A Novel Mechanism of Modulation of Hyperpolarization-activated Cyclic Nucleotide-gated Channels by Src Kinase*

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Hyperpolarization-activated cyclic nucleotide-gated channels (HCN1–4) play a crucial role in the regulation of cell excitability. Importantly, they contribute to spontaneous rhythmic activity in brain and heart. HCN channels are principally activated by membrane hyperpolarization and binding of cAMP. Here, we identify tyrosine phosphorylation by Src kinase as another mechanism affecting channel gating. Inhibition of Src by specific blockers slowed down activation kinetics of native and heterologously expressed HCN channels. The same effect on HCN channel activation was observed in cells cotransfected with a dominant-negative Src mutant. Immunoprecipitation demonstrated that Src binds to and phosphorylates native and heterologously expressed HCN2. Src interacts via its SH3 domain with a sequence of HCN2 encompassing part of the C-linker and the cyclic nucleotide binding domain. We identified a highly conserved tyrosine residue in the C-linker of HCN channels (Tyr476 in HCN2) that confers modulation by Src. Replacement of this tyrosine by phenylalanine in HCN2 or HCN4 abolished sensitivity to Src inhibitors. Mass spectrometry confirmed that Tyr476 is phosphorylated by Src. Our results have functional implications for HCN channel gating. Furthermore, they indicate that tyrosine phosphorylation contributes in vivo to the fine tuning of HCN channel activity.

Although identified only recently, the family of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels has generated great interest because it represents the molecular correlate of the hyperpolarization-activated cation current, termed Ih (syn. If or Iq) (1–4). This current plays a crucial role in the control of important biological functions, including cardiac and neuronal pacemaker activity, determination of resting membrane potential, dendritic integration, and synaptic transmission. Dysfunction of HCN channels has been linked to human diseases, including cardiac arrhythmia (5–6) and epilepsy (7–8). Structurally, the four members of the HCN channel family (HCN1–4) belong to the 6TM ion channel superfamily (9–11). In the plasma membrane, HCN channel subunits assemble to homo- or heterotetrameric complexes, thereby generating a large variety of channel subtypes with distinct biophysical properties (12). Further complexity is probably generated in vivo by the interaction of HCN channels with auxiliary subunits (13), interacting proteins (14–16), and by post-translational modifications (e.g. N-linked glycosylation) (17).

Whereas our knowledge of the structure and function of HCN channels has significantly increased over the last couple of years, there is only sparse information on the cellular regulation of these channels. It is well established that hormones and neurotransmitters can modulate Ih activity via G-protein pathways that modulate the cAMP concentration (1). Cyclic AMP enhances channel activity by direct binding to a cyclic nucleotide binding domain (CNBD) present in the C terminus of HCN channels. The region linking the last membrane-spanning domain (S6) to the CNBD (the C-linker) has been shown to play a key role in coupling cAMP binding with channel opening (18–20). Unlike in many other ion channels the modulation by cAMP does not seem to involve protein kinase A-mediated serine/threonine phosphorylation (21). By contrast, several experimental observations suggest that nonreceptor protein-tyrosine kinases (PTKs), especially members of the Src family, may regulate HCN channels (1). The HCN1 channel was originally identified in a yeast two-hybrid screen using the SH3 domain of Src as bait. However, it was not reported whether this interaction also occurs in native tissue and whether Src influences channel activity (22). Based on experiments with genistein, a broad spectrum PTK inhibitor, it was suggested that cardiac Ih is regulated by tyrosine phosphorylation (23–25). However, this finding was challenged by another group reporting that the genistein effect is caused through nonselective interactions with the channel molecule (26).

Src is widely expressed in neurons and heart cells and has been shown to be an important regulator of voltage- and ligand-gated ion channels (27). We therefore asked in the present study whether or not this kinase is involved in HCN channel modulation. Using genetic, biochemical, and electrophysiological methods, we demonstrate that Src specifically binds to the C terminus of HCN channels, phosphorylates the channels and thereby affects the activation kinetics of Ih. Moreover we identify a specific tyrosine residue in the C-linker region of HCN channels that is the molecular target of Src.
EXPERIMENTAL PROCEDURES

Expression Vectors

*Yeast Two-hybrid (YTH) Assay*—C-terminal portions of murine HCN2 (mHCN2) were subcloned into plasmid pEG202 (Clontech) in-frame with the LexA DNA binding domain to yield the following bait proteins (see also Fig. 4A): CT, residues 449–863; L, residues 449–522; BD, residues 523–646; L+B, residues 449–646; dC, residues 649–863; L–β, residues 449–607; C–BD, residues 487–646. The cDNAs of full-length chicken c-Src (residues 1–533) and the SH3 domain (residues 81–139) of c-Src were fused to B42 activation domain of the plasmid pG4–5 (Clontech).

*Bacterial GST Fusion Proteins*—C-terminal fragments of mHCN2 were subcloned into the EcoRI/BamHI site of pGEX2T (Amersham Biosciences) to generate the following GST-tagged proteins: CT, residues 448–863; L, residues 448–520; BD, residues 521–644; L+BD, residues 448–645; dC, residues 645–863. The SH3 domain of c-Src was cloned into the EcoRI/XhoI site of plasmid pET41a (Novagen) to produce a GST-SH3 fusion protein.

