Two adaptor proteins differentially modulate the phosphorylation and biophysics of Kv1.3 ion channel by Src kinase

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SUMMARY

The *Shaker* family K⁺ channel protein, Kv1.3, is tyrosine phosphorylated by v-Src kinase at Tyr¹³⁷ and Tyr⁴⁴⁹ to modulate current magnitude and kinetic properties. Despite two proline rich sequences and these phosphotyrosines contained in the carboxyl and amino terminals of the channel, v-Src kinase fails to co-immunoprecipitate with Kv1.3 as expressed in HEK 293 cells, indicating a lack of direct SH3- or SH2-mediated protein-protein interaction between the channel and the kinase. We show that the adaptor proteins, n-Shc and Grb10, are expressed in the olfactory bulb, a region of the brain where Kv1.3 is highly expressed. In HEK 293 cells, co-expression of Kv1.3 + v-Src with Grb10 causes a decrease in v-Src-induced Kv1.3 tyrosine phosphorylation and a reversal of v-Src-induced Kv1.3 current suppression, increase in inactivation time constant (\(j_{\text{inact}}\)), and disruption of cumulative inactivation properties. Co-expression of Kv1.3 + v-Src with n-Shc did not significantly alter v-Src-induced Kv1.3 current suppression but reversed v-Src induced increased \(j_{\text{inact}}\) and restored the right-shifted voltage at half-activation (\(V_{1/2}\)) induced by v-Src. The v-Src-induced shift in \(V_{1/2}\) and increased \(j_{\text{inact}}\) was retained when Y²²⁰, Y²²¹, and Y³⁰⁴ in the CH domain of n-Shc were mutated to F (triple Shc mutant) but was reversed back to control values when either wildtype Shc or the family member Sck, which is not a substrate for Src kinase, was substituted for the triple Shc mutant. Thus the portion of the CH domain that includes tyr²²⁰,²²¹,³⁰⁴ may regulate a shift in Kv1.3 voltage-dependence and inactivation kinetics produced by n-Shc in the presence of v-Src. Collectively these data indicate that Grb10 and n-Shc adaptor molecules differentially modulate the degree of Kv1.3 tyrosine phosphorylation, the channel’s biophysical properties, and the physical complexes associated with Kv1.3 in the presence of Src kinase.
INTRODUCTION

Voltage-gated potassium (Kv) channels form highly potassium (K)-selective pores that are conformationally switched open or closed by changes in membrane voltage (1, 2). Kv channels may not function in isolation in the plasma membrane but rather be intricately involved in protein-protein interactions (3). Such interactions build a molecular scaffold that may modulate the channel’s subcellular distribution in the neuron, regulate the level of expression of the channel, introduce signaling interchange with particular proteins contained in specific transduction cascades, and ultimately affect the functional properties of the channel (3-8). Protein-protein interactions occur between two or more proteins at discreet modules that may or may not require phosphorylation for recognition of the signaling motif.

Voltage and ligand-gated ion channels have been reported to interact with the src-homology 3 (SH3) domains of Src kinase by recognition of a proline-rich PXXP sequence motif in the channel review (9-10) to cause biophysical changes in channel function due to the interaction or to tyrosine phosphorylation by Src. Kv1.3, a mammalian homologue to the Shaker subfamily, contains two proline-rich stretches, one in the amino terminus (39-44 PLPPALP) and one in the carboxyl terminus (493-496 PXTP) of the channel protein that could interact with Src kinase SH3 domain. Kv1.3 also has a total of 16 tyrosine residues, 6 of which lie within good recognition motifs for tyrosine specific phosphorylation and could serve as potential interactive sites for Src kinase SH2 domain once phosphorylated by Src (see Fig. 1). Binding at SH2 or SH3 domains of Src kinase has been found to propagate down-stream signaling, and at times, inappropriate signaling by phosphorylation that has been linked to several pathological conditions including asthma, autoimmune disease, oncogenesis, Alzheimer’s, and allergies (11).

It has been previously shown that while a related Kv family member, Kv1.5, co-
immunoprecipitates with Src kinase at the Src SH3 domain, Kv1.3 fails to retain a strong interaction with Src kinase in HEK 293 cells by SDS-PAGE and Western blot analysis (5). We thus hypothesized that adaptor molecules could form the physical complexes bringing together Kv1.3 and v-Src kinase to modulate the electrical activity by tyrosine phosphorylation. Thus the repertoire of adaptor proteins expressed in a given neuronal cell type and the specific capacity for regulating the proximity of kinase to its effector (the ion channel) may influence the electrical phenotype of a neuron to the same extent that expression of various voltage-gated channel species shape the overall macroscopic current properties of a cell.

Kv1.3 is highly expressed in T-lymphocytes, the dentate gyrus of the hippocampus, the pyriform cortex, and the olfactory bulb (OB) (12). In the olfactory bulb, Kv1.3 is localized to the mitral and granule neurons, where it is a substrate for protein phosphorylation by multiple tyrosine kinases that induce change in its function as assessed electrophysiologically (13,14). Two adaptor proteins that have been shown to be substrates of Src kinase and which we found were well expressed in the olfactory bulb, are neuronal Src- and collagen-homology protein (n-Shc) and growth factor receptor binding protein 10 (Grb10) (15-18) (see Fig. 1). Thus the olfactory bulb represents a discrete brain region where channel-kinase-adaptor protein physiology could be investigated and where association of protein modules may influence channel function.

Herein we report that two adaptor proteins differentially modulate v-Src-induced Kv1.3 phosphorylation and modulation of channel function. Grb10 adaptor significantly reduces v-Src-induced Kv1.3 phosphorylation, whereas tyrosine phosphorylation of Kv1.3 is modestly increased in the presence of nShc adaptor. The proline rich sequences contained in Grb10 adaptor protein may compete for the SH3 domain of Src, to decrease the ability for Src to phosphorylate Kv1.3 and suppress Kv1.3 current magnitude, increase the inactivation time constant ($\tau_{\text{inact}}$), and disrupt
cumulative inactivation during repetitive voltage stimulation. N-Shc adaptor also acts to prevent the v-Src-induced increase in the Kv1.3 inactivation time constant ($J_{\text{inact}}$) but additionally reverses a shift in voltage-dependence typically observed for Kv1.3 in the presence of v-Src. Through site-directed mutagenesis of the regulatory Tyr residues in the CH domain of n-Shc and comparison of the activity of n-Shc with a close family member Sck, data suggest that a portion of the CH domain that includes tyr$^{221,222}$ and 304 and is homologous to Sck regulates a shift in Kv1.3 voltage-dependence and inactivation kinetics in the presence of v-Src kinase and Shc adaptor protein.

EXPERIMENTAL PROCEDURES

Solutions and Reagents

Human embryonic kidney cell (HEK 293) patch pipette solution contained (in mM): 30 KCl, 120 NaCl, 10 HEPES, and 2 CaCl$_2$ (pH 7.4). HEK 293 cell recording bath solution contained (in mM): 150 KCl, 10 HEPES, 1 EGTA, and 0.5 MgCl$_2$ (pH 7.4). Cell lysis buffer with protease and phosphatase inhibitors (PPI solution) contained (in mM): 25 Tris (hydroxymethyl) aminomethane (pH 7.5), 250 NaCl, 5 EDTA, 1% Triton X-100, 1 sodium orthovanadate, 1:100 dilution of Sigma Phosphatase Inhibitor Cocktail II (Catalog # P-5726), 1:500 dilution of Sigma Protease Inhibitor Cocktail (Catalog # P-8340). Wash buffer contained (in mM): 25 Tris (pH 7.5), 250 NaCl, 5 EDTA, and 0.1% Triton X-100. Homogenization buffer contained (in mM): 320 sucrose, 10 Tris base, 50 KCl, and 1 EDTA (pH 7.8). All salts were purchased from Sigma Chemical Company or Fisher Scientific. Tissue culture and transfection reagents were purchased from Gibco BRL.

