Reversible Tyrosine Protein Phosphorylation Regulates Large Conductance Voltage- and Calcium-activated Potassium Channels via Cortactin*

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Large conductance calcium- and voltage-activated potassium (BK) channels assemble as macromolecular signaling complexes and are potently regulated by reversible protein phosphorylation. However, although numerous studies have revealed regulation of BK channels through changes in direct phosphorylation of the pore-forming α-subunits the functional role of changes in phosphorylation of defined adapter/signaling proteins within the complex on channel function are essentially not known. Here, we demonstrate that mammalian BK channels are potently regulated by endogenous protein-tyrosine kinase and protein-tyrosine phosphatase activity closely associated with the channel. BK channel regulation was not dependent upon direct phosphorylation of the BK α-subunit, rather channel function was controlled by the tyrosine phosphorylation status of the adapter protein cortactin that assembles directly with the BK channel. Our data thus reveal a novel mode for BK channel regulation by reversible tyrosine phosphorylation and strongly support the hypothesis that phosphorylation-dependent regulation of accessory proteins within the BK channel signaling complex represents an important target for control of BK channel function.

Reversible protein phosphorylation of large conductance calcium- and voltage-activated potassium (BK) channels represents an important post-translational regulatory mechanism for the physiological control of these ion channels. An array of serine/threonine and protein-tyrosine kinases and cognate phosphatases has been demonstrated to control BK channel function. The majority of these studies have implicated direct phospho-regulation of the BK channel pore-forming α-subunit, and consensus phosphorylation sites have been proposed (1–7). However, an increasing body of evidence suggests that BK channels exist as a multimolecular signaling complex (8) suggesting that an alternative mechanism of BK channel regulation may be mediated through phosphorylation-dependent regulation of proteins closely associated with the BK channel pore-forming subunits. Indeed, in Drosophila the interaction of 14-3-3 proteins with the BK channel associated protein Slob is regulated by calmodulin dependent protein kinase II (7). The dynamic association of 14-3-3 proteins with Slob regulated by calmodulin kinase II controls the activity of BK channels dSlo. Although mammalian BK channel β-subunits have been implicated in the control of BK channels by serine/threonine phosphorylation (9, 10), the contribution of other accessory proteins that associate with mammalian BK channels in the control of BK channels by protein phosphorylation is essentially unknown.

Several studies have implicated tyrosine phosphorylation-dependent regulation of BK channels (1, 11–16). However, the functional effect of tyrosine phosphorylation on BK channel activity is diverse. Tyrosine-dependent protein phosphorylation has been reported to inhibit, activate, or have no effect on BK channel activity that likely depends on both the tyrosine kinase and cellular system under investigation. For example, overexpression of active c-Src with mSlo in HEK293 cells resulted in channel activation (11), in contrast expression of c-Src with hSlo resulted in an inhibition of BK channel activity (1). In both cases the effect of c-Src was only observed at intracellular free calcium concentrations in the micromolar range. In several native systems Src family kinases generally appear to play an inhibitory role as inhibition of kinase activity results in channel activation (1, 17, 18). However, tyrosine kinases have also been reported to activate BK channels in other systems (14, 15, 19).

These data suggest multiple pathways by which protein tyrosine phosphorylation can regulate BK channels. Firstly, several independent lines of evidence support direct tyrosine kinase-mediated phosphorylation of the BK channel α-subunit, including: (i) overexpression of active c-Src, pyk2, and HCK tyrosine kinases results in phosphorylation of the α-subunits expressed in HEK cell lines (1, 11, 12); (ii) site-directed mutagenesis of a C-terminal tyrosine residue (Tyr-762) abolishes channel phosphorylation and regulation by overexpressed c-Src (11); (iii) prostaglandin E₂ stimulation results in phosphorylation of BK channel α-subunits in osteosarcoma cells (13); (iv) overexpressed c-Src co-localizes with BK channels expressed in HEK293 cells (1) and endogenous c-Src co-immu-

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*This work was supported by the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: BK, large conductance calcium- and voltage-activated potassium channel; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetravalent; bpv(phen), potassium bisperoxo(1,10-phenanthroline) oxovanadate; [Ca²⁺], intracellular free calcium concentration; CD, cytochalasin D; HA, hemagglutinin; HEK293, human embryonic kidney 293 cells; Lav A, lavendustin A; Lav B, lavendustin B; LB, latrunculin B; PBS, phosphate-buffered saline; Pₜₜ, single channel mean open probability; PKA, protein kinase A.
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nprecipitates with BK channels in osteocarcinoma cells (13); and (v) Src kinase can bind and phosphorylate the Drosophila BK channel α-subunit C terminus in vitro (16) and the tyrosine kinase FAK associates directly with BK channels in osteoblasts (20). Secondly, in some systems, tyrosine kinase signaling pathways regulate BK channels indirectly through elevation of intracellular free calcium levels. For example, activation of the receptor tyrosine kinase erbB2 did not result in BK channel tyrosine phosphorylation in glioma cells, however, erbB2 activated BK channels in this system through elevation of intracellular free calcium (14). In osteocarcinoma cells although prostaglandin E2 stimulates tyrosine phosphorylation of BK channel α-subunits the prostaglandin E2 stimulation of channel activity appears to be mediated via an elevation of intracellular calcium rather than phosphorylation of the channel per se (13).

