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Phosphorylation-dependent Regulation of Kv2.1 Channel Activity at Tyrosine 124 by Src and by Protein-tyrosine Phosphatase ϵ*

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Voltage-gated potassium (Kv) channels are a complex and heterogeneous family of proteins that play major roles in brain and cardiac excitability. Although Kv channels are activated by changes in cell membrane potential, tyrosine phosphorylation of channel subunits can modulate the extent of channel activation by depolarization. We have previously shown that dephosphorylation of Kv2.1 by the nonreceptor-type tyrosine phosphatase PTPϵ (cyt-PTPϵ) down-regulates channel activity and counters its phosphorylation and up-regulation by Src or Fyn. In the present study, we identify tyrosine 124 within the T1 cytosolic domain of Kv2.1 as a target site for the activities of Src and cyt-PTPϵ. Tyr^{124} is phosphorylated by Src *in vitro*; in whole cells, Y124F Kv2.1 is significantly less phosphorylated by Src and loses most of its ability to bind the D245A substrate-trapping mutant of cyt-PTPϵ. Phosphorylation of Tyr^{124} is critical for Src-mediated up-regulation of Kv2.1 channel activity, since Y124F Kv2.1-mediated K^+ currents are only marginally up-regulated by Src, in contrast with a 3-fold up-regulation of wild-type Kv2.1 channels by the kinase. Other properties of Kv2.1, such as expression levels, subcellular localization, and voltage dependence of channel activation, are unchanged in Y124F Kv2.1, indicating that the effects of the Y124F mutation are specific. Together, these results indicate that Tyr^{124} is a significant site at which the mutually antagonistic activities of Src and cyt-PTPϵ affect Kv2.1 phosphorylation and activity.

Voltage-dependent K^+ (Kv)[1] channels are key regulators of cellular functions and affect parameters such as action potential wave forms, neuronal firing patterns, synaptic integration, neurotransmitter release, volume regulation, and cell proliferation (1). Proper function of Kv channels is vital to health and well being, as demonstrated by identification of mutations in genes that encode Kv channel subunits as causing cardiac and neurological disorders in humans (2–4).

Kv channels are composed of four α-subunits that span the cell membrane and that can be found in some cases in association with regulatory cytosolic β-subunits (1, 5, 6). All Kv channel α-subunits share a common core structure of six transmembrane segments (S1–S6) and a P-loop forming the pore region of the channel (7). α-Subunits contain several major structural domains. Among these is a voltage-sensing domain, composed of segments S1–S4 that folds in α-helical structure and surrounds the pore. This domain is responsible for energy transduction and for controlling gating behavior. A second major domain of α-subunits is the pore domain (segments S5-P-S6), whose structure is likely to be similar to the crystal structure of the bacterial KcsA K^+ channel, with the inner helix S6 lining much of the pore (7). Another major domain of α-subunits is the T1 region, which is part of the cytosolic N-terminal domain. In *Shaker*-related Kv channels, this domain comprises ~120 amino acids and is located between the N-terminal inactivation ball and S1 (8). The T1 domain is responsible for molecular segregation of Kv channels, in which tetramerization of α-subunits belonging to the same subfamily is preferred (8). In addition, this domain associates with the auxiliary β-subunits of *Shaker*-related Kv channels and is the site for regulation of channel activity by various cytosolic factors (8–11).

Significant evidence has shown that Kv channels are substrates of protein kinase activities and that phosphorylation can affect channel characteristics (12–15). Several studies have established a prominent role for Src family protein-tyrosine kinases (PTKs) in regulation of Kv channels. Among these, Src family kinases have been shown to phosphorylate Kv1.3 and to down-regulate its activity in heterologous expression systems as well as in Jurkat T cells and in rat olfactory bulb neurons (16–21). Similar effects were noted with Kv1.5 in transfected HEK 293 cells (22). Src- and Fyn-mediated phosphorylation of Kv1.5 and of Kv2.1 enhanced K^+ channel activity in mouse Schwann cells and in rat cortical astrocytes (23–25). Interestingly, in rat retinal pigment epithelial cells, Src family tyrosine kinases can activate or inhibit Kv1.3 channel activity in a manner dependent on protein kinase C activity (26). Last, an indirect role for the Kv1.5 α-subunits in phosphorylation of other subunits was recently shown. The Src homology 3 domains of Src family kinases can bind Kv1.5, thereby granting these PTKs access to Kv channel α-subunits that associate with Kv1.5 but that have no Src homology 3 binding sites of their own (27).