*Expression in HEK293 Cells*—The cDNAs of wild-type and mutant mHCN2 and hHCN4 were subcloned into the mammalian expression vector pcDNA3 (Invitrogen). A Myc-tagged c-Src was constructed in the pcDNA3.1/Myc-His vector (Invitrogen). The dominant-negative mutant of chicken c-Src (Src-K295M) was subcloned in the pIRES2-EGFP vector (BD Biosciences Clontech). Mutant HCN channels were generated by standard PCR techniques. All plasmid constructs were verified by automated DNA sequencing.

*Yeast Two-hybrid Screen*

Yeast strain *Saccharomyces cerevisiae* EGY48 was used for YTH assays. pEG202 and pG4–5 fusion plasmids, together with the lacZ reporter plasmid pSH18–34 (BD Biosciences, Clontech) were transformed into yeast by the lithium acetate method. To identify specific interactions transformants were grown on four different selective plates: 1) X-gal plates lacking uracil, histidine, and tryptophan, and containing galactose and raffinose as carbon source; 2) X-gal plates lacking uracil, histidine, and tryptophan, and containing glucose as carbon source; 3) plates lacking uracil, histidine, tryptophan, and leucine, and containing galactose and raffinose as carbon source; 4) plates lacking uracil, histidine, tryptophan, and leucine, and containing glucose as carbon source. An interaction was considered to be specific if a given transformant turned blue on plate condition 1, stayed white on plate condition 2, was able to grow on plate condition 3, and did not show growth on plate condition 4. Fig. 4B shows examples from selective plates 1 and 2.

*Expression and Purification of GST Fusion Proteins*

GST fusion proteins were expressed in the protease-deficient BL21 (DE3) strain of *Escherichia coli* by induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), for 1 h at 37 °C. Bacteria were pelleted by centrifugation (10 min, 4 °C, 5,000 × g) and resuspended in 4 ml of PBS supplemented with protease inhibitor mix (PI) containing 1 μg/ml leupeptin, 1 μM pepstatin A, 1 μg/ml antipain, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM orthophenanthroline, 1 mM benzamidine, 1 mM iodoacetamide. After a freeze-thaw cycle, lysozyme was added to a final concentration of 5 mg/ml. After incubation for 30 min on ice, 1% Triton X-100 was added, and the cell suspension was shaken for 30 min to improve solubility of the fusion proteins. Cell debris was pelleted by centrifugation (30 min, 10,000 × g, 4 °C). The supernatant was aliquoted and stored at −80 °C. For purification of GST fusion proteins, supernatant was incubated with 1 volume of 50% slurry glutathione-Sepharose beads (Amersham Biosciences) at 4 °C overnight. The loaded beads were pelleted by centrifugation (5 min, 500 × g, 4 °C) and washed twice with ice-cold PBS supplemented with PI. Beads were resuspended in 1 volume of PBS/PI and stored at 4 °C.

*GST Pull-down Assay*

About 3 μg of purified GST fusion protein was diluted into 1 ml of cell lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 5 mM EDTA (pH 8.0), 1% Triton X-100). 10 μl of glutathione-Sepharose beads were added, and the mixture was incubated for 3 h at 4 °C. Beads were centrifuged at 10,000 × g for 2 min and washed three times with 1× HNTG buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerin, 5 mM EDTA (pH 8.0), 0.1% Triton X-100). After removing the supernatant, beads were incubated with total lysates from transfected or untransfected HEK293 cells for 4 h at 4 °C. The beads were pelleted after repeating the steps of centrifugation and washing. 20 μl of SDS loading buffer were added, and the mixture was heated 5 min at 100 °C before loading onto an SDS-PAGE gel.

*In Vitro Kinase Assay*

Beads preloaded with about 2 μg of GST fusion proteins (see GST pull-down assay) were washed three times with 1× HNTG buffer and once with kinase buffer (20 mM HEPES (pH 7.5), 10 mM MgCl2, 1 mM dithiothreitol, 200 μM pervanadate, 0.35 mM ATP). The beads were then mixed with 40 μl of reaction buffer (kinase buffer supplemented with 0.05 mg/ml bovine serum albumin and 0.1% β-mercaptoethanol) with or without 1 unit of purified c-Src (Oncogene Research Products). The reaction was carried out by shaking at room temperature for 15 min. After centrifugation and discarding the supernatant, the reaction was stopped by adding SDS loading buffer. All samples were then subjected to SDS-PAGE and Western blotting. The monoclonal anti-phosphoryrosine antibody 4G10 (anti-pY) (Upstate Biotechnology) was used to detect tyrosine-phosphorylated proteins.

*Preparation of Mouse Brain Membranes and HEK293 Cell Lysates*

*Membrane Fractions*—Freshly isolated mouse brain tissue was washed with PBS and homogenized in ice-cold MOPS lysis buffer (0.3 mM sucrose, 20 mM MOPS, 1 mM EDTA) supplemented with 200 μM pervanadate and PI, using a glass cylinder and a Teflon plunger. Homogenates were centrifuged for 10 min at 5,000 × g. The pellet was rehomogenized and spun once more for 10 min. The combined supernatants were then centrifuged (45 min, 4 °C, 100,000 × g). The pellet comprising the total membrane fraction was resuspended in MOPS lysis buffer supplemented with 200 μM pervanadate and stored at −80 °C.