Taken together these data suggest multiple regulatory mechanisms by which tyrosine phosphorylation may regulate BK channel function. Importantly, few studies have addressed whether endogenous protein-tyrosine kinases/phosphatases closely associate with BK channels and whether direct BK channel α-subunit phosphorylation has an important functional consequence for BK channel regulation by endogenous tyrosine kinases/phosphatases.

We recently demonstrated that BK channels may assemble with the actin-binding protein, cortactin (21), which was originally identified as a major Src tyrosine kinase substrate of molecular weight 80–85 kDa and is a major tyrosine phosphoprotein in cells (22–24). Cortactin can be phosphorylated on multiple tyrosine residues, and cortactin phosphorylation status has been implicated in modulation of actin cytoskeletal dynamics (22–24). Intriguingly, in osteocarcinoma cells the major native tyrosine phosphorylated proteins that co-immunoprecipitate constitutively with native BK channels have a molecular mass of ~80–85 kDa (13) suggestive, albeit not demonstrated, of the major associated phosphoprotein being cortactin. This suggested that an important additional level of BK channel modulation by tyrosine phosphorylation-dependent signaling pathways may also be mediated by changes in tyrosine phosphorylation status of channel-associated proteins rather than through α-subunit phosphorylation and/or regulation by calcium. Furthermore, phosphorylation status is dependent upon the reciprocal action of protein kinases and phosphatases. However, the role of endogenous protein-tyrosine kinases and phosphatases that may closely associate with the BK channel complex are poorly understood.

In this study we have addressed the role of cortactin and endogenous Src family protein-tyrosine kinase activity and protein-tyrosine phosphatase activity in mediating regulation of BK channels expressed in HEK293 cells. Our studies demonstrate an important additional route for tyrosine-dependent phosphorylation regulation of BK channels, dependent upon BK channel assembly with cortactin, and further support the hypothesis that phosphorylation-dependent regulation of accessory proteins within the BK channel signaling complex represents an important target for control of BK channel function.

EXPERIMENTAL PROCEDURES

HEK293 Cell Culture and Transfection—HEK293 cells, which do not express endogenous BK channels as determined by reverse transcription-PCR, Western blot, and functional analysis, were subcultured essentially as described (4, 21). Briefly, cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in a humidified atmosphere of 95% air/5% CO2 at 37 °C. Cells were passaged every 3–7 days using 0.25% trypsin in Hanks’ buffered salt solution containing 0.1% EDTA. For biochemical studies cells were grown to 70–80% confluence in 25- or 75-cm2 flasks. For electrophysiological or imaging assays cells were plated on glass coverslips. Twenty-four hours prior to the experiment cells were washed, and medium was replaced with Dulbecco’s modified Eagle’s medium containing ITS serum replacement (Invitrogen). Cells were transiently transfected at 40–60% confluence with the respective cDNA using Lipofectamine 2000 (Invitrogen), essentially as described by the manufacturer. Medium was replaced after 24 h, and assays were performed 48–72 h post transfection. Stable cell lines were also created by selection using 1.0 mg/ml Geneticin (Invitrogen). Channel constructs contained a C-terminal hemagglutinin (-HA) epitope tag as previously described (25). For the majority of these studies the e22 murine splice variant of the BK channel α-subunit was used (25), although identical results were also observed with other variants (see “Results”). The cortactin binding-deficient mutant channel e22-P656:667A and the Src kinase tyrosine phosphorylation mutant e22-Y762F were created by QuikChange II site-direct mutagenesis (Stratagene), and mutation and sequence integrity were confirmed by sequencing.

Patch Clamp Electrophysiology—Experiments were performed by only selecting patches that contained low channel densities. To facilitate this when using stable cell lines, cells were not maintained in selection medium. Single channel current recordings were performed in the inside-out configuration of the patch clamp technique, at room temperature (20–24 °C), using physiological potassium gradients. The pipette solution (extracellular) contained (in mM): 140 NaCl, 5 KCl, 1 CaCl2, 2 MgCl2, 20 glucose, 10 HEPES, pH 7.4. The bath solution (intracellular) contained (in mM): 140 KCl, 5 NaCl, 2 MgCl2, 1 BAPTA, 30 glucose, 10 HEPES, 1 mM ATP, pH 7.3 with free calcium [Ca2+]i buffered to 0.2 μM, unless indicated otherwise. Channel activity was determined during 60-s depolarizations to the voltage indicated in the respective figure legends. For experiments in which [Ca2+]i was buffered to 10 μM, 1 mM dibromo-BAPTA was used as the calcium buffer. Solution exchange, or application of drugs to the intracellular face of patches, was by gravity-driven perfusion (10 volumes of the bath solution (bath volume, 0.5 ml) at a flow rate of 1–2 ml/min). For some experiments drugs were added directly to the bath.

