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Identification and characterization of small molecule modulators of KChIP/Kv4 function

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Abstract—Potassium channels and their associated subunits are important contributors to electrical excitability in many cell types. In this study, a yeast two-hybrid assay was used to identify inhibitors such as a diaryl-urea compound (CL-888) that binds to and modulates the formation of the Kv4/KChIP complex. CL-888 altered the apparent affinity of KChIP1 to Kv4.3-N in a Biacore® assay, but did not dissociate the two proteins in size-exclusion chromatography experiments. Kv4.2/KChIP1 current amplitude and kinetics were altered with compound exposure, supporting the hypothesis of a compound-induced conformational change in the protein complex. Fluorescence spectroscopy of a unique tryptophan residue in KChIP1 was consistent with compound binding to the protein. Molecular modeling using the KChIP1 crystal structure indicates that compound binding may occur in a small tryptophan-containing binding pocket located on the hydrophilic side of the protein.

1. Introduction

In the mammalian brain and heart, rapidly inactivating (A-type) voltage-gated potassium (Kv) channels operate at subthreshold membrane potentials to control the excitability of neurons and cardiac myocytes. The pore-forming α-subunits of the Kv4, or Shal-related, channel family, give rise to the somatodendritic A-type currents in neurons. Expression of Kv4 channels in heterologous cells results in currents that differ significantly from native A-type currents, possibly due to regulatory subunits that are missing in the heterologous system. We previously identified a family of calcium-binding proteins, called K^+ channel-interacting proteins (KChIPs) that bind to the cytoplasmic N-termini of Kv4 channels and dramatically enhance channel activity by modulating the density, inactivation kinetics, and rate of recovery from inactivation.[1] They also colocalize and coassociate with Kv4 α-subunits in mammalian brain and heart, indicating that they are integral components of native A-type channels. Four members of the family have been cloned, with KChIPs 1, 3, and 4 being expressed predominantly in the brain, while KChIP2 is present in brain and cardiac tissue. The KChIP family members serve a variety of functions in addition to their activity on Kv4 channels, including modulators of cardiac excitability,[2,3] presenilin-binding proteins,[4,5] and calcium-dependent transcriptional mediators.[6–8]

Yeast two-hybrid (YTH) technology was used to identify the KChIP family of proteins and to further characterize the interaction between Kv4.3 and KChIP1.[9] This technology was again used in a high-throughput screen, similar to that used previously with other channel proteins,[10] to identify small molecules that might disrupt the interaction between Kv4.3 and KChIP1. Such molecules could be useful for the treatment of epilepsy, stroke, neuropathic pain, or other disorders, where an enhancement of inhibitory or excitatory neuronal activity would be desirable.

One such compound, CL-888,[11] is described in detail in this study. CL-888 was identified in the high-throughput

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YTH screen using a luciferase reporter system and confirmed in a secondary YTH screen using a growth reporter. This may reflect a compound-induced alteration or disruption in the binding between KChIP1 and Kv4.3N, but (like any HTS) it does not necessarily predict a disruption in preformed complex of full-length proteins expressed in mammalian cells. A number of assays were thus developed to further study the interaction among this small molecule, KChIP1, and the KChIP1/Kv4.3 complex. In short, CL-888 was found to bind to KChIP1 and modulate the conformation and function of the channel complex.

2. Materials and methods

2.1. Chemistry

CL-888[11] was prepared using conventional techniques[11,12] for the synthesis of phenylureas from 2-amino-4-chloro-benzoic acid and 1,2-dichloro-4-isocyanato-benzene.

2.2. Biacore® assay

Surface plasmon resonance (SPR) technology is used to measure the mass concentration of molecules close to a surface, such as a chip. Interaction of two molecules can be measured with high-sensitivity by attaching one of the interacting partners to the chip. A solution of the other interacting partner is then flowed over the surface of the chip. A binding event between the two is detected as a change in the local concentration at the surface, and an SPR response is elicited.

A Biacore® CM5 chip was coated with ~5000 RU of the KChIP1 monoclonal antibody K59/19.5, which is similar to those described previously.[13] A solution of 50 μg/ml KChIP1/Kv4.3 protein complex in 10 mM Hepes, 150 mM NaCl, 0.005% surfactant P-20, pH 7.4, was flowed over the chip, and the response was measured. The complex was then preincubated with various concentrations of compound in this buffer, and this solution was flowed over the chip. Finally, a solution of KChIP1 alone was flowed over the chip. In a separate experiment, KChIP1 was bound to a Biacore® CM5 chip tagged with KChIP mAB K59/19.5. A solution of CL-888 in varying concentrations was flowed over the chip, and the response was measured. In all experiments, a blank surface was subtracted to control the nonspecific binding to the chip surface and positive compounds were run over the K59 anti-KChIP1 antibody alone as a control for antibody/KChIP1 binding disruption.

