Enhanced Activity of a Large Conductance, Calcium-sensitive K⁺ Channel in the Presence of Src Tyrosine Kinase.

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Running Title: Modulation of BK Channels by Src Tyrosine Kinase

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Summary

Large conductance, calcium-sensitive K⁺ channels (BKCa channels) contribute to the control of membrane potential in a variety of tissues, including smooth muscle, where they act as the target effector for intracellular ‘calcium sparks’ and the endothelium-derived vasodilator nitric oxide. Various signal transduction pathways, including protein phosphorylation can regulate the activity of BKCa channels, along with many other membrane ion channels. In our study, we have examined the regulation of BKCa channels by the cellular Src gene product (cSrc), a soluble tyrosine kinase that has been implicated in the regulation of both voltage- and ligand-gated ion channels. Using a heterologous expression system, we observed that co-expression of murine BKCa channel and the human cSrc tyrosine kinase in HEK 293 cells led to a calcium-sensitive enhancement of BKCa channel activity in excised membrane patches. In contrast, co-expression with a catalytically inactive cSrc mutant produced no change in BKCa channel activity, demonstrating the requirement for a functional cSrc molecule. Furthermore, we observed that BKCa channels underwent direct tyrosine phosphorylation in cells co-transfected with BKCa channels and active cSrc, but not in cells co-transfected with the kinase inactive form of the enzyme. A single Tyr to Phe substitution in the C-terminal half of the channel largely prevented this observed phosphorylation. Given that cSrc may become activated by receptor tyrosine kinases or G-protein coupled receptors, these findings suggest that cSrc-dependent tyrosine phosphorylation of BKCa channels in situ may represent a novel regulatory mechanism for altering membrane potential and calcium entry.
Introduction

In the large family of voltage-gated K⁺ channels, large conductance, calcium-sensitive potassium (maxi-K or BK<sub>Ca</sub>) channels represent a unique class whose gating depends primarily on membrane voltage, but which can be shifted in the negative direction by intracellular free calcium. A direct physiologic consequence of this behavior is that BK<sub>Ca</sub> channels act as ‘coincidence detectors’ and regulate, in a feedback fashion, cellular processes stimulated by close temporal changes in membrane potential and intracellular calcium. That BK<sub>Ca</sub> channels indeed play such a role is evidenced by the fact that blocking these channels increases the degree of myogenic tone observed in arterial smooth muscle (1-3) and enhances the presynaptic calcium-dependent release of neurotransmitter at neuromuscular junctions (4;5).

Given their potential to influence cellular processes, it is not surprising that BK<sub>Ca</sub> channels are also targets of cellular signaling pathways, including phosphorylation and dephosphorylation reactions (6-11), heterotrimeric GTP-binding proteins (12;13), and the endothelium-derived vasodilator nitric oxide (14). To date, however, many of the molecular aspects of these regulatory events remain poorly understood.

Of these various cellular pathways, protein phosphorylation remains as one of the most common forms of intracellular signaling and is critically involved in every aspect of eukaryotic cell function, from muscle contraction to gene transcription to cell division.

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1 The abbreviations used are: BK<sub>Ca</sub> channel, large conductance, calcium-sensitive K⁺ channel; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(b-aminoethyl ether) N,N',N''-tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.
Adding to this richness was the discovery in the late 1970s that tyrosine residues may also be phosphorylated, and since then, tyrosine phosphorylation has emerged as a critical process, particularly in the regulation of cellular growth and development (15). However, more recent observations have pointed to an additional role for tyrosine phosphorylation in the control of cellular excitability, via the modulation of both voltage-dependent and ligand-operated ion channels. Nonreceptor tyrosine kinases, *Src* or pp60c-Src in particular, have been shown to regulate a variety of membrane ion channels, including GABA$_A$ receptors (16;17), glutamate receptors (18), nicotinic acetylcholine receptors (19), voltage-gated calcium (20) and potassium channels (21-24). These effects range from enhancement to inhibition of channel activity, and in some cases, a *Src* tyrosine kinase has been shown to be physically associated with the ion channel complex (18;22). These findings thus expand our picture of ion channel regulation to include tyrosine along with serine/threonine phosphorylation as critical regulatory events in the control of membrane excitability.

In the current study, we have examined the effects of the human *Src* tyrosine kinase on the functional behavior of a murine BK$_{Ca}$ channel using a transient co-expression strategy. Our results indicate that the c*Src* tyrosine kinase can enhance BK$_{Ca}$ channel activity in a calcium-dependent manner and that BK$_{Ca}$ channels undergo tyrosine phosphorylation as a consequence of co-expression. Akhand et al. (25) have recently reported that nitric oxide can directly activate c*Src* via thionitrosylation; these results taken together with our own would suggest additional mechanisms by which c*Src* may be involved in the regulation of BK$_{Ca}$ channels. *Src*-dependent tyrosine phosphorylation
may thus represent a generalized cellular mechanism by which BK_{Ca} channel activity can be regulated in response to diverse cellular stimuli.