Data acquisition and voltage protocols were controlled by an Axopatch 200 B amplifier and pCLAMP9 software (Axon Instruments Inc., Foster City, CA). All recordings were sampled at 10 kHz and filtered at 2 kHz. Channel activity was allowed to stabilize for at least 10 min after patch excision before addition.
of drugs. BK channel activity was stable for ~1 h under the recording conditions used (data not shown), in the absence of channel modulators.

Single-channel open probability ($P_o$) was derived either from single-channel analysis using pSTAT (Axon Instruments) or WINEDR (Version 2.3.9, J. Dempster, University of Strathclyde, UK) for patches with <4 channels. In the case of patches with >4 channels, an integration-over-baseline algorithm was used using Igor Pro 4.1 (WaveMetrics, Lake Oswego, OR). In the latter case, $N_oP_o$ (number of functional channels × open probability of channel) values were determined as follows. All-point histograms were plotted to obtain the “offset,” i.e. leak current, as well as the single-channel current amplitude from the peak intervals. After subtraction of the offset from the traces there were integrated over 30- to 60-s segments. The integral divided by integration time and single-channel current amplitude gives $N_oP_o$.

To determine the mean percent (%) change in channel activity after a treatment, in patches with low to moderate levels of channel expression, mean $P_o$ or $N_oP_o$ was measured immediately before and 10 min after the respective drug treatment. Mean change in activity was expressed as a percentage (%) of the pre-treatment control ± S.E. In the respective figure legends and text a positive percentage (%) change in activity reflects activation, whereas a negative percentage (%) change reflects channel inhibition.

Cytochalasin D and Latrunculin B (Sigma) were dissolved in Me$_2$SO and used at final bath concentrations of 10 µM and 2 µM, respectively. Me$_2$SO vehicle controls (<0.01% and 0.05%, respectively) had no significant effect on channel activity (not shown). Lavendustin A (Calbiochem) and Lavendustin B (Calbiochem) were dissolved in ethanol, and used at a final concentration of 10 µM. Ethanol concentration was <0.01% and had no significant effect on channel activity (not shown). Sodium orthovanadate (Na$_2$VO$_4$) was activated for maximal inhibition of protein tyrosine phosphatases as previously described (26) and stored at ~20°C before use at a final concentration of 10 µM. The tyrosine phosphatase inhibitor, potassium bisperoxo(1,10-phenanthroline) oxovanadate (u) (bpv(phen)), was dissolved in dH$_2$O immediately prior to use and used at a final concentration of 10 µM.

**Immunoprecipitation and Western Blotting**—Cells were lysed at 4°C in buffer containing: 150 mM NaCl, 50 mM HEPES, pH 7.5, 1.5 mM MgCl$_2$, 10 mM sodium pyrophosphate, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 10% v/v glycerol, 1% Triton-X-100, and proteinase inhibitor mixture (Roche Applied Science). Na$_2$VO$_4$ was added in some experiments and/or patches. Cells were washed three times in ice-cold PBS, permeabilized with 0.1% Triton in PBS at room temperature for 10 min, washed three times, and blocked in PBS containing 1% bovine serum albumin and 0.05% Tween 20 for 1 h. Cells were incubated with Phalloidin-Alexa Fluor 488 conjugate (Molecular Probes) at a concentration of 5 units/ml for 45 min in the dark. After incubation, cells were washed with ice-cold 0.05% Tween 20 in PBS three times before mounting on microscope slides using Mowiol. Confocal images were acquired on a Zeiss LSM510 laser scanning microscope, using a 63× oil Plan Apochromat (numerical aperture = 1.4) objective lens, in multitracking mode to minimize channel cross-talk.

**Statistical Analysis**—All data are presented as means ± S.E. with $n$ = number of independent experiments and/or patches. Data were analyzed by analysis of variance (ANOVA) with post-hoc Student-Newman-Keuls test with significance set at $p < 0.01$, or by Students paired $t$ test, as appropriate.

**RESULTS**

**Closely Associated Endogenous Tyrosine Kinase/Phosphatase Activity Regulates BK Channels**—As a first step to interrogate the role of endogenous Src family tyrosine kinases, closely associated with BK channels, we selected inside-out patches from HEK293 cells in which BK channels were expressed at low levels to allow analysis at single channel resolution and to reduce the potential saturation of endogenous signaling cascades that may be inherent in highly overexpressing cells in which large (nano-amp) macropatch recordings are routinely analyzed.