Olfactory Bulb Membrane Solubilization

One set of olfactory bulbs (OB) from Sprague-Dawley rats at postnatal day 17 (P17) and P30
(adult) were quickly dissected using American Veterinary Medical Association (AVMA)- and National Institutes of Health (NIH)-approved methods. Harvested OB tissue was homogenized on ice using a Kontes tissue grinder (size 20; 50 strokes) and homogenization buffer containing 0.5% nonidet P-40 (NP-40) detergent and Sigma protease and phosphatase inhibitor cocktails (see Solutions and Reagents). The mixture was vortexed every 15 minutes for 4 hours, centrifuged at 14,000 x g for 10 minutes at 4°C, and the supernatant (lysate) was stored at -80 °C until subsequent use. Protein concentration was determined by Bradford protein assay; 10 or 40 mg of lysate was separated by 10% SDS-PAGE, proteins were electrotransferred to nitrocellulose, and Western blots were probed with antisera for Src, Shc, and Grb (see below).

cDNA Constructs and Antibodies

All channel, kinase, and adaptor protein coding regions were downstream from a cytomegalovirus (CMV) promoter. The v-Src cDNA construct was a generous gift from Dr. Richard Huganir (Johns Hopkins University, Baltimore, MD) and was located in a modified PRK5 vector. The R385A v-Src cDNA construct (v-Src with severely impaired tyrosine kinase activity) was a generous gift from Dr. M. Senften (Friedrich Miescher-Institute, Basel Switzerland; (19)). The entire R385A v-Src coding region was inserted into a modified PRK5 vector (20). Neuronal Shc (n-Shc), Sck, and n-Shc mutant cDNA (Y to F point mutations at tyr220, tyr221, tyr304) were generous gifts from Dr. T. Nakamura (Sumitomo Electric Industries, Yokohama, Japan) and were expressed in the vector pCMV1 (21). The n-shc mutant will be referred to as the “triple”mutant. Grb10 cDNA was a gift from Dr. R. Roth (Stanford University) and was expressed in pBlueScript SK (-) vector (Stratagene). AU13, a rabbit polyclonal Kv1.3 antiserum, was designed against the 46-amino acid sequence (478MVIEEGMNHSAPQTPFKTGNSATCTTNNPNDCVNIKKIFTDV523) representing the
unique coding region of Kv1.3 between the amino terminus and transmembrane domain 1. The purified peptide was produced by Genmed Synthesis (San Francisco, CA), and the antiserum was generated by Cocalico Biologicals (Reamstown, PA). This antibody was used for immunoprecipitation (1:1000) and Western blot detection (1:1500) of Kv1.3 as in Tucker and Fadool (submitted). The monoclonal antibody 4G10 (Upstate Biochemical) recognized phosphorylated tyrosine residues and was used at 3.5 \( : \) g/ml for immunoprecipitation and 1:1000 for Western analysis. Src monoclonal antibody, directed against the Src SH3 domain (Oncogene Ab-1), was used at 10 \( : \) g/ml for immunoprecipitation and 1:40-1:100 for Western analysis. Polyclonal antisera for Shc (H-108) and Grb10 (K-20) were from Santa Cruz; each was used at 20 \( : \) g/ml for immunoprecipitation and between 1:200-1000 for Western blot detection.

**Maintenance and Transfection of HEK 293 Cells**

HEK 293 cells were maintained in Minimum Essential Medium (MEM), 2% penicillin/streptomycin, and 10% FBS (Gibco BRL). Before transfection, cells were grown to 100% confluency (7 days), dissociated with trypsin-EDTA (Sigma) and mechanical trituration, diluted in MEM to a concentration of 600 cells/ml, and replated on Corning dishes (Catalog # 25000, Fisher Scientific). cDNA was introduced into HEK 293 cells with a lipofectamine reagent (Gibco BRL) 3-5 days after passage as previously described (13,14). Briefly, cells were transfected for 4-5 hours with 0.5-0.75 \( : \) g of each cDNA construct per 35-mm dish for electrophysiology or 3.5-5.0 \( : \) g of each cDNA construct per 60-mm dish for biochemistry. Plasmid DNA with no coding insert (control vector) served as the control to equalize total \( : \) g of cDNA added to each dish. Cells were either harvested for biochemical analysis or used for electrophysiological recordings approximately 30-40
hours after transfection.

For patch-clamp experiments, transfection efficiency was monitored by co-transfecting with pHook (Invitrogen) as a means of rapidly selecting cells expressing Kv1.3 channels. pHook encodes a transmembrane domain from the platelet-derived growth factor receptor (PDGF-R), which is then anchored on the extracellular side of the plasma membrane. Before patch recording, a brief incubation with an appropriate antibody linked to a 5- m polystyrene bead allowed recognition of transfected cells. Efficiency of co-transfection (greater than 95%) with our transfection method has been tested using double, sequential labeling and confocal microscopic visualization, as reported previously(14).

Electrophysiology

Patch electrodes were fabricated from Jencons glass (Jencons Limited, Bedfordshire, UK), fire-polished to approximately 1 m, and coated near the tip with beeswax to reduce the electrode capacitance. Pipette resistances were between 9 and 14 MS. Hoffman modulation contrast optics was used to visualize cells at 40x magnification (Axiovert 135, Carl Zeiss). Macroscopic currents in cell-attached membrane patches were recorded using an Axopatch-200B amplifier (Axon Instruments), filtered at 2 kHz, digitized at 2-5 kHz, and stored for later analysis. All voltage signals were generated and data were acquired with the use of either a Microstar DAP 800/2 board with in house written software (DapClamp; Microstar Lab, Bellevue, WA) or an Axon Digidata 1200 board with pClamp software (Axon Instruments). Data were analyzed using Microcal Origin software.

Outward macroscopic currents were recorded in the cell-attached rather than whole-cell configuration; Kv1.3 channel expression is so robust in the HEK 293 expression system that it is not
routinely possible to record whole-cell currents without saturating the amplifier (20, 22, 23). Patches were held routinely at a holding potential of -90 mV and stepped in 20 mV depolarizing potentials using a pulse duration of 1000 milliseconds. Pulses were generally delivered at intervals of 60 seconds or longer to prevent cumulative inactivation of the Kv1.3 channel (24). Kv1.3 peak current amplitude, channel inactivation ($I_{\text{inact}}$) and deactivation ($I_{\text{deact}}$) kinetics, voltage at one-half maximal activation ($V_{1/2}$), and slope of voltage dependence (6) were measured in the presence of Src kinase and the presence or absence of an adaptor protein (N-Shc or Grb10). Each biophysical property was analyzed in the form of non-normalized data by one-way ANOVA with a Student Newman Keuls (snk) follow-up test at the 95% confidence level to determine any statistical difference in Kv1.3 channel function in the presence of kinase or adaptor proteins. For graphical representations of peak current amplitudes among different transfection conditions, measurements were normalized to the transfection condition of Kv1.3 alone within a single recording session and respective transfection set. Kinetic properties of Kv1.3 have been reported to be independent of current magnitude (24), thus peak current amplitude but not kinetic data were normalized to adjust for differences in channel expression between transfections.

Fitting parameters for inactivation and deactivation kinetics were as previously described (14). Briefly, the inactivation of the macroscopic current, during a 1000 ms voltage step from -90 to +40 mV, was fit to the sum of two exponentials by minimizing the sums of squares using a bi-exponential function ($y = y_0 + A_1 e^{(x-x_0)J_1} + A_2 e^{(x-x_0)J_2}$). The two inactivation time constants ($J_1$ and $J_2$) were combined by multiplying each by its weight (A) and summing. The summed inactivation rate was calculated with the equation $\text{Tau Inactivation} (J_{\text{Inact}}) = [A_1 \times J_1] + (A_2 \times J_2)/(A_1 + A_2)$. The deactivation of the macroscopic current was fit similarly but to a single exponential ($y = y_0 + Ae^{(x-x_0)J_3}$).
Tail current amplitudes were plotted in a current-voltage relationship and fit to a Boltzmann sigmoidal curve ($Y = [(A_1 + A_2)/(1 + e^{(x-x_0)/\Delta x})] + A_2$) to calculate the slope of voltage dependence ($\Delta E$) and voltage at half-activation ($V_{1/2}$) for Kv1.3. To study cumulative inactivation of Kv1.3 under the control condition and in the presence of Src and/or an adaptor protein, patches were held at -90 mV and stepped to +40 mV twelve times using a similar pulse duration as above but recording using different interpulse intervals, including 60, 30, 10, 2, 1, and 0.5 seconds. Peak current amplitudes were normalized to the initial trace in each series for a given interpulse interval. Cumulative inactivation was always present with interpulse intervals less than 60 seconds in the control (Kv1.3 alone) transfection condition, and as much as 50% of channels were caught in the inactivated state with repeated stimulation less than 2 seconds.