2.3. Size-exclusion column chromatography

KChIP1/Kv4.3 complex was incubated for varying times at 37 °C with 10 μM CL-888, arachidonic acid (AA), or separately with 2 mM EDTA and the mixtures eluted on a Superdex 200 SEC analytical column (Pharmacia) equilibrated with 10 μM CL-888, AA, or 2 mM EDTA, respectively.

2.4. Fluorescence spectroscopy

Fluorescence emission of full-length KChIP1 protein was scanned as a function of wavelength in the absence and presence of inhibitor at various concentrations ranging from 0.2 to 50 μM. The fluorescence scans were performed at an excitation of 295 nm with a slit width of 2 nm and an emission slit width of 4 nm. The emission maximum of tryptophan fluorescence in KChIP1 protein occurs at 340 nm. Increasing concentrations of CL-888 quenched the fluorescence of the protein. The fluorescence spectra were recorded on a Fluoromax-3 (Jobin-Yvon, Edison, NJ, USA). The fluorescence intensity changes were fitted to a single binding site model using the equation

$$\Delta F = \frac{(K_d + [P_t] + [I_t]) - \sqrt{(K_d + [P_t] + [I_t])^2 - 4[P_t][I_t]}}{2c}, \quad (1)$$

where ΔF is the fluorescence intensity change at a fixed wavelength, K_d the binding affinity of the inhibitor for the protein, $[P_t]$ the total protein concentration, $[I_t]$ the total inhibitor concentration, and c a constant that relates the fluorescence intensity to concentration. Similarly, to monitor the changes in the intrinsic fluorescence of CL-888, the excitation wavelength was set at 305 nm with bandwidths as above. The emission maximum of the inhibitor occurred at 440 nm when free in solution and 430 nm when bound to KChIP1. The shift in the emission maximum was accompanied by an increase in the fluorescence intensity of the bound inhibitor. To determine the binding affinity using these intensity changes, increasing concentrations of the KChIP1 protein were titrated into the inhibitor and the resulting changes in the fluorescence intensity at the emission maximum of 430 nm were used to generate binding isotherms and the estimation of the affinity.

2.5. Electrophysiology

Xenopus oocytes were harvested from frogs under general anesthetic using aseptic techniques. Oocytes were treated with 2 mg/ml collagenase for 1–1.5 h, defolliculated, and injected with 40–50 nl cRNA (a mixture of hKv4.3 and hKChIP1 or hKv4.3 alone). The concentration of hKv4.3 and hKChIP1 cRNA varied but was generally about 5 ng/μl hKv4.3 and 100 ng/μl hKChIP1.

Currents were recorded from *Xenopus* oocytes 1–2 days post-injection (hKv4.3+KChIP1) or 2–5 days post-injection (hKv4.3) to obtain comparable current amplitudes, using standard voltage clamp amplifiers at room temperature. The time between cRNA injection and recording could be altered by changing the concentration of cRNA injected. Recordings were done in the ND-96 bath solution containing (in mM) 96 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 50 μg/ml gentamycin, pH 7.6. The pipette (electrode) solution generally consisted of 3 M KCl. Electrodes were made from borosilicate glass and typically had resistances of 0.5–2 MΩ. The standard current–voltage (I–V) pulse protocol consisted of voltage

steps from −60 to +50 mV, in 10 mV increments, for 100–200 ms, at a frequency of one *I–V* for every few minutes. Voltage protocols designed to measure recovery from inactivation consisted of pairs of voltage steps to +40 mV. The first pulse duration was sufficient to inactivate >90% of the channels, whereas the second pulse was applied at variable times after the first, such that at the longest recovery time point, >80% of the initial current was recovered. Data were digitized and stored via computer for later analysis by standard software packages.

Compounds were first dissolved in DMSO to an initial stock concentration of 1–100 mM. Working dilutions of 1:2000–1:500 were made into the bath solution for final concentrations in the micromolar range. Compounds were applied by bath perfusion using gravity or pump-driven flow. A minimum of 2 ml (10 times the bath volume) over 10 min was flowed for each compound concentration tested. In cases where no compound effect was observed with acute compound application, oocytes were incubated at 15 °C with compound (or solvent control) for +18 h and tested.