In excised inside-out patches from HEK293 cells expressing murine BK channels inhibition of endogenous Src family tyrosine kinases with 10 µM lavendustin A resulted in a robust stimulation of BK channel activity in the presence of submicromolar calcium concentrations (Fig. 1). Channel activation was associated with a significant ($p < 0.05$, paired $t$ test) increase in mean channel open time from 1.21 ± 0.32 ms, $n = 7$ under control conditions to 2.29 ± 0.79 ms, $n = 7$ in the presence of lavendustin A. The mean closed time was significantly reduced ($p < 0.01$, paired $t$ test) from 7.12 ± 0.20 ms, $n = 7$ to 0.35 ± 0.12 ms, $n = 7$ in the presence of lavendustin A. In three patches in which mean channel open probability was determined across a wide
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A

Control

+ Na3VO4

+ Lav A

Time (ms)

Activity (% of 0 mV)

B

C

Lav A

Lav B

Lav A + Na3VO4

% activation of control

C

Lav A

Lav B

Na3VO4

Lav A + Na3VO4

% activation of control

FIGURE 1. Endogenous protein-tyrosine kinase and tyrosine phosphatase activity regulates BK channels. A, representative single channel traces in excised inside-out patches from HEK293 cells, expressing the e22 splice variant. In the left-hand panels traces are shown before (control) and after application of the Src family protein-tyrosine kinase inhibitor lavendustin A (+ Lav A, 10 μM) to the intracellular face of the patch for 10 min. In the right-hand panels patches were pre-exposed to the broad spectrum tyrosine phosphatase inhibitor Na3VO4 (+ Na3VO4, 10 μM) and channel activity determined before (control) and after Lav A as above. Patches were exposed to 0.2 μM [Ca2+]i in the presence of Mg-ATP and recorded at +40 mV. B, time course plots of the effect of Lav A (circles) and its inactive analog lavendustin B (Lav B, 10 μM, triangles) on channel activity as determined in A. Channel activity normalized to the channel activity immediately prior to the addition of Lav A or Lav B (0 min = 100%). Pretreatment with 10 μM Na3VO4 blocked the activation observed with Lav A (squares). C, summary bar chart demonstrating the stimulation of BK channel activity by Lav A but not by Lav B, Na3VO4, alone had no significant effect on BK channel activity but blocked the stimulatory effect of Lav A. Data are mean ± S.E., n = 8/group expressed as the percentage activation compared with pre-treatment control.**, p < 0.01 ANOVA compared with the Lav A group.

Voltage range the half-maximal voltage for channel activation ($V_{0.5max}$) was shifted to the left by lavendustin A by 8.3 ± 2.3 mV with no significant change in the slope of the curves (slope in control 1.76 ± 0.31, in the presence of lavendustin A, 1.64 ± 0.41) suggesting the voltage sensor per se is not altered. The stimulatory effect of lavendustin A did not significantly reverse over 15–20 min in 3/3 patches following washout, in general agreement with the essentially irreversible (reversibility $t_{1/2} > 100$ min) effect of the slow binding inhibition reported for lavendustin A on epidermal growth factor receptor tyrosine kinase activity (27). The inactive analog lavendustin B, under identical conditions, had no significant effect on BK channel activity (Fig. 1). This suggests that Src family tyrosine kinase activity closely associated with the channel tonically inhibits BK channels expressed in HEK293 cells, an effect in general agreement with the effect of Src family kinase inhibitors in native cells (1, 17, 18). The stimulatory effect of lavendustin A was independent of the BK channel α-subunit splice variant used as both the zero and e22 splice variants were activated by similar extents (88.5 ± 3.8%, n = 7 and 95.3 ± 8.7%, n = 8 of pre-treatment control, respectively).

However, the stimulatory effect was only observed at submicromolar intracellular free calcium concentrations. When intracellular free calcium was buffered to 10 μM, lavendustin A had no significant effect on channel activity at any potential examined (for example in the zero variant, the mean percentage change in activity determined at 10 mV (close to the $V_{0.5max}$ under these conditions) was −6.9 ± 9.4%, n = 4) compared with pre-treatment control.

Because inhibition of Src family tyrosine kinase activity in the isolated inside-out patch stimulates BK channel activity this would also suggest that endogenous tyrosine phosphatase activity is also intimately associated with the channel complex to reverse the tonic inhibition mediated by kinase phosphorylation. In support of this idea, the broad-spectrum tyrosine phosphatase inhibitor, sodium orthovanadate prevented channel activation upon Src family kinase inhibition by lavendustin A (Fig. 1). Sodium orthovanadate alone had no significant effect on BK channel activity suggesting that the tonic inhibition (Fig. 1C) via closely associated tyrosine kinase is maximal and/or that the endogenous tyrosine phosphatase is not constitutively active.

Direct Phosphorylation of the BK Channel Pore-forming Subunit Is Not Required for Channel Regulation by Endogenous Tyrosine Phosphorylation—Several previous studies have reported both inhibitory and stimulatory effects of exogenously expressed tyrosine kinases on BK channel activity in HEK293 cells and that the effect of tyrosine phosphorylation is mediated via phosphorylation of the BK channel pore-forming subunit itself. Furthermore, tyrosine residue (Tyr-762) in the BK channel C terminus has been shown to be the predominant tyrosine residue phosphorylated by exogenous c-Src tyrosine kinase in vitro (11). We thus asked whether Tyr-762 is an important substrate for the tyrosine phosphorylation-dependent regulation of BK channels in our system. Mutation of Tyr-762 to phenylalanine had no significant effect on single channel open probability and/or half-maximal voltage of activation as previously reported (data not shown). However, the Src family kinase inhibitor lavendustin A still activated BK channels in which Tyr-762 had been mutated to phenylalanine (Y762F mutant) to the same extent as in wild-type (Tyr-762) channels (Fig. 2). Furthermore, the stimulatory effect of lavendustin A on Y762F channels was abolished by pre-treatment with the tyrosine phosphatase inhibitor, sodium orthovanadate (Fig. 2).

