Dual Phosphorylations Underlie Modulation of Unitary KCNQ K⁺ Channels by Src Tyrosine Kinase*

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Src tyrosine kinase suppresses KCNQ (M-type) K⁺ channels in a subunit-specific manner representing a mode of modulation distinct from that involving G protein-coupled receptors. We probed the molecular and biophysical mechanisms of this modulation using mutagenesis, biochemistry, and both whole-cell and single channel modes of patch clamp recording. Immunoprecipitation assays showed that Src associates with KCNQ2–5 subunits but phosphorylates only KCNQ3–5. Using KCNQ3 as a background, we found that mutation of a tyrosine in the amino terminus (Tyr-67) or one in the carboxyl terminus (Tyr-349) abolished Src-dependent modulation of heterologously expressed KCNQ2/3 heteromultimers. The tyrosine phosphorylation was much weaker for either the KCNQ3-Y67F or KCNQ3-Y349F mutants and wholly absent in the KCNQ3-Y67F/Y349F double mutant. Biotinylation assays showed that Src activity does not alter the membrane abundance of channels in the plasma membrane. In recordings from cell-attached patches containing a single KCNQ2/3 channel, we found that Src inhibits the open probability of the channels. Kinetic analysis was consistent with the channels having two discrete open times and three closed times. Src activity reduced the durations of the longest open time and lengthened the longest closed time of the channels. The implications for the mechanisms of channel regulation by the dual phosphorylations on both channel termini are discussed.

Regulation of neuronal activity by tyrosine kinases has emerged as an important theme in neurobiology. In addition to their well-documented role in neuronal proliferation and trophic responses, actions are apparent in several different types of ion channels that link alterations in electrical activity to neuronal plasticity. Examples are non-receptor tyrosine kinase modulation of Shaker family K⁺ channels (1–7) and growth factor receptor regulation of voltage-gated Ca²⁺ channels (8–10). The family of KCNQ genes make K⁺ channels composed of KCNQ1–5 subunits, which are responsible for M-type K⁺ currents in the nervous and auditory systems (11–14) and slow delayed rectifier K⁺ currents in the heart (15, 16). KCNQ1–5 channels have recently been incorporated into the Kv nomenclature as Kv7.1–5 (17). Compared with other Kv channels, KCNQ channels have an extended intracellular carboxyl terminus that seems to be the target of many modulatory signals. Examples are modulation by Ca²⁺ (18), using calmodulin as the channel Ca²⁺ sensor (19), and regulation by plasma membrane phosphoinositides (20–22) perhaps in concert with protein kinase C (23).

Src is the prototypical member of the family of non-receptor tyrosine kinases that also includes Fyn, Hck, Lck, and Yes. These proteins often act via two mechanisms: by binding to substrate via Src homology (SH)¹ and SH3 domains and/or by phosphorylation of target tyrosines (24). For example, distinct effects of Src actions by these two mechanisms have been described for Kv1.4 and Kv1.5 K⁺ channels (6). We recently showed subunit-specific modulation of KCNQ channels by Src and suggested that Src action was distinct from modulation by muscarinic acetylcholine receptor stimulation (25). Although we showed that tyrosine phosphorylation of a substrate is involved, we did not ascertain whether direct phosphorylation of the channels themselves is required and the location of any putative tyrosines involved. The strongest effect of Src activity in both the heterologous system involving cloned channels and sympathetic neurons is a strong voltage-independent suppression of whole-cell currents with other effects being a shift in the voltage dependence of activation and a slowing of activation kinetics (25). Here we focus on the major voltage-independent effect and probe the underlying molecular and biophysical mechanism by which it occurs. The observed depression of macroscopic KCNQ currents could be a reduction of the unitary properties of the channels or a reduction of channel number in the plasma membrane. In neurons, the M current, named for its suppression by muscarinic acetylcholine receptor agonists (26), is largely composed of KCNQ2/3 heteromultimers (11, 13), and we predominantly focused on these channels in this work. Using a heterologous expression system and site-directed mutagenesis, we suggest that two widely separated tyrosines, one in the amino terminus and the other in the carboxyl terminus, must be phosphorylated for Src-mediated modulation. In addition, by single channel recording and biotinylation assays we show that the suppression of whole-cell KCNQ2/3 currents by Src is caused by a reduced probability of channel opening. These results provide important insights into our understanding of regulation of K⁺ channels by protein tyrosine kinases. In particular, they shed further light on the molecular mechanisms regulating gating of KCNQ channels.