**Experimental Procedures**

*Reagents and Chemicals*

Lipofectamine and high glucose-containing DMEM cell culture medium were purchased from Gibco/BRL. DNA modifying enzymes were obtained from New England Biolabs. The anti-phosphotyrosine mouse monoclonal antibodies 4G10 and PY20 were purchased from Upstate Biotech. Inc and Transduction Labs, respectively. The anti-Src monoclonal antibody 327 was purchased from Calbiochem. The anti-mSlo rabbit polyclonal antibody and HRP-linked goat anti-mouse and goat anti-rabbit secondary antibodies were obtained from Chemicon International. The SuperSignal chemiluminescence detection reagent was purchased from Pierce Chemical Co. Chemicals used in the preparation of solutions for electrophysiological recordings were from Sigma-Aldrich Chemical Co.

*Construction and Transfection of cDNA Plasmids*

The cDNA encoding the mouse brain mSlo α subunit (26) was obtained from Dr. L. Pallanck (Univ. of Wisconsin) and a ~3.7 kb fragment was subcloned into the SV-40 promoter-based mammalian expression plasmid SRα (27) as follows: the EcoR I site of SRα and the BamH I site at the 5’ end of the mSlo cDNA were blunted with Klenow fragment and a Not I linker was ligated to these ends. The Xba I site at the 3’ end of the mSlo cDNA was then blunted with Klenow fragment and the insert cDNA was ligated between the Not I and EcoR V restriction sites in the plasmid’s polylinker region. The cDNA encoding the wild-type green fluorescent protein (GFP) (28) was subcloned in the SRα plasmid between the Pst I and
EcoR I sites. A full-length cDNA encoding human Src tyrosine kinase (Dr. D. Fujita, University of Calgary) was subcloned into SRα using the EcoR I site. Site-directed mutagenesis of both the mSlo α subunit and cSrc cDNAs was carried out using the ‘Transformer’ mutagenesis kit (Clontech Laboratories). An epitope-tagged form of mSlo was also prepared by insertion of the seven amino acid sequence ‘EEFMPME’ (29;30) at position 1121 near the C-terminus; the sequence of this modified mSlo α subunit reads HSIPSTAAEEFMPMENRPNR. The presence of this tag did not alter the intrinsic voltage- or calcium-dependent gating of these modified channels following expression (data not shown). The enzymatically ‘dead’ form of cSrc was prepared by a Lys to Met mutation at position 298 in the kinase’s catalytic domain (31).

HEK 293 cells (32) were obtained from Dr. M. Calos, Dep’t of Genetics, Stanford University and were maintained at 37°C in a 5% CO₂ incubator in DMEM containing L-glutamine, 4.5 g/L D-glucose and 10% (v/v) characterized fetal bovine serum (Hyclone Laboratories, Logan UT). Transient transfection of cells at 50-80% confluency was carried out in 35 mm tissue culture dishes using the lipofection technique. Briefly, 6-8 µl of Lipofectamine was mixed together with 1.2-1.5 µg of plasmid cDNA in 1 ml of serum-free DMEM and placed on cells for 5-6 hours at 37°C in a humidified incubator containing 5% CO₂. DNA-containing medium was then aspirated and replaced with serum-containing medium. The following day, cells were detached using 0.025% trypsin/0.5 mM EDTA in PBS and replated onto sterile glass coverslips and 10 cm culture dishes. Electrophysiological recordings were typically performed on days 2-4 following transfection. For Western blotting and enzymatic assays, cells were harvested on day 3 or 4 following transfection.
Enhancement of BK Channels by Src Tyrosine Kinase

**Electrophysiology**

Macroscopic currents were recorded at 35±0.5 °C from excised inside/out membrane patches of HEK 293 cells using an Axopatch 200B patch clamp amplifier and pClamp 6.03 software. BK\textsubscript{Ca} channel currents from native m\textit{Slo} or the epitope-tagged m\textit{Slo} (see above) were activated by voltage clamp pulses delivered from a holding potential of 0 mV to membrane potentials ranging from -180 to 240 mV; tail currents were recorded at +50 mV, -80 mV or -120 mV. Current traces were filtered at 2-5 kHz (4-pole Bessel filter) and acquired on a Dell Dimension XPS computer at a sampling frequency of 8-10 kHz using a Digidata 1200 analogue/digital interface. Recording micropipettes were pulled from thin-walled borosilicate glass capillaries (1.2 mm I.D., 1.5 mm O.D., World Precision Instruments, Sarasota, FL) using a Sutter P-89 horizontal electrode puller. Micropipettes were filled with a solution containing (in mM): 5 KCl, 140 KOH, 1 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, 10 HEPES, pH adjusted to 7.3 with methanesulfonic acid and had tip resistances of 2-4 MΩ. The bath solution contained 5 KCl, 140 KOH, 1 MgCl\textsubscript{2}, 2 EGTA or HEDTA, 10 HEPES, pH adjusted to 7.2 with methanesulfonic acid; variable amounts of a 0.1 M CaCl\textsubscript{2} solution were added to give the desired free calcium concentrations. The level of free calcium in each solution was confirmed using a calcium electrode (Orion model 93-20) with calibration standards (WPI, Sarasota, FL) ranging from pCa 8 to 2. The recording chamber (~0.3 ml volume) was perfused at a constant rate of 1-1.5 ml/min, using a set of manually controlled solenoid valves to switch between various solutions.

