Tyrosine Phosphorylation Modulates Current Amplitude and Kinetics of a Neuronal Voltage-Gated Potassium Channel

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Fadool, Debra A., Todd C. Holmes, Kevin Berman, Daniel Dagan, and Irwin B. Levitan. Tyrosine phosphorylation modulates current amplitude and kinetics of a neuronal voltage-gated potassium channel. J. Neurophysiol. 78: 1563–1573, 1997. The modulation of the Kv1.3 potassium channel by tyrosine phosphorylation was studied. Kv1.3 was expressed in human embryonic kidney (HEK 293) cells, and its activity was measured by cell-attached patch recording. The amplitude of the characteristic C-type inactivating Kv1.3 current is reduced by >95%, in all cells tested, when the channel is co-expressed with constitutively active nonreceptor tyrosine kinase, v-Src. This v-Src-induced suppression of current is accompanied by a robust tyrosine phosphorylation of the channel protein. No suppression of current or tyrosine phosphorylation of Kv1.3 protein is observed when the channel is co-expressed with R385A v-Src, a mutant with severely impaired tyrosine kinase activity. v-Src-induced suppression of Kv1.3 current is relieved by pretreatment of the HEK 293 cells with two structurally different tyrosine kinase inhibitors, herbimycin A and genistein. Furthermore, Kv1.3 channel protein is processed properly and targeted to the plasma membrane in v-Src cotransfected cells, as demonstrated by confocal microscopy using an antibody directed against an extracellular epitope on the channel. Thus v-Src-induced suppression of Kv1.3 current is not mediated through decreased channel protein expression or interference with its targeting to the plasma membrane. v-Src co-expression also slows the C-type inactivation and speeds the deactivation of the residual Kv1.3 current. Mutational analysis demonstrates that each of these modulatory changes, in current amplitude and kinetics, requires the phosphorylation of Kv1.3 at multiple tyrosine residues. Furthermore, a different combination of tyrosine residues is involved in each of the modulatory changes. These results emphasize the complexity of signal integration at the level of a single ion channel.

INTRODUCTION

Potassium channels play critical roles in setting a neuron’s resting potential, influencing the duration of the action potential, and determining the frequency of repetitive firing (Jan and Jan 1994). Ion channels are dynamic, responding to intercellular and intracellular modulatory signaling molecules. Protein phosphorylation is a ubiquitous mechanism for modulating the activity of many proteins including ion channels (Levitan 1994). Most studies to date have focused on serine/threonine phosphorylation and have described modulation of a wide variety of ion channels in neurons and other cell types (reviewed by Catterall 1993; Levitan 1994).

In contrast to the plethora of reports on ion channel modulation by serine/threonine phosphorylation, the functional role of tyrosine phosphorylation of ion channels is just beginning to be explored (for reviews, see Jonas and Kaczmarek 1996; Siegelbaum 1994). This is surprising in view of the fact that tyrosine kinases are expressed at exceptionally high levels in the nervous system (Nairn et al. 1985). Tyrosine phosphorylation can modulate ligand-gated ion channels, including nicotinic acetylcholine receptor/channels (Gillespie et al. 1996; Hopfield et al. 1988; Qu et al. 1990; Wallace 1995), inositol trisphosphate (IP3)-gated ion channels (Jayaraman et al. 1996), and glutamate (Wang and Salter 1994) and γ-aminobutyric acid-A (Moss et al. 1995) receptor/channels. Several cloned voltage-gated potassium (Kv) channels have been shown to be modulated by nonreceptor tyrosine kinases. Huang et al. (1993) found that direct tyrosine phosphorylation of Kv1.2, resulting from G-protein coupled muscarinic acetylcholine receptor activation, suppresses channel current without altering channel kinetics. Much, if not all, of this modulation is abolished by mutating a single tyrosine residue near the amino terminus of the channel. Lev et al. (1995) showed that the Kv1.2 current suppression can be attributed to a nonreceptor tyrosine kinase (PYK2) of the focal adhesion kinase (FAK) family. Protein tyrosine phosphorylation by a janus kinase (JAK) family tyrosine kinase has been reported to increase the mean open time for a cloned voltage- and Ca2+-activated potassium channel (Prevarskaya et al. 1995), and the insulin receptor tyrosine kinase can influence ion channel activity (Jonas et al. 1996) and peptide secretion (Jonas et al. 1997) in Aplysia bag cell neurons.

We have begun to explore the detailed molecular mechanisms of potassium channel modulation by tyrosine phosphorylation using the cloned rat Kv1.3 channel expressed heterologously in human embryonic kidney (HEK 293) cells. This system allows mutational and biochemical analysis in parallel with functional studies. In previous experiments, we demonstrated that treatment of HEK 293 cells with a tyrosine phosphatase inhibitor leads to tyrosine phosphorylation of Kv1.3 and that this is accompanied by suppression of Kv1.3 current (Holmes et al. 1996). We now show that co-expression of the v-Src tyrosine kinase suppresses Kv1.3 current and also modulates the channel’s inactivation and deactivation kinetics and that multiple tyrosine residues are involved in each of these modulatory phenomena. These findings demonstrate that phosphorylation of different combinations of tyrosine residues can result in complex modulatory changes in a single ion channel.