¹ The abbreviations used are: SH, Src homology; CA, constitutively active; CHO, Chinese hamster ovary; wt, wild-type; DN, dominant-negative; PIP₂, phosphatidylinositol 4,5-bisphosphate; pS, picosiemens; CMD, channel-modulatory domain.
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**EXPERIMENTAL PROCEDURES**

**cDNA Constructs**—Plasmids encoding human KCNQ2, rat KCNQ3, human KCNQ4, and human KCNQ5 (GenBank™ accession numbers AF110020, AF091247, AF105202, and AF249278, respectively) were kindly given to us by David McKinnon (State University of New York, Stony Brook, NY; KCNQ2 and KCNQ3), and Thomas Jentsch (Zeiss, Germany; KCNQ4) and Klaus Steinmeyer (Aventis Pharma, Frankfurt am Main, Germany; KCNQ5).

The c-Src (Src) clone used has been described previously (25). Src K298M (dominant-negative Src) was generated by using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. Constitutively active (Src-Ca) kinase has been generated as described previously (S. A. Courtneidge, Sugen Inc.) and has been described previously (27). KCNQ2 and KCNQ3 were subcloned into pcDNA3 (Invitrogen) as described previously (28). KCNQ4 and KCNQ5 were subcloned into pcDNA3.1zeo— as described previously (25). Myc-tagged KCNQ2–5 were generated by subcloning each channel in-frame into cytomegalovirus-eyc (pcMV) plasmid (Clontech) behind the Myc epitope. The tyrosine to phenylalanine KCNQ5 mutants were made commercially using the QuikChange mutagenesis kit (BioS&T, Montreal Canada) and were sequenced to verify mutagenesis. Rat wild-type and K298M Src were subcloned into pcDNA3.1zeo— using EcoRI.

**Cell Culture and Transfections**—Chinese hamster ovary (CHO) cells were grown in 100-mm tissue culture dishes (Falcon, BD Biosciences) in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum and 0.1% penicillin and streptomycin in a humidified incubator at 37 °C (5% CO2) and passaged every 3–4 days. Cells were cultured in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. Transfections of CHO cells were performed using Polyfect reagent (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. For electrophysiological and biochemical experiments, cells were used 48–96 h after transfection. As a marker for successfully transfected cells, cDNA encoding green fluorescent protein was co-transfected together with the cDNAs of interest. For successful transfections, cells were used 48–96 h after transfection. As a marker for successfully transfected cells, cDNA encoding green fluorescent protein was co-transfected together with the cDNAs of interest.

**Biotinylation of Cell Surface Protein and Immunoblottting**—Cells were grown in 100-mm culture dishes and individually transfected with Myc-tagged KCNQ2–5 and green fluorescent protein together with either wild-type (wt) or dominant-negative K298M rat Src (DN-Src). After 48 h, cells were washed three to five times with cold phosphate-buffered saline at room temperature (22–25 °C), and then cell surface proteins were biotinylated by EZ-link sulfo-N-hydroxysuccinimide-S-biotin (0.5 mg/ml, Pierce) in phosphate-buffered saline. After incubation at 4 °C for 1 h, cells were washed five times with ice-cold phosphate-buffered saline to remove any remaining biotinylation reagent. Cells were then harvested with a rubber policeman in gentle lysis buffer (GLB; 75 mM NaCl, 50 mM Tris-HCl, 2 mM EGTA, 1% Nonidet P-40, 10% glycerol plus the protease inhibitor phenylmethylsulfonyl fluoride (PMSF)) and homogenized using a Dounce (Sorenson) and were supernetted at 4 °C for 1–2 min. Inflow to the chamber was by gravity from several reservoirs, selective by activation of sole-noid valves (Vavelink 8, Automated Scientific). Bath solution exchange was complete by <30 s. To observe green fluorescent protein fluorescence, a mercury lamp was used in combination with an Eclipse TE 300 inverted microscope equipped with an HQ fluorescein isothiocyanate filter cube (Nikon, Melville, NY). To evaluate current amplitudes at saturating voltages, CHO cells were held at 0 mV, and 800-ms hyperpolarizing steps to −60 mV followed by 1-s pulses back to 0 mV were given. The amplitude of the current was usually defined as the difference between the holding current at 0 mV and the current at the beginning (after any capacity current has subsided) of the 1-s pulse back to 0 mV. All results are reported as mean ± S.E.