Compounds were usually screened initially at a single dose of 50–100 μM. Current traces were analyzed for peak current amplitude (at +50 mV), steady-state current amplitude (at +50 mV, defined as an equilibrium current at the end of the voltage pulse), inactivation time constant (inactivation τ), and recovery from inactivation time constant (recovery τ). If possible, compounds were washed out of the bath for up to 30 min to determine whether their action was reversible. The percent change (±SEM) in peak and steady-state current amplitudes and the percent change (±SEM) in time constants (negative number if faster, positive if slower) were calculated by dividing the data in the presence of a compound by the control data prior to compound application. A concentration–response relationship could be determined across three or more concentrations, and the data were fit to the logistic equation to determine the IC_{50}.

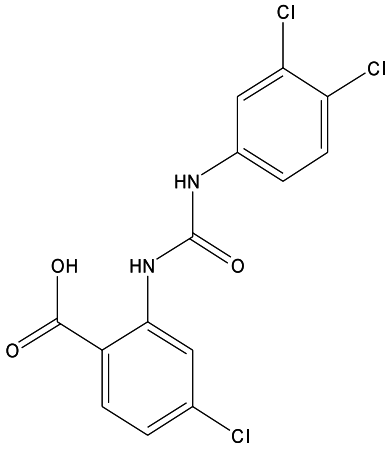
3. Results and discussion

3.1. Biacore®

An initial approach for the Biacore® analysis of KChIP1 and Kv4.3 involved the use of the individual protein components. In this paradigm, the GST-tagged KChIP1 (~50 kDa) was anchored to the CM5 chip by an anti-GST antibody and Kv4.3 AA 1–143 was applied via the flow system. Although some interaction did occur between the proteins, quantitative data collection would be problematic as the Kv4.3 1–143 protein is largely insoluble and aggregated in solution; hence, the stoichiometry was less than ideal. Attempts were made to circumvent the aggregation problem by using a more soluble Kv4.3 protein (AA 43–111); however, no interaction between KChIP1 and this protein was observed using the anti-GST paradigm.

A strategy utilizing the KChIP1/Kv4.3 complex (200 kDa molecule existing as a 1:1 octamer) was instead developed and validated. This assay utilizes an anti-KChIP1 monoclonal antibody (K59) developed in-house to pull down the complex. The antibody was chosen after comparing its binding ability to the complex with a monoclonal Kv4.3 antibody. This assay takes an advantage of the size difference between the intact complex octamer (200 kDa) and the resulting smaller fragments of KChIP1 after dissociation from Kv4.3 (25 kDa). Because KChIP1 alone or in complex with Kv4.3 is recognized by K59, the Biacore® reflects a dissociation of the complex by a diminution in signal, due to the decrease in mass between the full complex and ultimately free KChIP1. The binding characteristics of KChIP1 proteins were initially examined in the Biacore® system. KChIP1 bound well to the K59 anti-KChIP1 antibody attached to the surface of a Biacore® CM5 chip, as does the KChIP1/Kv4.3N (1–143) coexpressed complex. When this complex was preincubated for 10 min with ⩾1 μM CL-888 (Fig. 1), a decrease in signal was seen, reflecting less binding to the antibody-coated chip. This occurred in a concentration-dependent manner, with an IC_{50} of ~15 μM and saturating with 100 μM compound (Fig. 2A). The signal remaining with ⩾100 μM CL-888 was greater than the signal with KChIP1 alone (without GST), indicating that free KChIP1 may still be able to bind to the chip. A T7 antibody used to detect the remaining T7-tagged Kv4.3N on the chip revealed little remaining Kv4.3N. Thus, it appears that CL-888 was able to alter the complex binding characteristics to the KChIP1 antibody or dissociate the complex in a concentration-dependent manner. CL-888 was also flowed across the intact complex after it had been bound to the antibody, and no binding to, or alteration of, the complex was observed (data not shown).

Similar experiments were performed with an arachidonic acid (AA) analog ETYA, a known KChIP-dependent modulator of Kv4 currents.[14] ETYA also dose-dependently altered KChIP1/Kv4.3N binding, in a similar potency range as CL-888 (Fig. 2B). Arachidonic acid produced similar effects, but was less reproducible due to its chemical instability (data not shown).



CL-888

Figure 1. Structure of CL-888 (4-chloro-2-[3-(3,4-dichloro-phenyl)-ureido]-benzoic acid).