METHODS

Solutions and reagents

HEK 293 cell patch pipette solution contained (in mM) 30 KCl, 120 NaCl, 10 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic...
acid (HEPES), and 2 CaCl$_2$ (pH 7.4). HEK 293 cell bath recording solution contained (in mM) 150 KCl, 10 HEPES, 1 ethylene glycol-bis(β-aminoethyl ether)-N,N',N'′-tetraacetic acid, and 0.5 MgCl$_2$ (pH 7.4). Protease and phosphatase inhibitor (PPI) solution consisted of (in mM) 25 Tris, pH 7.5: 150 NaCl; 100 NaF; 5 EDTA; 1 Na$_3$VO$_4$; 1 phenylmethylsulfonyl fluoride; and 1% Triton X-100: 1 μg/ml leupeptin; and 2 μg/ml aprotinin. All salts were purchased from Sigma Chemical (St. Louis, MO). Tissue culture and transfection reagents were purchased from Gibco/BRL (Grand Island, NY). Genistein, daidzein, and herbimycin A were purchased from Calbiochem and made as concentrated stock solutions in dimethyl sulfoxide (DMSO). Care was taken to protect genistein and herbimycin A from light, both during storage (−20°C) and experimentation. Genistein and daidzein were stored under nitrogen.

cDNA constructs and antibodies

Kv1.3 channels were expressed using the In vitro vector pcDNA3 (In Vitrogen, San Diego, CA). The v-Src cDNA construct was a generous gift of Dr. Richard Huganir (Johns Hopkins University, Baltimore, MD) and was located in a modified PRK5 vector. The R385A v-Src cDNA construct (v-Src with severely impaired tyrosine kinase activity) was a generous gift of Dr. M. Senften (Friedrich Miescher-Institute, Basel Switzerland) (Senften et al. 1995). The entire R385A v-Src coding region was removed from the original PSV vector using the unique restriction sites HindIII and BglII and then was inserted into the modified PRK5 vector in its multiple cloning region at the HindIII site. All channel or kinase coding regions were downstream from a cytomegalovirus (CMV) promoter. Tyrosine phosphorylated proteins were immunoprecipitated and detected by Western blot analysis with the mouse monoclonal antibodies 4G10 (Upstate Biochemical, Lake Placid, NY) and PY20 (Transduction Laboratories, Lexington, KY) that recognize phosphotyrosine. Two rabbit polyclonal antisera, raised against MalE fusion proteins (New England BioLabs, Beverly, MA) containing either an intracellular or an extracellular sequence specific to Kv1.3, generously were provided by Dr. James Douglass (Vollum Institute, Portland, OR) (Cai and Douglass 1993). These antibodies were used for Western blot analysis and immunocytochemistry. Src expression and activity were verified by measuring Src protein levels and total cell protein phosphotyrosine levels in cell lysates, with anti-Src mouse monoclonal antibody MAb 327 (Oncogene Science, Cambridge, MA) and the antiphosphotyrosine antibody PY20.

Site-directed mutagenesis

The parent Kv1.3 clone was propagated in Escherichia coli DH-1. Plasmid DNA was prepared by standard methods using a Qiagen Maxi Kit (Qiagen, Chatsworth, CA) followed by phenol-chloroform extraction and ethanol precipitation (Sambrook et al. 1989). All Kv1.3 channel mutants were constructed using two sequential polymerase chain reactions (PCR), with the plasmid containing the channel gene serving as a template. For each phosphorylation mutant, three oligonucleotides, each 15–24 bases in length, were synthesized. Two of the oligonucleotides were complementary to sequences on opposite sides of the tyrosine residue to be mutated, and the third was a mutant primer with an appropriate base change to mutate tyrosine to phenylalanine. The first PCR reaction used the mutagenic primer and the upstream primer. The second PCR reaction used the amplified, gel purified product of the first reaction and the downstream oligonucleotide as primers. In this way, a stretch of mutant DNA flanked by two unique restriction sites was obtained: the product was double digested and ligated into the parent channel backbone. The resulting mutant construct was sequenced to verify the mutation and detect PCR errors.