**Phosphorysine Labeling**—CHO cells were grown in Ham’s F-12 medium (Invitrogen) supplemented with 10% newborn calf serum and 1% penicillin/streptomycin. Transfections of CHO cells were performed in 60-mm plates with 3 μg of total recombinant plasmid using Lipo-fectAMINE reagent according to the manufacturer’s protocol (Invitrogen).

**Cell-attached Patch/Single Channel Electrophysiology**—Single channel current amplitudes were calculated by fitting all-point histograms with single or multi-Gaussian curves.

**Phosphotyrosine Labeling**—CHO cells were grown in Ham’s F-12 medium (Invitrogen) supplemented with 10% newborn calf serum and 1% penicillin/streptomycin. Transfections of CHO cells were performed in 60-mm plates with 3 μg of total recombinant plasmid using LipofectAMINE reagent according to the manufacturer’s protocol (Invitrogen).

**RESULTS**

**Sites of Action of Src on KCNQ Channels**—We have previously reported that c-Src (Src) modulates both KCNQ currents from cloned channels heterologously expressed in CHO cells and endogenous KCNQ channels in rat sympathetic neurons. Src modulation is most easily observed by overexpression of Src in either system, which strongly depresses the whole-cell currents. In the heterologous system, currents from KCNQ3, KCNQ4, and KCNQ5 homomultimers and KCNQ2/3 heteromultimers, but not KCNQ1 or KCNQ2 homomultimers, are depressed by Src overexpression (25). We found that Src-de-
pended phosphorylation of a protein that may be the channel itself correlated with the subunit specificity of Src action and hypothesized that this is due to a subunit-specific phosphorylation of channel tyrosines. Alignment and inspection of the KCNQ1–5 amino acid sequences does not reveal conserved tyrosines among KCNQ3–5 that are not present in KCNQ1 and KCNQ2. However, there are five tyrosines conserved among KCNQ2–5 subunits that are predicted to be accessible from the intracellular side. These residues are depicted schematically in Fig. 1B. Using KCNQ3 as a background, we initially individually mutated those tyrosines to phenylalanines and assessed the effect of Src on those mutant channels.

Since KCNQ3 homomultimers usually express poorly, we co-expressed KCNQ2 and either the wt or mutant KCNQ3 subunits to form KCNQ2/3 heteromultimers. We compared the amplitudes of the currents in cells only expressing the channels with those co-expressed with Src. Since KCNQ2 homomultimeric currents are not depressed by Src (25), we reasoned that any suppression of the currents by Src must be on the KCNQ3 subunits in the tetramer. In these experiments, we often verified that the co-expressed subunits did form heteromultimers by assaying block by tetraethylammonium ions. At 10 mM, tetraethylammonium block of KCNQ2 or KCNQ3 homomultimers is nearly total or very slight, respectively, and block of KCNQ2/3 heteromultimers is ~60% (28, 30). As before, co-expression of KCNQ2 and wt KCNQ3 subunits resulted in robust currents that were greatly suppressed by overexpression of Src (Fig. 1A). For KCNQ2/wtQ3 channels, the mean current amplitude in control cells was 451 ± 92 pA, but in cells co-expressed with Src, it was reduced to 150 ± 42 pA (n = 37, 39; p < 0.005). We also observed slowing of activation kinetics as described previously (25). Heteromultimers formed by co-expression of KCNQ2 and the five Tyr-to-Phe mutant KCNQ3 subunits also expressed well and yielded currents similar in amplitude to those formed by wt heteromultimers. Tetraethylammonium (10 mM) block of currents produced by co-expression of KCNQ2 and the KCNQ3 mutants ranged from 52–72%, consistent with expression of heteromeric channels. Among the five mutants, we found that co-expression of two of them with KCNQ2 resulted in currents that were not suppressed by Src co-expression. The two were Y67F, located in a highly conserved region of the amino terminus, and Y349F, located in the carboxyl terminus in the first highly conserved region just downstream of the end of the sixth transmembrane domain (Ser) (Fig. 1A). For co-expression of KCNQ2/Q3–Y67F, the mean current amplitude in control cells was 264 ± 59 pA, and in cells co-expressed with Src, it was 297 ± 116 pA (n = 7, 23). For co-expression of KCNQ2/Q3–Y349F, the mean current amplitude in control cells was 294 ± 68 pA, and in cells co-expressed with Src, it was 248 ± 59 pA (n = 8, 9). Interestingly Tyr-349 is in the domain suggested to be a site for binding of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to the channels (22) and for calmodulin-mediated assembly (31). For the other mutants, Y88F, Y243F, and Y513F, overexpression of Src inhibited the heteromeric current in a way similar to the inhibition of currents from wt KCNQ2/3 channels (Fig. 1C). These data suggest that Src-mediated suppression of KCNQ2/3 channels might involve phosphorylation of both Tyr-67 in the amino terminus and Tyr-349 in the carboxyl terminus.

