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. Tyr124 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 Tyr124 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 Tyr124 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) 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...
channel activity following its inhibition by the G protein-coupled M1 muscarinic acetylcholine receptor in Xenopus oocytes and mammalian cells (35). The same PTP was later shown also to counter serotoninergic inhibition of Kv1.1 and Kv1.2 in a similar experimental system (34). We have demonstrated that the nonreceptor form of PTP epsilon (cyt-PTP) (35) dephosphorylates and down-regulates Kv1.5 and Kv1.2 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 Kv1.2 were hyperphosphorylated in primary Schwann cells and in sciatic nerve tissue from mice genetically lacking PTPs. These effects were correlated with increased Kv channel currents in PTP−/− deficient Schwann cells, as well as with transient but severe hypomyelination of sciatic nerve axons in young PTP−/− deficient mice (36). Strong support for Kv2.1 being a substrate of cyt-PTP was obtained in experiments, which established that a substrate-trapping mutant of cyt-PTP 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-PTP binding to at least one phosphoryrosine residue in Kv2.1. The identity of that residue, however, remained unknown.

In the present study, we identify Tyr124, 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-PTP, implying that it is dephosphorylated by this phosphatase. Phosphorylation of Tyr124 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 Tyr124 as an important site for mutually antagonistic regulation of Kv2.1 by Src and by cyt-PTP 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-PTP (35), D245A cyt-PTPs (36), rat Kv2.1 (gift of Drs. J. Barhanin and M. Laius tagged with T7 wild-type and Y257F Src (gift of Dr. S. Courtneidge). Both PTPs 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-PTP L (H9262) (38), polyclonal anti-Kv2.1, monoclonal anti-v-Src (H9280), monoclonal anti-FLAG M2 and/or polyclonal anti-Kv2.1, each diluted 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 Axiosvert microscope.

Protein Blotting and Substrate-Blocking—Cell lysates were from HEK 293 cells transfected with the relevant expression vectors, and stained with antibodies as described previously (40). Cell lysates were incubated with 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 Axiosvert microscope.

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FIG. 1. Tyr124 of Kv2.1 is phosphorylated by Src in vitro. A decapeptide containing Tyr124 and the surrounding residues (119GIDEIYLEC) 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-32P]ATP as described under “Experimental Procedures.” Aliquots of the phosphorylation reaction were analyzed by SDS-PAGE and blotting. Top, 32P 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.

RESULTS

Phosphorylation of Tyr124 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, Tyr124 of the N-terminal cytosolic T1 domain, is embedded in a sequence that is somewhat similar to the consensus sequence for Src phosphorylation (121DEIYLES versus consensus of EEIYG/EEF) (42).

In order to determine whether Src could phosphorylate Tyr124 of Kv2.1 in vitro, we examined the ability of Src to phosphorylate a peptide derived from the sequence of Kv2.1, 119GIDEIYLEC, which was centered around Tyr124 and in which this residue was the only tyrosine. A similar peptide in which Tyr124 was replaced with a nonphosphorylatable phenylalanine residue (119GIDEIFLEC) 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-32P]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 Tyr124 was present, was considerably higher than that of GST-Y124F (Fig. 1). This indicates that Tyr124 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 Tyr124 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, 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 Tyr124 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...
Y124F Kv2.1 phosphorylation is 30.7% of wild-type (WT) Kv2.1 cellular Kv2.1 protein expression. Data (average and S.E.) indicate that Y124F Kv2.1 binding to D245A cyt-PTP was 60% less than WT Kv2.1 in a manner similar to the nonmutant Tyr124 phosphorylation. These results, together with the in vitro Src-mediated phosphorylation of Tyr124 shown above, indicate that Tyr124 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 PTPε (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 Tyr124 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 Tyr124 Kv2.1 channels. However, mutant Y124F Kv2.1 channels are

![Image 1](https://example.com/image1.png)

**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 of Kv2.1 and of Src in cell lysates, respectively. 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).

![Image 2](https://example.com/image2.png)

**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).
severely and specifically impaired in their ability to undergo up-regulation by Src-mediated phosphorylation, underscoring the role of Tyr124 in regulating this process.

**DISCUSSION**

Results presented here demonstrate the importance of Tyr124 of Kv2.1 as a site that is phosphorylated by Src in vitro and in vivo. Phosphorylation at Tyr124 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. Nevertheless, cortical Kv2.1 channels were activated by depolarization 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). 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. 5B. Note different scales of vertical axes in graphs A and C.

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 Tyr124 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 results establish Tyr124 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 (Po), 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 Tyr124 and that affect properties of the membrane-spanning regions of Kv2.1.

Of note, Tyr124 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 Tyr124 may play a crucial role in regulating neuronal excitability in various regions of the brain.

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REFERENCES