HEK 293 cell culture and transfection

HEK 293 cells were maintained in modified Eagle medium (MEM), 2% penicillin/streptomycin, and 10% fetal bovine serum. Before transfection, cells were grown to confluence (7 days), dissociated with trypsin-EDTA and mechanical trituration, diluted in MEM to a concentration of 600 cells/μl, and replated on Corning No. 25000. Dow Corning, Corning, NY) dishes. cDNA was introduced into the HEK 293 cells with a lipofectamine reagent (Gibco/BRL) 3–4 days after cell passage. At the time of transfection the cells were ~20–30% confluent for electrophysiological and microscopy experiments or 80–90% confluent for biochemical experiments. Lipofectamine and DNA were allowed to complex for 30–40 min. Cells were transfected for 4.5–5 h with 1 μg DNA/35-mm dish (microscopy), 2 μg DNA/35-mm dish (electrophysiology), or 10 μg DNA/60-mm dish (biochemistry); the DNA/lipofectamine complex was diluted in 1 or 2 ml of serum depleted OptiMEM (Gibco/BRL), respectively. In co-expression experiments, equal amounts of channel and kinase cDNA were mixed. For channel-alone or kinase-alone treatment groups, plasmid DNA with no coding insert was added to the channel or kinase cDNA so the total amount of DNA added was constant for all experimental groups. Transfection efficiency was monitored in parallel plates by transfecting with a Lac Z expression plasmid and subsequently staining the β-galactosidase reaction product. Staining efficiency was routinely 70–90%, and physiological expression of Kv1.3 was observed in 40–60% of the cells. In later experiments, constructs were cotransfected with pHook (Invitrogen); before patch recording, a brief incubation with an appropriate antibody linked to a 5 μM polystyrene bead allowed rapid recognition of transfected cells (Gromwald et al. 1988). More than 85% of bead-labeled cells expressed Kv1.3 current. Typically single channel events were detected as early as 9 h posttransfection, and macroscopic currents were recorded in the range of 24–96 h. A critical variable for transfection efficiency is the substrate. HEK 293 cells exhibited considerably lower channel expression (14%) when they were plated on poly-D-lysine–coated glass coverslips.

Biochemistry

HEK 293 cells were harvested 2 days posttransfection by lysis in ice-cold PPI solution (see above). The cell lysates were clarified by centrifugation at 15,000 g for 5 min at 4°C. The clarified lysate was incubated for 1 h with protein-AG sepharose (Pharmacia, Piscataway, NJ; 3 mg/ml cell lysate) at 4°C, and the protein-AG sepharose was separated from the clarified lysate by centrifugation at 15,000 g for 5 min at 4°C. Immunoprecipitation of tyrosine phosphorylated proteins from the supernatant was done by overnight incubation with antibody (1:4 ratio 4G10/PY20; 5 μg antibody/ml cell lysate) at 4°C followed by a 2-h incubation with protein-AG sepharose (3 mg/ml cell lysate) at 4°C. The immunoprecipitates were washed three times with ice-cold modified PPI solution (0.1% Triton X-100). Lysate samples and washed immunoprecipitates were distributed in sodium dodecyl sulfate (SDS)-gel loading buffer (Sambrook et al. 1989) containing 1 mM Na$_3$VO$_4$.

Proteins were separated on 10% acrylamide gels by SDS-polyacrylamide gel electrophoresis (PAGE) at 200 V and electrottransferred to nitrocellulose for Western blot analysis (Sambrook et al. 1989). The nitrocellulose blot was blocked with 5% nonfat milk and incubated overnight in primary antibody against Kv1.3 at 4°C. It was then incubated with horseradish peroxidase-conjugated secondary antibody (Amersham, Arlington Heights, IL) for 2 h at room temperature. Enhanced chemiluminescence (ECL; Amersham) exposure on XAR-2 film (Kodak, Rochester, NY) was used to visualize labeled protein. The film autoradiograms were analyzed by densitometry using a Bio-Rad model GS-670 imaging densitometer (Bio-Rad, Hercules, CA).
Two days after transfection, HEK 293 cells were fixed lightly with 1% paraformaldehyde for 10 min at room temperature, then washed five times with phosphate-buffered saline (PBS). The cell-bearing coverslips were incubated overnight at 4°C in anti-Kv1.3 rabbit polyclonal antisera, directed against either an intracellular or an extracellular epitope on the channel protein. The antisera were diluted 1:50 with 10% normal goat serum. Coverslips were washed five times with PBS and incubated in secondary antibody diluted with 10% normal goat serum (anti-rabbit F(ab')2-fluorescein-isothiocyanate (FITC): 1:1000, TagoImmunologicals, Burlingame, CA, 30 min at 37°C). The coverslips were washed again and mounted on glass slides with GelMount (Biomeda, Foster City, CA). Indirect immunofluorescence images were acquired by laser-scanning confocal microscopy (MRC-600, Bio-Rad, Hercules, CA) using equal gain and aperture setting for all images. Intensity of immunofluorescence was quantified by measuring the FITC signal (in arbitrary units) within a uniform 100 × 100 pixel box surrounding a single HEK 293 cell. For staining with the extracellular antibody, a total of 56 cells were measured across three transfection groups: control, Kv1.3, and Kv1.3 and v-Src. The mean FITC signal of the control group was background subtracted from each cell of the Kv1.3 and Kv1.3 and v-Src co-transfected groups. The remaining signal in the Kv1.3 and Kv1.3 + v-Src groups was compared by Student’s t-test.