Protein-tyrosine phosphatases (PTPs) are generic antagonists of PTKs and play crucial roles in regulating physiological processes by affecting protein phosphorylation (28–30). However, in contrast with the wealth of information concerning the effects of PTKs on Kv channels, very little is known about how PTPs participate in these processes. The receptor-type PTPα (31, 32) physically associates with Kv1.2 and up-regulates

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[1] The abbreviations used are: Kv channel, delayed rectifier, voltage-gated potassium channel; cyt-PTPϵ, nonreceptor form of PTPϵ; GST, glutathione *S*-transferase; PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; WT, wild-type.

channel activity following its inhibition by the G protein-coupled M1 muscarinic acetylcholine receptor in *Xenopus* oocytes and mammalian cells (33). The same PTP was later shown also to counter serotonergic inhibition of Kv1.1 and Kv1.2 in a similar experimental system (34). We have demonstrated that the nonreceptor form of PTP epsilon (cyt-PTPϵ) (35) dephosphorylates and down-regulates Kv1.5 and Kv2.1 following their phosphorylation by Src or by Fyn in transfected cells and in *Xenopus* oocytes (36). In agreement with these results, both Kv1.5 and Kv2.1 were hyperphosphorylated in primary Schwann cells and in sciatic nerve tissue from mice genetically lacking PTPϵ. These effects were correlated with increased Kv channel currents in PTPϵ-deficient Schwann cells, as well as with transient but severe hypomyelination of sciatic nerve axons in young PTPϵ-deficient mice (36). Strong support for Kv2.1 being a substrate of cyt-PTPϵ was obtained in experiments, which established that a substrate-trapping mutant of cyt-PTPϵ bound and co-precipitated with Kv2.1 (36, 37). Interactions between this mutant and Kv2.1 were severely reduced by sodium pervanadate, indicating that they were mediated by the catalytic site of cyt-PTPϵ binding to at least one phosphotyrosine residue in Kv2.1. The identity of that residue, however, remained unknown.

In the present study, we identify Tyr^{124}, a tyrosine residue located in the T1 cytosolic domain of Kv2.1, as an important site for phosphorylation by Src. We also identify the same residue as a docking site for the substrate-trapping mutant of cyt-PTPϵ, implying that it is dephosphorylated by this phosphatase. Phosphorylation of Tyr^{124} has significant effects on Src-mediated regulation of Kv2.1 channel activity, since mutating this residue to a nonphosphorylatable phenylalanine abolishes most of the ability of Src to up-regulate channel activity without affecting other properties of the channel. These results establish Tyr^{124} as an important site for mutually antagonistic regulation of Kv2.1 by Src and by cyt-PTPϵ and highlight the role of post-translational modifications in the T1 domain in affecting Kv2.1 activity.

EXPERIMENTAL PROCEDURES

Reagents—The following cDNAs were used in this study, all cloned in the eukaryotic expression vector pcDNA3 (Invitrogen): mouse cyt-PTPϵ (35), D245A cyt-PTPϵ (36), rat Kv2.1 (gift of Drs. J. Barhanin and M. Lazdunski), and chicken wild-type and Y527F Src (gift of Dr. S. Courtneidge). Both PTPϵ cDNAs contained a FLAG tag at their C terminus. The Y124F mutation was introduced into the rat Kv2.1 cDNA by site-directed mutagenesis; the presence of the desired mutation and absence of other mutations were verified by DNA sequencing. Antibodies used in this study included polyclonal anti-PTPϵ (38), polyclonal anti-Kv2.1 (Alomone Laboratories, Jerusalem), monoclonal anti-v-Src (Calbiochem), anti-FLAG M2 affinity beads (Sigma), and anti-phosphotyrosine (clone PY20; Transduction Laboratories).

Cell Culture and Immunofluorescence—HEK 293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% (v/v) fetal calf serum (Invitrogen), 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were transfected using the calcium-phosphate method (39). For immunofluorescence, 293 cells were plated on glass cover slips previously coated with polylysine (Sigma), transfected with the relevant expression vectors, and stained with antibodies as described previously (40). Primary antibodies used were monoclonal anti-FLAG M2 and/or polyclonal anti-Kv2.1, each diluted 1:250; secondary antibodies included CY3- or fluorescein-conjugated anti-mouse or anti-rabbit IgG (1:300 dilution; Jackson Immunoresearch Laboratories). Stained cells were examined with the aid of a Bio-Rad model MRC 1024 confocal system and an argon/krypton mixed gas laser, mounted on a Zeiss Axiovert microscope.

