Modulation of the Voltage-gated Potassium Channel (Kv4.3) and the Auxiliary Protein (KChIP3) Interactions by the Current Activator NS5806*

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KChIP3 (potassium channel interacting protein 3) is a calcium-binding protein that binds at the N terminus of the Kv4 voltage-gated potassium channel through interactions at two contact sites and has been shown to regulate potassium current gating kinetics as well as channel trafficking in cardiac and neuronal cells. Using fluorescence spectroscopy, isothermal calorimetry, and docking simulations we show that the novel potassium current activator, NS5806, binds at a hydrophobic site on the C terminus of KChIP3 and modulates the interactions between this calcium-binding protein and the T1 domain of the Kv4.3 channels through reorientation of helix 10 on KChIP3.

Potassium is the most abundant ion in vertebrate cells, and its concentration is highly regulated by the coordinated activation and inactivation of Na+/K+ pumps and other K+ transporters, including voltage sensitive K+ channels. In excitable tissue such as brain and heart tissue, the higher intracellular concentration of K+ is used to restore the resting membrane potential after depolarization; this makes the regulation of K+ ions of utmost relevance to cell survival. Voltage-gated K+ channels are the most diverse and widespread cation-conducting membrane proteins and have been found to be involved in numerous biological processes such as apoptosis (1), neuron signaling (2), cell volume (3), and cardiac action potential (4). In neurons and cardiomyocytes the Kv4 subfamily of voltage-gated K+ channels regulate the fast-inactivating components of the I_{SA} and I_{TO} currents, respectively (5). These channels form octameric transmembrane complexes with accessory subunits such as potassium channel interacting proteins (KChIPs)2 (6), dipeptidyl peptidases (7), and Kvβ proteins (8). Interaction of KChIP2 with Kv4 channels in heart results in an increase in current density, acceleration of recovery from inactivation, and slower inactivation kinetics (6). Similar effects on the biophysical properties of neural fast-inactivating K+ currents have been observed for KChIP1 (10) and KChIP3 (11). Even though the molecular structures of the Kv4 T1 domain in the presence and absence of the regulatory KChIPs are known, the mechanism by which these auxiliary proteins mediate channel gating remains unknown. Patch clamp studies using heterologous expressed Kv4 channels highlight the relevance of the T1 domain in gating and mediating ancillary subunit interaction. Two fragments on the Kv4 T1 domain (amino acids 7–11 "site 1" and 71–90 "site 2") have been identified as being necessary for KChIP1 current regulation (9).

KChIP3, also known as DREAM or calsenilin, is expressed in the brain/testis and heart and has been shown to interact with presenilin (12), DNA (13), and Kv4.3 (6). KChIP3 contains four calcium binding domains "EF-hands," of which EF-3/4 binds Ca2+ with high affinity, EF-2 binds Mg2+ preferentially, and EF-1 is inactive (14). Similarly to other KChIPs, this protein contains a relatively polar surface, whereas the opposite side is predominantly hydrophobic with a large nonpolar cavity (15). The formation of Kv4–KChIP complex has been shown to be necessary for proper regulation of the fast component of the I_{TO} currents in the heart (4) and I_{SA} in the brain (10). Demod-

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2 The abbreviations used are: KChIP3, K+ channel interacting protein 3; 1,8-ANS, 8-anilinonaphthalene-1-sulfonic acid; ITC, isothermal titration calorimetry; NS5806, (1-[2,4-dibromo-6-(1H-tetrazol-5-yl)-phenyl]-3-(3,5-bis-trifluoromethyl-phenyl)-urea); Ahx, 6-amino hexacarboxonic acid.
ulation of I_{rTO} currents is a characteristic trait in hypertrophied and failing hearts (16) as well as in Brugada syndrome (17). Normal functioning heart tissue displays an increasing I_{rTO} current density gradient from endocardium to epicardium, which has been shown to correlate with a transmural KChIP density (18). Studies using canine models of failing hearts have shown that heart failure results in a decrease in phase 1 repolarization due to a decrease in I_{rTO} current as well as a remodeling of the gating kinetics of this current (19). Although numerous drugs have been shown to down-regulate the amplitude or modify the gating kinetics of I_{rTO} current, drugs that directly up-regulate the I_{rTO} current amplitude have not been reported.

Recently, Calloe et al. (17) introduced a new compound named NS5806, which was shown to modulate the I_{rTO} current kinetics and reverse the I_{rTO} current decrease induced by heart failure in canine models (20). Interestingly, this I_{rTO} activator has also been shown to decrease I_{SA} currents in cultured hippocampal neurons where its effects on gating kinetics are comparable to those observed in heart tissue (11). Moreover, the induced potassium current modulation by NS5806 has been shown to depend on the presence of Kv4 auxiliary KChIP protein (11, 21).

In this study we investigate the mechanism of NS5806 I_{rTO}/I_{SA} current regulation by determining the interaction between NS5806 and KChIP3 and how this interaction modulates the KChIP3-Kv4.3 association. We show that the I_{rTO} activator NS5806 binds to the Kv4 auxiliary protein KChIP3 at the C terminus near the calcium binding sites, EF-hand 3 and EF-hand 4, with an affinity comparable to the previously determined EC_{50} for current potentiation. We also characterize the association constants between KChIP3 and peptide fragments of the T1 domain of Kv4.3 to show that calcium binding induces an increase in the affinity between KChIP3 and the T1 domain of Kv4.3. In the presence of NS5806 the interaction between KChIP3 and the hydrophobic N terminus of Kv4.3 is enhanced, and the calcium dependence of this interaction is abolished. Furthermore, anisotropy data suggest that the complex KChIP3-T1 domain adopts an altered quaternary structure in the presence of NS5806. Kinetic data indicates that NS5806 decreases the rate of dissociation between KChIP3 and site 1 of Kv4.3, which results in an overall increase in affinity. These results support the idea that NS5806 binds at the interface between the hydrophobic N terminus of Kv4.3 and the hydrophobic cavity at the C terminus of KChIP3 and stabilizes the protein complex. These results are further supported by docking, molecular dynamic simulations, and site-directed mutagenesis. Overall, the studies shown here provide a roadmap for the elucidation of the precise mechanism by which Kv4 current kinetics is regulated by KChIPs and NS5806.

**EXPERIMENTAL PROCEDURES**

General—NS5806 (1-[(2,4-dibromo-6-(1H-tetrazol-5-yl)-phenyl]-3-(3,5-bis-trifluoromethylphenyl)-urea) >99% purity was purchased from Tocris Bioscience. 1,8-ANS (8-anilino-1-naphthalenesulfonic acid) was from Cayman Chemical Co and DMSO was purchased from Sigma. A 100 mM stock solution of NS5806 was prepared by dissolving NS5806 in DMSO (spectroscopic grade) and stored at −20 °C. 1,8-ANS stock solution was prepared by dissolving 1,8-ANS in deionized water. The sequence-specific fluorescent biarsenical probe FlAsH-EDT2 was synthesized and purified following a published protocol (22). The concentration of NS5806, FlAsH-EDT2, and 1,8-ANS stocks were determined spectrophotometrically using ε_{350nm} = 4995 M⁻¹cm⁻¹ for 1,8-ANS in water, ε_{498nm} = 69500 M⁻¹cm⁻¹ for FlAsH-EDT2 in 0.1 M NaOH, and ε_{295nm} = 2200 M⁻¹cm⁻¹ for NS5806 in 20 mM MOPS, pH 7.4. All measurements were conducted at room temperature. Emission intensities were corrected for dilution effects and inner filter effect. The amount of DMSO in the protein samples did not exceed 1.2% v/v. Calcium probes Quin-2, Calcium Green-5N, and CaGreen-2 were purchased from Invitrogen.

**Isolation and Purification of KChIP3 Constructs—Expression and purification of mouse KChIP3 recombinant proteins were carried out by following the previous established protocol with minor modifications (23). The gene sequence encoding for mouse KChIP3-(65–256) and KChIP3-(161–256) were inserted into pReceiver-B31, whereas KChIP3-(65–256)-CCPGCC and GST-Kv4.3-(1–152) were inserted into pReceiver-B01 and pReceiver-B04, respectively (GeneCopoeia). KChIP3(Y174A), -(F218A), and -(F252A) were obtained by site-directed mutagenesis of the KChIP3-(65–256) construct using the QuikChange Lightning kit (Agilent Technologies) and confirmed by DNA sequencing (Eurofins Genomics). These constructs were purified using a nickel-nitrilotriacetic acid column (Qiagen) followed by anion exchange chromatography (DEAE-Sepharose, GE Healthcare). Protein purity was assessed by SDS-PAGE.