**Src Binds to KCNQ2–5 Subunits but Induces Subunit-specific Phosphorylation**—The actions of non-receptor tyrosine kinases on effector proteins may involve binding of the kinase to the effector, its tyrosine phosphorylation by the kinase, or a combination of the two (32). In the case of Shaker K$^+$ channels, distinct effects of Src kinase have been attributed to both of these mechanisms (3, 6). Thus, we investigated whether the Src effects on KCNQ channels were due to binding of Src to the channels or to phosphorylations and, if the latter, whether direct phosphorylation of channel tyrosines are involved. To probe for binding of the kinase to the channels, we performed co-immunoprecipitation assays. KCNQ2–5 subunits were epitope-tagged by introduction of Myc epitope to their amino...
immunoprecipitated with anti-Myc antibodies (top panel) or with anti-Src antibodies preincubated with the immunizing peptide used to generate the antibodies (bottom panel), and the immunoprecipitates were separated by SDS-PAGE and probed with anti-Myc antibodies. B, shown are immunoblots from CHO cells individually transfected with Myc-tagged KCNQ2, KCNQ3, or KCNQ5 channels either alone or together with CA-Src. Whole-cell lysates were immunoprecipitated with anti-Myc antibodies (top panel), and the immunoprecipitates were separated by SDS-PAGE and probed with anti-phosphotyrosine antibodies. The lower panel shows immunoblots of the lysates with anti-Src antibodies without any immunoprecipitation. IP, immunoprecipitation; IB, immunoblot; P-Tyr, phosphotyrosine.

termini and individually expressed in CHO cells together with Src. The properties of the KCNQ2–5 channels were not affected by introduction of the Myc epitope (data not shown). Whole-cell lysates were immunoprecipitated with anti-Src antibodies, the precipitates were resolved by SDS-PAGE, and immunoblots were performed with anti-Myc antibodies. The immunoblots yielded signals for all KCNQ2–5 channels, and those signals were sharply reduced by preadsorbing the anti-Src antibodies with the immunizing peptide that was used to raise the antibodies (Fig. 2A). Similar results were seen in three such assays. Thus, we conclude that all KCNQ2–5 channels can associate with Src kinase either via some intermediary adaptor protein or by directly binding to the channels. Although these data suggest that Src can associate with KCNQ2, homomeric KCNQ2 currents are not suppressed by Src overexpression (25), indicating that association with channels is not sufficient for Src-mediated modulation of whole-cell currents.