**Electrophysiology**

Macroscopic currents in cell-attached membrane patches were recorded 36–72 h after transfection using an Axopatch-IB amplifier (Axon Instruments, Foster City, CA). Cells were visualized at ×40 magnification using a phase contrast water immersion lens (Zeiss, Thornwood, NY). Electrodes were fabricated from Jencons glass (No. M15/10, Bedfordshire, UK) fire-polished to 1-μm tip diam, and coated near the tip with beeswax to reduce the electrode capacitance. Tip diameter was standardized using the bubble number technique of Mittman et al. (1987), to ensure that patch membrane area was comparable in all experiments. Pipette resistances were between 9 and 14 MΩ. All voltage signals were generated and data were acquired using a microstar DAP 800/2 board (Microstar Lab, Bellevue, WA). The amplifier output was filtered at 2 kHz, digitized at 2–5 kHz, and stored for later analysis.

Patches were held routinely at a holding potential of −80 or −90 mV, and the voltage was stepped to depolarizing potentials for a pulse duration of 1,000 ms. Stimuli were delivered at ±45-s intervals to prevent cumulative inactivation of the Kv1.3 channel (Marom et al. 1993). To measure the voltage dependence of activation, patches were held at −90 mV and stepped to depolarizing potentials in 5-mV increments because of the steep activation curve characteristic of this channel. In these experiments the pulse duration was shortened to 50 ms, and patches were stimulated at 10-s intervals without cumulative inactivation of the channel. The acute effects of genistein (25 μM) or daidzein (26 μM) were tested by direct pipetting or laminar perfusion of the drugs into the recording chamber while patch-recording. The longer term effects of herbimycin A (870 nM) were measured by treating cells with either vehicle (DMSO in MEM) or herbimycin A diluted in MEM, 7–8 h posttransfection and then monitoring current levels in the cells 24–72 h after adding the inhibitor. In all cases, the final concentration of DMSO was ≤0.02%.

Data were analyzed using software written in our laboratory, in combination with the analysis packages Origin (MicroCal Software, Northampton, MA) and Quattro Pro (Borland International, Scotts Valley, CA). Data traces were subtracted linearly for leakage conductance. Functional expression of Kv1.3 current was defined as the presence of a voltage-dependent inactivating current. The inactivation of the macroscopic current, during a 1,000-ms voltage step from −80 to +40 mV, was fit to the sum of two exponentials by minimizing the sums of squares. The two inactivation time constants were combined by multiplying each by its weight and summing as described previously (Kupper et al. 1995). The deactivation of the macroscopic current was fit similarly but to a single exponential. Deactivation was measured on returning to −80 mV after a 1,000-ms step to +40 mV. To standardize for day-to-day variations in transfection efficiency, experimental treatments were compared statistically only with controls done on the same day. Differences between control and treatment groups within a particular transfection day then were averaged with those for other transfection days to determine overall statistical significance defined at the 0.95 confidence level.

**RESULTS**

**V-Src kinase suppresses cloned Kv1.3 channel current**

We have found that Src application modulates the outward current in rat olfactory bulb neurons (Fadool and Levitan, 1999). We have isolated patches from HEK 293 cells expressing Kv1.3, and confirmed the expression of Kv1.3 in cell-attached patches by an extracellular antibody, a total of 56 cells were measured across three transfection groups: control, Kv1.3, and Kv1.3 and v-Src. The remaining signal in the Kv1.3 and Kv1.3 + v-Src groups was compared by Student’s t-test.
unpublished observations). Because the Kv1.3 potassium channel is expressed prominently in the olfactory bulb (Kues and Wunder 1992), we investigated whether cloned Kv1.3 expressed in HEK 293 cells can be modulated by v-Src co-expression. HEK 293 cells provide a convenient assay system for the functional expression of cloned ion channels (Marshall et al. 1995): the cultured cells are maintained easily, have few contaminating endogenous ion channels, are amenable to patch recording, and express sufficient protein for parallel biochemical analyses. Channel expression is so robust that we routinely record macroscopic currents, corresponding to the activity of ≈1,000 channels, in standard (~1 μm² area) membrane patches. It was not possible to record in the whole cell configuration without saturating the amplifier.

Figure 1A illustrates the characteristic, C-type inactivating Kv1.3 current in a cell-attached patch in response to a series of depolarizing voltage steps to different pulse potentials. Such currents are observed routinely in about half of all HEK 293 cells tested (78 of 158 cells) 36–72 h after transfection with a plasmid encoding Kv1.3. In contrast, the outward current evoked by an identical voltage-step protocol is reduced by >95% (in all 84 cells tested) when Kv1.3 is co-expressed with the constitutively active nonreceptor tyrosine kinase, v-Src (Fig. 1B). The peak current magnitude in these Kv1.3 and v-Src cotransfected cells (Fig. 1B and Table 1) does not differ significantly from that observed in mock-transfected cells or in cells transfected with either v-Src alone (n = 6) or with plasmid containing no channel coding insert (control vector, n = 9). This suppression of Kv1.3 current is accompanied by robust phosphorylation of the channel, as measured by an immunoprecipitation/Western blot strategy (Holmes et al. 1996) employing antibodies specific for Kv1.3 and phosphorysotyrosine (Fig. 2A, top). In contrast, no tyrosine phosphorylation of Kv1.3 (Fig. 2A) or suppression of Kv1.3 current (Fig. 1C) is observed when the channel is co-expressed with R385A v-Src, a point mutant with severely impaired tyrosine kinase activity (Senften et al. 1995). The expression efficiency (percentage of patches containing inactivating Kv1.3-like current) in cell-attached patches from cells cotransfected with Kv1.3 and R385A v-Src is comparable with that observed in cells transfected with Kv1.3 alone. Furthermore, the peak current amplitude and various kinetic parameters of the Kv1.3 current are not affected significantly by co-expression with R385A v-Src (Table 1). Thus the suppression of Kv1.3 current by v-Src cotransfection requires the tyrosine kinase activity of v-Src.