**Immunoprecipitation and Electrophoretic Separation**

Transfected cells were harvested by lysis 2 days post-transfection in ice-cold PPI solution (see Solutions and Reagents). The lysates were clarified by centrifugation at 14,000 x g for 10 minutes at 4°C and incubated for 1 hour (h) with 0.2-0.3 mg/ml protein A-sepharose (Amersham-Pharmacia), followed by re-centrifugation to remove the protein A-sepharose. To test the phosphorylation state of Kv1.3 in the presence of v-Src kinase plus or minus an adaptor protein, tyrosine-phosphorylated proteins were immunoprecipitated from the clarified lysate by overnight incubation at 4°C with 3-4 µg/ml 4G10 antibody (Upstate Biochemical). Immunoprecipitated proteins were harvested by a 2-h incubation with protein A-sepharose and centrifugation as above. The immunoprecipitates (IPs) were washed 3 times with ice-cold wash buffer. Lysates and washed IPs were diluted in sodium dodecyl sulfate (SDS) gel loading buffer (25) containing 1 mM Na$_2$VO$_4$. 
Protein concentration was determined by Bradford protein assay. Proteins were separated on 10% acrylamide SDS gels and electrophoretically transferred to nitrocellulose for Western blot analysis. Nitrocellulose membranes were blocked with 5% nonfat milk and incubated overnight at 4°C in primary antibody against Kv1.3, v-Src, Shc, or Grb10. Membranes were then exposed to species-specific peroxidase-conjugated secondary (Amersham-Pharmacia or Sigma) for 90 minutes at room temperature. Enhanced chemiluminescence (ECL; Amersham-Pharmacia) exposure of Fuji RX film (Fisher) was used to visualize labeled protein.

Resultant bands were quantified by densitometry using a Hewlett-Packard Photosmart Scanner in conjunction with Quantiscan software (Biosoft, Cambridge, UK). Pixel densities for each band were normalized to Kv1.3 within the same cell passage, transfection, and autoradiograph. Mean pixel densities were then calculated across sets of normalized data contained in single autoradiographs. This type of quantification was designed to reduce inherent variability in cell culture, transient transfection efficiencies, and ECL exposure times. Statistical significance was set at the 95% confidence level for immunodensitometry data that were analyzed by one-way ANOVA with snk follow-up test.
RESULTS

In this study we have found that the modulation of the voltage-gated potassium channel, Kv1.3, by the cellular tyrosine kinase, v-Src, is differentially affected by the presence of two adaptor proteins, n-Shc and Grb10. A schematic of the protein interaction modules and tyrosine phosphorylation recognition motifs contained in Kv1.3, n-Shc, Grb10, and Src is presented in Figure 1.

**Signaling Proteins Are Expressed in the Olfactory Bulb**

Kv1.3 becomes robustly phosphorylated at tyr\(^{137}\) and tyr\(^{449}\) when co-expressed with v-Src kinase in HEK293 cells to induce current suppression and decreased kinetics of inactivation (increased \(J_{\text{inact}}\) time constant) (20). Likewise, patch pipette dialysis of c-Src\(^{pp60}\) plus MgATP induces similar biophysical changes in the outward whole-cell current of OB neurons, that is carried largely by Kv1.3 (13). Although Src kinase is ubiquitously expressed throughout the CNS, it was important to confirm the expression of Src in the OB in addition to its capacity for modulation of Kv1.3. Ten or forty g of OB lysate was separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were probed with a monoclonal antibody for viral Src (v-Src, 1:40-1:100) and Src expression was detected in the OB lysates at the expected molecular weight of 60 kDa (Fig. 2).

In HEK 293 cells, a direct protein-protein interaction has been observed between v-Src kinase and Kv1.5 and this protein-protein interaction causes current suppression of Kv1.5 (5). Although Src kinase modulates channel properties of Kv1.3 by tyrosine phosphorylation, the channel fails to co-immunoprecipitate with Src kinase in HEK 293 cells (5). We conjectured that perhaps adaptor protein modules might facilitate channel-kinase interactions in native neurons expressing Src and Kv1.3. We thus probed the SDS-PAGE separated OB lysates with antisera for Shc (H-108) and
Grb10 (K-20). Fig. 2 demonstrates the observed protein bands of the predicted molecular weight (46/52/66 kDa and 60 kDa, respectively) for these two adaptor proteins. Shc adaptor protein is not expressed in brain regions, however, a neuronal-specific member of the Shc family, n-Shc, is highly expressed in the brain and the OB, as shown by 

*in situ* hybridization (18). Since H-108 antibody detects all Shc isoforms as well as n-Shc, it is highly likely that we are detecting predominantly the n-Shc family member in the OB.

**v-Src kinase phosphorylation of Kv1.3 is altered by adaptor proteins, Grb10 and n-Shc**

Deletion of the c-Src activation loop produces the viral form of Src, v-Src, which has unlimited, constitutive activity in the absence of other cellular signaling (26). The rest of the experiments of this study were performed by transient co-transfection of v-Src plus Kv1.3 in an heterologous expression system (HEK 293 cells). This model facilitated the understanding of the potential mechanisms by which Src kinase could exert its effects on Kv1.3 in native OB neurons by allowing the experimenter to control the expression of putative protein-protein interacting partners and by having the ability to activate the kinase without induction of the endogenous upstream signaling cascade. Additionally, human embryonic kidney (HEK 293) cells are known to express high levels of cloned proteins (27), which facilitates good protein yields for immunoprecipitation. Untransfected HEK 293 cells also show no C-type inactivating K currents in response to voltage stimulation (20).

HEK 293 cells were co-transfected with cDNA for Kv1.3 + v-Src (see Experimental Procedures) plus or minus cDNA coding n-Shc or Grb10 adaptor proteins. Kv1.3 plus v-Src co-transfected HEK 293 cells demonstrated that v-Src kinase significantly increased tyrosine phosphorylation of Kv1.3 14-fold over that found in Kv1.3-alone transfected cells (n = 10) (Fig. 3).
Kv1.3 exhibited low levels of basal tyrosine phosphorylation, but as also observed by Holmes et al. (23), its tyrosine phosphorylation increased dramatically when co-expressed with v-Src. The basal value of Kv1.3 tyrosine phosphorylation was assigned an arbitrary value of 1.0 (Fig. 3B). This value was used as a normalization factor for all other transfection conditions. The predicted molecular weight (M_r) of the Kv1.3 channel (58 kDa) shows a decreased electrophoretic mobility, i.e. upward band shift, on SDS-PAGE under protein tyrosine phosphorylation conditions (23, 28, 29). The bracketed region (Fig. 3A, upper panel) from 55-72 kDa spanning all Kv1.3 immunoreactivity was thus quantified in assessing Kv1.3 tyrosine phosphorylation in this and all subsequent experiments. The expression of Kv1.3 protein in each condition, for this, and all remaining experiments, was verified by separating 5 μg of cell lysate for each transfection condition and probing the lysates with " -Kv1.3 antibody (Fig. 3A, lower panel). Uniform protein expression of the channel in the original lysate was confirmed by densitometry as reported in Figs 3,8.

Both Grb10 and n-Shc adaptor proteins have been shown to be substrates of Src kinase (17;18;30), and each contain several signaling motifs for protein-protein interactions (Fig. 1). We tested the effect of each adaptor protein on v-Src-induced Kv1.3 phosphorylation using immunoprecipitation and Western analysis as described above. Addition of Grb10 cDNA to v-Src plus Kv1.3 co-transfected HEK 293 cells caused a significant decrease in Kv1.3 tyrosine phosphorylation, which approximated that of basal levels (Kv1.3 in the absence of v-Src kinase) (ANOVA, snk, " = 0.05) (Fig. 3B). Addition of n-Shc cDNA, however, slightly increased Kv1.3 tyrosine phosphorylation but not significantly greater than that found in Kv1.3 plus v-Src co-transfected cells (ANOVA, snk, " = 0.05) (Fig. 3B).
v-Src-induced modulation of Kv1.3 is altered by adaptor proteins, Grb10 and n-Shc

v-Src-induced current suppression of Kv1.3 (20) was first confirmed by comparing the biophysical properties of Kv1.3-transfected cells with those of Kv1.3-plus-v-Src-co-transfected cells. We then tested whether changes in v-Src-induced Kv1.3 tyrosine phosphorylation caused by co-expression with Grb10 or n-Shc also altered Kv1.3 physiology. For these patch-clamp experiments, HEK 293 cells were transfected with Kv1.3 alone or co-transfected with Kv1.3 plus v-Src, plus or minus an adaptor protein. Approximately 40 hours post-transfection, macroscopic outward currents were recorded in the cell-attached configuration. Cells were voltage-clamped at -90 mV and stepped to a depolarizing potential of +40 mV for 1 second to activate Kv1.3. Peak current amplitude was 1094 +/- 88 pA (n = 67) in the Kv1.3-only control transfection condition and 88 +/- 14 pA (n = 34) in the Kv1.3 plus v-Src condition, confirming a statistically significant 92% suppression of Kv1.3 current in the presence of the kinase (Student’s t-test, " = 0.05) (Fig. 4A-B). This change in current magnitude appeared to be coincident with a shift in Kv1.3 voltage dependence. This is reflected in an ~10 mV change of voltage at half-maximum activation (V1/2) in the presence of v-Src as calculated from a Boltzman fit of the tail currents when cells were held at -90 mV and stepped to +20 mV in increments of 5 mV using a pulse duration of 50 ms (Table 1). v-Src significantly increased the J_{Inact} of Kv1.3 from 716 ms (n = 51) to 1475 ms (n = 29), as shown in the examples of Figure 4C-D. Because v-Src had no significant effect on the J_{Deact} of Kv1.3 by Student’s t-test (" = 0.05) (Kv1.3 alone = 22 ms (n = 47) versus Kv1.3 + v-Src = 20 ms (n = 26) this kinetic property was not further studied.