Transfected HEK 293 cells plated on coverslips were placed in a temperature-controlled recording chamber on the stage of a Nikon TE 300 Eclipse inverted
microscope. Individual cells expressing BKCa channels were then identified visually by co-expression of the marker protein GFP under epifluorescence using 480 nm excitation and 510 nm emission filters. Based on the number of green fluorescent cells, transfection efficiency was judged to be 20-30% in any given experiment.

*In Vitro* Phosphorylation Assay

Frozen pellets of transfected cells were thawed on ice and then resuspended in 1.0 ml of ice-cold RIPA buffer containing 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM Na3VO4, 10 mM NaF, 1 mM EDTA, 0.2 mM EGTA, 0.5% (v/v) NP-40, 0.1% (w/v) SDS, 0.3% (w/v) sodium deoxycholate, 2 µg/ml each aprotinin and leupeptin. The crude cell lysates were kept on ice for ~10 min, then centrifuged at 15,000 r.p.m. for 5 min at 4°C to remove insoluble materials. Following transfer to clean microcentrifuge tubes, the supernatants were pre-cleared by incubation with 20 µl of Protein A/G-agarose (Santa Cruz Biotech) on a rotator for 1 hour at 4°C. The agarose beads were pelleted by centrifugation at 15,000 r.p.m. for 5 min and the supernatants were again transferred to clean tubes, then incubated for 2 hours at 4°C with 4-5 µg/tube of the mouse anti-Src monoclonal antibody 327. Following addition of 20 µl of Protein A/G-agarose per tube, samples were again rotated at 4°C for an additional hour, then centrifuged for 2 min at 10,000 r.p.m. The pelleted materials were washed twice by gentle resuspension in 1 ml of RIPA buffer, then centrifuged as above. Following the second wash, the pelleted materials were washed in 1 ml of ice-cold buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM DTT, 0.1% (v/v) NP-40 and 10% (v/v) glycerol. Liquid from each sample was then aspirated, and the immunoprecipitates were assayed immediately for tyrosine kinase activity.
cSrc-dependent tyrosine kinase activity was measured at 30 °C in a final assay volume of 50 µl containing 50 mM HEPES, pH 7.8, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.2 mM Na₃VO₄, 4 mg/ml p-nitro-phenylphosphate, and 100 µM cdc2₅₂₀ peptide substrate (Upstate Biotech. Inc.). The reaction was started by addition of γ²²P-ATP (10 µM final, 2000-3000 cpm/pmol) and stopped at various times by adding a 40 µl aliquot from each reaction mixture to tubes containing 25 µl of 50% (v/v) acetic acid. A sixty microlitre aliquot from each tube was then spotted onto a P81 paper disc (Whatman), followed by washes for 5-10 min in 3 x 500 ml volumes of 0.5% (v/v) phosphoric acid. P81 paper discs were then briefly rinsed in acetone and dried. Radioactivity bound to the discs was measured by Cerenkov counting in a Beckman model LS6000SC liquid scintillation counter.

**Western Blotting**

Transfected cells were detached on day 3 by brief incubation with sterile PBS containing 0.05% trypsin/0.5 mM EDTA, centrifuged in 15 ml culture tubes at ~100xg for 5 min, and stored at -80 °C as intact cell pellets. These pellets were resuspended in 0.5-1 ml of ice-cold lysis buffer containing 1xTBS, 1% (v/v) Triton X-100, 1 mM EGTA, 2 mM EDTA, 10 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF and 5 µg/ml each of leupeptin and aprotinin, then sonicated for 5-10 sec to shear the genomic DNA. Lysates were mixed with Laemmli sample buffer containing 0.5% (v/v) β-mercaptoethanol, incubated for 20-30 min at 70°C and the proteins then separated by SDS-PAGE. The resolved proteins were electrotransferred to nitrocellulose membrane at 4 °C in a buffer containing 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol either for ~2 hours at 80-90 V or overnight at 35 V. Membranes were first dried in a fume hood to fix
proteins, then briefly rinsed in a buffer containing 20 mM Tris HCl, pH 7.4, 150 mM NaCl and 0.1% (v/v) Tween-20 (TTBS). Membranes were incubated at room temperature for 20-30 min in TTBS containing 5% (w/v) skim milk powder to block non-specific binding of antibodies, then rinsed 3 x 5 min in TTBS. Incubation of membranes with primary antibodies was carried out in TTBS containing 1% (w/v) skim milk powder for 1-2 hours at room temperature, followed by 3-5 x 10 min washes with TTBS alone. Membranes were then incubated for ~1 hour with the appropriate secondary antibody also diluted in TTBS/1% (w/v) skim milk powder, followed by 3-5 x 5 min washes with TTBS. After the final wash, blots were immediately developed by applying the SuperSignal chemiluminescence reagent for 2-3 min, then exposing to x-ray film (Hyperfilm, Amersham).