Protein Blotting and Substrate Trapping—Cells were lysed in Buffer A (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40), supplemented with 0.5 mM sodium pervanadate and protease inhibitors (1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 40 μM bestatin, 15 μM E-64, 20 μM leupeptin, 15 μM pepstatin; Sigma). SDS gel electrophoresis, blotting, and antibody hybridization were as described (40). Complete protein transfer following blotting was verified routinely by noting transfer of prestained molecular size marker proteins of the proper size range; the absence of lane-to-lane variations in blotting was verified by staining the blotted membranes with Ponceau S (Sigma). In control experiments, where known, graded amounts of protein were subject to SDS-PAGE and blotting, the intensities of signals obtained were proportional in a linear fashion to the amounts of antigen loaded on the gel. For substrate-trapping immunoprecipitation experiments, cells were lysed in Buffer A supplemented with 5 mM iodoacetic acid and protease inhibitors. One mg of cellular proteins was reacted with anti-FLAG M2 beads for 6–8 h, followed by two washes with buffer A and one wash with radioimmune precipitation buffer.

GST Fusion Protein Phosphorylation by Src—GST-Y124 was prepared by annealing the two complementary oligomers, Y124 sense (GATCCGGCATCGATGAGATCTACCTGGAGTCCTGCG) and Y124 antisense (AATTCGCAGGACTCCAGGTAGATCTCATCGATGCCG). The resulting double-stranded DNA fragment, which encoded a decapeptide centered around Tyr^{124} of Kv2.1 (^{119}GIDEIYLESC; accession number X16476) and which contained *Bam*HI and *Eco*RI overhangs, was cloned into the *Bam*HI and *Eco*RI sites of pGEX2TK (Amersham Biosciences). The mutated GST-Y124F construct, in which Tyr^{124} was replaced by an unphosphorylatable phenylalanine, was prepared in a similar manner using oligonucleotides Y124F sense (GATCCGGCATCGATGAGATCTTCCTGGAGTCCTGCG) and Y124F antisense (AATTCGCAGGACTCCAGGAAGATCTCATCGATGCCG). The structure of the constructs was verified by DNA sequencing. GST fusion proteins were grown in *Escherichia coli* DH5α bacteria, and purified by binding to glutathione-agarose beads (Sigma) and subsequent elution with 20 mM glutathione, 50 mM Tris-Cl, pH 8.0. Wild-type chicken Src was expressed in 293 cells and was immunoprecipitated using anti-v-Src antibodies (Calbiochem). GST fusion phosphorylation reactions were conducted in 25 μl of kinase buffer (20 mM MOPS, pH 7.0, 5 mM $MgCl_2$), to which equal amounts of precipitated Src, 1 μl (equal to 5 μCi) of [γ-^{32}P]ATP (3000 Ci/mmol, 10 mCi/ml; Amersham Biosciences), and equal amounts (~2 μg/5 μl) of eluted GST fusion proteins were added. Tubes were incubated at 30 °C for 30 min; samples were then separated on 12% SDS-PAGE gels and blotted onto membranes. Radioactivity present in GST fusion proteins was quantified with a phosphor imager (BAS 2500; Fuji, Japan). Verification of the amounts of GST fusion proteins present in the reactions was performed by Ponceau S staining of membranes and by probing membranes with anti-GST antibodies. For verification of amounts of Src present, blots were probed with anti-Src antibodies.

Electrophysiology in Xenopus Oocytes—*Xenopus laevis* frogs were purchased from *Xenopus* 1 (Dexter, MI). The procedures followed for surgery and maintenance of frogs were approved by the Animal Research Ethics Committee of Tel Aviv University. Frogs were anesthetized with 0.2% tricaine (Sigma). Pieces of the ovary were surgically removed and digested with 1 mg/ml collagenase (type IA; Sigma) in Ca^{2+}-free ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES titrated to pH 7.5 with NaOH) for 1 h to remove follicular cells. Stage V and VI oocytes were used for DNA nuclear injection and were maintained at 18 °C in ND96 (containing 1.8 mM $CaCl_2$), supplemented with 1 mM pyruvate and 50 μg/ml gentamycin. Nuclear injections utilized 0.5 ng/10 nl plasmid DNA encoding WT Kv2.1 or Y124F Kv2.1 per oocyte; when indicated, similar amounts of DNA for constitutively active Y527F Src were also injected.

Standard two-electrode voltage clamp measurements were performed as previously described (41) 2–4 days following nuclear DNA microinjection. Oocytes were bathed in a modified ND96 solution (containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 5 mM HEPES, titrated to pH 7.4 with NaOH) under constant perfusion using a peristaltic pump (Gilson) at a flow rate of 0.4 ml/min. $CaCl_2$ was reduced to 0.1 mM to virtually eliminate the contribution of endogenous Ca^{2+}-activated Cl^- currents. Whole cell currents were recorded at room temperature (20–22 °C) using a GeneClamp 500 amplifier (Axon Instruments). Glass microelectrodes (A-M Systems, Inc.) were filled with 3 M KCl and had tip resistances of 0.5–1.5 megaohms. Stimulation of the preparation and data acquisition and analyses were performed using the pCLAMP 6.02 software (Axon Instruments) and a 586 personal computer interfaced with a Digidata 1200 interface (Axon Instruments). Current signals were filtered at 0.5 kHz and digitized at 2 kHz. Unless specified, the holding potential was −80 mV. Leak subtraction was performed off-line, using the Clampfit program of the pCLAMP 6.02 software. Data analysis was performed using the Clampfit program (pCLAMP 8.1; Axon Instruments) and Axograph 4.0 (Axon Instruments). All data were expressed as mean ± S.E. Statistically significant differences between paired groups were assessed by Student's *t* test.