Upon co-transfection of Grb10 cDNA with Kv1.3 plus v-Src, Grb10 adaptor protein relieved v-Src-induced current suppression of Kv1.3 to return the peak current amplitude to 86% of the
control level (Kv1.3-only transfection) (n = 9) (Fig. 4A, Table 1). Grb10 adaptor protein relieved the v-Src-induced increase in $J_{\text{inact}}$ to values significantly less than those of the Kv1.3-only control condition (ANOVA, $snk$, $\alpha = 0.05$) (Fig. 4C,E). In contrast, co-transfection of n-Shc cDNA failed to relieve v-Src-induced Kv1.3 current suppression (Fig. 4B, Table 1, n = 36). Kv1.3 current in the presence of v-Src and n-Shc was 21% of the Kv1.3-only control condition (Table 1). Lastly, co-expression of n-Shc reversed the right-shifted $V_{1/2}$ of $-41 \pm 2$ mV (n=11) observed in the Kv1.3 + v-Src transfection condition back to values approximating the Kv1.3 control condition (Table 1; $-47 \pm 1$ mV, n=6)(Figure 5). In contrast, Grb10 had no effect on the v-Src-induced $V_{1/2}$ shift, but decreased the slope of the voltage-dependence ($k = 3.2$ versus 4.4; Table 1).

Taken together, the data from our combined SDS-PAGE-immunoprecipitation and patch-clamp electrophysiological experiments indicate that the adaptor protein Grb10 inhibits a large proportion of the v-Src-induced tyrosine phosphorylation of Kv1.3 and that this decreased phosphorylation correlates with a decrease in Src kinase modulation of Kv1.3 current amplitude. The adaptor molecule n-Shc does not significantly relieve v-Src-induced modulation of Kv1.3 current amplitude or the corresponding total tyrosine phosphorylation of the channel, which appears to be retained or increased in the presence of the adaptor protein. Although we do not know if different tyrosine residues in Kv1.3 are targeted by v-Src in the presence of n-Shc, clearly the structural differences between n-Shc and Grb10 could provide differential interaction with Kv1.3 to account for the differences in Kv1.3 physiology in the presence of Src kinase and n-Shc or Grb10. The fact that Grb10 inhibits most of v-Src-induced phosphorylation and functional modulation could suggest that Grb10 blocks access of v-Src kinase to Kv1.3 in the cell. Since both Grb10 and Kv1.3 contain proline-rich sequences specific for interaction with the SH3 domain of v-Src kinase, and the channel cannot tightly co-immunoprecipitate with Src at this domain (5), Grb10 may have a stronger affinity.
for this domain and physically block the kinase access to the channel.

\textit{v-Src kinase disrupts Kv1.3 cumulative inactivation, an effect that is restored by Grb10 and accentuated by n-Shc}

Kv1.3 ion channel inactivates slowly and remains in the inactivated state for up to 60 seconds (24, 31). When the interpulse interval is less than 60 seconds, Kv1.3 exhibits a cumulative inactivation upon repeated stimulation due to its long dwell time in the inactivated state. Kv1.3 cumulative inactivation has not been studied under a kinase-induced phosphorylated state. To test whether v-Src kinase affected Kv1.3 cumulative inactivation, cells were held at -90 mV and stepped to +40 mV 12 times for 1 second each at varying interpulse intervals. Peak current amplitudes were normalized to that of the first trace in each series of 12 traces and plotted against pulse number. Sample size for each interpulse interval varied slightly due to the technical difficulty of performing all needed recordings on a cell without losing the voltage-clamp. Kv1.3 showed the expected cumulative inactivation when expressed alone: no inactivation at 60 second interpulse intervals, slight cumulative inactivation was initiated at 30 second interpulse intervals, and marked cumulative inactivation was apparent at 10, 2, 1, and 0.5 s interpulse intervals (n = 22-26) (Fig. 6A). With an interpulse interval permitting only a 2 second recovery from inactivation, as much as 50% of the Kv1.3 channels were caught in the inactivated state. In the condition of v-Src co-transfection, Kv1.3 cumulative inactivation was not initiated until the interpulse interval was shortened to 2 seconds. A maximum of 20-25% of Kv1.3 channels were caught in the inactivated state, even when recovery from inactivation was only 500 milliseconds, compared with Kv1.3 alone transfections in which typically 75% of channels remain inactivated with such short recovery intervals (n = 19-23) (Fig. 6B). The severe decrease in Kv1.3 cumulative inactivation in the presence of the kinase was not due
to overall kinase-induced current suppression because the current amplitudes were normalized to that of the first trace in each series and thus percent decreases could be calculated, if present. These data indicate that v-Src kinase could contribute a permissive function in the excitability of cells by increasing the number of Kv1.3 channels available for activation during rapid repetitive stimulation. The Src-induced disruption of Kv1.3 cumulative inactivation would be consistent with either Kv1.3 failing to enter the inactivated state or Kv1.3 exiting the inactivated state more quickly in the presence of the kinase. These alternatives could be best deciphered at the unitary current level in future studies.

Co-transfection of v-Src plus Kv1.3 cDNAs in the presence of either Grb10 or n-Shc adaptor proteins differentially affected the disruption of cumulative inactivation of Kv1.3 by v-Src kinase (Fig. 6C-D). Addition of n-Shc adaptor protein failed to restore cumulative inactivation of Kv1.3 in Kv1.3 plus v-Src co-transfected cells (Fig. 6D). When recovery from inactivation was shortened to only 500 milliseconds, nearly 90% of Kv1.3 channels could still be available for reactivation upon voltage stimulation. Addition of Grb10 adaptor protein, however, re-established cumulative inactivation properties of Kv1.3 in the presence of v-Src kinase (Fig. 6C).

The effects of Grb10 and n-Shc on the phosphorylation and modulation of Kv1.3 by v-Src are phosphorylation-dependent

To determine the importance of v-Src kinase activity and to eliminate the possibility that this kinase exerts its effects on Kv1.3 only through its own non-catalytic interaction domains, we substituted a mutant Src kinase for v-Src in our transfection scheme described above. Mutation of arginine 385 of Src to alanine produces a severely impaired Src kinase (R385A Src) (19). To confirm that the mutant Src kinase is in fact significantly impaired, we tested its ability to
phosphorylate Kv1.3, and we show that this mutant only phosphorylates Kv1.3 to 4-fold above its basal level as opposed to the 14-fold increase by wild-type v-Src (n = 5) (Fig. 7B). The outward current of Kv1.3 in the presence of R385A Src kinase is 87% that of the controls, showing little suppression (n = 12) (Fig. 7A, Table 1). The R385A Src kinase does not change the inactivation or cumulative inactivation of Kv1.3 in comparison to the control condition of Kv1.3 alone (Table 1, Fig. 8A). When co-transfected with Kv1.3 plus R385A Src kinase, Grb10 decreases what little phosphorylation of Kv1.3 remains to less than that of the Kv1.3-only transfection condition (n = 3) (Fig. 7B). Physiologically, Kv1.3 peak current amplitude is increased 1.4-fold when R385A Src is substituted for Src kinase in the presence of Grb10, so there is no current suppression in this condition (n = 7) (Fig. 7A, Table 1). When R385A Src kinase is substituted for Src kinase, Grb10 continues to decrease the $J_{\text{inact}}$ but the $V_{1/2}$ shifts to approximately that of Kv1.3 only transfection conditions (Table 1). Kv1.3 exhibits normal cumulative inactivation in the presence of R385A Src and Grb10 adaptor protein (n = 4-6) (Fig. 7B). These results demonstrate that the effects of Grb10 on v-Src-induced Kv1.3 phosphorylation are dependent on the kinase activity of Src. Without the kinase activity of v-Src, Grb10 cannot modulate Kv1.3 voltage-dependence ($V_{1/2}$). However, in the presence of Grb10, inactive Src (R385A Src) can significantly increase Kv1.3 current magnitude and inactivation kinetics even in the absence of apparent phosphorylation (Table 1, Figure 7B). Therefore, a portion of v-Src that does not include the catalytic domain may modulate Kv1.3 in the presence of Grb10 as a phosphorylation independent interaction.