The intracellular C-terminal tail of the BK channel pore-forming subunit contains additional putative consensus site tyrosine residues that may be a target for tyrosine phosphorylation/dephosphorylation. However, we found no evidence for robust constitutive tyrosine phosphorylation of the BK channel pore-forming subunit in our system. For
example, we failed to detect BK channels, using several distinct tyrosine phosphorylation-specific antibodies in Western blots, from immunoprecipitates in which the BK channels had been immunoprecipitated from transfected HEK293 cells using antibodies directed against either the engineered HA epitope or using antibodies against the native BK channel protein. In contrast, robust tyrosine phosphorylation of BK channels was observed upon co-expression with c-Src as previously reported (1, 11) (data not shown). This is in general agreement with previous studies in which exogenous tyrosine kinase expression was required to detect robust tyrosine kinase phosphorylation of BK channel pore-forming subunits in HEK293 cells (1, 11, 12). Although we cannot definitively exclude low levels of tyrosine phosphorylation on other residues that are below the limits of detection of our assay, taken together these data suggested that the BK channel pore-forming subunit may itself not be the direct target for tyrosine phosphorylation/dephosphorylation under our conditions.

As a control for our phosphotyrosine antibodies in Western blots we assayed for the major tyrosine phosphorylated protein, cortactin that directly associates with BK channels (21). Cortactin was immunoprecipitated from HEK293 cells expressing the BK channel using an anti-cortactin antibody and immunoprecipitates then probed with a tyrosine phospho-specific antibody. These assays revealed robust tyrosine phosphorylation of cortactin in control immunoprecipitates (Fig. 3A). Reciprocal assays, in which tyrosine phosphorylated proteins were immunoprecipitated from HEK 293 cells and blots probed for cortactin, revealed robust immunoprecipitation of cortactin (Fig. 3A). Although the BK channel α-subunit co-immunoprecipitates with cortactin under these conditions (see for example Fig. 7) no tyrosine phosphorylated protein was detectable at 125 kDa corresponding to the BK channel α-subunit monomer in immunoprecipitates in which cortactin has been immunoprecipitated from HEK293 cells (Fig. 3A) using several distinct tyrosine phosphorylation-specific antibodies in Western blots.

Cortactin serves as a molecular bridge between the BK channel pore-forming α-subunit and actin and is an important determinant of BK channel regulation by the actin cytoskeleton (21). Furthermore, cortactin is a major substrate for Src family tyrosine kinases (22–24). We thus asked whether cortactin tyrosine phosphorylation was modified in cells treated with the Src family kinase inhibitor lavendustin A, which results in BK channel activation. Cortactin phosphorylation was significantly reduced in cells pretreated for 10 min with the Src family kinase inhibitor lavendustin A, which results in BK channel activation. Cortactin phosphorylation was significantly reduced in cells pretreated for 10 min with the Src family kinase inhibitor lavendustin A, which results in BK channel activation. Cortactin phosphorylation was significantly reduced in cells pretreated for 10 min with the Src family kinase inhibitor lavendustin A, which results in BK channel activation. Cortactin phosphorylation was significantly reduced in cells pretreated for 10 min with the Src family kinase inhibitor lavendustin A, which results in BK channel activation. Cortactin phosphorylation was significantly reduced in cells pretreated for 10 min with the Src family kinase inhibitor lavendustin A, which results in BK channel activation. Cortactin phosphorylation was significantly reduced in cells pretreated for 10 min with the Src family kinase inhibitor lavendustin A, which results in BK channel activation.

**FIGURE 2.** The BK channel C-terminal Src kinase phosphorylation motif (Y762F) is not required for regulation of BK channels by endogenous tyrosine phosphorylation activity. A, representative single channel traces in excised inside-out patches from HEK293 cells, expressing the Y762F BK channel mutant before (control) and after application of 10 μM lavendustin A (Lav A) to the intracellular face of the patch for 10 min. Patches were exposed to 0.2 μM [Ca²⁺], in the presence of Mg-ATP and recorded at +20 mV. B, summary bar chart demonstrating the stimulation of BK channel activity by Lav A and the blockade of the activation by Na₃VO₄. Data are mean ± S.E., n = 4/group expressed as the percentage activation compared with pre-treatment control. **, p < 0.01 ANOVA compared with the Lav A group.
immunoprecipitates in previously demonstrated to be dependent upon BK channel lin B, also activates BK channels (21) (Fig. 4), an effect we have the actin cytoskeleton, using either cytochalasin D or latrunculin B, also activates BK channels (21) (Fig. 4), an effect we have