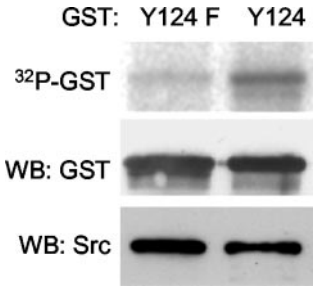

FIG. 1. **Tyr^{124} of Kv2.1 is phosphorylated by Src *in vitro*.** A decapeptide containing Tyr^{124} and the surrounding residues (^{119}GIDEIYLESC) was fused to GST (GST-Y124). This fusion protein and a similar one containing the Y124F mutation (GST-Y124F) were phosphorylated *in vitro* by Src in the presence of [γ-^{32}P]ATP as described under "Experimental Procedures." Aliquots of the phosphorylation reaction were analyzed by SDS-PAGE and blotting. *Top*, ^{32}P incorporated into either GST fusion protein. The *middle* and *bottom panels* depict hybridization of blots with anti-GST and anti-Src antibodies, respectively, to indicate the amount of GST fusion proteins and of Src kinase in the assays. Note that despite a slight excess of Src in the GST-Y124F lane, this protein is less phosphorylated than GST-Y124. Shown is one experiment representative of two performed.

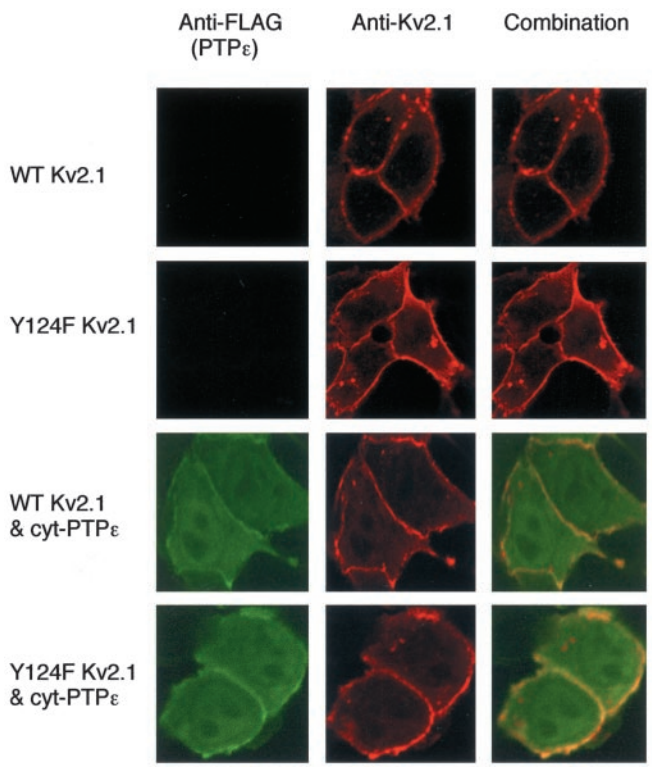

FIG. 2. **Kv2.1 and cyt-PTPε co-localize at the cell membrane.** Confocal microscopy analysis of expression of Kv2.1 (*red*) and cyt-PTPε (*green*) in HEK 293 cells. WT or Y124F Kv2.1 was expressed in the cells, either alone or together with FLAG-tagged cyt-PTPε, after which cells were processed as described under "Experimental Procedures." Note the exclusively membranal localization of WT and Y124F Kv2.1 molecules as well as nuclear, cytosolic, and membranal expression of cyt-PTPε. Original magnification was ×600. Identical staining pattern of cyt-PTPε was obtained in cells that did not express either form of Kv2.1 (40) (data not shown).