Transfection of n-Shc with Kv1.3 plus R385A Src results in no increase in phosphorylation over that of Kv1.3 + R385A Src (n = 3) compared with the 18-fold increase in channel tyrosine phosphorylation in the presence of the adaptor and live v-Src kinase (Fig. 7B). In contrast to
transfection of n-Shc with Kv1.3 + v-Src, substitution with R385A Src yielded a relief in v-Src-induced current suppression (Fig. 7A, Table 1) up to 97% of control Kv1.3 alone conditions (n=5). Kv1.3 exhibits normal cumulative inactivation in the presence of R385A Src and n-Shc adaptor protein (n = 2-3) (Fig. 8C). These results show that the n-Shc adaptor can only affect Kv1.3 phosphorylation and channel properties in the presence of active kinase activity by Src and has no functional effect on the channel alone.

The CH domain is responsible for n-Shc regulation of v-Src-induced phosphorylation and current suppression of Kv1.3

Members of the Shc family of adaptor proteins contain highly homologous SH2 and PTB domains, responsible for recognizing specific sequences of other proteins containing phosphotyrosines, and CH domains that are not highly conserved and contain different varieties of proline-rich regions and tyrosine phosphorylation-specific sequences (18). Within the CH domain, the Shc protein is known to be phosphorylated on three specific tyrosine residues, tyr239 and tyr240, and tyr317 (30, 32-35) that correspond to tyr220, tyr221, and tyr304 of n-Shc, respectively (18). Sck is a member of the Shc family, which is expressed predominantly in the peripheral nervous system, and differs from n-Shc in the structure of its CH domain. Sck is also NOT a substrate for Src kinase(18). Sck none the less contains homologous tyrosines in its CH domain. Dr. Takeshi Nakamura (Sumitomo Electric Industries) generously provided our laboratory with cDNA constructs encoding n-Shc, Sck, and a n-Shc mutant in which these three key tyrosines were conservatively mutated to phenylalanine (Y220F, Y221F, and Y304F). The cDNA construct in which all three tyrosine residues in the CH domain were collectively altered from tyrosine to
phenylalanine as a cassette, we have termed "n-Shc triple tyrosine mutant." We used this n-Shc triple tyrosine mutant and the Sck member of the Shc family to determine the contribution of the CH domain toward the n-Shc effect on v-Src-induced changes in Kv1.3 function and tyrosine phosphorylation. n-Shc triple tyrosine mutant lacks the tyrosine residues that would normally be phosphorylated by Src kinase to provide phosphotyrosine residues for interaction with SH2-containing proteins, whereas Sck contains the tyrosine residues, but these tyrosines are not normally substrates for Src kinase. We performed transfection of HEK 293 cells similar to that described for n-Shc above, substituting either Sck or the n-Shc triple tyrosine mutant for n-Shc.

When co-transfected with Kv1.3 and v-Src, Sck adaptor protein maintained an increased Kv1.3 phosphorylation by v-Src (n = 4) (Fig. 7B). Sck partially relieved v-Src-induced Kv1.3 current suppression to 49% of the control level (n = 21) (Fig. 7A, Table 1). Like n-Shc, Sck reversed the increased $J_{\text{inact}}$ found in Kv1.3 plus v-Src co-transfected cells to an inactivation rate approaching Kv1.3 alone (Table 1). Lastly, Sck, but not n-Shc, restored the cumulative inactivation of Kv1.3 in Kv1.3 plus v-Src co-transfected conditions (n = 10-11) (Fig. 7D). Results from these Sck experiments suggest that regions of the n-Shc CH domain that differ from those of the Sck CH domain are responsible for partially relieving Kv1.3 current suppression and disrupted cumulative inactivation by v-Src. Homologous regions between the n-Shc and Sck adaptors may mediate voltage-dependence or kinetics of inactivation in v-Src + Kv1.3 co-transfected cells.

When n-Shc triple tyrosine mutant was substituted for n-Shc in the transfection scheme of Kv1.3 plus v-Src kinase plus adaptor protein, phosphorylation of the ion channel was 8-fold over that of the control, slightly less than that of Kv1.3 plus v-Src kinase (n = 5) (Fig. 7B). Similar to that of wild-type n-Shc, mean v-src-induced Kv1.3 current suppression was 79% and the $V_{1/2}$ was
significantly right-shifted to $-37.6 \pm 2$ mV (Figs. 5, 7A, Table 1). Unlike that of n-Shc, the n-Shc triple tyrosine mutant had a similarly increased $J_{\text{inact}}$ approximating that Kv1.3 plus v-Src co-transfected cells. Also in contrast to n-Shc, but similar to Sck, the n-Shc triple tyrosine mutant restored cumulative inactivation of Kv1.3 to similar levels as that observed for Kv1.3 alone ($n = 2$) (Fig. 8E). The data from experiments performed with the n-Shc triple tyrosine mutant suggest that tyr$^{220}$, tyr$^{221}$, tyr$^{304}$ only marginally contribute to increasing Kv1.3 phosphorylation by v-Src.

These residues are apparently not necessary for the partial recovery of Src-induced Kv1.3 current suppression observed in the presence of Sck. Tyr$^{220}$, tyr$^{221}$, and/or tyr$^{304}$ in n-Shc, however, must participate in the loss of cumulative inactivation of Kv1.3 as disrupted by v-Src kinase, because removal of these residues (n-Shc triple tyrosine mutant) or their capacity to be phosphorylated by Src (Sck) reverses Src disruption of Kv1.3 cumulative inactivation. Removal of these residues also prevents n-Shc from returning v-Src modulated Kv1.3 channel to normal inactivation and voltage dependence states. Therefore, the ability for n-Shc to modulate current magnitude and $J_{\text{inact}}$ and voltage-dependence is likely to not to lie within its SH2 and/or PTB domains but with residues or signaling structures contained in its CH domain.

**DISCUSSION**

We have begun to investigate the mechanisms by which signaling modules could regulate ion channel physiology and subsequent sensory processing in the OB by using cloned signaling components in a controlled heterologous expression system. In determining the excitability and plasticity of single nerve cells, it will be important to consider not only the diversity of voltage-gated ion channels expressed in a neuron, but also the array of protein modules that could interact
with any given channel at discrete phosphorylation-dependent and -independent targets. The degree of excitability of OB neurons is dependent upon the activity of voltage-gated ion channels that may greatly shape the coding of olfactory information. Modulation of voltage-gated potassium (Kv) channels, in particular, by tyrosine phosphorylation is a dynamic means of changing neuronal excitability, and thus, the ability of the involved brain area to encode information (36). We have found that while Src kinase is expressed in the OB and modulates Kv1.3 current magnitude, kinetics of inactivation, voltage-dependence, and cumulative inactivation, adaptor proteins further shape the nature of the kinase-induced modulation. Regulation of ion channel modulation by adaptor proteins is most likely via protein-protein interactions across a channel-kinase-adaptor complex to alter tyrosine phosphorylation of the channel. While adaptor proteins fine tune multiple Kv1.3 channel properties, only current magnitude appears to be consistently correlated to degree of channel phosphorylation. Kv1.3 current magnitude is inversely proportional to total Kv1.3 phosphorylation.