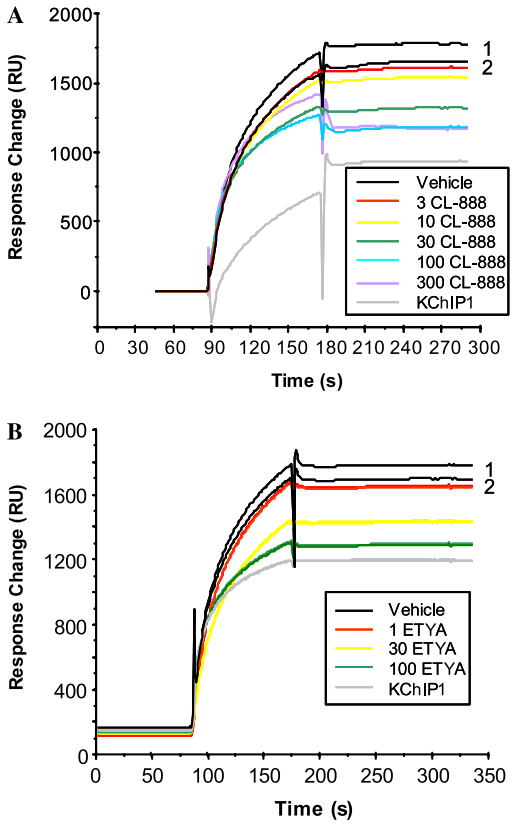


Figure 2. CL-888 and ETYA alteration of complex binding in Biacore®. (A) Increasing concentrations of CL-888 were incubated with the Kv4N/KChIP1 complex. Vehicle 2 was tested at the end of the compound applications. KChIP1 was applied to indicate the signal expected if all Kv4 protein was removed from the chip. $n = 2$ traces per condition, $n = 3$ experiments. (B) Experiment as in (A), but with increasing concentrations of ETYA. $n = 3$ experiments.

Compounds were also tested for binding to KChIP1 alone bound to the CM5 chip via the K59 anti-KChIP1 antibody. Both CL-888 and ETYA (at 100 μM) bound to KChIP1, as detected after background subtraction of binding to K59 on the chip (Fig. 3).

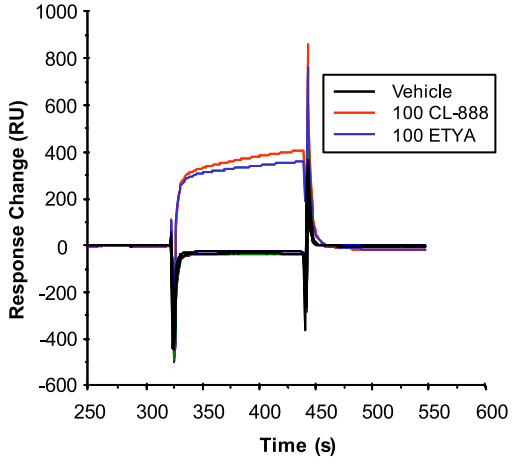


Figure 3. CL-888 binds to KChIP1 alone in Biacore®. CM5 Biacore® chip was treated as before, but KChIP1 protein was attached instead of the protein complex. CL-888 bound to the KChIP1 protein quickly, causing an increase in the signal, when compared to vehicle alone control. $n = 2$ for each condition per run; $n = 3$ experiments.

3.2. Size-exclusion chromatography

The KChIP1/Kv4.3N complex, when examined on a size-exclusion column, appears as a single monodispersed peak of ≈270 kDa (Fig. 4). Treatment of the complex with the divalent cation chelator EDTA (Fig. 4A) resulted in a time-dependent loss of the peak corresponding to the 270 kDa complex and an increase in the peaks corresponding to Kv4.3N alone (massive aggregates) and to a KChIP1 dimer (~55 kDa). Lengthy treatment (3 h) with EDTA resulted in the loss of most of the protein starting material, probably due to the precipitation of the components. In contrast, incubation of KChIP1/Kv4.3N with the compound CL-888, AA, or ETYA (latter not shown) did not significantly change the retention time, peak area, or peak distribution of the complex at any time point up to 3 h (Fig. 4B). Minor changes in the high-molecular weight shoulder of the complex were sometimes observed, but this result was