We then asked whether the channels are directly phosphorylated by Src kinase using another immunoprecipitation assay. In these experiments, we used the constitutively-active (CA)-Src-Y527F (33) to enhance Src-dependent phosphorylation signals. CHO cells were individually transfected with KCNQ2, KCNQ3, or KCNQ5 (we had technical problems in these assays with KCNQ4) either alone or together with CA-Src. Whole-cell lysates were precipitated with anti-Myc antibodies, the precipitates were separated by SDS-PAGE, and immunoblots were performed with anti-phosphotyrosine antibodies. In this assay, only direct phosphorylations of the channel proteins will result in a phosphotyrosine signal in the immunoblots. We found that both KCNQ3 and KCNQ5 were strongly tyrosine phosphorylated in a wholly Src-dependent manner but that KCNQ2 showed only a very faint phosphorylation signal that was not Src-dependent at all (Fig. 2B). Similar results were seen in three such assays. These results are in accord with the subunit-specific sensitivity of KCNQ channels to Src action and with our earlier phosphotyrosine assays that associated phosphotyrosine signals with KCNQ3–5 but not with KCNQ2 (25). The present data indicate that Src acts by direct phosphorylation of channel tyrosines and that binding of the kinase to the channels is not sufficient for the subunit-specific modulation of KCNQ currents by the kinase.

**Two Tyrosines Are Specifically Phosphorylated by Src—** Given that mutation of either Tyr-67 or Tyr-349 in KCNQ3 abolished Src modulation of KCNQ3-containing channels, we asked which of these tyrosines or the other tyrosines that we mutated are phosphorylated by Src. To answer this question, we performed immunoprecipitation assays similar to those shown in Fig. 2B using the mutant Myc-tagged KCNQ3 channels tested in Fig. 1. In these assays, we used wt KCNQ3 and KCNQ2 as positive and negative controls, respectively. Fig. 3 shows a representative experiment. As before, wt KCNQ3 was strongly tyrosine phosphorylated in a Src-dependent manner, and KCNQ2 showed little phosphorylation at all. The mutants whose currents exhibited undiminished suppression by Src overexpression showed a phosphotyrosine signal similar to wt KCNQ3 (data not shown). However, both the Y67F and Y349F mutants showed a modest Src-dependent signal that was consistently weaker than that of wt KCNQ3 but still apparent. Thus, we hypothesized that Src phosphorylates both of these two tyrosines. To test this, we constructed the double mutant KCNQ3-Y67F/Y349F and included this mutant in our phosphorylation assay. This double mutant also expressed well under whole-cell clamp when expressed in CHO cells (data not shown). Fig. 3 also includes the phosphorylation assay using KCNQ3-Y67F/Y349F and shows that this double mutant displayed no tyrosine phosphorylation signal at all. These results are representative of three to five experiments. From the functional measurements shown in Fig. 1 we know that mutation Y67F or Y349F in KCNQ3 abolishes suppression of KCNQ2/3 currents by Src. The phosphorylation assays strongly suggest that Src suppresses KCNQ2/3 current by phosphorylation of both Tyr-67 and Tyr-349 in KCNQ3, and we conclude that phosphorylation of both of these widely separated residues, one in the amino terminus and the other in the carboxyl terminus, are required for Src action.

**Src Does Not Act by Altering Cell Surface Abundance of the Channels**—We considered that the suppression of whole-cell currents by Src activity may be due to two possibilities: 1) a reduction in the number of functional channels in the plasma membrane or 2) alteration of the biophysical properties of the channels, such as open probability or unitary conductance. We tested the first possibility by performing biotinylation assays and immunoblots to specifically label the KCNQ channels on the plasma membrane. We compared biotinylated (cell surface) KCNQ channel protein from cells co-transfected with wt Src with cells co-expressed with dominant-negative Src K298M (DN-Src) (34). After labeling by biotin, cell surface proteins were isolated by allowing them to bind to streptavidin-coated beads. The biotinylated proteins were separated by SDS-PAGE and transferred to nitrocellulose. Anti-Myc antibodies specifically labeled the channels at the molecular masses of ~100 kDa for KCNQ2, 110 kDa for KCNQ3, 80 kDa for KCNQ4, and 125 kDa for KCNQ5. Lysate from cells expressed with non-Myc-tagged KCNQ3 was not labeled, showing the specificity of the Myc antibodies, and lysate from non-biotinylated cells was not retained by the streptavidin beads, showing specific binding to the beads (data not shown). Shown in Fig. 4A are immunoblots of proteins from the cell surface (top row) and the total lysate (bottom row) from cells individually transfected with Myc-tagged KCNQ2–5 channels together with wt Src or DN-Src. The amount of protein in the lanes of the blot was estimated by measuring the pixel intensity of their corresponding antibody-
labeled bars. The experiment shown in Fig. 4A reveals no increase in relative cell surface expression in wt Src-overexpressing cells compared with DN-Src-expressing cells. These experiments are summarized in Fig. 4B. For each channel tested, the ratio of cell surface protein of the wt Src group to that of the DN-Src group was normalized by the ratio of total channel protein in the corresponding lysate. We found that for KCNQ2–5 there was no significant increase in cell surface channel protein caused by overexpression of wt Src kinase, indicating that Src does not alter the surface abundance of channels at the plasma membrane. We thus investigated Src effects on KCNQ currents at the single channel level.