The differential degree of Kv1.3 tyrosine phosphorylation and modulation of Kv1.3 current in v-Src + Kv1.3 co-transfected cells in the presence of Grb10 and n-Shc may be attributed to the inherent uniqueness in signaling modules contained in these two adaptor proteins (Fig. 9). Unlike n-Shc, Grb10 decreases or eliminates v-Src-induced Kv1.3 tyrosine phosphorylation, current suppression, and disrupted cumulative inactivation properties. As described in the introduction, the Src SH3 domain recognizes and binds to proline-containing sequences in other proteins capable of forming a polyproline type II helix (37), minimally recognizing PXXP and most favorably recognizing sequences that conform to the canonical Src SH3 domain binding site RPLPXXP (38). Kv1.3 (amino acids 39-44 (PLPPALP) and 493-496 (PQTP)) and Grb10 (amino acids 30-36
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(PAGPGLP), 136-139 (PAIP), and 148-154 (PGSPPVL)) both contain two or more such proline-rich regions. Thus it is conceivable that the Kv1.3 and Grb10 proline-rich regions compete for interaction with the Src SH3 domain such that the Src SH3 domain preferentially binds to a Grb10 proline-rich target, allowing Src access to the Kv1.3 ion channel only in the absence of Grb10 expression or correct subcellular localization (Fig. 9A). Grb10 adaptor protein may therefore regulate v-Src modulation of Kv1.3 by blocking the access of the kinase to the ion channel.

Because v-Src induced Kv1.3 tyrosine phosphorylation is dramatically reduced in the presence of Grb10, tyr$^{137}$ and tyr$^{449}$, the normal molecular targets for Kv1.3 phosphorylation by v-Src, are unlikely interaction modules for either the Src SH2 or Grb10 SH2 domain (Fig. 9A). Secondarily, our data substituting R385A Src kinase for v-Src show that not all changes in Kv1.3 function must be phosphorylation dependent. Grb10 could alter channel function (inactivation, current magnitude) by interaction with catalytic inactive Src, that could provide an interactive domain with the channel or adaptor, but provide little to no kinase activity. Lastly, some effects of v-Src on Kv1.3, such as a shift in the voltage-dependence, were not prevented in the presence of Grb10. Residual phosphorylation of a different residue that is not abolished by Grb10 interacting with v-Src kinase cannot be excluded or explained by simple competition. Site specific phosphorylation mutations in the channel would be advantageous to clarify these alternatives in future studies.

Collectively these data indicate that Grb10 adaptor protein regulation of Kv1.3 modulation by src kinase is complex and highly likely to involve multiple modulatory sites.

The specific mechanism by which n-Shc may regulate the phosphorylation and subsequent modulation of Kv1.3 by v-Src in HEK 293 cells may also afford several alternatives. In addition to the SH3 domain, Src kinase also contains a SH2 domain, which recognizes and binds to
phosphorylated tyrosines of other proteins. Kv1.3 has at least six potential phosphotyrosine sites that are accessible to a SH2 domain from the cytoplasmic face of the channel: tyr$^{111,112,113}$, tyr$^{137}$, tyr$^{449}$, and tyr$^{479}$. N-Shc contains three key tyrosines in its central CH domain: tyr$^{220}$, tyr$^{221}$, and tyr$^{304}$. N-Shc also contains a SH2 domain at its C-terminus, as well as a phosphotyrosine binding (PTB) domain at its N-terminus. As mentioned above, Kv1.3 and n-Shc both contain numerous proline-rich sequences. Because of the many possible protein-protein interactions among Kv1.3, v-Src, and n-Shc due to their interaction motifs, we attempted to simplify our study by substituting two members of the Shc family for n-Shc: Sck, which differs from n-Shc at its CH domain, and the n-Shc triple mutant, which lacks the three key tyrosines. Our data from these experiments and those using R385A Src indicate that interactions that enable n-Shc to restore the shift in $V_{1/2}$ of Kv1.3 by 10 mV and restore the increased $J_{\text{inact}}$ to control values require the three key tyrosines of the CH domain of n-Shc to be intact. Finally, the ability of n-Sck to partially relieve Kv1.3 current suppression brought about by v-Src may result from residues in the CH domain that are not homologous to those in n-Shc, nor are the three tryosine residues important for this function. Taken together, our results suggest that n-Shc could exerts its effects in this system by using proline-rich regions and the three key tyrosines of its CH domain rather than its PTB domain or SH2 domain.

Phosphotyrosine signaling depends heavily upon protein-protein interactions mediated by adaptor proteins to carry out its function (39). Although the assemblage of Kv1.3, v-Src kinase, n-Shc, and Grb10 is expressed in the olfactory bulb, it is not known how Src kinase might be activated in native neurons and modulated by adaptor molecules. We do know that receptor-lined kinases expressed in the bulb differentially modulate Kv1.3 dependent upon stage of development,
the animal’s degree of satiety, and odor-sensory experience (14; Tucker and Fadool, submitted). Growth factors, such as IGF and BDNF, have been demonstrated to drive up Src kinase activity which subsequently alters voltage- or ligand gated channels (40). Given that production and release of growth factors from neurons are activity dependent (41, 42) provides a venue for activation of Src kinase with increased electrical activity.

There are several good examples of how adaptor proteins may play central roles in directing tyrosine kinase signaling. For example, the novel ZIP1 and ZIP2 adaptor proteins link PKC activity to an auxiliary potassium channel subunit, Kv$2$, thereby linking kinase activity to potassium channel octomers (43). PDZ-domain containing postsynaptic density protein 95 (PSD-95) is required for Src modulation of NMDA-sensitive glutamate receptors, and PSD-95 has also been shown to bind NMDA receptor subunits, the inward rectifiers Kir2.1 and Kir2.3, and the Shaker homolog Kv1.4 (44-47). The human homolog of the PDZ-containing Drosophila discs large protein (Hdlg) is able to bind both the Src-family member Lck and Kv1.3 (48). PSD-95 forms the bridge in a complex between Src kinase and Kv1.3 in cultured microglia (49). Finally, Nitabach et al. (50) demonstrate that Kv1.5 channels may act as adaptor molecules themselves by forming heteromultimeric complexes with Kv1.4 subunits otherwise insensitive to interaction with Src kinase by providing the necessary proline rich regions for Src kinase SH3 domain interaction through the Kv1.5 subunit. Future co-immunoprecipitation experiments in native olfactory bulb tissue combined with targeted-gene deletions in mice will be valuable in accessing the detailed function and contribution of adaptor molecules for encoding chemical communication.
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REFERENCES

1. Biggin, P. C., Toosild, T., and Choe, S. (2000) *Cur. Opin. Struct. Biol.* **10**, 456-461
2. Yi, B. A. and Jan, L. Y. (2000) *Neuron.* **27**, 423-425
3. Scannevin, R. H. and Trimmer, J. S. (1997) *Biochem. Biophys. Res. Com.* **232**, 585-589
4. Tiffany, A. M., Manganas, L. N., Kim, E., Hsueh, Y.-P., Sheng, M., and Trimmer, J. S. (2000) *J. Cell Biol.* **148**, 1-147
5. Holmes, T. C., Fadool, D. A., Ren, R., and Levitan, I. B. (1996) *Science* **274**, 2089-2091
6. Arnold, D. B. and Clapham, D. E. (1999) *Neuron* **23**, 149-157
7. Joiner, W. J., Khanna, R., Schlichter, L. C., and Kaczmarek, L. K. (2001) *J. Biol. Chem.* **276**, 37980-37985
8. Kim, E., Niethammer, M., Rothchild, A., Jan, Y. N., and Sheng, M. (1995) *Nature* **378**, 85-88
9. Pawson, T. and Scott, J. D. (1997) *Science* **278**, 2075-2080
10. Magoski, N. S., Wilson, G. F., and Kaczmarek, L. K. (2002) *J. Neurosci.* **22**, 1-9
11. Bradshaw, J. M., Mitaxov, V., and Waksman, G. (1999) *J. Mol. Biol.* **293**, 971-985
12. Kues, W. A. and Wunder, F. (1992) *Eur. J. Neurosci.* 4, 1296-1308
13. Fadool, D. A. and Levitan, I. B. (1998) *J. Neurosci.* 18, 6126-6137
14. Fadool, D. A., Tucker, K., Phillips, J. J., and Simmen, J. A. (2000) *J. Neurophysiol.* 83, 2332-2348
15. Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Pawson, T., and Pelicci, P. G. (1992) *Cell* 70, 93-104
16. Ooi, J., Yajnik, V., Immanuel, D., Gordon, M., Moskow, J. J., Buchberg, A. M., and Margolis, B. (1995) *Oncogene* 10, 1621-1630
17. Langlais, P., Dong, L. Q., Hu, D., and Liu, F. (2000) *Oncogene* 19, 2895-2903
18. Nakamura, T., Muraoka, S., Sanokawa, R., and Mori, N. (1998) *J. Biol. Chem.* 273, 6960-6967
19. Senften, M., Schenker, G., Sowadski, J. M., and Ballmer-Hofer, K. (1995) *Oncogene* 10, 199-203
20. Fadool, D. A., Holmes, T. C., Berman, K., Dagan, D., and Levitan, I. B. (1997) *J. Neurophysiol.* 78, 1563-1573
21. Nakamura, T., Sanokawa, R., Sasaki, Y., Ayusawa, D., Oishi, M., and Mori, N. (1996) *Oncogene* 13, 1121
22. Bowlby, M. R., Fadool, D. A., Holmes, T. C., and Levitan, I. B. (1997) *J. Gen. Physiol.* 110, 601-610
23. Holmes, T. C., Fadool, D. A., and Levitan, I. B. (1996) *J. Neurosci.* 16, 1581-1590
24. Marom, S., Goldstein, S. A., Kupper, J., and Levitan, I. B. (1993) *Receptors and Channels* 1, 81-88
25. Sambrook, J. and Russel, D. W. (2001) *Molecular Cloning*, 3 Ed., Cold Spring Harbor Press, New York