Results

In HEK 293 cells transiently transfected with cDNAs encoding green fluorescent protein (GFP) and a mouse brain mslo α subunit (26), the pore–forming subunit of a large conductance, calcium-sensitive K+ channel, voltage clamp steps ranging from –180 to 240 mV gave rise to large, outwardly rectifying macroscopic currents in excised inside/out membrane patches (Fig. 1).

[Insert Figure 1 about here]

In contrast, membrane patches from cells transfected with GFP alone displayed only negligible currents in response to the same voltage clamp steps (data not shown). Increasing the cytoplasmic free Ca²⁺ from 0 to ~120 µM was observed to have several effects on the macroscopic currents: 1) a shift in the voltage for half-maximal activation
of current to more negative membrane potentials, 2) an increase in the maximal current amplitude, and 3) an increase in the speed of current activation (Fig. 1A-E). Normalized conductance-voltage (G-V) relations derived from tail current measurements (Fig. 1F) document the left-ward shifts in channel gating along voltage axis with increasing free intracellular calcium. These observations are thus similar to those recently reported by others (33-39) for heterologous expression of mammalian Slo genes encoding BK<sub>Ca</sub> channels.

Several investigators have already reported that BK<sub>Ca</sub> channel activity can be modulated by G-protein and phosphorylation/dephosphorylation pathways (6-10), suggesting that these channels are important cellular targets for various regulatory mechanisms. More recently, much interest has been generated by observations that cellular tyrosine kinases, in particular c<sub>Src</sub>, are able to modulate the activity of both ligand- and voltage-gated ion channels (16-20), including K<sup>+</sup> channels (21-24). Such findings may now have broader implications, given that many G-protein coupled receptors (GPCRs), which can regulate cellular excitability, are also now recognized to activate various tyrosine kinases as part of their signal transduction cascades (40;41). To determine if BK<sub>Ca</sub> channel activity could also be modulated by tyrosine phosphorylation, we utilized our transient transfection strategy to co-express the BK<sub>Ca</sub> channel with cDNAs encoding active and catalytically inactive forms of the human c<sub>Src</sub> tyrosine kinase (42). Site-directed mutagenesis was used to generate the inactive form of the enzyme by a Lys to Met substitution in the catalytic domain (see Methods). The protein expression and tyrosine kinase activities of c<sub>Src</sub> transfected HEK 293 cells were then confirmed using two complementary approaches.
Figure 2A shows a Western blot of total cell lysates probed with an antibody (4G10) to detect proteins containing phosphorylated tyrosine residues.

In cells transfected with either the BKCa channel alone (lane 1, BK alone) or BKCa channel plus the inactive form of cSrc (lane 2, BK + dead Src), similar levels of phosphotyrosine-containing proteins were observed. The major phosphoprotein at Mr ~60 kDa in lane 2 likely represents the inactive cSrc, which still undergoes tyrosine phosphorylation in the intact cell. However, co-transfection of cDNAs encoding the BKCa channel and active cSrc produced a large increase in the level of phospho-tyrosine containing proteins (lane 3, Fig. 2A), indicating functional expression of cSrc tyrosine kinase activity in situ.

To directly confirm that both the active and inactive forms of the kinase were indeed expressed following transient transfection, a second Western blot of the same samples shown in Fig. 2A was performed, using a monoclonal antibody against the cSrc protein. In HEK 293 transfected with the mSlo cDNA alone, no signal was detected (lane 1, Fig. 2B). However, strong immunoreactive bands of Mr ~60 kDa were readily observed in cells co-transfected with cDNAs for mSlo and either the active or inactive forms of cSrc (lanes 2 and 3, Fig. 2B). This result thus directly confirms the expression of cSrc protein under the latter two transfection conditions.