Modulation of Unitary KCNQ2/3 Channels by Src—We investigated the actions of Src kinase on KCNQ2/3 heteromultimers by performing single channel recordings in cell-attached patches from CHO cells expressed with KCNQ2/3 channels either alone or together with CA-Src. We used CA-Src in these experiments to maximize Src activity so as to get as clear a picture as possible of Src actions at the single channel level. Unitary KCNQ2/3 channels were identified by having reversal potential near $E_K$ and a voltage dependence expected from their macroscopic behavior. As homomeric KCNQ2 or KCNQ3 channels express poorly, we are confident that the channels observed were nearly all KCNQ2/3 heteromultimers since it is rare to observe homomeric channels using the same sized pipette tips as used here on cells expressing only KCNQ2 or KCNQ3 subunits. Furthermore KCNQ2/3 heteromultimers exhibit a unitary behavior quite distinct from homomeric channels recently characterized in our laboratory (29).

Cell-attached patch recordings were performed at an assumed membrane potential of 0 mV, which is near the maximal open probability ($P_o$) for these channels (25, 28, 35). As shown in Fig. 5 and Table I, KCNQ2/3 channels exhibit at 0 mV a $P_o$ of $0.47 \pm 0.06$ ($n = 13$) and a single channel amplitude of $0.54 \pm 0.06$ pA ($n = 13$). The fitted conductance over a range of potentials was $7.1 \pm 0.5$ pS ($n = 10$). Both of these parameters are intermediate between those of KCNQ2 and KCNQ3 homomultimers (29) and generally similar to that described before (35). In cells co-expressed with CA-Src, the open probability was only $0.07 \pm 0.01$ ($n = 8$), nearly 7-fold lower than in cells only expressed with the channels, but the unitary conductance of $7.7 \pm 0.4$ pS ($n = 10$) was not significantly different from channels in control cells. The reduction of $P_o$ observed in the single channel experiments is probably greater than that in the
shown in Fig. 6 and Table II. In control cells, the open times distributions were best fit by a two-exponential equation as with the channels having two open states, we found the open times to have as great a tonic Src activity when co-expressed in the cells with the channels. Thus, analyzed at the single channel level, overexpression of CA-Src profoundly reduced the \( P_o \) of KCNQ2/3 channels but did not change their unitary conductance (Table I).

**Table I**

| \( P_o \) | \( t_o \) (pA) | \( t_s \) (pS) | \( t_m \) (ms) | \( t_c \) (ms) |
|-----|-----|-----|-----|-----|
| KCNQ2/3 | 0.47 ± 0.06 | 0.54 ± 0.06 | 7.06 ± 0.45 | 23.3 ± 3.7 | 61.0 ± 13.3 |
| KCNQ2/3 + CA-Src | 0.07 ± 0.01* | 0.57 ± 0.06 | 7.73 ± 0.41 | 9.5 ± 2.0 | 151 ± 34 |

* * \( p < 0.001 \).*

whole-cell measurements (Ref. 25 and this study) because the former used CA-Src and the latter used wt Src, which is likely to not have as great a tonic Src activity when co-expressed in the cells with the channels. Thus, analyzed at the single channel level, overexpression of CA-Src profoundly reduced the \( P_o \) of KCNQ2/3 channels but did not change their unitary conductance (Table I).