*Cell Lysates*—Three days after transfection, HEK293 cells were washed twice with PBS and lysed with 500 μl of cell lysis buffer supplemented with 200 μM pervanadate and PI. After 10 min on ice the lysed cells were scraped off the dish and transferred to a reaction tube. The lysate was centrifuged (15 min, 4 °C, 12,000 × g), and the supernatant was subject to coimmunoprecipitation or stored at −80 °C.

*Coimmunoprecipitation*

Total brain membrane fractions or cell lysates of HEK293 cells were incubated overnight at 4 °C with 25 μl of protein A-Sepharose (Sigma) and a specific antibody directed against one of the two examined proteins and 500 μl of HNTG buffer supplemented with 200 μM pervanadate and PI. Beads were pelleted by centrifugation (15 min, 4 °C, 12,000 × g) and washed three times with cold HNTG buffer supplemented with 200 μM pervanadate. Interacting proteins were visualized after boiling for 5 min in Laemmli sample buffer by SDS-PAGE and
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Western blot analysis. The following antibodies were used: anti-HCN2 (Alomone), anti-Src (GD11, Upstate Biotechnology Inc.), anti-GST (Amersham Biosciences), anti-Myc (9E10); anti-phosphotyrosine (4G10 anti-pY), Upstate Biotechnology Inc. and p-Tyr-100, Cell Signaling Technology. An antibody against the cyclic nucleotide-gated channel CNGB3 (28) was used as control.

Mass Spectrometry (MS) Analysis

The GST fusion protein containing the whole C terminus of HCN2 (amino acids 448 – 863) was in vitro phosphorylated by Src. Thereafter, the protein was boiled in SDS-PAGE sample buffer, separated on a 10% Tris-glycine gel, and stained with Coomassie Blue. The piece of gel containing the HCN2 fusion protein was excised and in-gel-digested with trypsin according to standard procedures. Tryptic peptides were extracted with 5% formic acid/50% acetonitrile, dried, and stored at -20 °C until analysis by mass spectrometry.

A surveyor liquid chromatography system (ThermoFinnigan, San Jose, CA), consisting of degasser, MS Pump, and autosampler, equipped with a C18 trap column (RP, 300 μm × 5 mm, Agilent Technologies) and PicoFrit™ column: 75 μm × 100 mm, 15-μm tip packed with a 5-μm Aquisil C18 (ThermoFinnigan, San Jose, CA) was used. The samples were loaded onto the column with an RP gradient of 2–98% B over 180 min. RP solvents were 0.1% formic acid in either water (A) or acetonitrile (B). The flow rate was 200 nl/min. A Finnigan LTQ linear ion trap mass spectrometer equipped with an ESI microspray source was used for MS/MS experiment with ion transfer capillary of 160 °C and NSI voltage of 1.8 kV. The mass spectrometer was set that one full MS scan was followed by ten MS/MS scans on the ten most intense ions from the MS spectrum. Spectra from each fraction were searched by SEQUEST algorithm against the mHCN2 sequence. In these searches, differential modifications of 80 daltons to tyrosine residues were selected. For all sequences reported here, spectra were manually validated and contained sufficient information to assign not only the sequence, but also the site of phosphorylation. All output results were combined using home made software to delete the redundant data.

Cell Culture and Heterologous Expression

HEK293 cells (DMSZ, Braunschweig, Germany) were maintained in DMEM medium (Invitrogen, Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, and incubated at 37 °C with 10% CO2. For transfection, HEK293 cells were seeded on 6-well plates (diameter 3.5 cm) at a density of 700,000 cells per well. After 6 h, cells were transfected with expression plasmid DNA (0.6 μg of each plasmid per well) using the FuGENE 6 transfection reagent (Roche Diagnostics). For electrophysiological measurements, transfected cells were detached using 0.05% trypsin/0.5 mM EDTA (Invitrogen, Life Technologies, Inc.) and replated onto 12-mm poly-l-lysine-coated coverslips in 24-well plates. HL-1 cardiomyocytes were obtained from Dr. W. C. Claycomb (Louisiana State University Health Science Center, New Orleans, LA) and maintained in Claycomb Medium (JRH Biosciences, Andover, UK), supplemented with 10% fetal bovine serum, 4 mM l-glutamine, 10 mM noradrenaline, and penicillin-streptomycin as previously described (29). Single HL-1 cells were detached from confluent cultures using trypsin-EDTA. Isolated cells were either replated or directly used for patch-clamp experiments.