RESULTS

Phosphorylation of Tyr^{124} of Kv2.1 by Src in Vitro—Previous studies have shown that tyrosine phosphorylation of Kv2.1 by Src and Fyn up-regulates channel activity, whereas dephosphorylation of Kv2.1 by cyt-PTPε counters this effect (36). This result suggests that both kinases, on the one hand, and cyt-PTPε, on the other hand, affect phosphorylation of the same set of tyrosine residues in opposite manners. The Kv2.1 α-subunit has 19 tyrosine residues, of which five are located in extracellular or in membrane-spanning segments and are presumably inaccessible to cytosolic or membrane-associated enzymes. Of the remaining 14 tyrosines, six are located in the N-terminal cytosolic domain, seven are within the C-terminal domain, and one is located in the short cytosolic loop between the S4 and S5 membrane-spanning domains. Examination of the tyrosine residues in Kv2.1 revealed that only one of these, Tyr^{124} of the N-terminal cytosolic T1 domain, is embedded in a sequence that is somewhat similar to the consensus sequence for Src phosphorylation (^{121}DEIYLES *versus* consensus of EEIYG/EEF) (42).

In order to determine whether Src could phosphorylate Tyr^{124} of Kv2.1 *in vitro*, we examined the ability of Src to phosphorylate a peptide derived from the sequence of Kv2.1, ^{119}GIDEIYLESC, which was centered around Tyr^{124} and in which this residue was the only tyrosine. A similar peptide in which Tyr^{124} was replaced with a nonphosphorylatable phenylalanine residue (^{119}GIDEIFLESC) served as a negative control. Both peptides were produced as glutathione *S*-transferase (GST) fusion proteins in bacteria and were purified by glutathione-agarose affinity chromatography and by subsequent elution as described under "Experimental Procedures." Equal amounts of both fusion proteins were mixed with Src and with [γ-^{32}P]ATP, and phosphorylation was allowed to proceed for 30 min. Some background phosphorylation of the GST-Y124F fusion protein was evident in these studies (Fig. 1). Since the Kv2.1-derived sequence of the GST-Y124F fusion protein lacks tyrosine residues, this finding indicated that some of the 14 tyrosine residues of GST were phosphorylated, in agreement with previous results (43). In agreement, similar weak phosphorylation levels were detected in experiments using purified GST protein to which no peptide had been added (not shown). In contrast, phosphorylation of the GST-Y124 fusion protein, in which Tyr^{124} was present, was considerably higher than that of GST-Y124F (Fig. 1). This indicates that Tyr^{124} can be phosphorylated by Src *in vitro* and is consistent with results presented below being mediated by phosphorylation at this residue.

Reduced Phosphorylation of Y124F Kv2.1 by Src in Vivo—In order to examine the potential role of Tyr^{124} in regulation of Kv2.1 in the context of the entire Kv2.1 protein, we mutated this residue to phenylalanine. Upon expression in cells, WT and Y124F Kv2.1 localized to the cell membrane in similar manners, as indicated by confocal microscopy analyses of cells expressing either protein (Fig. 2). Although cyt-PTPε is predominantly cytosolic, substantial amounts of cyt-PTPε are targeted to the cell membrane, and some cyt-PTPε is found within the cell nucleus (35, 40, 44). As seen in Fig. 2, both WT and Y124F Kv2.1 co-localized with membrane-associated cyt-PTPε, indicating that both had similar opportunities to interact with the phosphatase. In addition, both WT and Y124F Kv2.1 channels were expressed at similar levels following transfection into cells (Fig. 3). We conclude that the Y124F mutation did not significantly affect the subcellular localization or expression levels of Kv2.1.

Expression of WT Kv2.1 with constitutively active (Y527F) Src resulted in robust phosphorylation of the channel, in agreement with previous studies (36). In contrast, Src-mediated phosphorylation of Y124F Kv2.1 was reduced by ~70% under similar conditions (Fig. 3). Interestingly, co-expression of cyt-PTPε in the same system reduced phosphorylation of both WT and Y124F Kv2.1 by Src (not shown). These results confirm that cyt-PTPε can counter Src-mediated phosphorylation of Kv2.1 at Tyr^{124} and most likely at other sites as well. We have recently shown that PTPε can dephosphorylate and activate Src (45). The effect of cyt-PTPε on Kv2.1 in the above studies