Cells containing KChIP3-(65–256)-CCPGCC and GST-Kv4.3-(1–152) constructs were disrupted by sonication (Fisher, Model 100). However, these constructs were recovered as insoluble inclusion bodies that were collected by centrifugation. Inclusion bodies were washed extensively with 50 mM Tris buffer, pH 8.0, 1 M NaCl, 0.5% Triton X-100, 0.1% NaN₃, 1 mM EDTA, and 5 mM DTT, and portion aliquots were stored at −20 °C. 1,8-ANS stock solutions were prepared by dissolving 1,8-ANS in water, and Ca²⁺ and Mg²⁺ were prepared by dissolving 256)-CCPGCC with the biarsenical probe FlAsH-EDT2 was solubilized in 100 mM Tris buffer, pH 8.0, 2.0 M urea, and 5 mM DTT. Refolding was achieved by fast dilution of solubilized inclusion bodies into a 10× volume of 100 mM Tris buffer, pH 7.4, 250 mM sucrose, 30% glycerol, 20 mM lauryldimethylamine N-oxide, 1 mM 2-mercaptoethanol on an ice bath with constant stirring. Refolded protein was concentrated and dialyzed against 20 mM Tris buffer, pH 7.4. Labeling of KChIP3-(65–256)-CCPGCC with the biarsenical probe FlAsH-EDT2 was conducted by adding 2× stoichiometric excess probe under identical condition as reported before (22). Unbound probe was removed by size exclusion chromatography, and labeled protein was further purified through a nickel-nitrilotriacetic acid column followed by extensive dialysis in 20 mM MOPS, pH 7.4, 100 mM NaCl. The labeling efficiency and stock concentration were determined by comparing the protein concentration using the FlAsH and ReAsH absorbance in 0.1 M NaOH. Protein purity and proper folding before FlAsH labeling was assayed using SDS-PAGE electrophoresis, CD spectroscopy, and the Zn²⁺ and Ca²⁺-induced oligomerization changes as previously reported.
**Sample Preparation**—Protein samples were prepared by diluting a concentrated protein stock in 20 mM MOPS, pH 7.4, 100 mM NaCl, and 1 mM DTT. Final protein concentration was measured using $\varepsilon_{280\text{ nm}} = 19,000 \text{ M}^{-1}\text{ cm}^{-1}$ for KChIP3-(65–256) and KChIP3(F218A), $\varepsilon_{280\text{ nm}} = 17,200 \varepsilon_{280\text{ nm}}^{-1}\text{ cm}^{-1}$ for KChIP3(Y174A), $\varepsilon_{280\text{ nm}} = 10,500 \varepsilon_{280\text{ nm}}^{-1}\text{ cm}^{-1}$ for KChIP3-(161–256), and $\varepsilon_{508\text{ nm}} = 63,500 \varepsilon_{508\text{ nm}}^{-1}\text{ cm}^{-1}$ for KChIP3-(65–256)-CCPGCC labeled with FIAsh and corrected for the fraction labeled (22). The concentration of Ca$^{2+}$ and Mg$^{2+}$ was kept at 1 mM and 5 mM, respectively, and 1 mM EDTA or EGTA was added to obtain the calcium-free protein (herein referred as apo-state or apo-form) or Mg$^{2+}$-bound KChIP3 samples, respectively.

**Steady State Fluorescence Measurements**—Steady state emission spectra were recorded using a PCl-ChromosFD spectrofluorometer (ISS). Tryptophan emission spectra were measured using a 295-nm excitation, and titrations were carried out in a 0.1 × 1-cm path length quartz cuvette with excitation along the 0.1-cm path. KChIP3-NS5806 dissociation constants were determined by non-linear fit of the change in integrated fluorescence (310–400 nm) using a single binding site model, $\Delta F = (K_d + \{P_t\} + \{L_t\}) - \sqrt{(K_d + \{P_t\} + \{L_t\})^2 - 4\{P_t\}\{L_t\}} / 2c$ (Eq. 1)

where $\Delta F$ is the intensity change, $K_d$ is the dissociation constant, $n$ is the number of binding sites, $P_t$ is the total protein concentration, $L_t$ is the total ligand concentration, and $c$ is a proportionality constant. For 1,8-ANS displacement studies, the excitation wavelength was set at 350 nm along the 2-mm path of a 0.2 × 1-cm cuvette, and the integrated emission intensity (410–500 nm) of 1,8-ANS bound to KChIP3 was used to calculate the extent of displacement. The dissociation constant of NS5806 for KChIP3 was recovered using a single binding site displacement equation (24), $F_{\text{measured}} = F_{\text{max}} \left[ \alpha + \beta \frac{\{L_t\}}{(K_d + \{L_t\})(1 + \frac{\{L_t\}}{K_d})} \right]$ (Eq. 2)

where $\alpha$ and $\beta$ are proportionality constants, $K_d$ is the dissociation constant of 1,8-ANS (measured separately), $L_t$ is the total concentration of NS5806, and $K_d$ is its dissociation constant.

To investigate the association between KChIP3 constructs and Kv4.3-(2–22) site 1 or Kv4.3-(70–90) site 2, fluorescently labeled peptides were purchased from ThinkPeptides (Sarasota, FL). The fraction of Kv4.3-(2–22)-Ahx-FITC bound to KChIP3 constructs was determined based on the increase in steady state anisotropy and corrected for the increase in total intensity upon complex formation using, $f_b = \frac{r - r_b}{(r - r_b) + \frac{r}{f} (r_b - r)}$ (Eq. 3)

where $f_b$ is the fractional concentration of peptide bound, $r$ is the measured anisotropy, $r_F$ and $r_b$ are the anisotropy of the free and fully bound peptide, respectively (25). Due to the large change in fluorescence intensity of Kv4.3-(70–90)-Ahx-dansyl peptide upon binding to KChIP3 constructs, the fraction bound was determined by the change in integrated fluorescence intensity (450–650 nm) normalized by the maximum intensity change. The resulting titration curves were analyzed using a single site binding model, Equation 1.

For the determination of the calcium dissociation constants, a sample containing 1 μM KChIP3-(65–256) carrying the biarcsenial binding sequence (CCPGCC) at the C terminus and labeled with FIAsh was prepared in 20 mM MOPS, pH 7.4, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, and 1 mM nitrilotriacetic acid. All solutions were previously decalified by treatment with CHELEX resin (Bio-Rad). The amount of residual calcium was determined using Quin-2 and calculated to be ~200 nM. Calcium binding was monitored by the change in integrated fluorescence emission (510–580 nm) upon calcium binding to KChIP3-(65–256)-FIAsh. Calcium was added from a buffered 50 mM CaCl$_2$ stock prepared from a 100 mM CaCl$_2$ standard (Fisher), and the free calcium concentration was determined by obtaining the saturation curves for the calcium indicators Quin-2 ($K_d = 120$ nM), Calcium Green-5N ($K_d = 14$ μM), and CaGreen-2 ($K_d = 550$ nM). The determined free calcium concentrations were in agreement with the values calculated using the pCa calculator software (26). The macroscopic calcium binding constants in the presence of NS5806 were obtained under identical conditions. FIAsh-labeled KChIP3 was excited with a 470-nm laser diode, and the change in fluorescence intensity was used to probe the extent of calcium binding. The resulting titration curves were analyzed using a double-Hill equation, $f_{\text{bound}} = \frac{(f - 1)[Ca^{2+}]_t^{\text{local}}}{K_a^{\text{local}} + [Ca^{2+}]_t^{\text{local}}} + \frac{f[Ca^{2+}]_t^{\text{local}}}{K_b^{\text{local}} + [Ca^{2+}]_t^{\text{local}}}$ (Eq. 4)

where $f_{\text{bound}}$ is the fraction of proteins bound to calcium calculated from the intensity change, $f$ is the fraction of sites B with dissociation constant $K_b$ and showing a Hill coefficient $n_b$, with remaining sites showing a dissociation constant of $K_a$ and Hill coefficient $n_a$.