**Kv1.3 expression and targeting are not affected by v-Src**

To test the possibility that v-Src suppresses Kv1.3 current by inhibiting expression of the channel protein, we measured the amount of Kv1.3 protein in transfected HEK 293 cells by Western blot analysis with an anti-Kv1.3 antibody (Cai and Douglass 1993). As shown in Fig. 2A (bottom), similar amounts of Kv1.3 protein are detected in cells transfected with Kv1.3 alone and cells cotransfected with Kv1.3 and v-Src (or R385A v-Src). Similar results for the equivalence of Kv1.3 expression with and without v-Src co-expression were obtained in 17 other experiments. Densitometry of the autoradiograms was used to quantitate channel expression. For each experiment, the expression level of Kv1.3 transfected alone was set to 1.00. The relative Kv1.3 expression level when v-Src is cotransfected is 1.04 ± 0.04 (mean ± SE; n = 18). Another possible explanation for the suppression of current is that Kv1.3 protein is expressed normally in v-Src cotransfected cells but cannot be processed properly and targeted to the plasma membrane. To investigate this, we carried out immunocytocchemical analysis of HEK 293 cells, using an antibody directed against an extracellular epitope on the channel protein. The cells were fixed lightly to minimize cell permeabilization, so staining by this antibody (Fig. 2B) provides a measure of Kv1.3 channels that are located in the plasma membrane and hence are accessible to the antibody. As shown in the confocal microscopy images in Fig. 2B, the immunostaining of Kv1.3 channels with the extracellular antibody is comparable in cells transfected with channel alone and in those cotransfected with channel and v-Src, suggesting that membrane targeting of Kv1.3 is not affected substantially by tyrosine phosphorylation. These photomicrographs are representative of >30 sin-

| TABLE 1. Biophysical properties of wild-type and mutant Kv1.3 channels and their modulation by v-Src |
|---------------------------------|--------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Construct                      | Peak Current (pA) | Inactivation (τ), ms | Deactivation (τ), ms | Activation (V_{1/2}), mV | Voltage Dependence (k) |
| Kv1.3 + vector                 | 550 ± 96 (26)     | 668 ± 54 (26)       | 26 ± 3 (26)       | -46.1 ± 1 (3)     | 2.9 ± 0.4 (3) |
| Kv1.3 + v-Src                  | 37 ± 2* (37)      | 1,575 ± 328* (18)   | 14 ± 2* (26)      | 10.2* (3.3)       | 2.7 ± 0.2 (5)  |
| Kv1.3 + vector                 | 547 ± 157 (10)    | 707 ± 76 (8)        | 19 ± 3 (8)        | -44.3 ± 2 (5)     | 3.2 ± 0.3 (10) |
| Kv1.3 + R385A v-Src            | 418 ± 81 (14)     | 521 ± 61 (14)       | 24 ± 3 (13)       | -46.6 ± 1.4 (10)  | 2.3 ± 0.2 (3)  |
| YY1111+113FFF Kv1.3 + vector   | 504 ± 79 (18)     | 791 ± 71 (17)       | 22 ± 2 (17)       | -34.9 ± 2.1 (2)   | 3.2 ± 0.2 (3)  |
| YY1111+113FFF Kv1.3 + v-Src    | 204 ± 64* (8)     | 774 ± 141 (8)       | 12 ± 2* (9)       | -34.6 ± 2.5 (4)   | 4.4 ± 0.2 (4)  |
| Y137F Kv1.3 + vector           | 412 ± 84 (22)     | 665 ± 71 (20)       | 27 ± 3 (20)       | -40.7 ± 0.8 (8)   | 3.4 ± 0.5 (8)  |
| Y137F Kv1.3 + v-Src            | 195 ± 53 (7)      | 566 ± 189 (6)       | 26 ± 7 (6)        | -35.6 ± 2.8* (2)  | 1.9 ± 0.2 (2)  |
| Y449F Kv1.3 + vector           | 410 ± 123 (17)    | 766 ± 82 (7)        | 21 ± 4 (8)        | -46.2 ± 0.2 (2)   | 2.7 ± 0.1 (2)  |
| Y449F Kv1.3 + v-Src            | 604 ± 116 (11)    | 1,119 ± 110* (6)    | 25 ± 7 (6)        | -46.4 ± 1.2 (3)   | 2.6 ± 0.2 (3)  |
| Y479F Kv1.3 + vector           | 736 ± 147 (12)    | 763 ± 87 (12)       | 27 ± 6 (12)       | -43.5 ± 2.6 (6)   | 2.5 ± 0.3 (6)  |
| Y479F Kv1.3 + v-Src            | 231 ± 116* (5)    | 726 ± 137 (7)       | 15 ± 5* (7)       | -40.0 ± 1.4 (4)   | 2.5 ± 0.4 (4)  |