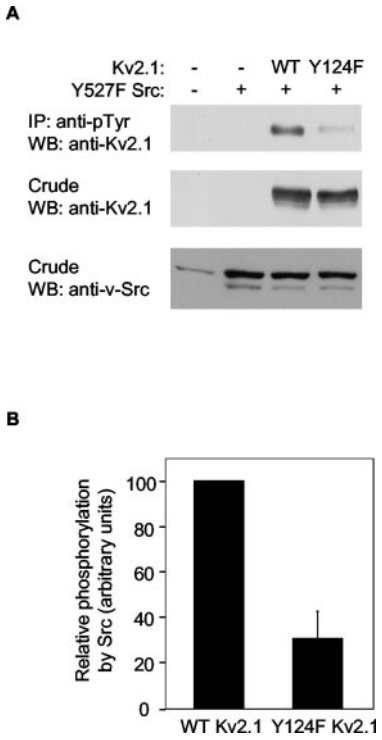

FIG. 3. **Reduced phosphorylation of Y124F Kv2.1 by Src.** HEK 293 cells were transiently transfected with activated (Y527F) Src, together with WT or mutant (Y124F) Kv2.1. Cells were lysed and immunoprecipitated with anti-phosphotyrosine antibodies, after which precipitates were analyzed on 7% SDS-PAGE gels. Shown is precipitated, tyrosine-phosphorylated Kv2.1 (*top panel*); the *middle* and *bottom panels* document expression of Kv2.1 and of Src in cell lysates, respectively. Blots are from an experiment representative of three performed. *B*, *bar diagram* showing intensity of Kv2.1 phosphorylation, normalized to cellular Kv2.1 protein expression. Data (average and S.E.) indicate that Y124F Kv2.1 phosphorylation is 30.7 ± 12.1% of that of WT Kv2.1 (n = 3, p = 0.00293 by Welch's t test).

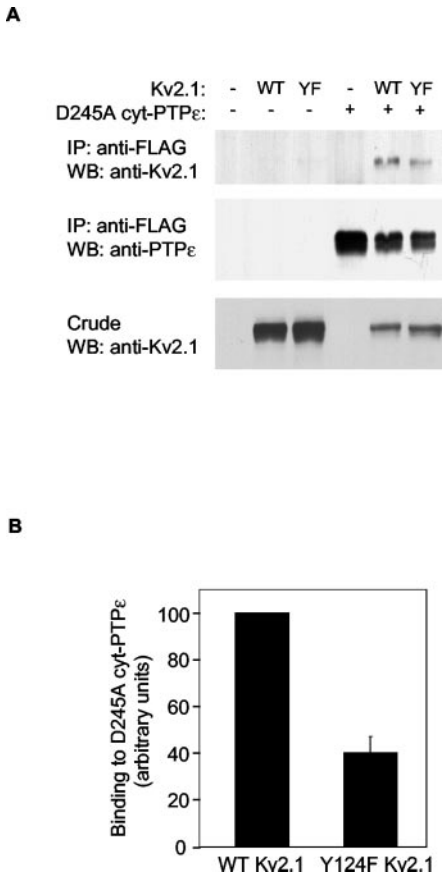

FIG. 4. **Reduced binding of Y124F Kv2.1 to a substrate-trapping mutant of cyt-PTPϵ.** *A*, HEK 293 cells were transiently transfected with D245A cyt-PTPϵ and with WT or Y124F Kv2.1 as indicated. Cells were lysed, cyt-PTPϵ was immunoprecipitated with anti-FLAG antibodies, and precipitates were analyzed on 7% SDS-PAGE gels. Shown are amounts of Kv2.1 that co-precipitated with D245A cyt-PTPϵ (*top panel*) as well as amounts of precipitated cyt-PTPϵ (*middle panel*) and expression of Kv2.1 (*bottom panel*). Blots are from an experiment representative of four performed. *B*, *bar diagrams* showing co-immunoprecipitated Kv2.1, normalized to Kv2.1 expression in the cells. Data (average and S.E.) indicate that Y124F Kv2.1 binding to D245A cyt-PTPϵ is 40.3 ± 7.0% of that of WT Kv2.1 (n = 4, p = 0.0034 by Welch's t test).

was not due to cyt-PTPϵ affecting Src activity, since this study utilized Y527F Src, which lacks the tyrosine that can be dephosphorylated by PTPϵ. Furthermore, dephosphorylation of Src by cyt-PTPϵ activates Src (45) and would have led to increased, rather than decreased, phosphorylation of Kv2.1. These results, together with the *in vitro* Src-mediated phosphorylation of Tyr^{124} shown above, indicate that Tyr^{124} is a significant site of phosphorylation by Src in Kv2.1.

Reduced Binding of Y124F Kv2.1 to a Substrate-trapping Mutant of cyt-PTPϵ—In a separate series of experiments, binding of the substrate-trapping mutant D245A cyt-PTPϵ to Y124F Kv2.1 was examined. Substrate-trapping mutants of this type are virtually inactive but in many cases can recognize and bind their phosphorylated substrates stably enough to allow co-precipitation of the trapping mutant with its associated substrate (37). Indeed, D245A cyt-PTPϵ bound and precipitated WT Kv2.1 from transfected cells (Fig. 4). However, ~60% less Y124F Kv2.1 was co-precipitated with D245A cyt-PTPϵ despite expression of similar levels of WT and Y124F Kv2.1 channels in these cells (Fig. 4). Of note, precipitation of either Kv channel with D245A cyt-PTPϵ was significantly reduced when these experiments were performed in the presence of sodium pervanadate (not shown). Pervanadate oxidizes the critical cysteine residue of the catalytic center of PTPs (46), thereby disrupting the binding of D-to-A type PTP mutants to their putative substrates (37). This last result indicated that the binding observed was due to specific association of the catalytic site of cyt-PTPϵ with phosphorylated tyrosines of Kv2.1. Importantly, immunoprecipitation experiments using D245A cyt-PTPϵ were performed without co-expression of exogenous Src, thereby avoiding possible bias in the results due to prior phosphorylation of specific tyrosines by the exogenous kinase. In all, these results indicate that Tyr^{124} is a significant site to which the catalytic center of cyt-PTPϵ binds and, by extension, dephosphorylates.