**Fluorescence Lifetime and Modulated Anisotropy Measurements**—Tryptophan fluorescence decay lifetimes were measured using a ChronosFD spectrofluorometer (ISS, Champaign, IL) in the frequency domain mode. Tryptophan was excited with the frequency modulated light (280-nm diode), and emission was collected using a 305-nm long pass filter and 400-nm short pass filter (Andover). The modulated anisotropy of tryptophan was calculated from the amplitude ratio of the parallel and perpendicular components of the modulated emission. The fluorescence decay lifetime of 1,8-ANS was determined by excitation with a 370-nm output of a frequency-modulated laser diode, and the emission was collected through 400–600-nm wide pass filters (Andover). Modulation-phase data were best fitted by a multiple-exponential decay model using GlobalsWE software, and the $\chi^2$ parameter was used as criterion for goodness of fit (27).
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**Isothermal Titration Calorimetry (ITC) Measurements—** Thermodynamic parameters for NS5806 binding to KChIP3-(65–256) and KChIP3-(161–256) were determined using a VP-ITC titration calorimeter (Microcal Inc. Northampton, MA). ITC buffer (20 mM MOPS, pH 7.4, 0.25 mM DTT, and 100 mM NaCl) was prepared using decalcified water filtered through Chelex-100 resin (Bio-Rad). The protein stock solution was dialyzed against ITC buffer overnight. To minimize artifacts from mismatched buffers, stock NS5806 solutions were prepared in the final ITC dialysate buffer. The cell sample and injection syringe were extensively cleaned with decalcified water and then with ITC buffer. The reaction cell was loaded with 10 μM KChIP3 (65–256 or 161–256) solution, and the concentration of NS5806 in syringe was 473 μM. 30 injections (9 μl each) of NS5806 were titrated into protein solution with 2-min intervals between injections. Parallel experiments were carried out for titration of NS5806 into the dialysate buffer as a control for heats of dilution of ligand. The temperature was kept at 25 °C, and stirring speed was at 307 rpm. ITC data were analyzed using Origin 7 ITC data analysis software (OriginLab Corp., Northampton, MA). The data obtained for NS5806 binding to both KChIP3 constructs were analyzed using a two binding site model (28).

**Molecular Modeling—** The NS5806 binding sites on KChIP3 surface were identified using AutoDock 4.2 software (29). The structure of Ca2+-bound KChIP3-(65–256) (PDB entry 2JUL, conformation # 1) and KChIP3-(161–256) (PDB entry 2E6W) were used as a rigid macromolecule, and docking grids were set to cover the entire protein surface (15, 30). The most favorable docking site among 200 docking simulation was selected based on their energy rankings. The structure of the NS5806-KChIP3 complex was further refined by running 10 ns of molecular dynamic simulation using AMBER03 force fields on the YASARA interface at 298 K, with a dynamic time step of 1.25 fs and trajectories recorded every 25 ps for further analysis (31). The molecular model for the KChIP3-Kv4.3 T1 domain was constructed by combining the crystal structure of KChIP1-Kv4.3 T1 domain (PDB entry 2NZ0) (32) and the KChIP3 or the NS5806-KChIP3 complex before molecular dynamic simulation. The energy-minimized KChIP3-Kv4.3 T1 domain or NS5806-KChIP3-Kv4.3 T1 domain complexes were then subjected to a 10-ns molecular dynamics simulation. The resulting protein and drug-protein complexes were aligned using the MUSTANG algorithm on the YASARA interface.

**RESULTS**

NS5806 Binds at the C Terminus of KChIP3—The Kv4-mediated current amplitude increase and slowing of inactivation associated with NS5806 have been reported to be enhanced or 161–256) solution, and the concentration of NS5806 in syringe was 473 μM. 30 injections (9 μl each) of NS5806 were titrated into protein solution with 2-min intervals between injections. Parallel experiments were carried out for titration of NS5806 into the dialysate buffer as a control for heats of dilution of ligand. The temperature was kept at 25 °C, and stirring speed was at 307 rpm. ITC data were analyzed using Origin 7 ITC data analysis software (OriginLab Corp., Northampton, MA). The data obtained for NS5806 binding to both KChIP3 constructs were analyzed using a two binding site model (28).

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**RESULTS**

NS5806 Binds at the C Terminus of KChIP3—The Kv4-mediated current amplitude increase and slowing of inactivation associated with NS5806 have been reported to be enhanced or dependent on the presence of the auxiliary KChIP proteins (11, 21). In this study we probe whether this dependence is due to direct interaction between NS5806 and the neuronal calcium sensor KChIP3. NS5806 interaction with KChIP3 was initially characterized by monitoring the steady state tryptophan fluorescence emission of KChIP3 upon the addition of NS5806. The single tryptophan residue (Trp-169) found in KChIP3-(65–256) sequence is located at the interface between EF-3 and EF-2, and thus its emission may probe NS5806 association to either N- or C-terminal domains. Upon the addition of a 2× excess of NS5806, a significant decrease in tryptophan emission intensity was observed (Fig. 1a). The quenching effect was present in the apo-state as well as in the calcium-bound state of KChIP3. The absorption spectrum of NS5806 in 20 mM MOPS buffer shows an absorption peak at 340 nm with an extinction coefficient of 105 M-1 cm-1. This absorption band overlaps with the Trp-169 emission spectrum, suggesting that resonance energy transfer between Trp-169 and NS5806 is responsible for the observed Trp emission quenching.

To determine the KChIP3 affinity for NS5806, the Trp emission intensity was plotted as a function of NS5806 concentration of KChIP3-(65–256) (Fig. 1b), and the dissociation constants were recovered using Equation 1 (see Table 3). The stoichiometry of the model was set to 1 as analysis of the tryptophan fluorescence by the continuous variation method yielded a stoichiometry of one to one (data not shown). The recovered dissociation constants show that in the Ca2+-bound form, KChIP3-(65–256) binds NS5806 with a dissociation constant of 4.4 ± 0.3 μM, whereas a separate experiment using the truncated C terminus domain, KChIP3-(161–256), shows roughly a 2-fold affinity increase to Kd = 2.7 ± 0.3 μM (data not shown). Upon Ca2+ removal, the affinity of KChIP3-(65–256) for NS5806 decreases, Kd = 30 ± 2 μM, whereas the affinity of KChIP3-(161–256) is reduced to Kd = 26 ± 4 μM. Because both constructs show similar affinities for NS5806 in the calcium-bound form and apo-state we propose that the C terminus of KChIP3 is the binding site for NS5806.

It is interesting to note that the decrease in fluorescence of apoKChIP3-(65–256) upon the addition of NS5806 is about twice as large as that observed for apoKChIP3-(161–256) (data not shown), and we hypothesize that this could be due to a structurally different conformation of the isolated C terminus in the apo-state. To investigate if this is the case, the quenching data were analyzed using a modified Stern-Volmer equation, which accounts for the presence of tryptophan residues that may not be quenched by NS5806 (Fig. 1d). The results show that both KChIP3-(65–256) and KChIP3-(161–256) in the apo-form bind to NS5806 with the same affinity and that the observed differences in the extent of quenching are due to the fact that in the apo-state, KChIP3-(161–256) populates a second conformation (~40%) that is not quenched by NS5806. This additional population may be present due to a lower stability of the truncated C terminus domain.

To probe if resonance energy transfer is responsible for the observed fluorescence quenching, we measured the tryptophan fluorescence lifetime of both KChIP3 constructs in the calcium-bound form. The average lifetime of Trp-169 decreases from 3.3 ns to ~1.3 ns in Ca2+-KChIP3-(65–256) and from 4.3 ns to 3.2 ns in Ca2+-KChIP3-(161–256) as the concentration of NS5806 is increased (shown in Fig. 1c). The fluorescence lifetime decrease indicates that the quenching mechanism happens during the excited state of tryptophan and correlates well with a dynamic quenching process such as resonance energy transfer.