**FIGURE 3.** Cortactin tyrosine dephosphorylation controlled by actin-depolymerization and inhibition of endogenous Src family tyrosine kinases. A, representative Western blots from immunoprecipitations of HEK293 cell lysates. In the left-hand panels cortactin was immunoprecipitated (IP) using a mouse monoclonal anti-cortactin antibody. The resultant immunoprecipitates were probed for tyrosine-phosphorylated proteins using the rabbit polyclonal anti-phosphotyrosine antibody (pY) and for total cortactin protein using a rabbit polyclonal anti-cortactin antibody. In the right-hand panels total phosphotyrosine proteins were immunoprecipitated using the mouse monoclonal anti-phosphotyrosine antibody pY99 and immunoprecipitates probed for total cortactin as above, and the bottom right panel is total cortactin protein in the cell lysate used for the pY99 immunoprecipitate above. Cells were treated for 10 min with vehicle control (Control) or 10 μM of the actin depolymerizing drug cytochalasin D (+CD) or 10 μM of the Src family tyrosine kinase inhibitor lavendustin A (Lav A). B, summary bar chart of the tyrosine-phosphorylated cortactin levels determined from the respective immunoprecipitates in A. Data are mean ± S.E., n = 4/group expressed as tyrosine-phosphorylated cortactin levels as a percentage of control (control = 100%). **, p < 0.01 ANOVA compared with the control group. C, representative confocal projection images of HEK293 cells expressing the e22 variant BK channel and stained for F-actin using phalloidin in control (vehicle-treated) or cells pretreated with Lav A or Na3VO4. Subsequent exposure of cells to cytochalasin D (+CD) resulted in robust F-actin disassembly under all conditions.

body, revealed a reduced level of cortactin in immunoprecipitates supporting the reduced tyrosine phosphorylation status of cortactin (Fig. 3). Thus, a decrease in cortactin tyrosine phosphorylation is correlated with the increased activation of BK channels by Src family tyrosine kinase inhibitors.

Activation of BK Channels by Actin Disassembly Is Paralleled by Tyrosine Dephosphorylation of Cortactin—Disassembly of the actin cytoskeleton, using either cytochalasin D or latrunculin B, also activates BK channels (21) (Fig. 4), an effect we have previously demonstrated to be dependent upon BK channel interaction with cortactin in this system (21). Channel activation upon actin disruption by cytochalasin D was also associated with a significant increase (p < 0.05, paired t test) in mean channel open time, from 0.96 ± 0.30 ms (n = 5) under control conditions to 1.94 ± 0.64 ms in the presence of cytochalasin D. Mean channel closed time also significantly decreased (p < 0.01, paired t test) from 9.04 ± 2.17 ms (n = 5) to 4.34 ± 0.99 ms. Cytochalasin D also resulted in a small but significant left shift in V0.5max by 6.2 ± 2.3 mV (n = 3). The effect of actin disruption on channel kinetics thus markedly resembled that observed under the same conditions upon inhibition of endogenous protein-tyrosine kinase activity with lavendustin A. Furthermore, as for lavendustin A, when intracellular free calcium was buffered to 10 μM, cytochalasin D had no significant effect on channel activity at any potential examined. For example, in the zero variant, the mean percentage change in activity determined at 10 mV (close to the V0.5max under these conditions) was 2.1 ± 1.6%, n = 4.

Actin disruption has also been proposed to activate both tyrosine kinases as well as tyrosine phosphatases (28–30). We thus asked whether (i) actin disassembly also reduced cortactin tyrosine phosphorylation and (ii) whether cortactin dephosphorylation could explain the effect of Src family kinase inhibitors on BK channel activation. In cells treated for 10 min with the actin-distabilizing drug cytochalasin D total cortactin levels in immunoprecipitates was not affected; however, the tyrosine phosphorylation of cortactin in immunoprecipitates was significantly reduced compared with vehicle control (Fig. 3). Similar decreases in cortactin tyrosine phosphorylation in cells treated with cytochalasin D were also observed in assays in which total tyrosine-phosphorylated proteins in cell lysates were first immunoprecipitated using anti-pY99, and immunoprecipitates were then probed for total cortactin levels using anti-cortactin antibody (Fig. 3, A and B).

In functional assays, exposure of inside-out patches to 10 μM cytochalasin D stimulated BK channel activity, an effect
that was blocked by pretreatment with the tyrosine phosphatase inhibitor sodium orthovanadate (Fig. 4). Similarly, disruption of the actin cytoskeleton with 2 μM latrunculin B also stimulated channel activity that was again blocked by sodium orthovanadate (Fig. 4C). Actin depolymerization-induced activation of BK channels was also blocked by the tyrosine phosphatase inhibitor bpv(phen) (Fig. 4). This suggests that actin cytoskeletal disruption, in excised patches of HEK293 cells, either activates or makes target proteins accessible to a tyrosine phosphatase that results in stimulation of BK channels. Cytochalasin D (Fig. 3C) resulted in a robust disassembly of F-actin filaments in intact HEK293 cells. Importantly, pre-treatment of intact cells with tyrosine phosphatase (or tyrosine kinase) inhibitors did not prevent actin disruption by cytochalasin D (Fig. 3C) (or latrunculin B, data not shown). Thus the inability of cytochalasin D to activate BK channels in the presence of tyrosine phosphatase inhibitors does not result from an inability of cytochalasin D to destabilize actin filaments.