Reduced Stimulation of Y124F Kv2.1 Channel Activity by Src—To investigate the impact of the Y124F mutation on Kv2.1 channel activity, we expressed WT and Y124F Kv2.1 channels in *Xenopus* oocytes in the presence or absence of constitutively active (Y527F) Src. Fig. 5 shows that expression of WT Kv2.1 channels generated delayed rectifier outward K^+ currents that activated above −20 mV. Active Src up-regulated WT Kv2.1 current amplitude by more than 3-fold, with no significant changes in activation kinetics and voltage dependence of activation. Mutant Y124F Kv2.1 channels produced K^+ currents that were very similar to those generated by WT Kv2.1 with no significant differences either in amplitude or in kinetics and voltage dependence of activation (Fig. 5). In contrast to WT Kv2.1 channels, active Src increased Y124F Kv2.1 current amplitude by only 35% (Fig. 5). The Y124F Kv2.1 channels are therefore functional and are activated by depolarization in the absence of Src in a manner similar to the nonmutant Tyr^{124} Kv2.1 channels. However, mutant Y124F Kv2.1 channels are

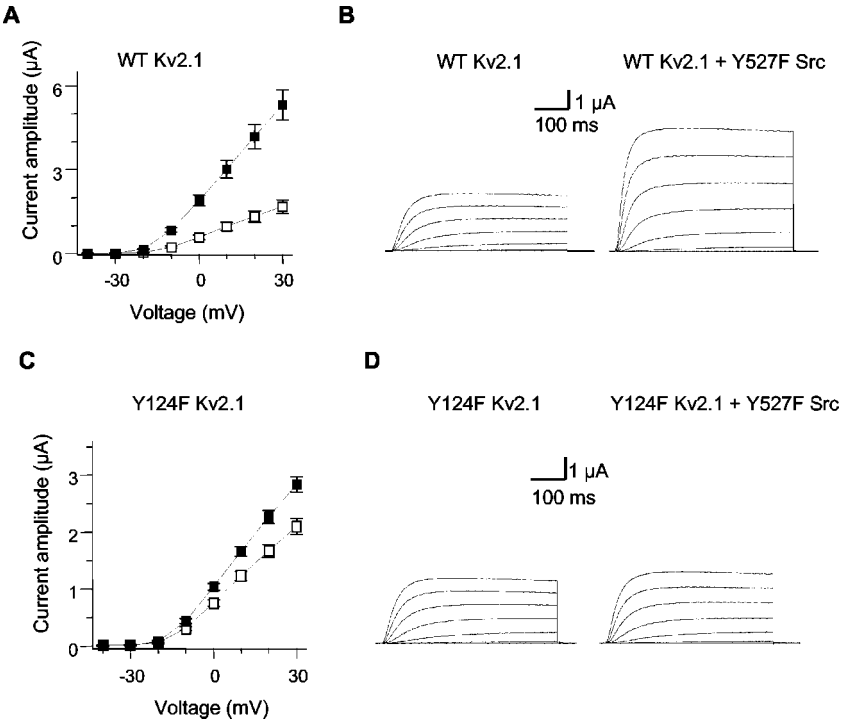

FIG. 5. **Effects of activated (Y527F) Src on WT and on Y124F Kv2.1 K^+ currents expressed in *Xenopus* oocytes.** *A*, current-voltage relations of WT Kv2.1 channels expressed in the absence ($n = 11$, *empty squares*) or presence of Y527F Src ($n = 13$, *solid squares*). Y527F Src significantly increases Kv2.1 currents by more than 3-fold ($p < 0.01$). *B*, macroscopic K^+ currents recorded from oocytes expressing WT Kv2.1 (*left*) or WT Kv2.1 and Y527F Src (*right*) were elicited by depolarizing pulses (500 ms) from a −80 mV holding potential to +30 mV in 10-mV increments. *C*, current-voltage relations of Y124F Kv2.1 channels expressed in the absence ($n = 15$, *empty squares*) or presence of Y527F Src ($n = 15$, *solid squares*). Active Src increases Kv2.1 currents by only 35% ($p < 0.05$). *D*, macroscopic K^+ currents recorded from oocytes expressing Y124F Kv2.1 (*left*) or Y124F Kv2.1 and Y527F Src (*right*) were elicited as in Fig. 5*B*. Note different scales of *vertical axes* in *graphs A* and *C*.

severely and specifically impaired in their ability to undergo up-regulation by Src-mediated phosphorylation, underscoring the role of Tyr^{124} in regulating this process.