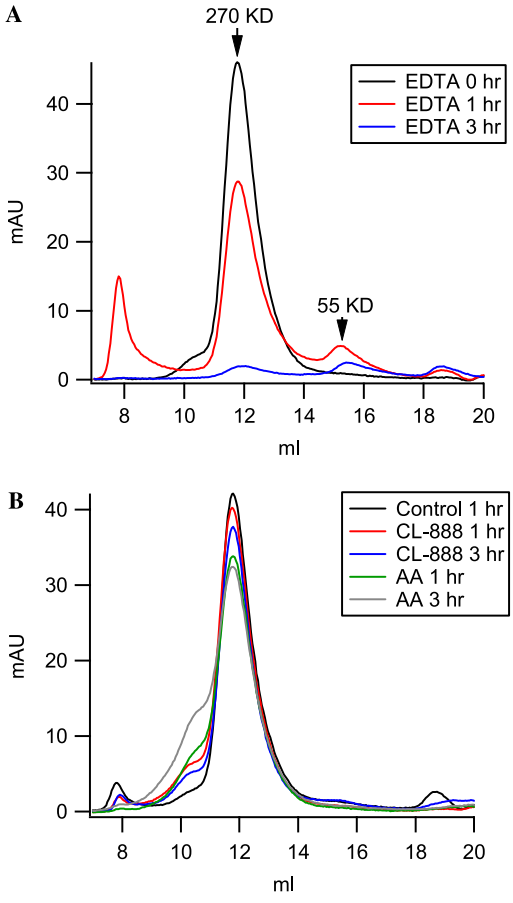


Figure 4. Size-exclusion chromatography of KChIP/Kv4N proteins. (A) EDTA treatment disrupts the complex with time. Treatment with 2 mM EDTA for 0, 1, and 3 h is shown. Longer incubations (3 h) with EDTA cause a loss of material from the column, due to insolubility of the individual proteins. $n = 2$ experiments. (B) Control treatment in buffer for 1 h indicates that the complex is relatively stable at 37 °C. The complex is incubated for 1–3 h at 37 °C with AA (10 μM), CL-888 (10 μM), or buffer alone (control). No significant change in the retention time, peak area, or peak distribution of the complex was observed with these treatments. $n = 3$ experiments.

not consistent with the behavior of the dissociated proteins (even as multimers) observed in other experiments.

The protein complex is eluted unchanged after incubation with the tested compounds, thus supporting the conclusion that CL-888, AA, and ETYA do not dissociate the complex, but rather change the conformation upon binding. This conformational change likely results in the changes observed both in Biacore® and in the functional electrophysiology experiments (see Section 3.4).

Previous work has identified the behavior of the KChIP1/Kv4N proteins coexpressed in bacteria and separated on SEC.[9] The current results are consistent with an octamer containing $Kv4_4$/$KChIP1_4$ eluting at 270 kDa, and match the stoichiometry observed for full-length, functional channels extracted from cellular membranes.[15] When dissociated from the complex or expressed alone, Kv4.3N exists as a massive aggregate, and KChIP1, when stripped of its structural calcium by EDTA, is also insoluble.[9] KChIP1 alone with divalent metal ions can exist as a monomer and/or dimer,[9,16] yet these forms were not found with compound exposure. Instead, virtually no changes in the protein complex were observed, thus indicating a lack of dissociation of the proteins.

3.3. Evaluation of CL-888 binding by fluorescence

Two fluorescent binding assays, changes in the intrinsic protein fluorescence and the inhibitor fluorescence, were employed to determine the nature of the interactions of CL-888 with the KChIP1 protein. The intrinsic protein fluorescence and the inhibitor fluorescence do not overlap and therefore provide a means to estimate the dissociation constant by the two methods independently. In the first method, CL-888 caused a saturable quenching of the fluorescence of the single tryptophan fluorescence present in KChIP1. The resultant fit of the changes in the fluorescence as a function of the inhibitor concentration yield a K_d of 0.4 ± 0.04 μM (Fig. 5). In the inhibitor fluorescence assay, the CL-888 fluorescence was enhanced 12-fold when bound to the KChIP protein. The binding affinity estimated from this method is comparable to the affinity determined using the protein fluorescence method (data not shown). These data, together with the Biacore® experiments with KChIP alone, indicate that CL-888 can bind to KChIP1 either alone or when complexed with Kv4.3N.

3.4. Electrophysiology

The functional effects of CL-888 were examined on currents produced by hKv4.3 and hKChIP1 (full-length proteins) expressed in *Xenopus* oocytes. CL-888 at 50 μM produced a significant (*t* test, $P < 0.05$) decrease in peak and steady-state currents (−65% and −68%, respectively), and a 305% slowing in the recovery from inactivation time constant compared to control (Fig. 6, Table 1). In oocytes expressing hKv4.3 alone, the compound effects were much less pronounced, producing only a 18% decrease in peak and steady-state currents