Dorsal root ganglion (DRG) neurons were isolated from adult mice as described by Wu et al. (30). Briefly, thoracic and lumbar DRGs were dissected and transferred immediately into DMEM medium. After removal of attached nerves and connective tissues ganglion fragments were placed in a microtube containing 1 ml of DMEM supplemented with trypsin (type I, 0.5 mg/ml, Sigma), collagenase (type I, 1 mg/ml, Sigma) and DNase (type I, 0.1 mg/ml, Sigma) and were incubated at 34 °C for 30 min. Thereafter, soybean trypsin inhibitor (type II-S, 1.25 mg/ml, Sigma) was added to stop trypsin digestion. The cell suspension was centrifuged (500 rpm, 3 min) to remove the supernatant and replenished with DMEM. Cells were then plated onto a 35-mm culture dish precoated with poly-l-lysine and kept in an incubator (37 °C, 10% CO2) for at least 1 h before electrophysiological recordings. Medium sized DRG neurons (30 – 45 μm) were selected for recordings. Pacemaker cells were prepared from sino-atrial node of adult mice as described by us previously (31).

Electrophysiology

Currents of heterologously expressed HCN channels were measured at room temperature 2–3 days after transfection using whole cell voltage clamp technique. The extracellular solution was composed of (in mM): 110 NaCl, 0.5 MgCl2, 1.8 CaCl2, 5 HEPES, 30 KCl, pH 7.4 adjusted with NaOH. The intracellular solution contained (in mM): 130 KCl, 10 NaCl, 0.5 MgCl2, 1 EGTA, 5 HEPES, pH 7.4 adjusted with KOH. For measurement of HCN currents of HL-1 cells the pipette solution was composed according to Sartiani et al. (32) (in mM): 120 potassium aspartate, 10 TEA-Cl, 0.4 Na3GTP, 2 MgCl2, 11 EGTA, 5 CaCl2, 10 HEPES, pH adjusted to 7.4 with KOH. The bath solution contained (in mM): 110 NaCl, 30 KCl, 1.8 CaCl2, 0.5 MgCl2, 2 BaCl2, 5 HEPES, pH adjusted to 7.4 with NaOH. For measurement of Ih currents of DRG neurons the pipette solution was composed (in mM): 130 potassium aspartate, 10 NaCl, 0.5 MgCl2, 1 EGTA, 1 CaCl2, 3 Mg-ATP, 5 HEPES, pH adjusted to 7.4 with KOH. The bath solution contained (in mM): 110 NaCl, 30 KCl, 1.8 CaCl2, 0.5 MgCl2, 5 BaCl2, 5 HEPES, pH adjusted to 7.4 with NaOH. For measurement of Ih currents of sino-atrial node cells were measured as described previously (31). Pipettes were pulled from borosilicate glass capillaries (GC150TF, Harvard Apparatus LTD) and had resistances of 2–3 MΩ when filled with the intracellular solution. Src kinase inhibitors (PP2 and genistein) and their respective inactive derivatives (PP3, daidzein) were purchased from Calbiochem (Germany). Stock solutions of these substances were prepared in Me2SO and were freshly diluted at least 1:1,000 in bath solution before using in experiments. The effects of Src inhibitors were determined after incubation of cells with the substances for at least 10 min.

Data were acquired at 10 kHz using an Axopatch 200B amplifier and pClamp 8 (Axon Instruments). Voltage clamp data were stored on the computer hard disk and analyzed off-line by using Clampfit 8 (Axon Instruments). Steady-state activation curves were determined by hyperpolarizing voltages of -140 mV to -30 mV from a holding potential of -40 mV for 3.2 s (for wild-type and mutant HCN2) and 4.8 s (for HCN4) followed by a step to -140 mV. Tail currents measured immediately after the final step to -140 mV, were normalized by the maximal current (Imax) and plotted as a function of the preceding membrane potential. The data points were fitted with the Boltzmann function: (I / Imin) / (1 + [Vm – V1/2] / k) where V1/2 is an offset caused by a nonzero holding current, Vm is the test potential, V1/2 is the membrane potential for half-maximal activation, and k is the slope factor. Times of constant channel activation (τact) of wild-type and mutant HCN2 and HCN4 channels were determined by monoexponential (or biexponential in the case of HCN2-Y476F and Ih of DRG neurons) function fitting the current evoked during hyperpolarizing voltage pulses to -140 mV unless otherwise specified. As has been described earlier (33) the initial lag in the activation of HCN channels was excluded from the fitting procedure. Statistical analyses were performed with Origin6.1
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The finding that two structurally unrelated PTK inhibitors affected HCN2 in the same fashion argued against a nonspecific action of these agents. More likely, the effect of PP2 and genistein was caused by specific inhibition of Src and subsequent channel dephosphorylation by cellular tyrosine phosphatases. To further strengthen this hypothesis, we cotransfected HEK293 cells with HCN2 and a catalytically inactive Src mutant, Src-K295M (Src-KM) (34). Indeed, HCN2 currents obtained from these cells activated with slower kinetics than control currents ($\tau_{act} = 353 \pm 7.4$ (n = 6); Fig. 1, E and F). The effect induced by Src-K295M was somewhat weaker than that of PP2, probably because of the high levels of endogenous wild-type Src. Inhibition of Src also led to inhibition of deactivation kinetics (Fig. 2, A and B). In contrast, neither PP2 nor PP3 did affect the voltage dependence of activation (Fig. 2, C and D). Regulation of HCN2 by cAMP was principally preserved in the presence of PP2. Cyclic AMP shifted the half-maximal activation voltage ($V_{acc}$) by about +10 mV and speeded up activation kinetics (TABLE ONE). Interestingly, however, the activation kinetics in the presence of saturating cAMP + PP2 was consistently slower than that observed with cAMP alone ($\tau_{act(cAMP)} = 272 \pm 23$ ms (n = 12); $\tau_{act(cAMP)} = 163 \pm 6.8$ ms (n = 8)) indicating that the decelerating effect of PP2 persisted in the presence of cAMP.

