase in whole cell recordings or transfecting v-Src implies an association between the channel and kinase that can withstand cytoplasmic disruption. The in vivo interaction between endogenous ERG1 and Src proteins in MLS-9 cells will position the signaling molecule close to its target channel, which should promote efficient phosphorylation and increase the selectivity of this process.

Our experiments do not distinguish between direct binding and indirect association within a multiprotein complex. Some K⁺ channels interact with Src family tyrosine kinases through SH2, SH3, or proline-rich domains (47, 48). The N and C termini of r-ERG1 contain several sequences similar to the optimal motif for binding Src SH2 domains (49, 50) and r-ERG1 has several proline-rich sequences that are similar to the optimal motif for binding Src SH3 domains (50). In principle, a multimolecular complex could also involve Src interaction with adaptor and/or scaffold proteins (50). For instance, PSD-95 can bind to some K⁺ channels (51) and we recently reported that, in microglia, PSD-95 binds to Src and the Kv1.3 channel (25). However, this seems less likely than a direct interaction since r-ERG1 lacks the C-terminal hydrophobic motif that is normally required for binding to a PDZ domain in PSD-95.

**Physiological Implications of Tyrosine Phosphorylation of ERG**—In excitable cells, K⁺ channels set the membrane potential and are crucial for regulating action potentials, Ca²⁺ influx, and secretion. Although their roles in non-excitable cells are not as well understood, the membrane potential contributes to ion homeostasis, Ca²⁺ signaling, and cell-volume regulation. Specific voltage-gated and Ca²⁺-activated K⁺ channels are important for lymphocyte proliferation, cytotoxic killing, and volume regulation (see Ref. 29 and references therein), and in proliferation, differentiation, and apoptosis in other non-excitable cells (52–55).

How might a tyrosine phosphorylation-regulated ERG current contribute to microglia functions? By helping to stabilize the membrane potential, ERG current should counteract depolarization influences, for instance, when purinergic receptor stimulation (56) or Cl⁻ channels (57) are activated. We recently reported that Kv1.3 is important for proliferation of hippocampal microglia (58) and for the respiratory burst in cultured microglia (59). We found that Kv1.3 in microglia is strongly inhibited by activation of endogenous Src and subsequent channel tyrosine phosphorylation, effects that are mimicked by oxygen-glucose deprivation in an in vitro model of stroke (25). The inverse regulation of Kv1.3 and ERG that we have observed raises the possibility that PTK-signaling pathways determine which K⁺ channels are active and able to contribute to microglia functions. Microglia express numerous PTKs, including src-family members (Src, Yes, and Lyn), non-receptor (Syk, Fak, and pyk2) and receptor-linked kinases (c-Fms and c-Kit) (22–24, 26, 27). It is intriguing that β-amyloid peptides stimulate the microglial respiratory burst through PTKs that include Lyn, Src, and Fak (23, 24). Hence, it will be interesting to determine whether any of these PTKs, other than Src, up-regulate the erg current in microglia.

Our findings may also be relevant to oncogenesis, since ERG currents are aberrantly expressed in numerous cancer cell lines compared with their normal counterparts (17). Although the related EAG channel has been implicated in malignant transformation (60), the potential role of ERG in cancer has not been specifically addressed. It is noteworthy that MLS-9 cells express large ERG currents and are highly proliferating. The mechanisms of K⁺-channel involvement in proliferation are not well understood; however, in many cell types, there is a correlation between K⁺-channel expression, membrane potential, and mitotic activity. In general, terminally differentiated cells (in G0 phase) have the most negative membrane potentials, quiescent cells that require mitogen stimulation to enter the cell cycle (e.g. lymphocytes) are less negative, and cycling cells (e.g. tumor cells) that do not enter G0 phase are even more depolarized (54). The biophysical properties of ERG channels are poised to produce the moderately depolarized membrane potentials typical of cancer cells.

ERG is activated and inactivated by depolarization and in, MLS-9 cells, it displays significant steady-state activity between about –60 and –20 mV (1). Since MLS-9 cells lack the Kv1.3 and inward-rectifier currents (1, 25) that are prevalent in primary cultured microglia from which they were derived (25, 57, 58), ERG is expected to dominate the membrane potential of MLS-9 cells and maintain it at a moderately depolarized level, particularly after Src activation. It is notable that the membrane potential of cells with large ERG currents (17) is typically near the activation threshold of some Ca²⁺ channels. Ca²⁺ influx regulates numerous Ca²⁺-dependent enzymes, and voltage-gated Ca²⁺ channels can rapidly activate Src, as well as Ras and mitogen-activated protein kinase signaling cascade (61, 62), which is downstream of growth factor receptors involved in proliferation. This suggests a possible feedback cycle between Ca²⁺ entry, Src activation, increased ERG current and proliferation.

HERG is also present in excitable cells, most notably in cardiac muscle where it contributes to repolarization after an action potential. Loss-of-function mutations can cause afterdepolarization and arrhythmias (48) and HERG current is decreased under normal physiological conditions by protein kinases A and C (19, 20, 46). HERG up-regulation is expected to speed repolarization and shorten the action potential, which could decrease the inter-spike interval and accelerate the heart rate. Thus an intriguing possibility is that up-regulation of HERG activity by tyrosine phosphorylation opposes the effects of PKA and PKC signaling pathways in regulating the cardiac action potential.

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