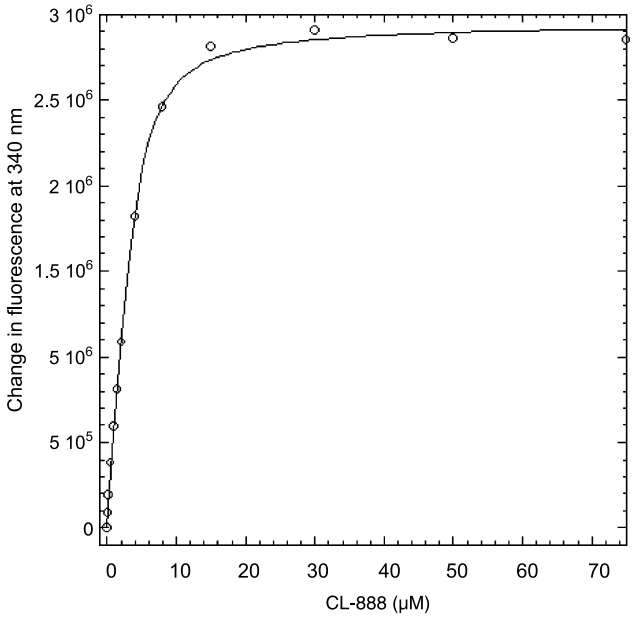


Figure 5. Binding isotherm of CL-888 binding to KChIP1. Five micromolars of the KChIP protein was titrated with an increasing concentration of the inhibitor. The change in the tryptophan fluorescence at 340 nm was used to determine the extent of quenching ($F_0 - F$). The change in fluorescence as a function of the increase in inhibitor concentration was fitted to a quadratic equation. The solid line reflects the curve fit to a single binding isotherm. $n = 3$ experiments.

(*t* test, $P > 0.05$), and a 97% slowing in the recovery time constant. No effects were observed on the inactivation time constant, either with or without KChIP1 expression.

The functional effects of CL-888 were similar in neuronal cultures of cerebellar granular neurons (CGN), which naturally express A-type currents. Peak current was reduced 75% and recovery from inactivation slowed by 27% in the presence of 50 μM compound, while no effect was observed on the inactivation kinetics (Table 1). This supports the hypothesis that Kv4/KChIP proteins underlie the A-type current in neurons. This conclusion is similar to that obtained previously using ETYA,[14] although the specific compound effects differ, and further supports the role for KChIP proteins in the function of native A-type currents.[3,17] Indeed, this study further links the idea of KChIP's ability to modulate the kinetics and amplitude of Kv4 channels, and supports the concept that small molecule binding to the KChIP1/Kv4 complex can modulate channel kinetics and current amplitude.

The electrophysiology results are also consistent with a mechanism of partially interfering with KChIP effects on the channel. As is the case for many small molecules at micromolar concentrations, some compound effects are observed on the Kv4.3 channels themselves (without KChIP1). This may occur via direct compound binding to Kv4.3, resulting in block of the channel pore and/or alteration of gating. Given the high degree of homology among the members of the KChIP family, it is also not surprising that CL-888 was observed to have similar

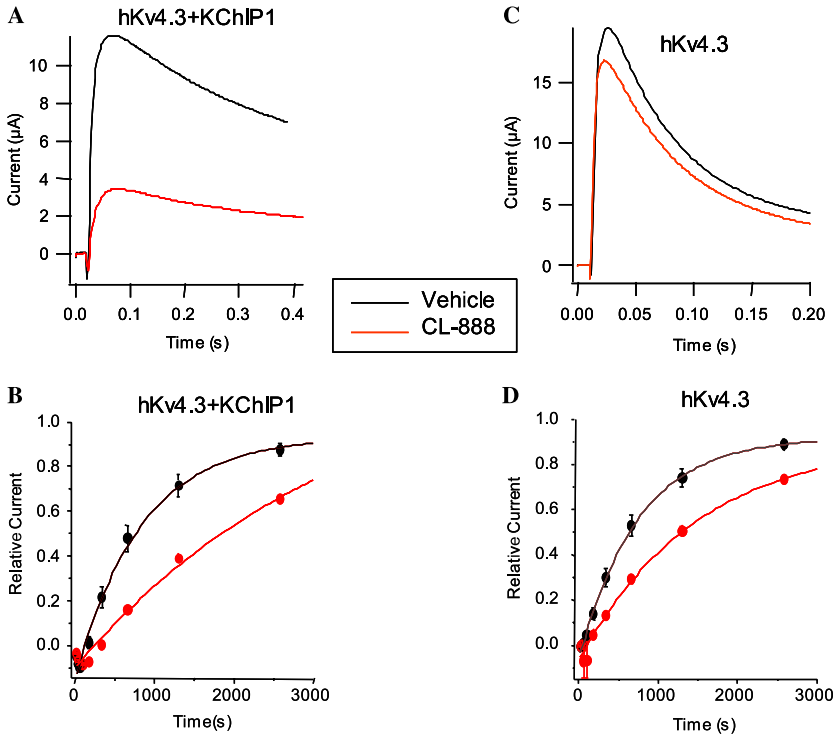


Figure 6. CL-888 (50 μM) affects current peak and kinetics in a KChIP predominant manner. Kv4.3 with (A,B) or without (C,D) KChIP1 expressed in *Xenopus* oocytes. Current traces (A,C) and exponential fits to recovery from inactivation (B,D) from the two conditions. Cells were held at −80 mV and depolarized to +40 mV. $n = 3$ experiments for each condition/treatment.