Furthermore, it is possible to use the fluorescence emission spectra of both KChIP3 constructs, the tryptophan fluorescence quantum yield, and the absorbance spectra of NS5806 while assuming a random NS5806-Trp-169 orientation to obtain the overlap function and calculate the Förster distance...
between the NS5806-Trp-169 pair for KChIP3-(65–256) and KChIP3-(161–256). The Förster distances were calculated to be 12 Å and 14 Å for the apo-form KChIP3-(65–256) and KChIP3-(161–256), respectively, whereas a Förster distance of 12 Å was recovered for the Ca²⁺/H₉₂₆₀ bound form of KChIP3-(65–256) and 13 Å for Ca²⁺/H₉₂₆₀ KChIP3-(161–256) (Table 1). Due to the location of Trp-169 within the protein matrix, a random orientation value for H₉₂₆₀ is not appropriate. However, an upper (H₁₁₀₀₂ max) and lower (H₁₁₀₀₂ min) limit for the orientation factor can be obtained from measurement of the frequency-modulated anisotropy of Trp-169 for both KChIP3 constructs (data not shown). These values were used to calculate the upper and lower limits for NS5806-Trp-169 distances as shown in Table 1. The recovered NS5806-Trp-169 distances using fluorescence emission and fluorescence lifetime of Trp-169 range from 5 Å to 13 Å and from 8 Å to 20 Å, respectively. The larger values for the lifetime measurements support the presence of an additional static quenching process induced by binding of NS5806, which results in smaller distances being resolved in the steady state quenching measurements. Additional contribution due to quenching of residual tyrosine fluorescence cannot be ruled out. Overall, these results show that NS5806 binds at the C terminus of KChIP3 near EF-hands 3 and 4 and quenches the single tryptophan residue through resonance energy transfer. NS5806 also showed binding and quenching of tryptophan residues on a Kv4.3-(1–152)-GST fusion protein; however, the dissociation constant for binding to this construct was 11 M (Fig. 1, e and f). The low solubility of the full T1 domain prevented us from further characterization of this interaction and from unequivocally showing that NS5806 binds at the T1
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FIGURE 2. a, fluorescence emission of 1,8-ANS–KChIP3-(65–256) complexes as a function of increasing concentrations of NS5806; the inset shows the shift in emission spectra maximum as a function of NS5806, b, replacement curves for the 1,8-ANS–KChIP3-(65–256) complex in the apo- and calcium-bound states as a function of NS5806 concentration. The dissociation constants recovered using Equation 2 are $K_d = 23 \pm 7 \mu M$ and $K_d = 2.5 \pm 0.3 \mu M$ for apo- and calcium-bound forms, respectively. Conditions: 95 mM 1,8-ANS, 10 mM Ca$^{2+}$-KChIP3-(65–256) in 20 mM MOPS, pH 7.4, and 100 mM NaCl. a.u., arbitrary units. c, change in fluorescence lifetime pre-exponential parameters $\alpha_1$ and $\alpha_2$ as a function of NS5806 concentration. Pre-exponential parameters were recovered using a triple discrete exponential pre-exponential parameters $\alpha_1$ and $\alpha_2$ as a function of NS5806 concentration. Pre-exponential parameters were recovered using a triple discrete exponential decay model from analysis of emission decay data of 10 mM 1,8-ANS bound to 10 mM KChIP3-(65–256) in the apo-form and Ca$^{2+}$-bound state. The lifetime of unbound ANS was set as a fixed value at 0.28 ns (measured separately), whereas the second and third decay lifetimes were set as linked variables with lifetimes of 5.7 ± 0.4 ns and 15.4 ± 0.9 ns in the apo-form ($\chi^2 = 2.7$) and 6.3 ± 0.5 ns and 16.7 ± 0.5 ns in the calcium-bound state ($\chi^2 = 1.7$).

domain. Nonetheless, based on the more favorable binding of NS5806 with KChIP3, it is likely that the site on KChIP3 is populated before binding at the T1 domain.

NS5806 Binding on KChIP3 Displaces 1,8-ANS Bound at the Hydrophobic Cavity Near EF-hand 4—Previously, we have identified two binding sites for the solvent-sensitive fluorophore 1,8-ANS at the hydrophobic cavities located on the C terminus of KChIP3-(65–256) as well as on KChIP3-(161–256) (33). To determine the nature of interactions between NS5806 and KChIP3, displacement studies were carried out in the presence of the hydrophobic fluorophore 1,8-ANS. Upon binding of 1,8-ANS to calcium-bound KChIP3-(65–256), an intense fluorescence emission at 475 nm was observed. The emission intensity of bound 1,8-ANS decreased sharply as the concentration of NS5806 was increased, indicating that NS5806 displaces 1,8-ANS from the hydrophobic cavity. The decrease in 1,8-ANS emission intensity was accompanied with a red shift of the emission maxima from 475 to 484 nm (Fig. 2a). Using the decrease in fluorescence intensity and the previously determined affinities of 1,8-ANS for KChIP3-(65–256), the affinities for NS5806 binding to KChIP3-(65–256) were determined (Equation 2) to be $K_d = 23 \pm 7 \mu M$ in the apo-form and $K_d = 2.5 \pm 0.3 \mu M$ in the Ca$^{2+}$-bound form (Fig. 2b), which are in good agreement with the quenching data.

Previously we identified that 1,8-ANS binds to at least two different hydrophobic sites on KChIP3, each bound 1,8-ANS characterized by distinct lifetimes. Therefore, it is possible to employ the fluorescence lifetime of 1,8-ANS in the presence of KChIP3-(65–256) and the associated pre-exponential factors to pinpoint the NS5806 binding site. The 1,8-ANS–Ca$^{2+}$-KChIP3-(65–265) complex exhibits a fluorescence decay that is best modeled by the sum of three exponential decays. We associated the fastest lifetime ($\tau_0 = 0.28$ ns) as corresponding to unbound 1,8-ANS. The two additional decay lifetimes correspond to 1,8-ANS binding to a partially polar cavity on the protein surface with lifetime $\tau_1 \sim 6$ ns and 1,8-ANS bound to a predominantly hydrophobic site/cavity on the KChIP3 surface with a lifetime of $\tau_2 \sim 16$ ns. The addition of NS5806 to the 1,8-ANS–Ca$^{2+}$KChIP3 complex did not alter the 1,8-ANS lifetimes; however, a systematic decrease in the pre-exponential factor for the long lifetime ($\alpha_2$) and an increase of the pre-exponential parameter for 1,8-ANS in solution ($\alpha_0$) were observed, whereas the pre-exponential factor for 1,8-ANS bound at the site with 6-ns lifetimes ($\alpha_1$) remained fairly constant (Fig. 2c). These results indicate that NS5806 binds to the hydrophobic site/cavity on KChIP3, which results in a displacement of 1,8-ANS bound at that site.

NS5806 Binding to KChIP3 Is Enthalpy-driven—The thermodynamic properties associated with the formation of NS5806–KChIP3 complexes were probed using isothermal calorimetry. Isotherms for the titration of NS5806 into Ca$^{2+}$ KChIP3-(65–256) and Ca$^{2+}$ KChIP3-(161–256) in the
presence of 5 mM Mg\(^{2+}\) are shown in Fig. 3. The isotherms were analyzed using a two-site binding model (solid line). The binding of NS5806 to KChIP3 was characterized with two dissociation constants with the specific binding site associated with a \(K_d\) of 0.8 \(\mu M\) and a nonspecific binding site that showed a very weak affinity with \(K_d \approx 150 \mu M\) (data not shown). This weak binding site was not observed in the Trp-169 quenching and 1,8 ANS displacement studies, probably due to an inefficient quenching or different binding site than that of 1,8-ANS. ITC thermodynamic parameters for NS5806 specifically binding to KChIP3 constructs are summarized in Table 2. The association of NS5806 to KChIP3-(65–256) and KChIP3-(161–256) exhibited an exothermic binding with \(\Delta H = -4.4 \text{ kcal mol}^{-1}\) and \(\Delta H = -6.9 \text{ kcal mol}^{-1}\), respectively. Association of NS5806 to KChIP3-(65–256) showed different enthalpic and entropic contributions than KChIP3-(161–256), which may indicate that the structural rearrangements induced by NS5806 are not localized solely on the C terminus domain. An exothermic interaction indicates that the overall interaction may involve a structural reorganization of the protein-drug complex upon association with the NS5806.

**NS5806 Increases the Affinity between Kv4.3 Site 1 and KChIP3 and Abolishes Its Calcium Dependence**—Previous structural and electrophysiological studies have identified that the T1 domain at the N terminus of Kv4 mediates binding of KChIPs (6, 34). The association of KChIP1 was also shown to involve two binding sites on the Kv4.2 T1 domain, site 1 comprising mainly nonpolar residues 2–22 and the more polar site 2 on the T1 domain surface consisting of residues 70–90 (35). Due to the poor solubility of the T1 domain of Kv4.3 (residues 1–152), we decided to investigate the interactions by employing the fluorescently labeled peptides Kv4.3-(2–22)-Ahx-FITC and Kv4.3-(70–90)-Ahx-Dansyl. A representative titration curve for KChIP3 binding to site 1 is shown in Fig. 4a, with the recovered parameters listed on Table 3.