Actin-induced depolymerization also stimulated the activity of BK channels in which the Src kinase tyrosine phosphorylation motif Tyr-762 was mutated to phenylalanine (Y762F mutant, Fig. 5). Thus the stimulatory effect of actin disruption, as well as Src family tyrosine kinase activity, is not dependent upon this site.

BK channels, including the e22 and zero variants used here (25), can be activated by protein kinase A (PKA)-dependent serine/threonine phosphorylation, which is also closely associated with the channel (2, 4, 6, 25). We thus verified that the stimulatory effect of actin disruption was not a result of channel activation by PKA phosphorylation. PKA activation is dependent upon a conserved C-terminal PKA consensus motif (Ser-899) in the BK channel α-subunit (2, 4, 6). However, cytochalasin D also robustly activated the e22 variant in which the consensus serine was mutated to alanine (e22-S899A). Cytochalasin D stimulated channel activity by 87.1 ± 6.5% (n = 4) similar to that observed for cytochalasin D on wild-type e22 channels (Fig. 4). Furthermore, in contrast to the e22 and ZERO splice variants, the STREX variant is inhibited by endogenous PKA (2, 4). Actin depolymerization also stimulated STREX channel activity to the same extent as for ZERO or e22 channels (90.2 ± 1.4%, n = 3). Taken together these data suggest that actin depolymerization does not activate BK channels through stimulation of the PKA pathway.

Assembly of Cortactin with the BK Channel Pore-forming Subunit Is Required for Tyrosine Phosphorylation-dependent Regulation of BK Channels—Because cortactin tyrosine phosphorylation status was regulated by both actin depolymerization and inhibition of Src family tyrosine kinases in HEK293 cells, we thus asked whether cortactin assembly with the BK channel-pore forming subunit was required to mediate the effect of endogenous tyrosine kinase/phosphatase on BK channel activity. Cortactin interacts with the BK channel pore-forming subunit via a non-canonical proline-rich domain in the BK channel C terminus, and this interaction is blocked by a double proline mutant (P656:667A) of the channel (21). The P656:667A mutant is completely insensitive to the actin depolymerizing drugs cytochalasin D (Fig. 6) or latrunculin B (not shown) and shows a small left shift in V_{0.5\text{max}} (~6 mV) similar to that observed in wild-type channels exposed to actin-disrupting agents (21). Importantly, the stimulatory effect of the Src kinase inhibitor lavendustin A was also abolished in the P656:667A mutant (Fig. 6) supporting a role for cortactin as the target for endogenous tyrosine-dependent phosphorylation regulation of BK channels. Because the tyrosine phosphorylation status of cortactin has been reported to control its interaction with F-actin and other proteins (22–24) we asked whether changes in cortactin tyrosine phosphorylation disrupted the...
DISCUSSION

These studies reveal an important additional level of BK channel regulation by reversible protein tyrosine phosphorylation: through regulation of the tyrosine phosphorylation status of accessory proteins within the BK channel macromolecular complex. We demonstrate that endogenous Src family tyrosine kinase and protein tyrosine phosphatase activities are intimately associated with BK channels. Control of cortactin tyrosine phosphorylation status likely represents an important pathway by which tyrosine phosphorylation signaling cascades as well as signaling pathways that modify actin cytoskeletal function control BK channel function.

Clearly, a major challenge for the future is to elucidate the relative contribution of the distinct modes by which BK channels may be regulated by tyrosine phosphorylation pathways: direct channel phosphorylation (1, 11, 16) and the indirect mechanisms, including changes in intracellular free calcium (14) and the role of adapter proteins within the channel complex as demonstrated here. Intriguingly, previous studies demonstrating a role for direct BK channel α-subunit phosphorylation by Src family tyrosine kinases have revealed that the functional effect of tyrosine phosphorylation is only observed at micromolar intracellular free calcium levels (1, 11). In contrast, the effect we observe, mediated via cortactin, is manifest at submicromolar calcium concentrations. This suggests a dual mechanism for BK channel regulation by tyrosine phosphorylation of the channel complex dependent upon the subplasmalemmal calcium concentration. In this model tyrosine phosphorylation of cortactin would act as a tonic inhibitory factor to regulate BK channel activity in the presence of submicromolar calcium. Because subplasmalemmal calcium levels are elevated into the micromolar range, which would be associated with actin cytoskeletal disruption and activation of protein tyrosine phosphatase activity, the mode of BK channel regulation may now switch to a mechanism dependent upon direct phosphorylation of the BK channel α-subunit. In support of this model, the effect of tyrosine kinase inhibition by lavendustin A on channel activity was completely abolished when intracellular free calcium levels were raised to 10 μM. At these levels of calcium previous studies have demonstrated a robust effect of exogenous Src kinase on BK channel activity (1, 11). At supramicromolar calcium levels calcium-dependent disruption of the actin cytoskeleton may now allow access of tyrosine kinases to the channel α-subunit to elicit direct phosphorylation. A similar model of kinase accessibil-