Table 1. Effects of CL-888 (50 μM) on currents evoked at 50 mV in *Xenopus* oocytes and cerebellar granular neurons

	Kv4.3	Kv4.3 +CL-888	Kv4.3/KChIP1	Kv4.3/KChIP1 +CL-888	CGN	CGN +CL-888
Peak current (μA)	21.2 ± 0.85	17.5 ± 0.35	9.4 ± 1.3	3.3 ± 0.54*	4.4 ± 0.76	1.1 ± 0.37*
Steady-state current (μA)	5.5 ± 0.96	3.5 ± 0.12	5.6 ± 0.79	1.8 ± 0.36*	—	—
Inactivation time constant (ms)	105 ± 10	86.5 ± 0.88	217 ± 4.4	189 ± 6.6*	45.7 ± 4.0	53.6 ± 9.9
Recovery from inactivation, time constant (ms)	749 ± 105	1478 ± 322*	830 ± 137	3358 ± 924*	73.6 ± 18.6	101 ± 11.4

* Significantly different from control (*t*-test, $P < 0.05$).

effects on currents composed of KChIP2/Kv4.3 channels expressed in oocytes (data not shown). Compounds active on this channel complex would be expected to have effects on cardiac tissue, but this was not tested in this study. The compounds described in this study are likely to have effects on other non-Kv4/KChIP proteins as well, as diaryl ureas have been reported to modulate other receptors,[12,18] chloride channels,[19,20] caspase activation,[21] and IL-8 receptor binding,[22] and AA and ETYA have diverse effects in many biological systems. Indeed, experiments in hippocampal slices indicate that acute compound application suppresses synaptic transmission and/or is toxic to the slices (data not shown). Thus, further exploration of these chemical series would need to occur prior to any further elucidation of the physiological effects of KChIP1/Kv4.3 inhibitors in more complex systems.

3.5. Modeling

The KChIP1 crystal structure has been recently reported.[9] Several attempts were made to cocrystallize CL-888 with KChIP1 and with the KChIP1/Kv4.3 complex, with little or no success. In the absence of X-ray crystallographic data, modeling efforts were initiated in an attempt to 'dock' CL-888 into the KChIP1 crystal structure. A MOLCAD surface of the KChIP1 crystal structure, colored by lipophilicity, is strongly lipophilic on one face, while the rest of the surface is more hydrophilic (SYBYL® 6.0, Tripos Inc., St. Louis, MO, USA). It is thought that the hydrophobic proximal N-termini of Kv4 subunits interact primarily with the hydrophobic face of KChIP1. In this orientation, the hydrophobic residues are masked, and the hydrophilic face would be oriented toward the cytoplasm. We therefore scanned the hydrophilic face for small cavities suitable for binding a small molecule. Several potential binding sites were detected, with one site being especially attractive due to its depth of cavity and relative degree of lipophilicity (Fig. 7). We found CL-888 could be easily accommodated in this site, using the MCDOCK routine within the FLO program,[23] if a small amount of flexibility is allowed for the Lys138 and Trp129 side chains (Fig. 8). The orientation found for CL-888 showed good overlap