Binding of site 1 to Ca\(^{2+}\) KChIP3-(65–256) was marked by an increase in anisotropy from \(r = 0.072\) to \(r = 0.238\) and a strong calcium dependence with a dissociation constant for Kv4.3 site 1 in the apo-form of \(K_d = 70 \pm 3 \mu M\) and \(K_d = 2.7 \pm 0.1 \mu M\) in the calcium-bound form. A stronger calcium dependence was observed for binding of site 2 to KChIP3-(65–256), with a dissociation constant of \(\sim 500 \mu M\) in the apo-state and \(K_d = 10 \pm 1 \mu M\) upon calcium binding. Similarly, binding of Site 1 to the KChIP3-(161–256) construct, which lacks the N terminus domain, showed an increase in anisotropy to \(r = 0.230\) in the presence of calcium as well as a strong calcium dependence with \(K_d \sim 450 \mu M\) in the apo-form to \(K_d = 24 \pm 1 \mu M\) in the calcium-bound form. On the other hand, binding of site 2 to calcium-bound KChIP3-(161–256) was weak (\(K_d \sim 390 \mu M\)), indicating that binding of this peptide is specific to the N terminus domain as proposed previously (32, 36). Furthermore, upon the addition of 150 \(\mu M\) NS5806, the binding affinity of

### TABLE 2
Thermodynamic parameters for NS5806 binding to Ca\(^{2+}\)KChIP3 constructs determined using ITC

| KChIP3 construct | \(K_d\) | \(\Delta H\) | \(-T\Delta S\) |
|------------------|---------|-------------|--------------|
| KChIP3-(65–256)  | 0.8 ± 0.4 | -4.4 ± 0.8 | -3.9 |
| KChIP3-(161–256) | 2.5 ± 1.0 | -6.9 ± 0.7 | -1.3 |

### TABLE 3
Dissociation constant for Kv4.3-(2–22) site 1 and Kv4.3-(70–90) site 2 binding to KChIP3 constructs

| KChIP3 construct | NS5806 \(K_d\) | Site 1 \(K_d\) | Site 2 \(K_d\) |
|------------------|----------------|----------------|----------------|
|                  | \(\mu M\)       |                |                |
| KChIP3-(65–256)  | Apo 30 ± 2     | 70 ± 3         | 1.9 ± 0.1      |
|                  | Ca\(^{2+}\) 4.4 ± 0.3 | 2.7 ± 0.1 | 1.9 ± 0.2 |
|                  | KChIP3-(161–256) | Apo 26 ± 4 | ~450 | 5.6 ± 0.2 |
|                  | Ca\(^{2+}\) 2.7 ± 0.3 | 24 ± 1 | 5.3 ± 0.3 |
|                  | KChIP3-(Y174A) | Apo 17 ± 0.8 | 90 ± 16 | 25 ± 3 |
|                  | Ca\(^{2+}\) 8.3 ± 0.8 | 6.9 ± 0.4 | 3.1 ± 0.2 |
|                  | KChIP3-(F218A) | Apo 28 ± 3 | 90 ± 31 | 130 ± 28 |
|                  | Ca\(^{2+}\) 13 ± 1.3 | 21 ± 0.2 | 7.8 ± 0.3 |

* Determined by the integrated intensity decrease of Trp-169 and fitted using Equation 1.
* Determined using steady state anisotropy change and corrected for change in fluorescence intensity using Equation 3.
* Determined by the integrated intensity increase of dansyl labeled peptide upon binding.
Interestingly, in the presence of Ca\(^{2+}\), the association rate for the fast and slow phase of site 1 binding kinetics of Kv4.3 site 1 to KChIP3-(65–256) and KChIP3-(161–256); a representative trace is shown on Fig. 4 and legend. On the other hand, the addition of Ca\(^{2+}\) restricted access to the hydrophobic cavity (Fig. 4 and legend). The association rate constants for the fast phase showed an increase upon binding of calcium from 0.02 in the apo-form to 0.13 in the calcium-bound state. The parameters of the fluorescence intensity modulation were mainly unaffected by binding of calcium. An increase in the pre-exponential factor associated with the slow phase rate of the site 1 peptide.

The change in fluorescence intensity associated with binding of Kv4.3 site 1 to KChIP3-(161–256) was also best modeled with a double exponential function (Table 4). Similarly to KChIP3-(65–256), the association rate constants for the fast phase were mainly unaffected by binding of calcium. An increase in the pre-exponential factor associated with the slow phase rate of the site 1 peptide.

The calculated dissociation rates decreased upon the addition of NS5806 and became partially calcium-independent with \(k_{\text{off}} = 24 \pm 3 \times 10^{-3} \text{s}^{-1}\) and \(k_{\text{slow}} = 0.03 \pm 0.01 \times 10^{-3} \text{s}^{-1}\) in the apo-form and \(k_{\text{fast}} = 17 \pm 2 \times 10^{-3} \text{s}^{-1}\) and \(k_{\text{off}} = 0.02 \pm 0.01 \times 10^{-3} \text{s}^{-1}\) in the presence of calcium.

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identify the docking site of 1,8-ANS to KChIP3-(65–256) and KChIP3-(161–256) and found that the same hydrophobic cavity at the C terminus was the most favorable binding site (33). The fact that docking simulations identify the same docking site for 1,8-ANS and NS5806 correlate well with the 1,8-ANS displacement studies shown above. The direct role of Phe-218 and Tyr-174 in binding of NS5806 was confirmed by independent mutation of these residues to alanine. We observed that mutation of either residues results in a 2–3-fold decrease in the affinity of NS5806 in the calcium-bound state, whereas the affinity in the apo-state for the Tyr-174 mutant increases about 2-fold to 17 ± 0.8 μM (Table 3). The binding affinity in the presence of calcium of the Kv4.3 site 1 peptide decreased 3-fold for the Tyr-174 mutant while remaining unchanged for the Phe-218 mutant. In the presence of NS5806, KChIP3(Y174A) showed an increased affinity for the site 1 peptide in the apo- and calcium-bound forms but still weaker than KChIP3-(65–256). On the other hand, KChIP3(F218A) in the presence of NS5806 showed lower affinity for the site 1 peptide in both apo and calcium states. Overall, these results support the docking simulation and the idea that these hydrophobic residues play an important role in NS5806 binding and its observed effect on Kv4.3 site binding.

Furthermore, given that the binding site of NS5806 is located in the vicinity of the EF-hand 3 and 4, we tested whether drug association alters the KChIP3 interactions with Ca2+. Titration curves for Ca2+ binding to KChIP3-(65–256)-FlAsH and NS5806:KChIP3-(65–256)-FlAsH are shown in Fig. 6; the resulting titration curves were best analyzed using a model that assumes two protein populations in the apo-state, each with at least two calcium binding sites (Equation 4). The recovered parameters show a dual population of KChIP3-(65–256)-FlAsH in the apo-state with the larger population (f = 0.82) having a macroscopic binding constant Kd = 170 ± 20 nM (Hill coefficient nH = 1.5) and the minor population having a Kd = 10 ± 2 μM (Hill coefficient nH = 1.1). The apparent dissociation constants are comparable to the calcium binding constants reported in previous studies, ranging from 0.1 to 10 μM (14, 37), indicating that the addition of the amino acid sequence CCVPGCC at the C terminus and labeling with the biarsenical fluorophore FlAsH did not alter the calcium affinity. Titration of calcium into KChIP3-(65–256)-FlAsH in the presence of 150 μM NS5806 resulted in titrations curves that were also best fitted with a double Hill equation. The macroscopic binding constant recovered for the high affinity sites was Kd = 120 ± 20 nM (Hill coefficient nH = 1.1) with f = 0.66 and a dissociation constant for low affinity sites of Kd = 21 ± 4 μM (Hill coefficient nH = 1.4), indicating that the overall calcium affinity of KChIP3-(65–256) is marginally affected by binding of NS5806.

**DISCUSSION**

The precise mechanisms underlying the gating regulation of Kv4-mediated K+ currents are yet to be completely understood; however, it is widely accepted that these channels do not undergo open state inactivation involving the N terminus (38–40). It has also been shown that the association of KChIPs at the N terminus of Kv4 channels impairs open-state inactivation (41). Electrophysiological studies support the idea that both the N and C termini of Kv4 channels interact to regulate the inactivation kinetics (40, 42), opening the possibility that such interactions are modulated by binding of KChIPs at the N terminus (34, 38). It is also proposed that the N terminus functions as a membrane transport control, which in the absence of KChIPs anchors Kv4 channels near the perinuclear region of the cell (43, 44). The association of KChIPs at the T1 domain of Kv4 results in translocation to the membrane and a concomitant increase in total K+ current. In addition, the presence of KChIPs is necessary for recovery of K+ current with similar biophysical properties as those found in vivo.

Recent reports by Calloe (17) on the ITO-activating properties of a novel diphenyl urea compound (NS5806) whose activ-
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...ivity depends on the presence of KChIPs has highlighted the role of these auxiliary proteins in regulation of I_{to} currents. This drug was shown to reverse the effect of induced heart failure in canine models (17, 19). On the other hand, Wittel et al. (11) hypothesized that the observed current inhibition in hippocampal neuronal cells was likely due to a higher concentration of NS5806 used (20 μM) compared with the concentration used in canine cardiomyocytes. Indeed, at NS5806 concentrations >100 μM a reversal of activation was observed in HEK293 cells. This highlights the possibility of multiple binding sites of NS5806 on the Kv4- auxiliary subunit complex. Nonetheless, NS5806 still resulted in a slowing of inactivation and accelerated recovery from inactivation in both cardiac and neuronal cells at concentrations below 20 μM.