To provide further evidence that cSrc was most likely responsible for the large increase in cellular phosphoproteins observed following co-transfection (lane 3, Fig. 2A), we directly measured the expressed cSrc tyrosine kinase activity by first immunoprecipitating cSrc and then performing an in vitro phosphorylation assay. In
cells transfected with mSlo alone or mSlo plus the inactive form of cSrc, very little protein kinase-dependent phosphorylation of the selective cSrc peptide substrate cdc25-20 (43) was observed (Fig. 2C). However, the immunoprecipitate from cells co-transfected with mSlo and active cSrc demonstrated a robust, time-dependent phosphorylation of the substrate under the same assay conditions. This observation thus strongly suggests that the increased level of phospho-tyrosine containing proteins detected in cSrc transfected cells (lane 3, Fig. 2A) is due primarily to the activity of the cSrc tyrosine kinase itself.

Figure 3 shows macroscopic BKCa channel currents recorded in an excised membrane patch from a cell co-transfected with cDNAs for mSlo and active cSrc (panels A-D) or mSlo and dead cSrc (panels E-H).

[Insert Figure 3 about here]

In the absence of free cytoplasmic calcium (ie. 2 mM EGTA in the bath), BKCa channel currents resembled those observed with expression of the BKCa channel alone (data not shown, refer to Fig. 1A). However, upon increasing free calcium from 0.9 µM to 120 µM, we observed that BKCa channel gating was left-ward shifted to a much greater degree in the presence of cSrc (Fig. 3A-D) compared to currents from mSlo channels either in the absence of cSrc co-expression (Fig. 1B-E) or co-expressed with the inactive form of the enzyme (Fig. 3E-H). In contrast, expression of just cSrc alone had no significant effect on the level of endogenous membrane currents observed under the same recording conditions (see Fig. 3J). Thus, at levels of free cytosolic calcium ≥ 4 µM, the open probability (Popen) of BKCa channels is greater at any given voltage in the presence of cSrc compared to channels expressed alone (Fig. 1) or in the presence of dead cSrc (Fig 3E-H). Most interesting, raising cytoplasmic calcium to 120 µM (Fig. 2E) leads to
very strong activation (ie. Popen near 1) of BK$_{Ca}$ channels over the entire range of membrane potentials from -180 to 90 mV. By comparison, in the absence of cSrc expression, BK$_{Ca}$ channels did not reach their half-maximal open probability until a membrane potential of ~ -70 mV at the same level of free calcium (Fig. 1F). These observations demonstrate that BK$_{Ca}$ channel gating can be enhanced by co-expression with the human cSrc tyrosine kinase. The observed lack of effect of the inactive form of cSrc on the voltage-dependence of gating and the ability of free calcium to shift gating left-ward along the voltage axis suggest that the enhancement of BK$_{Ca}$ channel activity observed following co-transfection of mSlo and cSrc is dependent upon cellular tyrosine kinase activity, and not simply the presence of the cSrc protein itself.

If the observed enhancement of BK$_{Ca}$ channel activity following co-expression of mSlo and active cSrc were due to tyrosine phosphorylation of the channel protein itself, as suggested by the maintained activity in excised membrane patches, it may be possible to demonstrate this directly. To test this possibility, HEK 293 cells were first transfected with mSlo alone, mSlo together with active cSrc or mSlo plus inactive cSrc. Following protein expression, cells were lysed and phospho-tyrosine containing proteins were immunoprecipitated from each dish of cells. The immunoprecipitates were then separated by SDS-PAGE and a Western blot was performed. Using a polyclonal antibody against the BK$_{Ca}$ channel $\alpha$ subunit, we detected a major band of M$_r$ ~125 kDa in the immunoprecipitate from cells transfected with mSlo and cSrc, but no immunoreactivity was observed under either of the other two conditions (Fig. 4A).

[Insert Figure 4 about here]
This finding thus provides direct evidence that the BK$_{Ca}$ channel $\alpha$ subunit undergoes direct tyrosine phosphorylation in the presence of active c$Src$. To exclude the possibility that this observation may be due to variable expression of the BK$_{Ca}$ channel itself, aliquots of the initial total cellular lysates were probed for expression of BK$_{Ca}$ channel protein. Figure 4B shows that under all three transfection conditions, similar amounts of immunoreactive BK$_{Ca}$ channel were detected using the same antibody. Taken together, these results support our initial hypothesis that in the presence of active c$Src$ tyrosine kinase, the BK$_{Ca}$ channel $\alpha$ subunit undergoes direct tyrosine phosphorylation. This phosphorylation may thus be responsible for the sustained enhancement of BK$_{Ca}$ channel activity observed in excised membrane patches in the presence of micromolar concentrations of free calcium.

In an attempt to identify the site(s) of tyrosine phosphorylation, site-directed mutagenesis was used to make Tyr to Phe substitutions at three potential c$Src$ phosphorylation sites (42;44) within the BK$_{Ca}$ channel $\alpha$ subunit. Following single substitutions at Tyr766 (GSIEY$^{766}$LKRE), Tyr935 (DTELY$^{935}$LTQP) or Tyr1027 (DGGCY$^{1027}$GDLF), mutant BK$_{Ca}$ channels were co-expressed in the presence of c$Src$ and then examined for their level of tyrosine phosphorylation. Using the same experimental strategy as described for Figure 4, we observed that Tyr to Phe substitutions at positions 935 and 1027 had no effect on c$Src$-dependent phosphorylation of BK$_{Ca}$ channels (Fig. 5A).