Values are means ± SE with sample size in parentheses. Time constant (τ) values were estimated from exponential fits to the inactivating or deactivating portions of the current. The V_{1/2}, the voltage at which half of the channels were activated, was calculated by fitting normalized peak tail currents at different holding potentials to a Boltzmann function. The slope of this function, or the value for the steepness of the voltage dependence, is reported as k. An ANOVA was performed for each of the biophysical properties of the wild-type vs. Kv1.3 mutants; there were no significant differences across any of the singly expressed constructs. Student’s t-test were performed across matched construct pairs in the co-expression experiments. *, significantly different, Student’s t-test (with pooled standard deviation or t statistic as deemed necessary by unequal sample sizes or F distribution).
Tyrosine kinase inhibitors relieve the current suppression by v-Src

The results described above suggest that Kv1.3 current suppression by v-Src results from modulation of Kv1.3 channels that are in the plasma membrane. To test this further, we asked whether inhibition of ongoing tyrosine kinase activity can reverse the suppression of Kv1.3 current. Most cells express high levels of tyrosine phosphatases that participate in a rapid turnover of protein phosphotyrosine (Hunter 1995; Nairn and Shenolikar 1992). When cells are pretreated with the irreversible tyrosine kinase inhibitor herbimycin A (Levitzi and Gazit 1995; Uehara et al. 1989) for 24–72 h before recording, Kv1.3 current suppression by v-Src (Fig. 3B) is relieved (Fig. 3C). C-type inactivating Kv1.3 current is observed in 53% of cotransfected cells pretreated with herbimycin A comparable with the efficiency of expression seen when Kv1.3 is transfected alone (49%). The current-voltage relationship for the peak Kv1.3 current is also the same in Kv1.3 transfected cells and in Kv1.3 and v-Src cotransfected cells pretreated with herbimycin A (Fig. 3D), indicating that the inhibitor does not evoke other currents. Although current suppression by v-Src clearly is relieved in herbimycin A-pretreated cells (Fig. 3E), the peak current amplitude is not as high as in cells expressing Kv1.3 alone (compare Fig. 3, A and C).

We also tested the action of a structurally different tyrosine kinase inhibitor, genistein (Akiyama et al. 1987), that acts more rapidly than herbimycin A. In 4 of 17 cells, genistein relieved the suppression of current in Kv1.3 and v-Src cotransfected cells within the duration of a patch recording. For these experiments, patches were held at −80 mV and depolarized to +40 mV at 1-min intervals. Twelve successive current traces from a Kv1.3-transfected cell, in response to such depolarizations in a Kv1.3 and v-Src cotransfected cell, are shown in Fig. 4A. Note that the peak current amplitude is stable over time (Fig. 4C). In contrast, little current is evoked by such depolarizations in a Kv1.3 and v-Src cotransfected cell (Fig. 4B, left). When 25 μM genistein is added to the medium bathing cotransfected cells, inactivating Kv1.3 current appears within 6.5–9.5 min and increases over time (middle traces in Fig. 4, B and C). Daidzein, a structural analogue of genistein that does not inhibit tyrosine kinase activity (Akiyama et al. 1987), does not relieve the suppression of current in cotransfected cells (right traces in Fig. 4, B and C). The peak current at 15 min in cells treated with genistein (251 ± 31 pA, n = 4) is significantly higher (paired t-test) than the current at time 0. In contrast, the peak current in cells treated 15 min with daidzein (50 ± 11 pA, n = 5) or vehicle alone (59 ± 13 pA, n = 11) is not significantly higher than the currents at time 0 (paired t-tests). The rapid appearance of Kv1.3 current after genistein treatment suggests strongly that modulation occurs for channels in (or near) the plasma membrane. Although the peak current ampli-
Tyrosine kinase inhibitor, herbimycin A, relieves the suppression of Kv1.3 current by v-Src. A: Kv1.3 currents in representative cell-attached patches on HEK 293 cells cotransfected with Kv1.3 or (B and C) Kv1.3 and v-Src and then treated for 24 h with either 0.02% dimethyl sulfoxide (DMSO) carrier (B) or 870 nM herbimycin A (C). Voltage protocol as in Fig. 1 except the pulse potentials ranged from −60 to +80 mV. All the recordings in A–C were made in parallel plates on the same transfection day. D: current-voltage relation in patches from 4 herbimycin treated Kv1.3 and v-Src cotransfected cells (●) in comparison with that in a Kv1.3 singly transfected cell (○) recorded on the same transfection day. Data are normalized to the peak current at +40 mV and fit with a locally weighted regression (lines). E: mean ± SE total current in patches from Kv1.3 and v-Src cotransfected cells treated with 0.02% DMSO carrier (●) or 870 nM herbimycin A (■). Peak current was measured with holding potential −80 mV and pulse potential +40 mV. *, significantly different, Student’s t-test (t' statistic).