Given the dependence of K+ current modulation by NS5806 on the presence of KChIPs, in this report we investigated whether KChIPs could be a target binding partner for NS5806. Titration data show that both the apo- and calcium-bound forms of KChIP3-(65–256) were quenched by NS5806 with an affinity 5-fold lower in the apo-form. The hydrophobic nature of NS5806 supports the idea that the binding site on KChIP3 is located in a solvent-restricted hydrophobic cavity and that calcium binding to KChIP3 increases accessibility of the drug to this cavity. Moreover, the similar dissociation constants recovered for both KChIP3 constructs indicate that in the calcium-bound and apo-state of the protein the binding site of NS5806 is located at the C terminus. Interestingly, the dissociation constants recovered for NS5806 binding to Ca2+ KChIP3-(65–256) are very similar to the EC50 value observed for current potentiation in CHO-K1 cells expressing Kv4.3/KChIP2 (EC50 = 5.3 μM) (21) as well as in HEK293 cells expressing Kv4.2/KChIP3 (EC50 = 6.9 μM) (11). Displacement studies of the extrinsic fluorophore 1,8-ANS bound to KChIP3-(65–256) confirm that NS5806 binds at a hydrophobic cavity. The emission spectrum of 1,8-ANS bound to KChIP3 shows a maximum at 475 nm. The addition of NS5806 to 1,8-ANS-KChIP3 complexes resulted in a fluorescence intensity decrease in the presence or absence of calcium as shown in Fig. 2b. Interestingly, upon the addition of ~40 μM NS5806, the total emission intensity of 1,8-ANS-KChIP3 complex is identical in the presence or absence of calcium. A fluorescence signal is still detected even at saturating amounts of NS5806 (150 μM). This is likely due the presence of additional 1,8-ANS bound to a site distinct from that occupied by NS5806. Furthermore, the emission maxima of 1,8-ANS bound to calcium or apo-form KChIP3-(65–256) in the presence of 150 μM NS5806 shows a maximum at 485 nm (Fig. 2a, inset). These results are in agreement with fluorescence lifetime data which show that the addition of NS5806 results in a concomitant decrease in the pre-exponential parameter α1 associated with 1,8-ANS bound at the solvent-restricted cavity (Fig. 2c), whereas the pre-exponential parameter that corresponds to 1,8-ANS bound to a solvent-exposed site with emission maximum at 485 nm remains constant. These results suggest that 1,8-ANS binds at a physiologically active hydrophobic cavity on KChIP3 and that displacement of 1,8-ANS could be used as a fluorescence-based high throughput method for the discovery of new drugs that bind at this hydrophobic cavity and could induce similar effects to that observed for NS5806. Indeed, an analogous 1,8-ANS screening assay has been previously reported for other drug targets (45).

Isothermal calorimetric titrations of NS5806 into KChIP3 further confirm the strong association between NS5806 and KChIP3. The recovered thermodynamic parameters show that both enthalpic and entropic contributions stabilize the complex formation, with the enthalpic contribution becoming dominant in the KChIP3-(161–256) construct. The large enthalpy contribution is in contrast to the idea that the binding site of NS5806 on KChIP3 is mainly hydrophobic (entropy-driven binding); however, it is likely that binding of NS5806 triggers larger exothermic structural rearrangements on the protein.

Characterization of the interaction of KChIP3 and peptides homologous to Kv4.3-(2–22) and Kv4.3-(70–90) indicate that the interactions between site 1/site 2 and KChIP3 are modulated by calcium (Table 3). In the presence of calcium KChIP3-(65–256) showed a 26-fold increase in affinity for site 1, whereas KChIP3-(161–256) showed an 18-fold increase compared with the apo-form. Furthermore, the dissociation constant recovered for site 1 binding to KChIP3-(161–256) in the presence of calcium was about 10-fold weaker than that of site 1 binding to KChIP3-(65–256). The determined dissociation constants can be utilized to calculate the role of the N- and C-terminal domains of KChIP3 on binding of site 1 of Kv4.3. The energetic contribution of the N and C termini of KChIP3 to the association with site 1 calculated from the dissociation constants in the calcium-bound state are −1.3 and −6.1 kcal mol−1, respectively. The larger contribution of the C terminus highlights the role of this domain on KChIP3 as mediator in protein-protein interaction. Interestingly, the Y134E mutation on KChIP1 (Y174E on KChIP3) has also been shown to completely abolish current modulation by KChIP1 (46). However, the contribution of the N terminus hydrophobic cavity of KChIPs is necessary for Kv4 translocation to the membrane and KChIP-Kv4 complex formation (47, 48). This is in agreement with structural studies, where the N terminus of Kv4.3 was determined to bind across the hydrophobic face of KChIP1, contacting residues spanning both the N and C termini of KChIP1 (32, 36). The dissociation constant for site 2 binding to KChIP3-(65–256) was also dependent on calcium, with an ~50-fold increase in affinity but still weaker than the site 1 interaction. The fact that calcium binding at the C terminus EF-3 and EF-4 of KChIP3 modulates binding of site 2 is remarkable, suggesting that the structural changes associated with the calcium binding to the C-terminal domain are propagated into the N-terminal domain of the protein. However, it is also possible that the calcium-induced dimerization of KChIP3 is responsible for the increase in affinity.

The addition of saturating amounts of NS5806 completely abolished the calcium dependence of site 1 association with KChIP3 as well as further increased its affinity. These results are surprising given that the experiments above support the idea that NS5806 and site 1 share the same binding site at the C terminus of KChIP3. Furthermore, a clear dose-dependent increase in KChIP3-site 1 association was observed upon the addition of NS5806 in the absence of calcium (Kd = 21 ± 1 μM), but no further increase was observed upon the addition of...
excess calcium. On the other hand, at saturating amounts of calcium, the addition of NS5806 induced a further increase in anisotropy, suggesting that in the presence of NS5806 the KChIP3-site 1 complex adopts an altered tertiary structure, as evident from the larger anisotropy upon binding in the presence of NS5806 (Fig. 4b). These results are comparable with those obtained for the Kv4.3(1–143)-KChIP1 complex using size exclusion chromatography in the presence of the diphenylurea compound CL-888 (49). A similar effect due to the presence of NS5806 for site 1 bound to KChIP3-(161–256) on anisotropy was observed, indicating that structural rearrangement is localized on the C terminus domain of KChIP3.

A better understanding of the mechanism by which NS5806 modulates the affinity of site 1 peptide binding to KChIP3 construct is gained from the kinetics associated with binding (Table 4). The biphasic nature of the binding kinetics is potentially due to the presence of KChIP3 proteins that populate partially distinct conformations or oligomerization state. Overall, these results revealed that saturating amounts of NS5806 decreased the rate of dissociation between KChIP3 and site 1 of Kv4.3 independent on the presence of calcium. A possible explanation for the stabilization of the protein peptide complex is that binding of NS5806 at the C-terminal cavity destabilizes this domain and increases the accessibility of the hydrophobic residues to interact with hydrophobic residues on site 1. Also, the fact that the affinity of KChIP3 in the apo-form for NS5806 is lower than in the presence of calcium indicates that there is a larger energy barrier for access to the hydrophobic cavity at the C terminus. However, once NS5806 binds at the hydrophobic cavity in the apo-form, the resulting structural changes are comparable to those present in the calcium-bound form. This results in similar affinity and dissociation constants for the N terminus of Kv4.3. On the other hand, the observed decrease in dissociation rate in the presence of NS5806 seems to indicate that the KChIP3-NS5806 populates a conformation that is different from that of the apo-form and calcium-bound KChIP3 and forms a more stable complex with site 1 of Kv4.3.