HCN2 Binds Src and Undergoes Tyrosine Phosphorylation—We performed a series of immunoprecipitation experiments to determine whether HCN2 is a substrate of Src (Fig. 3A). In the first set of experiments, cells expressing HCN2 were preincubated with the tyrosine phosphatase inhibitor pervanadate (PV) prior to immunoprecipitation with anti-HCN2. A specific band corresponding to the molecular mass of HCN2 (105 kDa) was detected with a specific anti-phosphotyrosine antibody (anti-pY). This band was significantly weaker when cells were pretreated with both PV and PP2. Similarly, the intensity of the band was reduced in HCN2/Src-KM-coexpressing cells pretreated with PV.

RESULTS

Inhibition of Src Slows Down Kinetics of Expressed HCN2 Channel—We investigated the effect of Src on HCN2 channels expressed in HEK293 cells. These cells endogenously express substantial amounts of Src making them a suitable system for our purpose (Fig. 1A). Preincubation of HCN2-expressing cells with the Src kinase inhibitor PP2 (10 µM) led to a profound deceleration of HCN2 activation kinetics (Fig. 1, B and F). The activation time constant ($\tau_{act}$) at −140 mV increased from 277 ± 14 ms (n = 13) at control conditions to 538 ± 29 ms (n = 19) in the presence of PP2. In contrast, PP2 had no effect on current densities (pA/pF; PP2, −78.9 ± 12.4 (n = 25); control, −84.4 ± 12.5 (n = 27); p > 0.05). The effect on $\tau_{act}$ was present over the whole voltage range (supplemental Fig. S1). By contrast, PP3, an inactive analogue of PP2, did not affect current kinetics (Fig. 1, C and F; supplemental Fig. S1). Similarly, another Src kinase inhibitor, genistein (Fig. 1, D and F), but not its inactive analogue daidzein (Fig. 1F), also slowed down the HCN2 current.

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Identification of Interacting Sites of HCN2 and Src—We performed a YTH screen to determine the domains conferring interaction between HCN2 and Src. Specific interaction was seen between the full-length C terminus of HCN2 and Src (Fig. A and B). Using a series of C-terminal bait vectors, we identified the sequence reaching from the C helix of the C-linker to the C terminus of the CNBD as the minimal interaction site required for binding of Src. We obtained the same result when only the SH3 domain of Src was tested for interaction (Fig. B). To confirm YTH data, we performed GST pull-down assays with full-length Src and fusion proteins corresponding to the bait vectors used in YTH (Fig. 4C). Again, the C-linker/CNBD (L-BD) region was required for specific interaction. In a reverse experiment, specific interaction between the SH3 domain of Src and the full-length HCN2 was demonstrated (Fig. 4D). Finally, in vitro kinase assays showed that Src does not only bind the L-BD region but that it also phosphorylates HCN2 in mouse brain and in HEK293 cells. A, Src-mediated tyrosine phosphorylation of HCN2 in HEK293 cells. Cells transfected with HCN2 were treated with 200 μM pervanadate (PV) for 15 min (lane 1) or with 10 μM PP2 for 30 min prior to PV treatment (lane 2). Lane 3 represents cells cotransfected with HCN2 and Src-SH3 (Src-KM) and treated with PV. Lysates of each group of cells were immunoprecipitated with anti-HCN2 and analyzed in immunoblots with anti-phosphotyrosine antibody (anti-pY) or with anti-HCN2. B, interaction of HCN2 with Src in mouse brain. Mouse brain membranes (1 mg) were immunoprecipitated with either anti-HCN2 or a control antibody (anti-CNGB3), blotted, and probed with anti-Src. C, detection of tyrosine-phosphorylated HCN2 in mouse brain. Mouse membranes (1 mg of protein) prepared in the presence of 200 μM pervanadate were immunoprecipitated with a mixture of two anti-phosphotyrosine antibodies (10 μg anti-pY +10 μg p-Tyr-100) and analyzed in immunoblots with anti-HCN2. Lane 2 shows a blot of 50 μg of total brain lysates using anti-HCN2.

Taken together, these findings suggested that HCN2 undergoes tyrosine phosphorylation by Src and is dephosphorylated by cellular phosphatases if Src is inhibited. We next studied whether an interaction between Src and HCN also occurs in native tissue. To this end, immunoprecipitations with mouse brain membrane fractions were performed (Fig. 3B). Src was present in immunoprecipitates obtained with anti-HCN2 but not with a control antibody (anti-CNGB3) indicating that Src is bound to HCN2 in vivo. Moreover, HCN2 was immunoprecipitated with anti-phosphotyrosine antibodies from mouse brain membranes prepared in the presence of PV, indicating that HCN2 is tyrosine-phosphorylated in brain tissue (Fig. 3C).