FIGURE 5. The BK channel C-terminal Src kinase phosphorylation motif (Y762F) is not required for actin depolymerization-induced activation of BK channels. A, representative single channel traces in excised inside-out patches from HEK293 cells, expressing the Y762F BK channel mutant before

BK-channel-cortactin-actin complex. However, neither lavendustin A nor sodium orthovanadate prevented co-immunoprecipitation of the complex from HEK293 cells (Fig. 7).
ity to cortactin has been proposed upon disruption of the actin cytoskeleton in other systems (22–24, 29). The predominant mode of channel complex regulation by tyrosine phosphorylation is thus likely to be both cell- and context-specific. Thus, the assembly of BK channels with the actin cytoskeleton via cortactin, expression of endogenous tyrosine kinases and phosphatase in the channel complex, and prevailing subplasmalemmal calcium concentrations will all be determining factors in BK channel regulation in distinct native systems.

Although our assays reveal a clear role for endogenous tyrosine kinases and tyrosine phosphatase activities in regulating BK channel function, the identification of the kinases/phosphatases that may be responsible remains elusive. Multiple tyrosine kinases and phosphatases exist, and they represent some of the largest gene families in the genome (31–34).

In this regard several tyrosine kinases, including c-Src, FAK, and Pyk2 are reported to co-immunoprecipitate with BK channels in different systems (1, 12, 20). Irrespective of the precise identity of the kinases and/or phosphatases involved in BK channel regulation in this system, distinct kinases and/or phosphatases may contribute to the same fundamental mechanism elucidated here in different cell types.

A fundamental question for the future is how changes in cortactin phosphorylation status exerts a functional effect on BK channel activity. Both lavendustin A and actin disruption significantly modified single channel kinetics resulting in an increase in mean open time and decrease in mean closed time with a significant left shift in $V_{0.5\text{max}}$ of $7\text{ mV}$ with no change in the slope of the curve. Taken together these data suggest the effect of actin disruption and tyrosine kinases is not via a direct effect on the voltage sensor per se. Cortactin tyrosine phosphorylation status had no significant effect on the ability of cortactin to assemble with the BK channel pore-forming $\alpha$-subunit, nor on its ability to interact with actin in the complex. Cortactin phosphorylation is under multifactorial control, although the functional effect of cortactin phosphorylation is very poorly understood. For example, cortactin phosphorylation can modify its interaction with other proteins as well as its ability to regulate F-actin assembly and dynamics (22–24). Thus whether tyrosine phosphorylation of cortactin controls mechanical coupling between BK channels and dynamic rearrangements of the actin cytoskeleton remains to be determined. Furthermore, whether other signaling pathways that disrupt the actin cytoskeleton ultimately regulate BK channel function through activation of a protein tyrosine phosphatase (or inhibition of a kinase) remains to be examined.

![FIGURE 6. Interaction of cortactin with the BK channel $\alpha$-subunit is required for endogenous tyrosine phosphorylation dependent regulation of BK channels. A, representative single channel traces in excised inside-out patches from HEK293 cells, expressing the P656:667A BK channel mutant that is unable to interact with cortactin, before (control) and after application of 10 $\mu\text{M}$ of the Src family tyrosine kinase inhibitor lavendustin A (Lav A) to the intracellular face of the patch for 10 min. Patches were exposed to 0.2 $\mu\text{M}$ $\text{[Ca}^{2+}\text{]}_{i}$ in the presence of Mg-ATP and recorded at $40\text{ mV}$. B, summary bar chart demonstrating the loss of BK channel regulation channel activity by actin depolymerization (+CD, 10 $\mu\text{M}$) or inhibition of endogenous Src family tyrosine kinases with Lavendustin A (Lav A) in the cortactin-binding deficient BK channel $\alpha$-subunit mutant P656:667A compared with the wild-type channel (WT). Data are mean ± S.E., $n = 6–10$/group expressed as the percentage activation compared with pre-treatment control. **, $p < 0.01$ ANOVA compared with the effect of CD or Lav A on wild-type channels.](https://doi.org/10.1074/jbc.M800750200)
Importantly, because increasing evidence suggests BK channels assemble as large macromolecular signaling complexes in mammalian cells (8), our data further support the hypothesis that phosphorylation-dependent regulation of accessory proteins within the BK channel signaling complex represents an important target for control of BK channel function. Elucidation of the role of other proteins within the BK channel signaling complex in the control of BK channel function by reversible protein phosphorylation represents a significant challenge for the future.

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