**Src Modulates the Kinetics of Gating of KCNQ2/3 Channels**—The effect of Src activity in lowering channel \( P_o \) could be due to a reduction in the open times of unitary events, a prolongation of their closed times, or some combination of the two. The gating of heteromeric KCNQ2/3 channels has been described by a linear state model involving two open states and three closed states (35–37). We performed kinetic analysis of unitary KCNQ2/3 currents at a constant (assumed) voltage of 0 mV to ascertain the kinetics of how Src acts to lower \( P_o \). Consistent with the channels having two open states, we found the open distributions were best fit by a two-exponential equation as shown in Fig. 6 and Table II. In control cells, the open times were 3.5 ± 0.3 and 34.7 ± 5.3 ms, and the distribution between the two was 28.5 and 71.5%, respectively (n = 6). In cells co-expressed with CA-Src, the longer open time was significantly reduced with that value decreasing to 12.5 ± 5.6 ms (p < 0.01, n = 6) and a reduction in the contribution to openings with long open times to 46.6% (n = 6). The overall mean open time was significantly reduced from 23.3 ± 3.7 to 9.5 ± 2.0 ms (p < 0.01, n = 6).

The closed time distributions of KCNQ2/3 channels were best fit by a three-exponential equation, corresponding to short, medium, and long closed times (Fig. 7 and Table II). In control cells, they were 1.9 ± 0.4, 17.7 ± 3.6, and 168 ± 59 ms, respectively (n = 6). The predominant effect of Src was to increase the longest closed time to 452 ± 76 ms with no significant effect on the short and medium closed times. The distributions of closed times between the three time constants also were not significantly altered. The mean closed time was increased from 61.0 ± 13.3 to 151 ± 34 ms. In summary, the effect of modulation of channel \( P_o \) by Src is reduction of the
mean time spent in the longer lived open state, a redistribution toward briefer openings, and an increase in the duration of the longest lived closed state of the channels.

DISCUSSION

Our results shed light on the molecular mechanisms of Src actions on KCNQ channels using biochemical and biophysical measurements. The biochemistry shows that Src modulates the channels by phosphorylation of two tyrosines, Tyr-67 and Tyr-349, located on termini on opposite ends of the transmembrane part of the channel and that the effect of Src activity is not on abundance of channels in the plasma membrane. The biophysical experiments show that both Tyr-67 and Tyr-349 must be present for channel modulation to occur and that the modulation consists of a reduction in channel $P_o$ and determine which kinetic states have altered occupancies by tyrosine phosphorylation. The longer open time is reduced, and the longest closed time is made longer.

Our previous work reported that the effects of Src were due to tyrosine phosphorylation since 1) tyrosine kinase inhibitors reversed all the Src effects, 2) blockade of tyrosine phosphatases mimicked Src overexpression, and 3) overexpression of the Src K298M point mutant that abolishes kinase activities but not Src binding to substrates was without any effect on KCNQ2/3 currents. Previous work on Src modulation of Kv1.4 and Kv1.5 K⁺ channels has shown distinct regulations due to Src binding or to Src-mediated phosphorylation (6). In the present study, we add channel mutagenesis and immunoprecipitation experiments to the previous line of evidence that suggests that Src binding alone does not alter KCNQ channel function. We demonstrate that Src directly phosphorylates two channel tyrosines (Tyr-67 and Tyr-349). The mutation of either one of these tyrosines is sufficient to ablate the suppression of KCNQ currents by Src. Thus, phosphorylation of both tyrosines seems to be required for channel modulation to occur, and the double mutation (Y67F/Y349F) completely abolishes the phosphorylation signals caused by CA-Src overexpression. Surprisingly these two tyrosines localize to opposite intracellular termini of KCNQ3. As shown schematically in Fig. 1B, Tyr-67 localizes to a conserved domain in the amino terminus, and Tyr-349 localizes to the first conserved domain in the carboxyl terminus just after the S6 transmembrane domain.