Identification of Interacting Sites of HCN2 and Src—We performed a YTH screen to determine the domains conferring interaction between HCN2 and Src. Specific interaction was seen between the full-length C terminus of HCN2 and Src (Fig. 4A and B). Using a series of C-terminal bait vectors, we identified the sequence reaching from the C helix of the C-linker to the C terminus of the CNBD as the minimal interaction site required for binding of Src. We obtained the same result when only the SH3 domain of Src was tested for interaction (Fig. 4B). To confirm YTH data, we performed GST pull-down assays with full-length Src and fusion proteins corresponding to the bait vectors used in YTH (Fig. 4C). Again, the C-linker/CNBD (L-BD) region was required for specific interaction. In a reverse experiment, specific interaction between the SH3 domain of Src and the full-length HCN2 was demonstrated (Fig. 4D). Finally, in vitro kinase assays showed that Src does not only bind the L-BD region but that it also phosphorylates

| TABLE ONE
| Effects of cAMP on HCN2 current in the absence and presence of PP2 |

| Channels | 0 mm cAMP | 1 mm cAMP |
|----------|-----------|-----------|
|          | $V_{n.s.}$ | $k$ | $n$ | $\tau_{act}$ | $n$ | $V_{n.s.}$ | $k$ | $n$ | $\tau_{act}$ | $n$ |
| HCN2     | $-94.2 \pm 0.89$ | $6.88 \pm 0.38$ | $12$ | $250 \pm 14$ | $10$ | $-84.6 \pm 0.94$ | $8$ | $7.91 \pm 0.44$ | $8$ | $164 \pm 6.84$ | $8$ |
| HCN2 + PP2 | $-95.6 \pm 0.73$ | $5.65 \pm 0.29$ | $8$ | $633 \pm 25$ | $8$ | $-85.4 \pm 1.13$ | $11$ | $7.35 \pm 0.53$ | $11$ | $272 \pm 23$ | $12$ |

**FIGURE 3.** Full-length Src and the SH3 domain of Src (Src-SH3) bind to the C terminus of HCN2. A, schematic representation of the HCN2 C terminus (CT). S6, sixth transmembrane segment; L, C-linker with helices A F; BD, cyclic nucleotide binding domain with helices A C and B sheets B B; dC, distal C terminus. B, yeast two-hybrid screen to analyze the interaction between HCN2 and Src. Bait vectors expressing the CT or portions of CT as indicated were tested for interaction with full-length Src or Src-SH3. Shown are transformants growing on (+) X-gal/galactose/raffinose/Ura/His/Trp and ( ) X-gal/Glu/Ura/His/Trp selection plates. Interactions were considered specific if transformants turned blue on + and stayed white on ( for further details see “Experimental Procedures”). The C linker fusion protein (L) led to nonspecific transcription of lacZ reporter gene. C, GST pull-down assay. Extracts from HEK293 cells overexpressing Myc-tagged Src or transfected with empty vector were incubated with glutathione beads coupled to GST or GST-CT, GST-L + BD, and GST-dC fusion proteins. Immunoblotting was performed with an antibody against the Myc epitope fused to Src. The last lane represents 30 μg of total lysate of HEK293 cells overexpressing Src. D, full-length HCN2 binds to the SH3 domain of Src. Extracts of HEK293 cells expressing HCN2 were incubated with GST or GST-SH3 fusion proteins. Immunoblotting was performed with anti-HCN2. E, in vitro phosphorylation assay. GST fusion proteins bound to glutathione beads were incubated with purified Src (1 unit) or with kinase buffer alone and subjected to Western blot analysis. The blot was probed with anti-pY (upper panel) to detect phosphorylated proteins and reprobed with anti-GST (lower panel) to determine total protein levels. CT (70 kDa), L + BD (~45 kDa) fusion proteins are phosphorylated, whereas dC is not. The arrow in the upper panel marks the 60-kDa band of autophosphorylated Src.
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**FIGURE 5.** Replacement of Tyr476 with phenylalanine abolishes the effect of PP2 on activation kinetics of HCN2. A, model of HCN2 with positions of all 17 tyrosine residues present in the two cytoplasmic loops and the CT. B, summarized data for the effects of 10 µM PP2 on the activation kinetics (τact) of various HCN2 channel mutants. ΔN130 is a truncation mutant in which the N-terminal 130 amino residues were deleted; Y259F and Y331F are point mutations; Y449–481F is a 6-fold mutant in which Tyr449, Tyr453, Tyr459, Tyr476, Tyr477, and Tyr481 are substituted by phenylalanine; Y476–618F is a 9-fold mutant in which Tyr476, Tyr477, Tyr481, Tyr543, Tyr555, Tyr579, Tyr600, Tyr604, and Tyr618 are substituted by phenylalanine. In Y670/766F the two tyrosines of the distal C terminus have been mutated to phenylalanine. C, normalized current of HCN2-Y476F in the absence and presence of 10 µM PP2. D, summarized data of effects of 10 µM PP2 and 100 µM genistein (Gen) on τact of HCN2, HCN2-Y476F, HCN2-Y477F, and HCN2-Y481F. The τact(Y476F) refers to the predominant fast component of the current. E and F, effect of 10 µM PP2 on the deactivation time constants (τdeact) of HCN2 and HCN2-Y476F. Following a 3.2-s prepulse to −140 mV tail currents were evoked by the potentials from −10 to +50 mV. The τdeact was determined by monoexponential fitting of tail currents. The number of experiments in B, D, E, and F is given in parentheses. *p < 0.05; **, p < 0.01 versus control in this and subsequent figures.