However, substitution of Tyr766 dramatically decreased the level of in situ BK$_{Ca}$ channel tyrosine phosphorylation in the presence of c$Src$. A Western blot of the total cell lysates
(Fig. 5B) demonstrates that following transfection, the expression levels of both the native and mutant channels are very similar, indicating that this observed difference in the degree of tyrosine phosphorylation is not due to differential expression of the BK$_{\text{Ca}}$ channel constructs.

Figure 6 shows BK$_{\text{Ca}}$ channel currents recorded in an excised inside-out membrane patch from a cell co-expressed with cSrc and BK$_{\text{Ca}}$ channels containing the Tyr766 to Phe substitution. In response to increasing concentrations of free cytosolic calcium, mutant BK$_{\text{Ca}}$ channel activity appeared to resemble that from native channels in the absence of cSrc, with no clear enhancement observed.

This finding suggests that phosphorylation of Tyr766 is likely responsible for the enhanced gating of BK$_{\text{Ca}}$ channels observed in the presence of cSrc. The effects of co-expression of cSrc or inactive cSrc on the gating of native and mutant BK$_{\text{Ca}}$ channels are summarized in a plot of the half maximal voltages of activation ($V_{1/2}$ values) versus the free cytosolic calcium concentrations (Fig. 7). Slope values derived from single Boltzmann fits of G-V curves did not vary significantly for wild-type and mutant BK$_{\text{Ca}}$ channels in the absence and presence of cSrc (range 20.4±3.8 at 120 μM free Ca to 28.3±5.1 at 4 μM Ca). This plot quantifies the calcium-induced left-ward shifts of channel gating for each of the expression conditions described above.
Discussion

The regulation of membrane ion channel activity by signal transduction pathways has long been considered an important mechanism for the alteration of cellular excitability and physiologic function. Examples of such events include: 1) the β-adrenergic regulation of cardiac L-type calcium channels (45;46) and epithelial CFTR chloride channels (47;48), 2) the regulation of inwardly rectifying K⁺ channels and neuronal calcium channels by G-protein βγ subunits (49;50), and 3) the enhancement of neuronal NMDA and AMPA receptors by protein phosphorylation and dephosphorylation (51;52). The results of our study now demonstrate for the first time that the pore-forming α subunit of the mammalian BKCa channel can undergo direct tyrosine phosphorylation in the presence of the human cSrc tyrosine kinase (see Fig. 4) and that this phosphorylation correlates with an enhancement of channel gating (see Figs. 3 and 6). This phosphorylation was entirely dependent upon cSrc tyrosine kinase activity and was not observed in the absence of cSrc or with co-expression of an inactive form of the enzyme. The simplest interpretation of these results is that cSrc itself is directly responsible for this tyrosine phosphorylation of the BKCa channel protein and the subsequent enhancement of calcium-sensitive gating. However, we can not exclude the possibility that the observed phosphorylation of BKCa channels is mediated by a different tyrosine kinase which is regulated by cSrc, such as Fak or Pyk2 (53). Alternatively, it is also possible that the observed enhancement of BKCa channel activity may result from a direct protein-protein interaction between the BKCa channel and a SH2 domain-containing protein, such as cSrc, for example, that is promoted by tyrosine phosphorylation and maintained in excised inside-out membrane patches.
An important aspect of our findings is that this enhancement was not observed under all experimental conditions, but rather, it occurred in a calcium-sensitive manner. In the presence of 2 mM EGTA with no added free calcium in the bath solution, BKCa channels demonstrate largely voltage-dependent gating, with conductance-voltage relations shifted strongly right-ward along the voltage axis (refer to Figs. 1A and F). Under these same recording conditions (ie. 2 mM EGTA only), co-transfection of BKCa channels with either the active or inactive forms of cSrc had no effect on channel gating properties (Fig. 7). However, in the presence of mSlo plus active cSrc, an enhancement of BKCa channel activity was observed as the level of free cytosolic free calcium was subsequently raised from 0.9 to 120 μM (Fig. 3B-E). This observation indicates that the enhancement of BKCa channel gating by cSrc co-expression occurs in a calcium-sensitive manner. Therefore, the magnitude of enhancement by tyrosine phosphorylation on channel gating appears to be dependent upon calcium binding to the channel.