Much work on the mechanisms of regulation of KCNQ channels has focused on the carboxyl terminus as a channel-modulatory and assembly domain with little focus on the role of the
amino terminus. Thus, regulation by phosphoinositides and Ca\textsuperscript{2+} has been suggested to be mediated by PIP\textsubscript{2} and Ca\textsuperscript{2+}/calmodulin binding to regions in the carboxyl terminus (22, 25), and tetrameric assembly has likewise been shown to be mediated by carboxyl-terminal domains (31, 38, 39). Our laboratory has shown KCNQ3 in particular to have a much greater maximal \( P_0 \) than KCNQ2, KCNQ4, or KCNQ5 and that this difference also localizes to the carboxyl terminus of the channel (29). What role might the amino terminus play? Indications that it might be important come from KCNQ3 mutants in which deletions or charge neutralizations of the conserved domain that contains Tyr-67 resulted in non-functional channels, although surface channel protein was observed by immunostaining.\textsuperscript{2}

One possibility would be a role analogous to the role of the amino terminus of olfactory cyclic nucleotide-gated channels that involves the amino terminus interacting with the main regulatory domain in the carboxyl terminus to control gating (40). Applying that scenario to KCNQ channels, the carboxyl terminus of the channels would function as a channel-modulatory domain (CMD) with putative interactions between the CMD and the gating machinery of the channel, and the amino-terminal would function as a “latch” to facilitate the regulatory interaction. Phosphorylation of Tyr-67 in the amino terminus and Tyr-349 in the carboxyl terminus might be necessary to disrupt the latching interaction between amino and carboxyl termini. Without the assisting role of the amino terminus in CMD action, stabilization of opening does not occur, and \( P_0 \) is lowered by Src phosphorylation. Precedent for dual tyrosine phosphorylation of \( K_\text{v} \) channels involved in tyrosine kinase modulation comes from \( K_v1.3 \) channels, which are doubly phosphorylated by v-Src at a tyrosine in each terminus of the channel, for which a kinase-dead v-Src mutant has little effect (1, 7). In that study, adapter proteins were shown to act as controllers of Src action either facilitating or blocking the signal.

A surprising result of this study is that phosphorylation of both tyrosines seems to be required for any Src action; i.e. the effect was all or none with mutation of only one residue sufficient to abrogate the effect. In the context of a role of the CMD in stabilizing opening, the phosphorylation of both tyrosines may be necessary to disrupt the latching interaction between amino and carboxyl termini. Without the assisting role of the amino terminus in CMD action, stabilization of opening does not occur, and \( P_0 \) is lowered by Src phosphorylation. Precedent for dual tyrosine phosphorylation of \( K^+ \) channels involved in tyrosine kinase modulation comes from \( K_v1.3 \) channels, which are doubly phosphorylated by v-Src at a tyrosine in each terminus of the channel, for which a kinase-dead v-Src mutant has little effect (1, 7). In that study, adapter proteins were shown to act as controllers of Src action either facilitating or blocking the signal.

The need for dual phosphorylations as the switch for KCNQ channel modulation by Src may arise from the need to maintain specificity in the signal, a need especially acute for the case of non-receptor tyrosine kinases that bind to targets using SH3 domains, a docking motif of demonstrated poor specificity (42). KCNQ3–5 channels have multiple PXPP motifs, the minimum SH3-binding pocket required for Src, but none of the preferred RPLPXXP motifs or the preferred SH2-binding sequences exist in these channels. Thus, some mechanism is required to min-

\textsuperscript{2} M. S. Shapiro, unpublished observations.
imize unintentional phosphorylation-mediated actions on the channel substrates. One mechanism for selectivity could be strict compartmentalization of Src kinase to their channel targets, but another powerful solution would be that the switch be composed of multiple simultaneous interactions between the kinase and effector (for a review, see Ref. 43). Thus, we suggest that dual widely separated tyrosine phosphorylations act as a coincidence detector. Only when both tyrosines are phosphorylated at the same time, a highly improbable occurrence if unintentional, does the M-channel get the message, and opening is modulated accordingly. Many of the mechanisms described for tyrosine kinase modulation of channels, including compartmentalization of signaling components in microdomains at the membrane, the use of simultaneous signals to achieve fidelity, and the involvement of adaptor or docking proteins, are common to many signaling pathways now being elucidated and finally to their intended intracellular targets.

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