Regulation of HCN Channels by Src Kinase

**A** [Image 231x26 to 258x38]

**B** [Image 206x354 to 564x733]

**C** [Image 327x136]/H11006

**D** [Image 304]/H11002

**E** [Image 327]/H11001

**F** [Image 327]/H11005

this domain (Fig. 4E). In contrast, a GST fusion protein covering the distal C terminus neither bound Src nor was phosphorylated by this kinase (Fig. 4, C and E).

**Src Regulates HCN2 by Phosphorylation of Tyrosine 476—HCN2 contains seventeen tyrosine residues in cytosolic domains, all of which could principally serve as phosphorylation sites for Src (Fig. 5A). We systematically mutated each of these residues individually to phenylalanine, and in addition constructed mutants containing multiple exchanges. All mutants were tested for their sensitivity to PP2. Fig. 5B summarizes τact values obtained for some of the mutants. A mutant lacking the N terminus (ΔN130), and mutants carrying mutations in the cytosolic loops (Y259F, Y331F) or distal C terminus (Y670/766F) maintained sensitivity to PP2. By contrast, a 6-fold mutant covering all tyrosines present in the C-linker (Y449–481F) and a 9-fold mutant covering the last three tyrosines of the C-linker and all six tyrosines of the CNBD (Y476–618F) were no longer affected by this agent. We reasoned that one of the three replacements present in both mutants (Y476F, Y477F, and Y481F) was crucial for the PP2 effect. Indeed, muta-

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Next, we determined the localization of phosphotyrosines in HCN2 by mass spectrometry (Fig. 6). Analysis of peptides obtained after trypsin digestion of an in vitro phosphorylated GST fusion protein containing the whole HCN2 C terminus revealed two phosphotyrosine peptides. The two peptides contained phosphate groups at position Tyr554 (Fig. 6A) and Tyr476 (Fig. 6B), respectively. No other phosphorypeptide was detected. Together with the electrophysiological data the identification of phosphorylated Tyr476 strongly suggested that this residue confers the modulatory action of Src. By contrast, phosphorylation of Tyr554 was very likely not involved in this process since the Y459F mutant was fully sensitive to PP2 (τact(control) = 164 ± 17 ms, n = 5; τact(+PP2) = 271 ± 12 ms, n = 7).

**Mutation of Tyr554 in HCN4 Prevents Regulation by PP2—**Tyr476 is localized in the B′-helix of the HCN2 C-linker (Fig. 7 and supplemental Fig. S2). In the crystal structure of the HCN2 C-linker-CNBD tetramer, Tyr476 contributes to intersubunit interaction by forming a hydrogen bond with a carbonyl backbone group (Glu494) present in the C′-helix of a neighboring subunit (Fig. 7A and supplemental Fig. S2). Importantly, Tyr476 is highly conserved within the HCN channel family (supplemen-
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FIGURE 6. Mass spectrometric analysis of the Src-phosphorylated HCN2 C terminus. Shown are the MS/MS spectra of the doubly charged form of the two identified phosphopeptides 455-QVEQpYMSFHK-464 (A) and 473-HDpYYEHR-480 (B). The b- and y-ion series used for the phosphopeptide identification are indicated. Those observed are underlined. Note that the mass difference between b4 and b5 in A and y4 and y5 in B is 243 Da, corresponding to phosphotyrosine residues.

Native Ih Is Regulated by Src—We finally asked whether native Ih is modulated by Src. To this end we studied the effect of PP2 on Ih in HL-1 cardiomyocytes (32), in sino-atrial node (SAN) pacemaker cells and in DRG neurons (Fig. 8). We selected these cell types because they endogenously express Src (not shown) and because their respective Ih reveals fast (DRG), intermediate (HL-1), and slow (SAN) kinetics. Like heterologously expressed channels native Ih was profoundly slowed down by PP2, confirming that regulation by Src is a general feature of Ih channels.

DISCUSSION

The activation and deactivation of Ih channels is tightly controlled in the cell. Importantly, a rise in cellular cAMP level speeds up these channels whereas hydrolysis of cAMP has the opposite effect (1). Here, we show that Src-mediated tyrosine phosphorylation is another crucial mechanism controlling kinetics. Inhibition of Src by pharmacological blockers as well as transfection with a catalytically inactive Src mutant profoundly slowed down activation kinetics of native and expressed Ih channels indicating that the phosphorylation/dephosphorylation state determines channel kinetics. Our data indicate that the dephosphorylated channel activates significantly slower than the phosphorylated channel.