DISCUSSION

Results presented here demonstrate the importance of Tyr^{124} of Kv2.1 as a site that is phosphorylated by Src *in vitro* and *in vivo*. Phosphorylation at Tyr^{124} accounts for approximately two-thirds of Src phosphorylation of Kv2.1, for a similar fraction of binding to D245A cyt-PTPϵ, and for ~80% of the ability of Src to enhance Kv2.1 activation by membrane depolarization. Whereas not all of the effects of Src are mediated by Tyr^{124}, the role of this residue is nonetheless very significant. Of note, Kv channels are activated by membrane depolarization, with phosphorylation acting as a modulator of this effect. It is therefore not surprising that mutant Y124F and the wild-type Tyr^{124} Kv2.1 channels were activated by depolarization to the same extent in the absence of Src and that the differential effect of Src phosphorylation was limited to their current amplitude without affecting other electrical parameters. In fact, this behavior, together with the conservative nature of the Y124F mutation and the normal expression levels and correct subcellular localization of Y124F Kv2.1 channels, underscores the specific but otherwise limited nature of the change introduced into Kv2.1 by the Y124F mutation. The same results establish Tyr^{124} as a significant target site for the mutually opposing effects of Src and cyt-PTPϵ on Kv2.1.

Identification of particular tyrosine residues as sites for PTP activity reflects substrate specificity of the PTP in question but also specificities of PTKs that phosphorylate these residues beforehand. Strong preference on the part of a PTK toward phosphorylating a particular tyrosine may limit the range of phosphotyrosines available to the PTP and bias the result obtained. For this reason, binding studies of D245A cyt-PTPϵ were performed without co-expression of exogenous Src, relying entirely on phosphorylation of Kv2.1 by endogenous PTKs present in HEK 293 cells. These cells express moderate levels of endogenous Src as well as other tyrosine kinases. The fact that under these circumstances the Y124F mutation was found to profoundly affect cyt-PTPϵ binding indicates the importance of this site also in the absence of strong Src activity.

Despite its significant effect on current amplitude, results presented here indicate that Src phosphorylation does not affect voltage-dependence and kinetics of activation of either WT or Y124F Kv2.1. It is therefore possible that Src family tyrosine kinases control the number of available active channels or, alternatively, that tyrosine phosphorylation increases the channel open probability (P_o), as previously described for *N*-methyl-D-aspartate receptors (47). The mechanism behind this effect is not known at present, but it is likely mediated by conformational changes induced in the cytosolic T1 domain by phosphorylation of Tyr^{124} and that affect properties of the membrane-spanning regions of Kv2.1.

Of note, Tyr^{124} is conserved in the only other known Kv2 family member, Kv2.2, as well as in Kv11.1. This residue is not conserved in most other families of Kv channel α-subunits. For example, all known Kv1 family members contain an alanine residue at the analogous position; Kv3 proteins contain aspartic acid, whereas Kv4 proteins contain leucine or isoleucine residues. Nevertheless, cyt-PTPϵ has been shown to counter activation of Kv1.5 by Src and by Fyn in HEK 293 cells and in *Xenopus* oocytes despite the absence of a tyrosine at the analogous position (36). This suggests that a similar mechanism of mutually antagonistic regulation by cyt-PTPϵ and a Src family PTK may operate via another tyrosine residue in other Kv channels.

Modulation of Kv2.1 channels by PTKs and PTPs may be functionally relevant for the control of cell excitability in different types of neurons. In both pyramidal and inhibitory interneurons of the cortex and hippocampus, Kv2.1 channels are clustered primarily on somata and proximal dendrites but not on axons (48). Recent studies showed that Kv2.1-containing channels play a role in regulating pyramidal neuron somatodendritic excitability primarily during episodes of high frequency synaptic transmission (49). In this context, the fine tuning of Kv2.1 current strength by PTKs and PTPs may play a role in synaptic efficacy during high frequency synaptic transmission. Similarly, Kv2.1-containing channels, which are found to regulate the tonic firing of sympathetic neurons (50)

and the discharge pattern of globus pallidus neurons (51), may be potentially subject to such modulation by PTKs and PTPs. In all, the fine tuning of Kv2.1 channel activity through the tyrosine phosphorylation of its amino-terminal residue Tyr^{124} may play a crucial role in regulating neuronal excitability in various regions of the brain.

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