Computational simulation allowed us to pinpoint the potential docking site for NS5806 as being at a hydrophobic cavity between EF-3, EF-4, and the H10 helix. This docking site is also in good agreement with the distances from Trp-169 determined using resonance energy transfer and with the displacement studies. However, previous docking studies of a similar diphenyl-urea compound (CL-888) identified a site on the hydrophilic surface of KChIP1 (49). The proposal that this is the docking site for NS5806 is not supported by the fact that similar dissociation constants were recovered for KChIP3-(65–256) and KChIP3-(161–256) even though the C-terminal construct lacks half of the residues involved in the proposed docking site for CL-888. We also conducted a docking simulation with NS5806 where we limited the docking grid to cover only the proposed binding site at the hydrophilic surface. The predicted

FIGURE 7. Reorganization of the hydrophobic pocket in the KChIP3 C terminus upon binding of NS5806 and/or site 1. Top left, position of helix-10 (shown in yellow) in the C-terminal hydrophobic pocket of KChIP3-(65–256) is stabilized by stacking interactions among Phe-218, Phe-252, and Tyr-174. Top right, binding of NS5806 to the KChIP3-(65–256) leads to the displacement of Phe-252, and NS5806 is stabilized by hydrophobic interactions with Phe-218 and Tyr-174. Bottom left, orientation of site 1 in the C-terminal hydrophobic pocket of KChIP3-(65–256) with a focus on the interaction of W19 from site 1 with hydrophobic residues from KChIP3-(65–256). Bottom right, the reorientation of the hydrophobic domain at the KChIP3 C terminus upon concomitant binding of site 1 and NS5806.
association energy of this site was 4 kcal mol$^{-1}$ weaker than the predicted energy for the docking at the hydrophobic cavity near EF-3 and EF-4. Indeed, the role of Tyr-174 and Phe-218 identified in docking simulation as being involved in stabilization of NS5806 and Kv4.3 site 1 peptide interactions is supported by site-directed mutagenesis studies, which show that mutation of either residue to alanine completely abolishes the effect of NS5806 (Fig. 5c). We also observed that a KChip3(F252A) mutant readily forms aggregates (data not shown), supporting the idea that this residue plays an important role in the stability of the protein.

Therefore, we propose that binding of NS5806 at the C-terminal hydrophobic cavity results in an increase in flexibility of the H10 helix that is likely facilitated by displacement of the hydrophobic residues on this helix. This is supported by molecular dynamic simulations which show that in the absence of NS5806 the aromatic residue Phe-252 on the H10 helix is stacked between Tyr-174 and Phe-218, inherently reducing the flexibility of this helix (Fig. 7). In contrast, simulations in the presence of NS5806 show a displacement of Phe-252 residue and concomitant motion of the H10 helix to accommodate NS5806 in the hydrophobic cavity. Furthermore, simulation of the Kv4.3-KChIP3 complex show that Trp-19 of Kv4.3 is positioned in an identical stacking position between Tyr-174 and Phe-218 as that found for Phe-252 and NS5806, whereas in the presence of NS5806 the cavity at the C terminus of KChIP3 expands to accommodate both NS5806 and the hydrophobic N terminus of Kv4.3 while pushing Trp-19 deeper into the hydrophobic cavity (Fig. 7). The motion of Trp-19 into the hydrophobic cavity of the KChIP3 C terminus may contribute to the slower rate of dissociation of the hydrophobic site 1 peptide in the presence of NS5806. The structural rearrangement observed using molecular dynamics is consistent with the idea that binding of NS5806 induces structural rearrangements in the Kv4.3-KChIP3 protein complex. Whether these structural changes are isolated on KChIP3 and the N terminus of Kv4.3 or they propagate across the T1 domain remains to be determined. Previous studies favor the idea that such structural changes could propagate toward the K$^+$ channel inner vestibule near the pore and modulate the K$^+$ currents (50, 51).

The results presented here could potentially explain the observed increase in K$^+$ current and the slowing of inactivation in the presence of NS5806 in cardiac, neuronal, and heterologous expression studies. The elimination of the calcium dependence of association between KChIP3 and site 1 of Kv4.3 as well as the increase in affinity in the presence of NS5806 support the idea that the observed increase in K$^+$ current amplitude is due to an enhanced translocation of Kv4.3-KChIP3 protein complexes to the cell membrane induced by the presence of NS5806. We further hypothesize that the resulting decrease in inactivation kinetics is due to the decrease in accessibility of the N terminus of Kv4.3 channel to interact with the cytoplasmic C terminus of Kv4. Indeed, this interaction has been shown to be necessary for modulation of gating currents of Kv4.2 (35). The fact that the dissociation constant was also reduced in the presence of calcium also highlights the potential role of this ion in K$^+$ current regulation. In support of the role of calcium in regulating K$^+$ current are studies which have shown that in rat stellate cells a protein complex is formed involving the voltage-gated calcium channel Cav3, the potassium channel Kv4.2, and the auxiliary protein KChIP3 (52).

Addendum—A recent report also reported that the association constant between KChIP1/KChIP4 and the T1 domain of Kv4.3 is enhanced 10-fold in the presence of calcium (53).

REFERENCES

1. Szabó, I., Zoratti, M., and Gulbins, E. (2010) Contribution of voltage-gated potassium channels to the regulation of apoptosis. FEBS Lett. 584, 2049–2056
2. Song, W. J., Tkatch, T., Baranaukas, G., Ichinose, N., Kitai, S. T., and Surmeier, D. J. (1998) Somatodendritic depolarization-activated potassium currents in rat neostriatal cholinergic interneurons are predominantly of the A type and attributable to coexpression of Kv4.2 and Kv4.1 subunits. J. Neurosci. 18, 3124–3137
3. Lang, T., Föllmer, M., Lang, K., Lang, P., Ritter, M., Vereninov, A., Szabo, I., Huber, S. M., and Gulbins, E. (2007) Cell volume regulatory ion channels in cell proliferation and cell death. Methods Enzymol. 428, 209–225
4. Niwa, N., and Nerbbonne, J. M. (2010) Molecular determinants of cardiac transient outward potassium current (ito) expression and regulation. J. Mol. Cell Cardiol. 48, 12–25
5. Liss, B., Franz, O., Sewing, S., Bruns, R., Neuhoff, H., and Roeppe, J. (2001) Tuning pacemaker frequency of individual dopaminergic neurons by Kv4.3L and KChip3.1 transcription. EMBO J. 20, 5715–5724
6. An, W. F., Bowbyl, M. R., Betty, M., Cao, J., Ling, H. P., Mendoza, G., Hinson, J. W., Mattsson, K. I., Strassle, B. W., Trimmer, J. S., and Rhodes, K. I. (2000) Modulation of A-type potassium channels by a family of calcium sensors. Nature 403, 553–556
7. Nadal, M. S., Ozaita, A., Amarillo, Y., Vega-Saenz de Miera, E., Ma, Y., Mo, W., Goldberg, E. M., Misumi, Y., Ikehara, Y., Neuhoff, T. A., and Rody, B. (2003) The CD26-related dipeptidyl aminopeptidase-like protein DPPX is a critical component of neuronal A-type K$^+$ channels. Neuron 37, 449–461
8. Martens, J. R., Kwak, Y. G., and Tamkun, M. M. (1999) Modulation of Kv channel αβ subunit interactions. Trends Cardiovasc. Med. 9, 253–258
9. Sakeun, R. H., Wang, K., Jow, F., Megules, J., Kopsco, D. C., Edris, W., Carroll, K. C., Lu, Q., Xu, W., Zhu, K., Katz, A. H., Olland, S., Lin, L., Taylor, M., Stahl, M., Malakian, K., Somers, W., Mosyak, L., Bowbyl, M. R., Chanda, P., and Rhodes, K. J. (2004) Two N-terminal domains of Kv4 K$^+$ channels regulate binding to and modulation by KChIP1. Neuron 41, 587–598
10. Bourdeau, M. L., Laplante, I., Laurent, C. E., and Lacaille, J. (2011) KChIP1 modulation of Kv4.3-mediated A-type K$^+$ currents and repetitive firing in hippocampal interneurons. Neuroscience 176, 173–187
11. Witzel, K., Fischer, P., and Bähring, R. (2012) Hippocampal A-type current and Kv4.2 channel modulation by the sulfonylurea compound NS5806. Neuropepharmacology 63, 1389–1403
12. Buxbaum, J. D., Choi, E. K., Luo, Y., Lilliehook, C., Crowley, A. C., Merriam, D. E., and Wasco, W. (1998) Calsenilin: a calcium-binding protein that interacts with the presenilins and regulates the levels of a presenilin fragment. Nat. Med. 4, 1177–1181
13. Carrión, A. M., Link, W. A., Ledo, F., Mellström, B., and Naranjo, J. R. (1999) DREAM is a Ca$^{2+}$-regulated transcriptional repressor. Nature 398, 80–84
14. Craig, T. A., Benson, L. M., Venyaminov, S. Y., Klimtchuk, E. S., Bajzer, Z., Prendergast, F. G., Naylor, S., and Kumar, R. (2002) The metal-binding domain of DREAM: implications for Ca$^{2+}$-dependent DNA binding and protein dimerization. Biochemistry 41, 2252–2264
15. Lusin, J. D., Vanarotti, M., Li, C., Valiveti, A., and Ames, J. B. (2008) NMR structure of DREAM: implications for Ca$^{2+}$-dependent DNA binding and protein dimerization. Biochemistry 47, 2252–2264
16. Kääb, S., Dixon, J., Duc, J., Ashen, D., Näbauer, M., Beuckelmann, D. J., Steinbeck, G., McKinnon, D., and Tomaseg, G. F. (1998) Molecular basis of transient outward potassium current downregulation in human heart failure: a decrease in Kv4.3 mRNA correlates with a reduction in current density. Circulation 98, 1383–1393
17. Calloe, K., Cordeiro, J. M., Di Diego, J. M., Hansen, R. S., Grunnet, M., Olesen, S. P., and Antzelevitch, C. (2009) A transient outward potassium current activator recapitulates the electrocardiographic manifestations of Brugada syndrome. Cardiovasc. Res. 81, 686–694