Multiple tyrosine residues in Kv1.3 are important for current suppression by v-Src

Kv1.3 contains a number of tyrosine residues (Figs. 5A and 6A) that lie within consensus sequences favorable for tyrosine phosphorylation (Songyang et al. 1995). We mutated these six tyrosine residues to phenylalanine at positions 111–113 (these 3 adjacent tyrosines were treated as a unit and mutated together), 137, 449, and 479 and examined the properties of each Kv1.3 mutant channel (Table 1). The biophysical properties measured, including peak current amplitude, inactivation and deactivation rate, and voltage dependence of channel activation, are not significantly different in any of the mutant channel constructs compared with the wild-type Kv1.3 [one-way analysis of variance (ANOVA), α = 0.05, n = 12–26].

We then examined the effects of v-Src cotransfection on the current magnitude of the various mutant channels (Figs. 5B and 6B, and Table 1). v-Src is still able to suppress both the YYY111–113FF and Y479F Kv1.3 mutant channels, suggesting that phosphorylation of these tyrosine residues is not necessary for the Src-induced current suppression (Fig. 5, B and C). In contrast, the peak current of the Y137F and Y449F Kv1.3 mutant channels is not modulated significantly (Student’s t-test) by v-Src cotransfection (Fig. 6, B and C). This indicates that phosphorylation of both of these tyrosine residues is necessary for current suppression by v-Src, and mutation of either one of them is sufficient to eliminate this modulation.

**v-Src modulates inactivation kinetics of Kv1.3**

In addition to suppressing Kv1.3 current, v-Src cotransfection slows the C-type inactivation of the residual current (Fig. 7).
In those cells in which there is sufficient residual current to fit the inactivation kinetics, the inactivation time constant of the wild-type (WT) channel is significantly greater in the presence of v-Src (Fig. 7B, left, and Table 1). This can be seen even more clearly in the Y449F mutant channel (Fig. 7A), in which the current is not suppressed by v-Src (see Fig. 6) and the time constant of inactivation can be fit more accurately (note the smaller error bar for Y449F in Fig. 7B). In contrast, no such slowing of C-type inactivation by v-Src is observed with the YYY111–113FFF, Y137F, or Y479F mutant Kv1.3 channels (Fig. 7B and Table 1). These results suggest that the slowing of C-type inactivation is an unusually complex modulatory phenomenon requiring the participation of at least three distinct tyrosine residues in different parts of the channel protein (see insets in Fig. 7B).

v-Src modulates deactivation kinetics of Kv1.3

The kinetics of deactivation of Kv1.3 at the end of a depolarizing pulse also can be modulated by Src (Fig. 8). v-Src co-expression causes a speeding of the deactivation of the WT Kv1.3 channel, expressed as a decrease in the deactivation time constant (Fig. 8B, left, and Table 1). A similar speeding of deactivation also is observed with the YYY111–113FFF and Y479F mutant Kv1.3 channels (Fig. 8, A and B), suggesting that these tyrosine residues do not play a critical role in this modulation. In contrast, no effect of Src on deactivation kinetics is observed with the Y137F or Y449F mutant Kv1.3 channels (Fig. 8B), indicating that Y137 and Y449 are important for modulation of deactivation.
channel inactivation and deactivation. This R385A v-Src mutant expresses the same amount of protein as wild-type v-Src, but its tyrosine kinase activity is very much decreased (Senften et al. 1995). This result provides strong evidence that the current suppression does not result simply from the expression of another protein together with the channel but requires the tyrosine kinase activity of wild-type v-Src. The second line of evidence is pharmacological and involves the demonstration that suppression of Kv1.3 current can be relieved by treatment of the cells with tyrosine kinase inhibitors. Although caution must be exercised in interpreting data with pharmacological agents that may have side effects, the similar results with two structurally distinct inhibitors suggest that their relief of current suppression does indeed result from the inhibition of tyrosine kinase activity.

The expression of v-Src can influence many cellular functions, including protein synthesis and cell proliferation (e.g., Neel et al. 1995; Roche et al. 1995). Thus it seemed possible that suppression of Kv1.3 current by v-Src involves a decrease in the synthesis of the channel protein or its targeting

**FIG. 5.** Effect of v-Src co-expression on the YYY111–113FFF and Y479F Kv1.3 mutant channels. *A:* schematic representation of the positions of 4 tyrosine residues within 2 tyrosine phosphorylation recognition motifs in the Kv1.3 channel sequence (left). Currents in representative cell-attached patches on HEK 293 cells transfected with the wild-type (WT) Kv1.3 channel (Control) or cotransfected with WT Kv1.3 and v-Src (+v-Src) (right). Holding voltage, −80 mV; the patch voltage was stepped to +40 mV. *B:* same as in A but for the Kv1.3 channel mutants, YYY111–113FFF and Y479F Kv1.3. *C:* histogram plot of the peak current amplitude (mean ± SE) of the WT, and YYY111–113FFF and Y479F Kv1.3 mutant channels, when patches were held at −80 mV and stepped to +40 mV. Solid bar = Kv1.3 construct alone. Hatched bar = Kv1.3 construct cotransfected with v-Src. Data were normalized to the singly transfected condition for each construct. *, significantly different, Student’s t-test. For current amplitudes, sample sizes, and statistical comparisons, see Table 1.