26. Maroney, A. C., Qureshi, S. A., Foster, D. A., and Brugge, J. S. (1992) *Oncogene* 7, 1207-1214

27. Marshall, J., Molloy, R., Moss, G. W., Howe, J. R., and Hughes, T. E. (1995) *Neuron* 14, 211-215

28. Shi, G., Kleinklaus, A. K., Marrion, N. V., and Trimmer, J. S. (1994) *J. Biol. Chem.* 269, 23204-23211

29. White, M. F. and Kahn, C. R. (1994) *J. Biol. Chem.* 269, 1-4

30. Sato, K., Gotoh, N., Otsuki, T., Kakumoto, M., Aoto, M., Tokmakov, A. A., Shibuya, M., and Fukami, Y. (1997) *Biochem. Biophys. Res. Com.* 240, 399-404

31. Marom, S. and Levitan, I. B. (1994) *Biophysical J.* 67, 579-589

32. van der Geer, P., Wiley, S., Gish, G. D., and Pawson, T. (1996) *Cur. Biol.* 6, 1435-1444

33. Ishihara, H., Sasaoka, T., Ishiki, M., Takata, Y., Imamura, T., Usui, I., Langlois, J., Sawa, T., and Kobayashi, M. (1997) *J. Biol. Chem.* 272, 9581-9586

34. Ishihara, H., Sasaoka, T., Wada, T., Ishiki, M., Haruta, T., Usui, I., Iwata, M., Takano, A., Uno, T., Ueno, E., and Kobayashi, M. (1998) *Biochem. Biophys. Res. Com.* 252, 139-144

35. Walk, S. F., March, M. E., and Ravichandran, K. S. (1998) *Eur. J. Immunol.* 28, 2265-2275

36. Hille, B. (2001) *Ion channels of excitable membranes*, 3 Ed., Sinauer Associates, Sunderland

37. Hubbard, S. R. and Till, J. H. (2000) *Annu. Rev. Biochem.* 69, 398

38. Sparks, A. B., Rider, J. E., Hoffman, N. G., Fowlkes, D. M., Quillam, L. A., and Kay, B. K.
(1996) *Proceedings of the National Academy of Sciences USA* **93**, 1540-1544

39. Faux, M. C. and Scott, J. D. (1996) *Cell* **85**, 9-12

40. Bence-Hanulec, K. K., Marshall, J., and Blair, L. A. C. (2000) *Neuron* **27**, 121-131

41. Balkowiec, A. and Katz, D. M. (2000) *J. Neurosci.* **20**, 7417-7423

42. Tongiorgi, E., Righi, M., and Cattaneo, A. (1997) *J. Neurosci.* **17**, 9492-9505

43. Gong, J., Xu, J., Bezanilla, M., van Huizen, R., Derin, R., and Li, M. (1999) *Science* **285**, 1565-1569

44. Kornau, H.-C., Seeburg, P. H., and Kennedy, M. B. (1997) *Cur. Opin. Neurobiol.* **7**, 368-373

45. Liao, G.-Y., Kreitzer, M. A., Sweetman, B. J., and Leonard, J. P. (2000) *J. Neurochem.* **75**, 282-287

46. Nehring, R. B., Wischmeyer, E., Döring, F., Veh, R. W., Sheng, M., and Karschin, A. (2000) *J. Neurosci.* **20**, 156-162

47. Shin, H., Hsueh, Y.-P., Yang, F.-C., Kim, E., and Sheng, M. (2000) *J. Neurosci.* **20**, 3580-3587

48. Hanada, T., Lin, L., Chandy, K. G., Oh, S. S., and Chishti, A. H. (1997) *J. Biol Chem.* **272**, 26899-26904

49. Cayabyab, F. S., Khanna, R., Jones, O. T., and Schlichter, L. C. (2000) *Eur. J. Neurosci.* **12**, 1949-1960

50. Nitabach, M. N., Llamas, D. A., Araneda, R. C., Intile, J. L., Thompson, I. J., Zhou, Y. I., and Holmes, T. C. (2001) *Proceedings of the National Academy of Sciences USA* **98**, 705-710
FIGURE LEGENDS

FIG. 1. **Diagrammatic representation of the tyrosine residues (Y) and potential protein-protein interactive modules (PTB, SH2, SH3, CH, and proline rich domains) between v-Src kinase, the Kv1.3 ion channel, and the adaptor proteins, n-shc and grb10.** For molecular details and abbreviations, please see text (INTRODUCTION).

FIG. 2. **Expression of Src kinase and related adaptor proteins in the rat olfactory bulb.** Western blot analysis of olfactory bulb (OB) lysates collected from adult (postnatal day 30) rats and homogenized in a NP40-based buffer. 10 : g (left) or 40 : g (right) of protein was loaded per set, separated by SDS-PAGE, electrotransferred to nitrocellulose, and probed with antisera against v-Src (1:100), Shc (1:800), or Grb10 (1:800).

FIG. 3. **Effect of adaptor proteins on the phosphorylation of Kv1.3 by v-Src kinase.** Human embryonic kidney (HEK 293) cells were transiently transfected with Kv1.3 cDNA plus or minus v-Src kinase and plus or minus adaptor protein (grb10 or n-shc). A, *(top panel)*, Kv1.3 protein was immunoprecipitated from Triton X-100-soluble cell lysates with "-Kv1.3 antiserum (IP:"Kv1.3), separated by SDS-PAGE, electrotransferred to nitrocellulose, and probed with "-phosphotyrosine antibody (IB:"P) that recognizes phosphorylated tyrosine residues. Upper arrow indicates M, of Kv1.3 and lower arrow indicates the heavy chain of IgG (IgG). The box defines the area that was integrated (55-72 kDa) to generate the quantitative immunodensity data in B. A, *(lower panel)*, Cell lysates used in the above immunoprecipitation experiment were probed with "-Kv1.3 antiserum to insure equal protein expression of the channel under varying transfection conditions. Normalized immunodensity values for the example shown in A are given below the various transfection conditions. B, Histogram plot of the tyrosine phosphorylation of Kv1.3 (Kv1.3), as measured by quantitative immunodensitometry, indicates an increase in phosphorylation in the presence of v-src (+v-src) or v-src plus n-shc (+v-src + n-shc). This increase in v-src induced tyrosine phosphorylation of Kv1.3 is inhibited in the presence of grb10 (+v-src + grb10). Pixel counts for each band were normalized to the value of the Kv1.3-only band within single gels for graphical representation. Non-normalized values from each transfection condition were compared statistically with one-way randomized design ANOVA, " = 0.05. * = significantly different from control (Kv1.3) by snk follow-up test.

FIG. 4. **Effect of adaptor proteins on the modulation of Kv1.3 by v-Src kinase.** Human embryonic kidney (HEK 293) cells were transiently transfected with Kv1.3 cDNA +/- v-src +/- grb10 or n-shc, as in Fig. 3. A-B, Representative cell-attached patch-clamp recordings from HEK 293 cells that were held at -90 mV (Vh) and stepped to +40 mV (Vc) for 1000 msec. Three different HEK 293 cells are shown in each, demonstrating Kv1.3 alone (Kv1.3), Kv1.3 + v-src (+ v-src) and Kv1.3 + v-src + adaptor protein (+ v-src + grb10) or (+v-src + n-shc) transfection conditions. C-D, Same transfection and recording conditions as in A-B, respectively, but traces are normalized to peak current value to visualize rate of inactivation (Jinact). J inact was determined by fit to a bi-exponential function as described in Experimental Procedures (red, dashed line). A-D, Values from the transfection conditions were compared statistically by one-way completely randomized design
ANOVA, snk follow-up test, " = 0.05. * = significantly different from control (Kv1.3). ** = significantly different from Kv1.3 + v-src co-transfection.