18. Zicha, S., Xiao, L., Stafford, S., Cha, T. I., Han, W., Varro, A., and Nattel, S. (2004) Transmural expression of transient outward potassium current subunits in normal and failing canine and human hearts. J. Physiol. 561, 735–748

19. Cordeiro, J. M., Calloe, K., Moise, N. S., Kornreich, B., Giannandrea, D., Di Diego, J. M., Olesen, S. P., and Antzelevitch, C. (2012) Physiological consequences of transient outward K⁺ current activation during heart failure in the canine left ventricle. J. Mol. Cell Cardiol. 52, 1291–1298

20. Calloe, K., Soltysinska, E., Jespersen, T., Lundby, A., Antzelevitch, C., Olesen, S. P., and Cordeiro, J. M. (2010) Differential effects of the transient outward K⁺ current activator NS5806 in the canine left ventricle. J. Mol. Cell Cardiol. 48, 191–200

21. Lundby, A., Jespersen, T., Schmitt, N., Grunnet, M., Olesen, S. P., Cordeiro, J. M., and Calloe, K. (2010) Effect of the I(to) activator NS5806 on cloned Kv4.6 channels depends on the accessory protein KChIP2. Br. J. Pharmacol. 160, 2028–2044

22. Adams, S. R., and Tsien, R. Y. (2008) Preparation of the membrane-permeant biarsenicals FlAsH-EDT2 and ReAsH-EDT2 for fluorescent labeling of tetracysteine-tagged proteins. Nat. Protoc. 3, 1527–1534

23. Osawa, M., Tong, K. I., Lilliehook, C., Wasco, W., Buxbaum, J. D., Cheng, H.-Y., Penninger, J. M., Ikura, M., and Ames, J. B. (2001) Calcium-regulated DNA binding and oligomerization of the neuronal calcium-sensing protein, calsenilin/DREAM/KChIP3. J. Biol. Chem. 276, 41005–41013

24. Chung, S., Velkov, T., Horne, J., Porter, C. J., and Scanlon, M. J. (2008) Characterization of the drug binding specificity of rat liver fatty acid binding protein. J. Med. Chem. 51, 3755–3764

25. Lakowicz, J. R. (2010) Principles of Fluorescence Spectroscopy, pp. 372–374, Springer, New York

26. Dweck, D., Reyes-Alfonso, A., Jr., and Potter, J. D. (2005) Expanding the range of free calcium regulation in biological solutions. Anal. Biochem. 347, 303–312

27. Beechem, J. M. (1989) A second generation global analysis program for the characterization of comparative models with YASARA NOVA? A self-parameterizing force field. Protein Sci. 5, 403–402

28. Wang, H., Yan, Y., Liu, Q., Huang, Y., Shen, Y., Chen, L., Chen, Y., Yang, Q., Hao, Q., Wang, K., and Chai, J. (2007) Structural basis for modulation of Kv4.0 channels by auxiliary KChIP2 subunits. J. Mol. Biol. 367, 32–39

29. Gonzalez, W. G., and Miklos, J. (2014) Application of ANS fluorescent probes to identify hydrophobic sites on the surface of DREAM. Biochim. Biophys. Acta 1844, 1472–1480

30. Bähring, R., Dannenberg, J., Peters, H. C., Leicher, T., Pongs, O., and Isbrandt, D. (2001) Conserved Kv4 N-terminal domain critical for effects of kv channel-interacting protein 2.2 on channel expression and gating. J. Biol. Chem. 276, 23888–23894

31. Callsen, B., Isbrandt, D., Sauter, K., Hartmann, L. S., Pongs, O., and Bähring, R. (2005) Contribution of N- and C-terminal channel domains to kv channel interacting proteins in a mammalian cell line. J. Physiol. 568, 397–412

32. Pioletti, M., Findeisen, F., Hura, G. L., and Minor, D. L., Jr. (2006) Three-dimensional structure of the KChIP1-Kv4.3 T1 complex reveals a cross-shaped octamer. Nat. Struct. Mol. Biol. 13, 987–995

33. Osawa, M., Dace, A., Tong, K. I., Valiveti, A., Ikura, M., and Ames, J. B. (2005) Mg²⁺ and Ca²⁺ differentially regulate DNA binding and dimerization of DREAM. J. Biol. Chem. 280, 18008–18014

34. Gebauer, M., Isbrandt, D., Sauter, K., Callsen, B., Nolting, A., Pongs, O., and Bähring, R. (2004) N-type inactivation features of Kv4.2 channel gating. Biophys. J. 86, 210–223

35. Bähring, R., and Covarrubias, M. (2011) Mechanisms of closed-state inactivation in voltage-gated ion channels. J. Physiol. 589, 461–479

36. Jergh, H. H., and Covarrubias, M. (1997) K⁺ channel inactivation mediated by the concerted action of the cytoplasmic N- and C-terminal domains. Biophys. J. 72, 163–174

37. Beck, E. J., Bowby, M., An, W. F., Rhodes, K. J., and Covarrubias, M. (2002) Remodelling inactivation gating of Kv4 channels by KChIP1, a small-molecular-weight calcium-binding protein. J. Physiol. 538, 691–706

38. Jergh, H. H., Shahidullah, M., and Covarrubias, M. (1999) Inactivation gating of Kv4 potassium channels: molecular interactions involving the inner vestibule of the pore. J. Gen. Physiol. 113, 641–660

39. Foeger, N. C., Marionneau, C., and Nerbonne, J. M. (2010) Co-assembly of Kv4 a subunits with Kv⁺ channel-interacting protein 2 stabilizes protein expression and promotes surface retention of channel complexes. J. Biol. Chem. 285, 33413–33422

40. Cui, Y. Y., Liang, P., and Wang, K. W. (2008) Enhanced trafficking of tetrameric Kv4.3 channels by KChIP1 clamping. Neurochem. Res. 33, 2078–2084

41. Martin, M. P., Alam, R., Betzi, S., Ingles, D. J., Zhu, J. Y., and Schönbrunn, E. (2012) A novel approach to the discovery of small-molecule ligands of CDK2. Chembiochem 13, 2128–2136

42. Wang, K., and Chai, J. (2009) Structural basis for auxiliary KChIP modulation of Kv4 channels. In Structure, function, and modulation of neuronal voltage-gated ion channels (Gribkoff, V. K., Kaczmarek, L. K., eds.) pp. 343–361, John Wiley & Sons, Inc., New York

43. Zhou, W., Qian, Y., Kunjiwilark, K., Pfaffinger, P. J., and Choe, S. (2004) Structural insights into the functional interaction of KChIP1 with shal-type K⁺ channels. Neuron 41, 573–586

44. Kunjiwilark, K., Qian, Y., and Pfaffinger, P. J. (2011) Functional stoichiometry underlying KChIP regulation of Kv4.2 functional expression. J. Neurochem. 126, 462–472

45. Bowby, M. R., Chanda, P., Edris, W., Hinson, J., Iow, F., Katz, A. H., Kennedy, J., Krishnamurthy, G., Pitts, K., Ryan, K., Zhang, H., and Greenblatt, L. (2005) Identification and characterization of small molecule modulators of KChIP/Kv4 function. Bioorg. Med. Chem. 13, 6112–6119

46. Wang, G., and Covarrubias, M. (2006) Voltage-dependent gating rearrangements in the intracellular T1-T1 interface of a K⁺ channel. J. Gen. Physiol. 127, 391–400

47. Cushman, S. J., Nanao, M. H., Jahng, A. W., DeRubeis, D., Choe, S., and Pfaffinger, P. J. (2000) Voltage-dependent activation of potassium channels is coupled to T1 domain structure. Nat. Struct. Mol. Biol. 7, 403–407

48. Anderson, D., Mehaffey, W. H., Itinca, M., Rehak, R., Engbers, J. D., Hameed, S., Zamponi, G. W., and Turner, R. W. (2010) Regulation of neuronal activity by Cav3-Kv4 channel signaling complexes. Nat. Neurosci. 13, 333–337

49. Li, M., Lei, L., Jia, L., Ling, X., Zhang, I., Zhao, Y., and Wang, K. (2014) Interactions of KChIP4a and its mutants with Ca²⁺ or Kv4.3 N-terminus by affinity capillary electrophoresis. Anal. Biochem. 449, 99–105