**DISCUSSION**

We have examined the molecular details of tyrosine phosphorylation of Kv1.3 by a co-expressed tyrosine kinase in a heterologous expression system. Co-expression of Kv1.3 with the nonreceptor tyrosine kinase, v-Src, causes a prominent suppression of Kv1.3 current in membrane patches of HEK 293 cells. Two lines of evidence indicate that the suppression of Kv1.3 current by v-Src requires tyrosine phosphorylation. First, when Kv1.3 is co-expressed with a v-Src construct in which the arginine at position 385 is substituted by alanine, the channel is not phosphorylated and there is no change in channel properties measured, including current amplitude, voltage dependence of activation, and rate of

**FIG. 6.** Effect of v-Src co-expression on the Y137F and Y449F Kv1.3 mutant channels. *A:* same as for Fig. 5 but showing the positions of the Y137 and Y449 residues. *B:* same as for Fig. 5 but for the Kv1.3 channel mutants, Y137F and Y449F Kv1.3. *C:* same as for Fig. 5. *, significantly different, Student’s t-test. Although there is some variability, the peak current of the Y137F and Y449F mutant Kv1.3 channels is not altered significantly by v-Src co-expression. For current amplitudes, sample sizes, and statistical comparisons, see Table 1.
TYROSINE PHOSPHORYLATION MODULATES Kv1.3

Tyrosine phosphorylation modulates Kv1.3. Treatment of tyrosine phosphatases with pervanadate in HEK 293 cells (Holmes et al. 1996), indicating that Y449 is also a target for endogenous tyrosine kinases. Huang et al. (1993) found that suppression of the Kv1.2 channel after activation of a muscarinic acetylcholine receptor can be relieved almost completely by mutation of a single tyrosine residue, Y132, in this channel. This residue may be homologous to Y137 in Kv1.3.

Our findings suggest that suppression of Kv1.3 current by v-Src requires phosphorylation of multiple tyrosine residues. Removal of either of two tyrosine residues, in different parts of the channel, removes some or all of the suppression caused by v-Src cotransfection. It remains to be determined whether cooperation among multiple phosphorylation sites, as appears to exist for Kv1.3, is a general feature of channel suppression by tyrosine phosphorylation or if a single tyrosine residue is the major target in other ion channels. Phosphorylation of multiple serine residues can contribute to the modulation of both voltage-gated (Li et al. 1992) and ligand-gated (Roche et al. 1996) ion channels.

Although tyrosine phosphorylation of Kv1.2 by a FAK family tyrosine kinase causes suppression of current without any apparent effects on channel kinetics (Huang et al. 1993), tyrosine phosphorylation by v-Src causes changes in both deactivation and C-type inactivation kinetics of Kv1.3. Here again, mutational analysis implicates multiple (and different) tyrosine residues in each of these modulatory phenomena. Modulation of channel kinetic properties can have important physiological consequences. In the case of Kv1.3 and probably other voltage-gated potassium channels (De-...
Coursey 1990; Marom and Levitan 1994; Marom et al. 1993), C-type inactivation during a prolonged depolarization is another manifestation of the cumulative inactivation seen in response to a series of closely spaced short depolarizations, such as action potentials (Aldrich 1981; Marom and Abbott 1994). Thus modulation of the rate of C-type inactivation, as shown here for Kv1.3, will have a profound influence on such features of neuronal activity as firing rate and delay of firing in response to a prolonged depolarization (Marom and Abbott 1994; Rahamimoff et al. 1992; Storm 1988). It is interesting that the rate of C-type inactivation of Kv1.3 increases in a characteristic fashion when the environment on the intracellular side of the channel is altered (Cahalan et al. 1985; Kupper et al. 1995; Marom et al. 1993; Oleson et al. 1993). Although the molecular mechanism of this phenomenon is not yet understood (Kupper et al. 1995), the present results raise the intriguing possibility that it may involve tyrosine dephosphorylation.

The involvement of tyrosine phosphorylation in cell division, differentiation, and signaling has been studied extensively. It is really quite striking, in contrast, how little information is available about the role of the exceptionally high levels of tyrosine kinases in postmitotic neurons (Brugge et al. 1985; Hanley 1988), where presumably they are not required for growth control. Our present results emphasize that phosphorylation of an ion channel by a tyrosine kinase can produce different modulatory effects depending on the particular combination of tyrosine residues phosphorylated. Elucidation of further details of the mechanism of Kv1.3 channel modulation will provide important insights into the complexity of kinase modulation of ion channels and help to define the role of tyrosine phosphorylation in neurons.

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