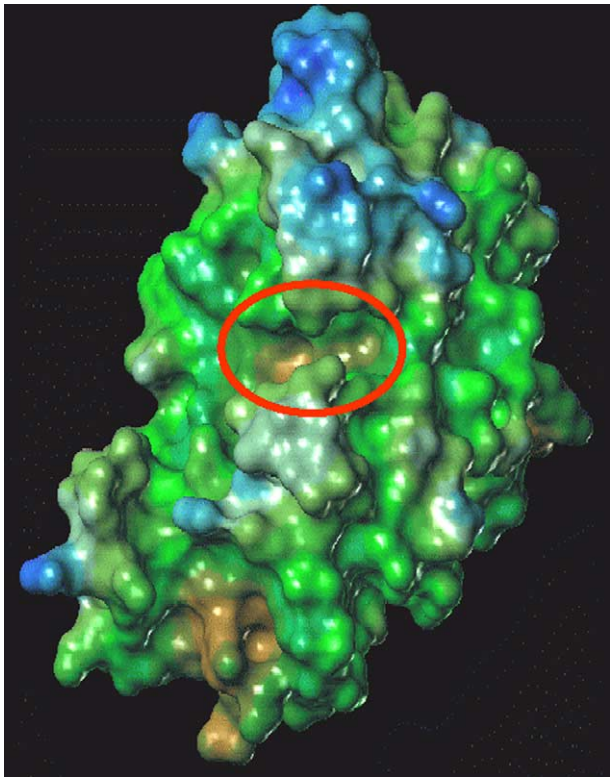


Figure 7. Space-filling model of the KChIP crystal structure[9] with the proposed binding site of CL-888 circled. MOLCAD surfaces were calculated for the structure, and surfaces are color coded by lipophilicity, where blue is highly hydrophilic and brown is highly lipophilic.

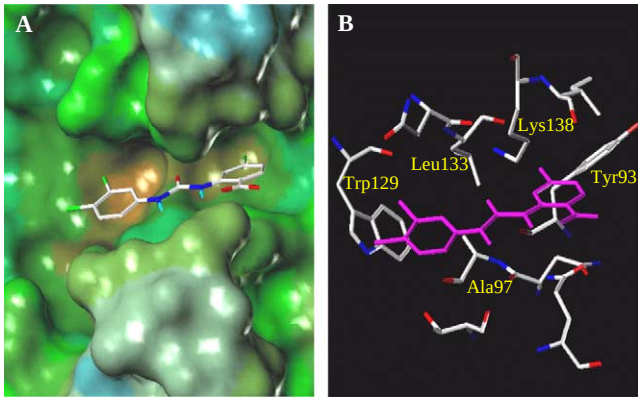


Figure 8. Detailed view of modeling of CL-888 binding to KChIP1. (A) MOLCAD calculated surface as in Figure 7, with CL-888 docking calculated with the MCDOCK routine within the FLO program. (B) The same image with the MOLCAD surface stripped away showing CL-888 in magenta along with the most closely interacting residues.

with hydrogen bond accepting, donating, and lipophilic regions in the protein using the GRID program[24] (Fig. 9). Proximity to the sole tryptophan residue in the protein, Trp129, was seen as an opportunity for fluorescence spectroscopy to detect compound binding and support our hypothesis. Indeed, fluorescence spectroscopy results were consistent with binding to KChIP1 at the proposed site on the hydrophilic face. This site of binding is also more consistent with a modulation of protein function, rather than a dissociation of the complex, an outcome more likely if compound binding occurred on the protein–protein interface.

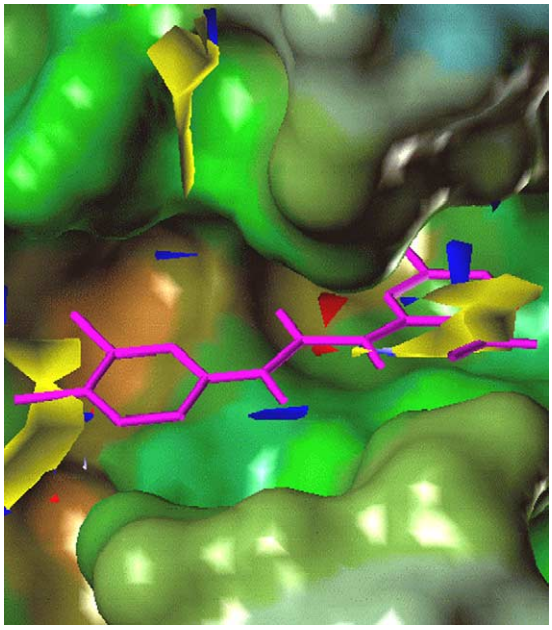


Figure 9. CL-888 model showing overlap with hydrogen bond accepting (carbonyl O: red), donating (amidic NH: blue), and liphophilic (aromatic CH: yellow) regions in the protein, identified using the GRID program.

4. Conclusion

A small-molecule diaryl-urea compound has been identified, which binds to and modulates the potassium channel KChIP/Kv4. Compound binding appears to alter the conformation of the KChIP/Kv4.3N complex and modulates the functional effects of KChIP1/Kv4.3 ion channels. Compound binding may occur near a single tryptophan residue in a small hydrophobic cleft of KChIP1, as illustrated by molecular modeling. This discovery may lead to novel methods of altering the function of this potassium channel complex in excitable tissues.

Acknowledgments

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