The Src kinase binds via its SH3 domain to the HCN2 channel. As a minimal requirement for high affinity binding of Src, we identified the sequence reaching from the C'-helix of the C-linker to the C terminus of the CNBD. This region is highly conserved among HCN1–4, suggesting that binding of Src is a common feature of HCN channels. Interestingly, Santoro et al. (22) reported binding of the SH3 domain of n-Src to the full-length C terminus of HCN1 whereas no interaction was seen for c-Src, the Src splice variant used in our study. n-Src differs from c-Src only by 6 or 11 amino acid insertions (35). The structural determinants conferring the isoform specificity of Src binding to HCN1 versus HCN2 remain to be determined.

We were unable to further narrow down the target sequence of Src, probably because the residues that directly interact with the SH3 domain are distributed in both C-linker and CNBD. It is very likely that only in the context of the correctly folded C-linker/CNBD domain these key residues are in the right spatial position required for SH3 binding. Notably, the identified sequence does not contain classic proline-rich (PXXP) SH3 binding motifs (36). However, there are several studies showing that SH3 domains can also bind to non-P ligand sequences (37–39). HCN2 represents another example of a protein interacting with SH3 via a non-classic binding sequence.
Mass spectrometry identified two tyrosine residues (Tyr^{459} and Tyr^{476}) in the C-linker of HCN2 that are phosphorylated by Src. The electrophysiological analysis revealed that only one of these residues, Tyr^{476}, is important in controlling activation kinetics. When this residue was mutated to phenylalanine activation kinetics were no longer sensitive to Src inhibitors. We cannot totally exclude that the loss of Src regulation in Y476F was caused by some nonspecific steric effect. However, it should be noted that mutation of any other of the seventeen regulatory sites and experimental parameters is a well known property of Ih. For example, activation curves of Ih are shifted by 30–40 mV to more hyperpolarized voltages when currents are measured in excised patches instead of whole cell mode (48). Recently, Yu et al. (25) reported effects of genistein on HCN channels expressed in Xenopus oocytes. Like in HEK293 cells, genistein slowed down HCN2 and HCN4 activation kinetics. However, in contrast to our findings, genistein also reduced current densities of both channels and induced in HCN2 a shift of \( V_{1/2} \) to more negative voltage. In addition, current modulation was only observed in excised patches after preincubation with blockers prior to establishing the whole cell mode, whereas we observed modulation only after preincubation with blockers.

We also studied the functional interaction between tyrosine phosphorylation and cAMP-dependent modulation of HCN2. Cyclic AMP shifted the activation curve of HCN2 in the presence of Src blockers by the same value (about \(+10\) mV) as at control conditions. Moreover, cAMP speeded up activation kinetics when Src was inhibited. However, in the presence of Src inhibitors the \( \tau_{act} \) values obtained at saturating cAMP were consistently bigger than in the absence of these agents. Collectively, these findings suggest that regulation of HCN channel activity by cAMP operates principally independent of the phosphorylation state of Tyr^{476}. However, the phosphorylation state of Tyr^{476} determines the extent by which a given cAMP concentration can accelerate activation kinetics. The complex modulation of channel activation resulting from the interplay between tyrosine phosphorylation and cellular signaling needs to be determined. Unfortunately, we were not able to reliably measure Ih after preincubation with the phosphatase inhibitor pervanadate because this agent induced profound cell detachment and led to a destabilization of the plasma membrane. Moreover, the effect of Src inhibitors was only seen when intact cells were preincubated with these agents, but was not observed in excised patches or when cells were perfused after establishing the whole cell mode. These findings suggest that the phosphatase required for dephosphorylation is either readily lost and/or that channel regulation requires the intact intracellular milieu including the presence of cytoskeletal and anchoring proteins. High sensitivity to cellular factors and experimental parameters is a well known property of Ih. For example, activation curves of Ih are shifted by 30–40 mV to more hyperpolarized voltages when currents are measured in excised patches instead of whole cell mode (48).
Regulation of HCN Channels by Src Kinase

Src is ubiquitously expressed in brain and heart cells. Thus, regulation of Ih channels by this kinase may be a general feature of this ion channel class. In agreement with this notion we demonstrated that HCN2 binds Src and is tyrosine-phosphorylated in mouse brain. Moreover, we provide evidence that Src regulates Ih from murine DRG neurons and sinoatrial node cells which mainly express HCN1/HCN2 (49–50) and HCN4 (51), respectively. Native Ih shows a wide range of activation kinetics (52). It was assumed that this diversity results from the subunit compositions of the individual channels, the CAMP levels and physical parameters like pH, ionic milieu, and temperature. The control of the phosphorylation state of Tyr476 by Src and not yet defined phosphatases represents another mechanism controlling kinetics. Importantly, unlike the cAMP system, Src affects activation speed without altering the voltage dependence of activation. This finding suggests that the control of activation kinetics by Src may reflect an important regulatory mechanism to adjust the properties of Ih to the individual requirements of different types of neurons and heart cells.

Src is regulated by a complex network of upstream signals. For instance, pathways involving Ras, epidermal growth factor, and integrin receptors were shown to regulate the activity of Src bound to the NMDA receptor (53). Ion channel activity may also be regulated by pathways that involve protein phosphatases opposing Src. IGF-1 was shown to regulate the activity of the rod CNG channel via such a pathway (54).

Activation kinetics by Src may reflect an important regulatory mechanism to adjust the properties of Ih to the individual requirements of different types of neurons and heart cells.

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