FIG. 5. **Voltage at half-activation (V1/2)** is right-shifted by v-Src Kinase and restored in the presence of n-Shc adaptor protein. A-E, HEK 293 cells were transfected with cDNAs for Kv1.3 +/- v-src +/- the adaptors, grb10, n-shc, or triple n-shc mutant (triple) as in Fig. 3. Cells were voltage-clamped in the cell-attached configuration with V_h = -90 mV and V_c = stepped from -70 mV to 0 mV in 5 mV increments with a pulse duration of 50 msec. F, Peak tail current amplitudes (dashed line) were normalized to the current measured at 0 mV and plotted against voltage. Points were fit to a Boltzman function. Arrow = Note right-shifted voltage at half-activation (V1/2) produced in the presence of v-src and restored in the presence of n-Shc.

FIG. 6. **Cumulative inactivation of Kv1.3 is interrupted by v-Src Kinase and re-established in the presence of grb10 adaptor protein.** HEK 293 cells were transfected. Cells were voltage-clamped in the cell-attached configuration with V_h = -90 mV and V_c = +40 mV for 1000 msec with different interpulse intervals including, 30, 10, 2, 1, 0.5 seconds. Peak current amplitudes were normalized to that of the first voltage stimulation (I_i/I_1) and this normalized current was plotted against pulse number (n). Error bar represents s.e.m. for 19-26 cells recorded at a given interpulse interval.

FIG. 7. **Phosphorylation-dependence of adaptor protein affecting v-src-induced phosphorylation and modulation of Kv1.3.** HEK 293 cells were transiently transfected with cDNAs for Kv1.3 +/- v-src or R385A v-src +/- grb10, n-shc, sck, or triple n-shc mutant as in Fig. 3. A, Normalized peak current amplitude for cells recorded with the same voltage paradigm and patch configuration as Fig. 4. Amplitudes for each transfection condition were normalized to that of control (Kv1.3) for each experiment to control for variation in cell passage and tranfection efficiency. Normalized values were then averaged across experimental days. Error bar represents s.e.m., number represents sample size for recordings. B, (top panel), Western-blot demonstrating the degree of tyrosine phosphorylated Kv1.3 under various transfection conditions. Protocol and abbreviations as in Fig. 3. Normalized densitometry values are given for the example blot. B, (lower panel), Histogram plot of the tyrosine phosphorylation of Kv1.3 (Kv1.3), as measured by quantitative immunodensitometry for several experiments as in B, (top panel). Error bar represents s.e.m. with number representing sample size of Western blots.

FIG. 8. **Phosphorylation-dependence of v-src induced interruption of Kv1.3 cumulative inactivation and the participation of the CH domain of n-shc.** HEK 293 cells were transfected as in Fig. 7 and patch recorded using the voltage-stimulation paradigm described in Fig. 6. Peak current amplitudes were normalized to that of the first voltage stimulation (I_i/I_1) and this normalized current was plotted against pulse number (n). Error bar represents s.e.m. for 4-11 cells recorded at a given interpulse interval. R385A = mutation of v-Src kinase at arginine 385; severely impaired v-Src kinase. Triple = n-shc triple mutant; Y to F mutation of n-shc at tyr220, 221, 304 in the CH domain. Sck = she family member with intact CH domain but not a substrate for v-Src kinase.
FIG. 9. Putative model of n-Shc and Grb10 modulation of v-src-induced changes in Kv1.3 channel properties and upregulation in phosphorylation. Src kinase is known to phosphorylate (P) Kv1.3 at tyr^{137} and tyr^{449} (noted by a ) to induce changes in channel function.  

**A, Grb10 regulation of v-Src-induced Kv1.3 modulation.** Kv1.3 and Grb10 proline-rich regions may compete for interaction with the Src SH3 domain such that the Src SH3 domain preferentially binds to a Grb10 proline-rich target (arrows), allowing Src access to the Kv1.3 ion channel only in the absence of Grb10 expression or correct subcellular localization. Grb10 adaptor protein may therefore regulate v-Src modulation of Kv1.3 by blocking the access of the kinase to the ion channel and inhibiting phosphorylation (P). This permits normal Kv1.3 current magnitude and cumulative inactivation in the presence of v-Src but voltage-dependence remains modulated by v-Src.

**B, N-Shc regulation of v-Src-induced Kv1.3 modulation.** V-Src phosphorylation (P) of Kv1.3 (*) is retained in the presence of n-Shc adaptor protein. Src likely phosphorylates Tyr residues of the n-Shc CH domain to permit normal Kv1.3 $I_{\text{inact}}$ and voltage-dependence in the presence of v-Src. The retention of Kv1.3 phosphorylation at tyr^{137} and tyr^{449} by v-Src in the presence of Shc does not permit Kv1.3 current levels to return to normal.
### Table 1. The Effect of Adaptor Proteins on v-Src-Induced Kv1.3 Current Properties

| Transfection Condition | Normalized Peak Current | \( \tau_{\text{inact}} \) | \( V_{1/2} \) | \( \kappa \) |
|------------------------|-------------------------|-----------------------|-------------|---------|
| Kv1.3                  | 1.00(67)                | 716+/−23(51)          | -49.6+/−0.9(36) | 2.7+/−0.2(39) |
| Kv1.3 + v-Src          | *0.08(34)               | *1475+/−187(29)       | *-41.1+/−1.6(11) | 3.2+/−0.3(11) |
| Kv1.3 + R385A          | 0.87(12)                | 693+/−42(10)          | -49.2+/−1.8(5)  | 3.3+/−0.5(5)  |
| Kv1.3 + Grb            | 1.12(6)                 | 782+/−46(5)           | -44.8+/−1.5(4)  | 2.7+/−0.2(4)  |
| Kv1.3 + v-Src + Grb10 | 0.86(9)                 | *539+/−54(9)          | *-43.0+/−1.7(8) | *4.4+/−0.5(8) |
| Kv1.3 + R385A + Grb10 | *1.35(7)                | *403+/−22(9)          | *-52.6+/−1.4(7) | 3.0+/−0.2(7)  |
| Kv1.3 + n-Shc          | 0.80(8)                 | 816+/−54(6)           | -44.1+/−1.7(7)  | 3.4+/−0.1(7)  |
| Kv1.3 + v-Src + n-Shc  | *0.21(36)               | 805+/−91(27)          | -47.3+/−1.0(6)  | 2.9+/−0.2(8)  |
| Kv1.3 + R385A + n-Shc  | 0.97(5)                 | 799+/−53(5)           | -50.6+/−2.3(3)  | 2.2+/−0.4(3)  |
| Kv1.3 + v-Src + Sck    | 0.49(10)                | 770+/−45(7)           | -55.0+/−1.0(6)  | 2.3+/−0.2(6)  |
| Kv1.3 + v-Src + triple | *0.21(5)                | *1168+/−116(5)        | *-47.6+/−2.1(3) | 3.1+/−0.6(3)  |

HEK 293 cells were transfected with cDNAs encoding Kv1.3 +/- v-Src or R385A Src kinase +/- Grb10 adaptor protein. Several voltage-stimulating paradigms were completed (see text) to collect the tabled biophysical properties by cell-attached patch-clamp recording. Peak current amplitudes were normalized to the control condition (Kv1.3) for each cell passage prior to averaging. Mean values (+/− s.e.m.) for inactivation time constant (\( \tau_{\text{inact}} \)), voltage at half-activation (\( V_{1/2} \)), and slope (\( \kappa \)) of the voltage-dependence were compared across various transfection conditions. Data were analyzed by completely randomized one-way ANOVA with Student Newman Keuls (snk) followup test as described in the text. * = Significantly different from Kv1.3 (control).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

(A) Kv1.3

(B) + v-Src

(C) + v-Src + n-Shc

(D) + v-Src + triple

(E) + v-Src + Grb10

(F) Normalized Peak Tail Current

- Kv1.3
- + v-Src
- + v-Src + triple
- + v-Src + n-Shc
- + v-Src + Grb10

Voltage (mV)

Normalized Peak Tail Current

V_{1/2}
Figure 6
Figure 7
Figure 8
Figure 9
Two adaptor proteins differentially modulate the phosphorylation and biophysics of Kv1.3 ion channel by Src kinase
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