What may be the physiologic consequences of cSrc-dependent tyrosine phosphorylation of BKCa channels in situ? In vascular smooth muscle cells and neurons, along with other cell types which utilize BKCa channels to dampen cellular activity, enhancing BKCa channel gating by tyrosine phosphorylation would tend to further decrease excitability, by turning off calcium influx through voltage-dependent calcium channels. In blood vessels and the nervous system, this would lead to vasodilation and reduced neurotransmitter release, for example. However, in many inexcitable cells, such haematopoietic and endothelial cells, which can produce a sustained calcium influx in response to a stimulus, an enhancement of BKCa channel gating by tyrosine phosphorylation would be predicted to support the rise in intracellular calcium by causing
greater membrane hyperpolarization and increasing the driving force for calcium entry. Therefore, tyrosine phosphorylation of BK$_{Ca}$ channels may lead to either an increase or decrease of cellular activity, depending upon the presence of other concomitant ionic fluxes. Alternatively, tyrosine phosphorylation may have a more important role in the density and/or distribution of BK$_{Ca}$ channels at the cell surface or the binding of regulatory/signaling molecules, such as protein kinases and/or phosphatases, to BK$_{Ca}$ channels. Ultimately, examination of these predictions in native cells will help determine the physiologic significance of BK$_{Ca}$ channel tyrosine phosphorylation.

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Figure Legends

**Figure 1** – Functional expression of BK$_{Ca}$ channels in HEK 293 cells. Macroscopic currents recorded from an excised inside/out membrane patch containing murine BK$_{Ca}$ channels (mSlo $\alpha$ subunits alone) transiently expressed in HEK 293 cells. The concentration of free calcium in the bath solution during voltage clamp recordings is indicated above each panel. A normalized conductance-voltage relation of each family of current traces is shown in panel F. Solid lines represent fits of the data points by single Boltzmann functions. The free calcium concentrations are indicated by symbols as
follows: ●, 0 Ca; □, 0.9 µM Ca; ▲, 4 µM Ca; △, 12 µM Ca; ◆, 120 µM Ca. In the presence of 0 Ca (panel A), voltage clamp steps ranged from -30 to +240 mV; tail currents were measured at +50 mV. For 0.9 and 4 µM free Ca (panels B and C), steps ranged from -90 to +180 mV and tail currents were measured at -80 mV. For 12 and 120 µM free Ca (panels D and E), steps varied from -180 to +90 mV, with tail currents measured at -120 mV. Refer to the diagrams of voltage clamp protocols shown below the panels. Scale bars shown in panel A apply to all current traces.

Figure 2 - Immunoblot analyses and *in vitro* kinase assay of HEK 293 cells transiently transfected with cDNAs encoding the human cSrc tyrosine kinase, an inactive form of *Src* and BK<sub>Ca</sub> channels. To verify both the expression and catalytic activity of recombinant human *Src* tyrosine kinase expressed in HEK 293 cells, Western blots of cells transfected with cDNA for BK<sub>Ca</sub> α subunits alone, or together with cDNAs for either catalytically inactive (’dead’) *Src* or wild-type cSrc were probed with the antiphosphotyrosine monoclonal antibody, 4G10 (panel A), or the anti-*Src* monoclonal antibody, 327 (panel B). Positions of molecular weight markers (in kDa) are shown on the right-hand side. Panel C shows *in vitro* phosphorylation of the selective *Src* tyrosine kinase peptide substrate cdc26-20 derived from the cSrc substrate, p34<sup>cdc2</sup>. *Src* tyrosine kinase activity was immunoprecipitated from HEK 293 cells transfected with either BK<sub>Ca</sub> α subunits alone or together with either dead or active *Src*. The time course of cdc26-20 peptide phosphorylation by immunoprecipitated kinase activity is plotted for all three transfection conditions; these conditions are indicated by symbols as follows: ○, BK<sub>Ca</sub> channels expressed alone; □, BK<sub>Ca</sub> channels co-expressed with the catalytically inactive cSrc mutant; △, BK<sub>Ca</sub> channels co-expressed with wild-type cSrc. Background substrate
phosphorylation (ie. no immunoprecipitate added) has been subtracted from all three conditions.

**Figure 3** – Macroscopic currents recorded from excised inside-out membrane patches containing \( \text{BK}_{\text{Ca}} \) channels co-expressed with either wild-type human \( S_{\text{rc}} \) (panels A-D) or catalytically inactive \( S_{\text{rc}} \) tyrosine kinase (panels E-H). The concentration of free cytosolic calcium in the bath solution during voltage-clamp recordings is indicated between each set of traces. Panel J shows currents recorded in the presence of 12 \( \mu \text{M} \) free calcium from a cell transfected with wild-type \( \text{cS}_{\text{rc}} \) alone. Normalized conductance-voltage relations of the various current traces are shown in panel I. Solid lines represent fits of the data points by single Boltzmann functions. The free calcium concentrations are indicated by symbols as follows: ◊, 0.9 \( \mu \text{M} \) Ca; □, 4 \( \mu \text{M} \) Ca; △, 12 \( \mu \text{M} \) Ca; ▽, 120 \( \mu \text{M} \) Ca. Filled symbols represent \( \text{BK}_{\text{Ca}} \) channels co-expressed with ‘dead’ \( \text{cS}_{\text{rc}} \), open symbols are for \( \text{BK}_{\text{Ca}} \) channels co-expressed with wild-type \( \text{cS}_{\text{rc}} \). In the presence of 0.9 and 4 \( \mu \text{M} \) free Ca (panels A,B and E,F), steps ranged from -90 to +180 mV; tail currents were measured at -80 mV. For 12 and 120 \( \mu \text{M} \) free Ca (panels C,D and G,H), steps varied from -180 to +90 mV, with tail currents measured at -120 mV. Scale bars shown in panel J apply to all current traces.

**Figure 4** - \( \text{BK}_{\text{Ca}} \) channels undergo tyrosine phosphorylation in the presence of \( \text{cS}_{\text{rc}} \). \( \text{BK}_{\text{Ca}} \) channels were expressed in HEK 293 cells either alone (BK alone) or co-expressed together with either catalytically inactive \( S_{\text{rc}} \) (BK+ dead \( S_{\text{rc}} \)) or wild-type \( \text{cS}_{\text{rc}} \) (BK+ \( \text{cS}_{\text{rc}} \)). Following expression, cells from each group were lysed and phosphotyrosine-containing proteins were immunoprecipitated using the monoclonal antibodies 4G10 and PY20 as described. Panel A shows a Western blot of immunoprecipitated proteins that
have been probed with a BK<sub>Ca</sub> channel-specific antibody. Panel B shows a Western blot of the total cellular lysates from each group probed using the same BK<sub>Ca</sub> channel antibody.

**Figure 5** – Mutation of Tyr766 prevents phosphorylation of BK<sub>Ca</sub> channels co-expressed with wild-type cSrc. Site-directed mutagenesis was used to make Tyr to Phe substitutions at positions 766, 935 and 1027 in the BK<sub>Ca</sub> channel α subunit. Wild-type and mutated channels were then individually co-expressed with cSrc and immunoprecipitation of phosphotyrosine-containing proteins was performed as described. Panel A shows a Western blot of immunoprecipitated proteins probed with the anti-BK<sub>Ca</sub> channel antibody. Mutant BK<sub>Ca</sub> channels are denoted as Y766F, Y935F and Y1027F above the corresponding lanes. Panel B shows the levels of expression of the native and mutant BK<sub>Ca</sub> channel proteins in the total cellular lysates from each of the transfection conditions shown in panel A.

**Figure 6** – Mutation of Tyr766 prevents enhancement of BK<sub>Ca</sub> channel gating by cSrc. The BK<sub>Ca</sub> channel mutant Tyr766 to Phe was co-transfected with wild-type cSrc and channel activity was recorded from inside-out membrane patches in the presence of 0.9 µM (panel A), 4 µM (panel B) and 12 µM (panel C) concentrations of free cytosolic calcium. For 0.9 µM and 4 µM free calcium, voltage-clamp steps ranged from -90 to +180 mV; for 12 µM free calcium, steps ranged from -180 to 90 mV. The scale bars shown in panel A apply to all current traces. Panel D shows normalized conductance-voltage curves calculated for the families of traces in panels A-C; the free calcium concentrations are represented by symbols as follows: ○, 0.9 µM; □, 4 µM; △, 12 µM.
Figure 7 – Plot of half-maximal voltages of activation for BK$_{Ca}$ channel activity versus the concentration of free calcium. Wild-type BK$_{Ca}$ channels were expressed either alone (○), or together with active cSrc (□) or dead cSrc (△) and the half-maximal voltages of activation ($V_{1/2}$ values) were derived from single Boltzmann functions fit to the normalized conductance-voltage curves. For the mutant BK$_{Ca}$ channel Y766F co-transfected with cSrc, half-maximal voltages are denoted by the symbol ▽. The values plotted at a free calcium concentration of 10$^{-8}$ M represent recordings made in the presence of 2 mM EGTA; the free calcium concentration of this solution was estimated to be 1-10 nM. Plotted values are expressed as means ± s.e.m. of 3-8 individual measurements under each of the conditions described.
A. IP: αp-Tyr
   Blot: α-BK

B. Blot: α-BK.
A. IP: αP-Tyr
Blot: α-BK

B. Blot: α-BK
A  0.9 μM Ca$_i$

B  4 μM Ca$_i$

C  12 μM Ca$_i$

D  

- Membrane Potential (mV)
- G/G$_{max}$

Graph D shows the relationship between membrane potential and conductance normalized to maximum conductance (G/G$_{max}$) for different concentrations of Ca$_i$: 0.9, 4, and 12 μM.
Enhanced Activity of a Large Conductance, Calcium-sensitive K⁺ Channel in the Presence of Src Tyrosine Kinase
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